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Research Papers

Determination of vitamin D by isomerisation

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Conditions have been established in which the spectral change produced by the isomerisation of vitamin D_2 and D_3 to the trans-isomers can be used as an accurate measurement of vitamin D. Vitamin D was successfully determined by isomerisation in commercial products containing only vitamin D at a relatively high potency, without the use of chromatographic procedures.

VERLOOP, Koevoet & Havinga (1955) have showed that calciferol, like other partly cis-polyenes, can be isomerised to the all *trans*-isomer. They detailed the conditions for the isomerisation of calciferol by iodine, and followed its conversion to *trans*-calciferol by the change in the absorption spectrum of the solutions.

We have studied the isomerisation of calciferol and crystalline vitamin D_3 by iodine in hexane solutions, and established conditions under which the spectral change was sufficiently characteristic and reproducible to yield a quantitative measurement of the vitamin D.

A simple method was, therefore, developed, which yielded satisfactory assays of vitamin D in some commercial products, without using chromatographic procedures for the removal of materials that usually interfere in the colorimetric measurement of the vitamin D with antimony trichloride.

This method was not successful for assaying low potency vitamin D products or those containing vitamin A. These products when diluted to suitable extinction values for spectrophotometric measurement do not provide sufficient vitamin D for an accurate measurement of the spectral change produced by the isomerisation of the vitamin D.

Experimental and results

REAGENTS

Vitamin D_2 solution. Prepared with Calciferol B.P. and spectro grade hexane to yield a concentration of 20 μg/ml and stored in low actinic glass containers.

Vitamin D_3 solution. Prepared with crystalline D_3 and spectro grade hexane to yield a concentration of 20 μg/ml and stored in low actinic glass containers.

Iodine solution. Prepared with re-sublimed iodine and spectro grade hexane to yield a concentration of 1 μg/ml.

Verloop & others (1955) reported that isomerisation of calciferol in light petroleum causes the absorption maximum of the solution to shift from 265 to 270 mμ with an increase in extinction. They specified that

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the iodine concentration should not exceed 2% of the weight of the calciferol in solution to avoid degradation of the calciferol.

We have determined the effect of iodine concentration, light, and time on the isomerisation of vitamin D in the following manner: Aliquots of the vitamin D solutions, equal to 100 μg of vitamin D, were placed in 10 ml flint glass volumetric flasks. Iodine solution was added to the flasks to yield a concentration from 0 to 5% of the weight of vitamin D, and each flask brought to 10·0 ml with hexane. Three or more solutions were prepared for each level of iodine.

EFFECT OF LIGHT AND IODINE CONCENTRATION

One set of solutions representing all levels of iodine was stored in the dark, and a duplicate set was exposed to fluorescent light. The absorption spectrum of each solution was determined in a ratio recording spectrophotometer at intervals of 15 to 20 min.

In the absence of light, no spectral change occurred over 4 hr in the solutions without iodine or containing iodine to D ratios of 0·1 : 100 or less. In solutions containing iodine to D ratios greater than 0·1 : 100, a spectral change occurred that appeared to be proportional in both magnitude and rate to the concentration of iodine. In solutions containing iodine to D ratios greater than 2 : 100, a gradual decrease in the extinction values occurred after 2 to 4 hr storage.

In the presence of light, no spectral change occurred in solutions without iodine during 16 hr exposure to light. Solutions containing iodine to D ratios of 0·1, 0·2 and 0·5 : 100 showed gradual spectral changes over a period of 30 to 60 min. After this time the extinction at 270 mμ of the solutions, reached the same values, and did not alter during 16 hr exposure to light.

In solutions containing iodine to D ratios of 1 and 2 : 100, the same spectral shift and extinction values at 270 mμ were produced in 15 to 30 min. A slow decrease in the extinction values of these solutions occurred during further exposure to light.

The spectral change occurred rapidly in solutions containing iodine to D ratios of 3 : 100 or greater. The extinction at 270 mμ of these solutions did not reach the maximum value shown by solutions containing smaller concentrations of iodine, and decreased rapidly on further exposure to light.

The spectral change produced by the isomerisation of calciferol to *trans*-calciferol in our tests is similar to that found by Verloop & others (1955). The spectral change produced by the isomerisation of vitamin D_3 is identical with that produced by the isomerisation of calciferol, except that the extinction value at 270 mμ is lower than the value obtained by the isomerisation of an equal weight of calciferol.

MEASUREMENT OF VITAMIN D

Because the extinction of solutions at a wavelength other than that at which a maximum occurs does not vary exactly in proportion with the

concentration of solute, we derived the quantitative factors for the spectral change produced by the isomerisation of vitamin D_2 and D_3 by means of several replicate determinations at one concentration of each vitamin.

Based on the results obtained in the preceding tests, we used a ratio of iodine to D of 0·2 : 100, which we judged to be the most reliable for the isomerisation of vitamin D.

5-ml aliquots of either vitamin D_2 or D_3 solution, representating 100 μg or 4000 units of the vitamin, were placed in 10 ml flint glass volumetric flasks, containing 0·2 μg of iodine, and the solution made to 10·0 ml with spectro grade hexane. Replicates of these solutions were made without iodine. All the solutions were exposed to fluorescent light and their extinction determined at 15 to 20 min intervals over the spectral range 255 to 280 mμ. The rate of spectral change varied to some extent in the solutions containing iodine. A constant extinction value at 270 mμ was reached by these solutions, which did not alter during exposure of the solutions to light during 16 hr. The solutions of vitamin D_2 gave a higher extinction value than the solutions of vitamin D_3.

The extinction at 270 mμ of the solutions treated with iodine was subtracted from that at 270 mμ of the corresponding untreated solutions of the vitamin D and the mean differences (10 estimations) in these values are for vitamin D_2, 400 units/ml, 0·127 $\pm$ 0·012 s.d., and for D_3, 400 units/ml, 0·103 $\pm$ 0·003 s.d.

The calciferol we used showed a molar extinction $\epsilon = 18{,}300$ and yielded an $\epsilon = 23{,}377$ for *trans*-calciferol after isomerisation. Our crystalline vitamin D_3 showed an $\epsilon = 17{,}731$ and yielded an $\epsilon = 21{,}692$ for *trans*-vitamin D_3 after isomerisation.

Fieser & Fieser (1959) give a value of $\epsilon = 23{,}600$ for *trans*-calciferol. Inhoffen, Quinkert, Hess & Hirschfield (1957) reported an $\epsilon = 25{,}400$ at 272 mμ for *trans*-calciferol, and an $\epsilon = 24{,}300$ at 272 mμ for *trans*-vitamin D_3.

We have, over a period of time, been able to confirm the factor 0·127 for the isomerisation of 400 units of vitamin D_2 in a number of different batches of B.P. calciferol. Only one batch of crystalline vitamin D_3 was available, and was used in deriving this factor.

APPLICATION OF THE METHOD

We measured vitamin D by isomerisation in the various products listed in Table 1. In these assays, the materials were treated by one of the following methods.

Method A. For solutions of vitamin D in vegetable oil or other solvents.

The product is diluted with spectro grade hexane to a concentration of 20 μg or 800 units/ml. This solution is diluted with spectro grade hexane to a concentration of 10 μg or 400 units/ml, and its extinction determined over the spectral range of 240 to 280 mμ. If an extinction between 0·50 and 0·90 at 265 mμ is given by the solution, transfer 5·0 ml aliquots at 20 μg/ml to 4 $\times$ 10-ml flint glass volumetric flasks. To

2 flasks add 0·2 μg of iodine and make each flask to 10·0 ml with spectro grade hexane. Expose the flasks to fluorescent light and determine their extinction over the spectral range 240 to 280 mμ at hourly intervals. When a constant extinction is not reached in 4 to 5 hr, the solutions can be exposed to light overnight (16 hr) and examined again at hourly intervals, until a constant value is reached. Should the extinction of the solutions show a decrease between consecutive readings, fresh solutions are prepared with the concentration of iodine reduced to 0·15 or 0·1 μg.

We found the isomerisation of vitamin D occurred very slowly in the assays of a number of commercial products. We believe this is due to the action of materials other than vitamin D competing for iodine. We chose to maintain the selected ratio of iodine to D and allow more time for isomerisation, rather than risk degrading vitamin D by using too high a concentration of iodine.

TABLE 1. THE VITAMIN D CONTENT OF VARIOUS PRODUCTS DETERMINED BY ISOMERISATION OF THE VITAMIN D

Product	Claim* units D	Found units D av. 2 assays	% of claim
Vitamin D_2 capsules	50,000	48,000	96·0
Vitamin D_2 capsules	50,000	54,500	109·0
Vitamin D_2 capsules	50,000	60,882	121·0
Vitamin D_2 capsules	50,000	47,410	94·8
Vitamin D_2 capsules	50,000	47,200	94·4
Vitamin D_2 oil	30,800	31,884	103·0
Vitamin D_2 oil	30,800	28,828	94·0
Vitamin D_2 oil	1,000,000	1,040,000	104·0
Vitamin D_2 oil	1,000,000	1,031,239	103·12
Vitamin D_2 oil	1,000,000	1,049,000	104·9
Vitamin D_2 oil	3,000,000	2,679,024	89·0
Vitamin D_2 powder	200,000	227,000	113·5
Vitamin D_2 powder	850,000	952,000	112·0
Vitamin D_2 powder	850,000	1,067,000	125·0
Vitamin D_2 powder	850,000	1,169,000	137·6
Vitamin D_2 powder	850,000	969,474	114·0
Vitamin D_3 oil	400,000	416,000	104·0
Vitamin D_3 oil	400,000	469,454	117·2
Vitamin D_3 oil	500,000	515,000	103·0
Vitamin D_3 oil	2,000,000	2,676,000	133·5
Vitamin D_3 oil	2,000,000	2,345,000	117·5

* The suppliers established their claim in the products by either biological or colorimetric assay with antimony trichloride.

Method B. When the products treated as described in Method A gave extinction values greater than 0·90 at 265 mμ, or for products in solid form, the following procedure was used.

Take a weight or volume of the product to yield 500 μg or 20,000 units of vitamin D. Place the sample in a 100 ml flask, add 3 ml of 50% aqueous potassium hydroxide, 40 ml of spectro grade ethanol, and reflux for 20 min. Cool the solution and transfer with 100 ml of water to a separatory funnel. Extract with 4 × 20 ml portions of hexane. Wash the combined hexane extracts with water until the last washing is neutral to litmus. Evaporate the hexane solution to dryness at 35° under vacuum, and transfer the residue to a 25 ml volumetric flask with spectro grade hexane and make to volume with the same solvent. Place 5·0 ml aliquots of this solution in four 10 ml flint glass volumetric flasks. To 2 flasks

add 0·2 μg of iodine and make to volume with spectro grade hexane. The course of isomerisation of the vitamin D is followed in the same way as described in Method A.

Calculations. The amount of vitamin D in the product as determined by either Method A or B is obtained by the following equation.

$$\frac{(AI - A) \times 400}{0{\cdot}127 \text{ or } 0{\cdot}103} = \text{units of vitamin D per ml of solution.}$$

AI: is the average extinction at 270 mμ of the duplicate solutions containing iodine.

A: is the average extinction at 270 mμ of the duplicate solutions without iodine.

0·127 or 0·103 are the factors for the increase in extinction at 270 mμ produced by the isomerisation of 10 μg or 400 units of vitamin D_2 or D_3. The appropriate factor is used depending on which vitamin is claimed to be in the product.

The value in units of vitamin D obtained by this equation is divided by the volume or weight of the product present in 1·0 ml of the solutions used in the isomerisation measurements, to yield the number of units of vitamin per gram or ml of the product.

Discussion

The isomerisation of vitamin D_2 and D_3 by iodine has been found to be reproducible, and was applied successfully to the measurement of vitamin D in a number of commercial products containing only vitamin D.

This assay method did not require the use of chromatographic procedures to remove substances that usually interfere in the determination of vitamin D by the antimony trichloride colorimetric method. It does require that a hexane solution of the saponified product containing 400 units of vitamin D per ml, be sufficiently transparent to allow measurement of the extinction. Because of this requirement, the assay was not applied to products containing vitamin A and low concentrations of vitamin D, which require chromatographic procedures to reduce the extinction contributed by materials other than vitamin D.

References

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