Journal of Chromatography, 153 (1978) 219–226

CHROM. 10,683

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY DETERMINATION OF POTENTIAL CONTENT OF VITAMIN D_2 (ERGOCALCIFEROL) AND VITAMIN D_3 (CHOLECALCIFEROL) IN RESINS, OILS, DRY CONCENTRATES AND MULTIVITAMIN FORMULATIONS

RENÉE VANHAELEN-FASTRÉ and MAURICE VANHAELEN

Institute of Pharmacy, Free University of Brussels, Campus Plaine, B-205/4, Boulevard du Triomphe, B-1050 Brussels (Belgium)

(First received August 25th, 1977; revised manuscript received October 25th, 1977)

SUMMARY

The determination of potential vitamin D concentrations in raw materials and in multivitamin formulations by high-performance liquid chromatography is reported. Simple experimental conditions allow the rapid separation of the vitamins from their respective pro- and pre-vitamins, from their irradiation side-products and from some of their overirradiation products.

Vitamin extraction is performed directly from dry concentrates, tablets and capsules, without saponification; all procedures are carried out at room temperature so as to preserve the ratio of vitamin D and pre-vitamin D present in the preparation.

The relative standard deviation is less than 1.5%.

INTRODUCTION

High-performance liquid chromatography (HPLC) allows the rapid separation and quantitation of the potential vitamin D* content in raw materials[1–7].

This paper describes a new convenient method for

(1) The separation of the D vitamins from their respective precursors (pro-vitamins: ergosterol and 7-dehydrocholesterol), their corresponding isomeric forms (pre-vitamins D*), the irradiation side-products of the pre-vitamins (lumisterols and tachysterols) and some of their overirradiation products[8,9].

(2) The simultaneous determination of the content in bioactive principles, pre-vitamin D and vitamin D.

(3) The extraction and determination steps to be carried out at room temperature so as to preserve the ratio of pre-vitamin D and vitamin D.

* Vitamin D is used here as a generic term for vitamin D_2 (ergocalciferol) or D_3 (cholecalciferol); in the same way, pre-vitamin D is used for pre-vitamin D_2 or D_3. Vitamin D is more closely related to a steroid hormone than to a vitamin in the classical sense.

(4) A simple extraction procedure especially convenient for rapid analysis of multivitamin formulations.

EXPERIMENTAL

Equipment

A high-performance liquid chromatograph (Model 6000M, solvent delivery system) operating at room temperature, equipped with an UV detector at 254 nm (Model 440), a septumless injector (Model U6K) and a 30 cm × $^1/_4$ in. I.D. stainless-steel column pre-packed with μ-Porasil (mean particle size, 10 μm), were all supplied by Waters Assoc. (Milford, Mass., U.S.A.).

The number of theoretical plates of the column was *ca.* 11,000 per metre.

A 25-μl capacity syringe was supplied by Precision Sampling Corporation (Baton Rouge, La., U.S.A. —Series B-110 Pressure Lok Liquid Syringe).

A Bransonic Ultrasonic Cleaner (Model 32, 50–55 Kc), supplied by Branson (Stamford, Conn., U.S.A.) was used for solvent degassing, vitamin extraction and preparation of standard solutions.

Amber glassware (stoppered volumetric flasks, centrifuge tubes and vials) was used.

Solvents

All solvents were analytical grade and anhydrous (water: max. 0.01 %) (Merck, Darmstadt, G.F.R.). The light petroleum (b.p. 40–60°) was distilled twice.

Just before analysis, 2,6-di-*tert.*-butyl-4-methylphenol (BHT) was added (0.02 %) to all solvents used for the extraction and dissolution of vitamin and pre-vitamin D [N,N-dimethylformamide, dimethyl sulphoxide, isooctane (2,2,4-trimethylpentane), benzene and light petroleum (b.p. 40–60°)].

Solvent system A was made up as follows: light petroleum (b.p. 40–60°)–1,2-dichloroethane–tetrahydrofuran (85:8:7, v/v/v), and used for the separation of the isomeric forms, except tachysterol, and determination of the potential content of vitamin D.

Solvent system B was as follows: light petroleum (b.p. 40–60°)–1,2-dichloroethane–*p*-dioxane (90:8:2, v/v/v) for the separation of the isomeric forms, including tachysterol, and determination of the potential content of vitamin D.

Reagents

The reagents used were vitamin D_2 (Merck, biochemical grade); vitamin D_3, 7-dehydrocholesterol, ergosterol, BHT (Aldrich, Milwaukee, Wisc., U.S.A.); lumisterol$_3$ (Roth, Karlsruhe, G.F.R.).

Tachysterol$_3$ was prepared from tachysterol$_3$-3,5-dinitro-4-methylbenzoate (Philips-Duphar, Weesp, Holland), following the procedure of Hanewald *et al.*[10].

Pre-vitamins D were prepared in our laboratory by isomerization of the corresponding vitamins D, dissolved in isooctane and heated at 100° for 60 min.

Vitamin D standard solutions

Vitamin D was dissolved in light petroleum. The required concentrations and dilutions were calculated from the sample concentrations obtained after extraction or dissolution and were, as far as possible, similar to these values.

Determination of the pre-vitamin response factors to HPLC detector

About 10 mg of vitamin D were dissolved in 50 ml of isooctane (solution A_1), or in 50 ml of benzene (solution B_1); 10 ml of each solution (initial solutions A_1 and B_1) were stored at −18°.

Isomerization was performed by reflux of the remaining solutions A_1 and B_1 (40 ml), in darkness, on a steam bath (100°), for 60 and 180 min, respectively, to afford the isomerized solutions A_2 and B_2. After heating, solutions A_2 and B_2 were abruptly cooled in an ice bath.

Just before analysis, all four solutions were allowed to warm up to room temperature, diluted ten times with light petroleum and injected alternatively (A_1/A_2; B_1/B_2) into the column using solvent system A.

Peak heights were measured with the following attenuation settings (UV detector at 254 nm): 0.05 a.u.f.s. for vitamin D; 0.01 or 0.02 a.u.f.s. for pre-vitamin D.

The response factor for pre-vitamin D is given by the peak height of vitamin D in the initial solution *minus* the peak height of vitamin D in the isomerized solution, *divided by* the peak height of pre-vitamin D in the isomerized solution (corrected by the attenuation setting).

Response factor values calculated from solutions A_1/A_2 and B_1/B_2 must be identical; furthermore, the concentrations of vitamin and pre-vitamin D in isomerized solutions must agree with the theoretical values at equilibrium (Table I)[10,11].

TABLE I

EQUILIBRIUM RATIO OF VITAMIN D AND PRE-VITAMIN D; COMPARISON BETWEEN EXPERIMENTAL (HPLC) AND REPORTED VALUES[10,11]

	Vitamin D_3		*Pre-vitamin D_3*		*Vitamin D_2*	*Pre-vitamin D_2*
	found	*calculated*	*found*	*calculated*	*found*	*found*
Solution A_2 (equilibrium at 100°)	71.9*	71.5[10] 72[11]	28.1*	28.5[10] 23[11]	72.7*	27.3*
Solution B_2 (equilibrium at 80°)	80.6*	78[11]	19.4*	22[11]	80.2*	19.8*

* Relative standard deviations, 1.5%.

The calculated response factors under our experimental conditions were 1.13 for pre-vitamin D_2 and 1.08 for pre-vatimin D_3 (relative standard deviation: 3%).

Preparation of sample solutions

Resins and oils. The resin or oil was dissolved in light petroleum to give a solution containing *ca.* 2000 I.U./ml.

Dry concentrates. A quantity of dry concentrate equivalent to *ca.* 20,000 I.U. of vitamin D was accurately weighed into a centrifuge tube (40 ml capacity), and 12 ml of N,N-dimethylformamide or dimethyl sulphoxide was added. After vigorous shaking, the tube was placed in an ultrasonic bath for 15 min and shaken every 5 min. Then 10 ml of isooctane and 8 ml of ice-cooled water were added to the dispersion (the centrifuge tube being kept in an ice bath). The tube was then vigorously shaken for 3 min and centrifuged at 4000 r.p.m. for 10 min. A few millilitres of the upper phase were pipetted and *immediately* used for HPLC injection.

Multivitamin formulations. Oily solutions were diluted with light petroleum to *ca.* 200 I.U./ml.

With aqueous solutions, a volume equivalent to *ca.* 2000 I.U. of vitamin D was pipetted into a centrifuge tube (40 ml capacity) and diluted to 8 ml with ice-cooled water. Then 10 ml of isooctane and 12 ml of N,N-dimethylformamide or dimethyl sulphoxide were added. Further extraction steps were similar to those described for dry concentrates.

With multivitamin tablets and coated tablets, if they did not disintegrate in N,N-dimethylformamide or dimethyl sulphoxide after shaking and ultrasonic treatment, they were ground and the resulting powder was *immediately* used for the determination. A number of tablets or a weight of powder equivalent to *ca.* 2000 I.U. of vitamin D were treated as described for dry concentrates. (Disintegration time in the ultrasonic bath: 15–30 min*.)

With multivitamin capsules, a number of capsules equivalent to *ca.* 2000 I.U. of vitamin D were introduced into a centrifuge tube and extracted as described for dry concentrates. (Disintegration time in the ultrasonic bath: 15–30 min*.)

High-performance liquid chromatography

The HPLC column was eluted at a constant flow-rate of 2 ml/min (resulting pressure: *ca.* 500 p.s.i.) and equilibrated for 60 min before analysis. The flow-rate was reduced to 1.5 ml with solvent system A when a higher resolution was required for very complex multivitamin formulations.

The standard solution was injected until the peak height response was reproducible to less than 1%.

Standard solutions (25 μl) and 25-μl samples were alternately injected. Peak heights were measured for vitamin D and pre-vitamin D, using a smaller attenuation setting for the pre-vitamin (5–20 times according to their relative concentrations).

Sample solutions were first analysed with solvent system B: when tachysterol was not detected or was present in negligible amount (in most cases), solvent system A was then used in order to reduce analysis time.

Calculations

The peak height of pre-vitamin D (corrected for the attenuation setting, the calculated response factor and the bioactivity factor) is added to the peak height of vitamin D and compared with the peak height of the standard solution injected immediately before or after the sample solution, to give the potential vitamin D content, as shown in the following equation:

$$\text{Potential vitamin D content (I.U./g)} = \frac{(H_D + H_P \cdot RF \cdot BF) \cdot C_S \cdot V \cdot 40{,}000\ (\text{I.U./mg}) \cdot 10^3}{H_S \cdot SW}$$

* Dissolution time in the ultrasonic bath is given for guidance. It will be longer for some multivitamin formulations, for which the dissolution will be improved by using larger volumes of N,N-dimethylformamide or dimethyl sulphoxide (in the second case, isooctane and water volumes will be readjusted).

where H_D is the peak height of vitamin D in the sample: H_P is the peak height of pre-vitamin D in the sample (corrected for the attenuation setting); RF is the calculated response factor of pre-vitamin D; BF is the bioactivity factor to convert pre-vitamin D bioactivity into vitamin D bioactivity (min. 0.34; max. 0.56[10–14]); C_S is the vitamin D concentration in the standard solution (mg/ml); V is the corrected final volume (ml) including all dilution factors and the eventual dilution of isooctane used for the extraction by oils present, for example, in capsules formulations; H_S is the peak height of vitamin D in the standard solution; SW is the sample weight (mg).

Irradiation procedure

A 15-mg amount of vitamin D dissolved in 100 ml of isooctane was heated at 100° for 60 min. After isomerization, a stream of nitrogen was passed through the solution for 60 min. Irradiation was carried out with an Hanau TNW 15 W lamp (254 nm) plunged in this solution, for 5 min at 15°.

RESULTS AND DISCUSSION

The HPLC conditions used permit the separation of vitamin D_3 from pre-vitamin D_3, provitamin D_3 (7-dehydrocholesterol), lumisterol$_3$, tachysterol$_3$ and some

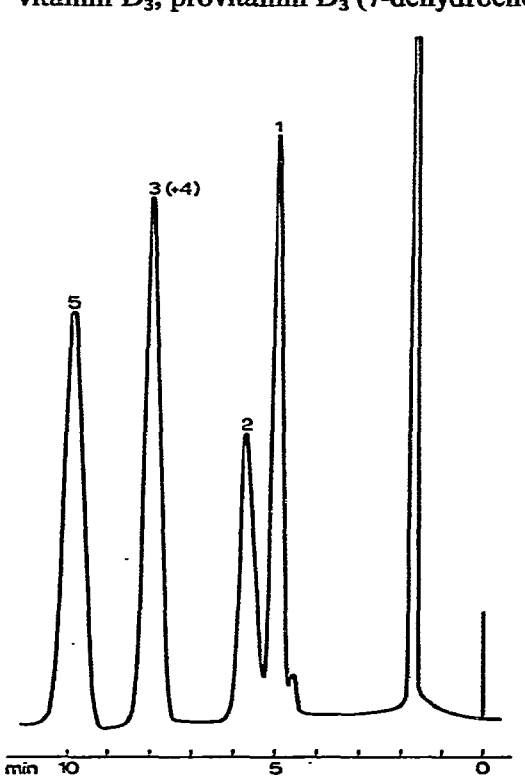

Fig. 1. High-performance liquid chromatogram of a synthetic mixture of vitamin D_2/D_3 and tachysterol$_3$ (3 + 4), pre-vitamin D_2/D_3 (1), lumisterol$_3$ (2) and ergosterol/7-dehydrocholesterol (pro-vitamin D_2/D_3; 5). Column, μ-Porasil (mean particle size, 10 μm). Solvent system, light petroleum (b.p. 40–60°)–1,2-dichloroethane–tetrahydrofuran (85:8:7, v/v/v) (flow-rate, 2 ml/min).

of the overirradiation products of pre-vitamin D_3 (Figs. 1 and 2). Under the same conditions, vitamin D_2 is separated from pro- and pre-vitamins D_2 with retention times identical with those observed for vitamin D_3 (Figs. 1 and 2). We did not attempt to separate vitamin D_2 from lumisterol$_2$ and tachysterol$_2$ because authentic samples were not available, but a similar chromatogram might be expected. Vitamins D_2 and D_3 were not distinguished under these conditions, but other fat-soluble vitamins, such as α-tocopherol, α-tocopheryl acetate, vitamin A alcohol and vitamin A esters, were well separated from vitamin and pre-vitamin D. These experimental conditions are thus suitable for the determination of vitamin D in multivitamin formulations.

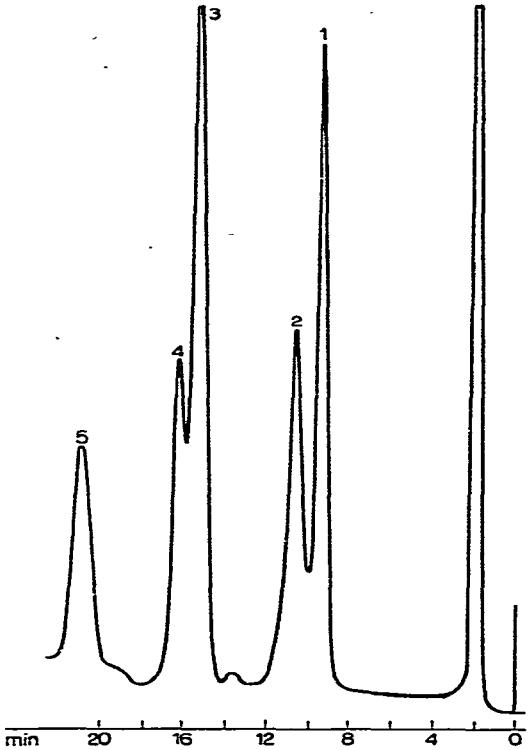

Fig. 2. High-performance liquid chromatogram of a synthetic mixture of vitamin D_2/D_3 (3), pre-vitamin D_2/D_3 (1), lumisterol$_3$ (2), tachysterol$_3$ (4) and ergosterol/7-dehydrocholesterol (pro-vitamin D_2/D_3; 5). Column, μ-Porasil (mean particle size, 10 μm). Solvent system, light petroleum (b.p. 40–60°)–1,2-dichloroethane–*p*-dioxane (90:8:2, v/v/v) (flow-rate, 2 ml/min).

The absorption maximum of vitamin D is 265 nm, so a wavelength of 254 nm was selected because it is suitable for most liquid chromatograph detectors.

The linearity of vitamin D and pre-vitamin D responses in HPLC was investigated. Beer's law is followed at least within the range 2–100 μg/ml (25-μl sample; sensitivity limit: 1 μg/ml).

In contrast to oily vitamin solutions and resins, which are readily soluble in petroleum light and can be directly injected after dilution, dry concentrates, tablets, coated tablets, capsules and aqueous solutions require an extraction step. This step

has been highly simplified: N,N-dimethylformamide or dimethyl sulphoxide (according to the formulation) have been found to be very suitable for the disintegration and subsequent dissolution of vitamin D; ultrasonic treatment improves the disintegration so that in most cases, grinding is not necessary.

Addition of water to N,N-dimethylformamide or dimethyl sulphoxide dispersions induces vitamin D insolubilization. Extraction of vitamin D from these mixtures is achieved in one step with isooctane. The recovery was checked by quantitative analysis performed on a solution of vitamin D in isooctane before and after extraction with N,N-dimethylformamide or dimethyl sulphoxide diluted with water, as described in the experimental section (recovery from N,N-dimethylformamide: 100.5%; from dimethyl sulphoxide: 100.2%; relative standard deviation: 0.5%).

Possible degradation of vitamin D in the ultrasonic bath was also investigated. Complete analysis of pure vitamin D, dissolved in N,N-dimethylformamide or dimethyl sulphoxide, was performed. No significant differences were observed with or without the ultrasonic treatment (30 min) (recovery from N,N-dimethylformamide: 100.4%; from dimethyl sulphoxide: 99.1%; relative standard deviation: 1.2%).

A powerful antioxidant (BHT) is added to the vitamin D solutions to avoid any oxidation process during analysis; it does not interfere in HPLC.

As the entire procedure is carried out at room temperature, and without saponification, the actual content in vitamin D and in pre-vitamin D in the formulations is preserved.

The method described for the accurate determination of the pre-vitamin response factors to HPLC detection should be carried out in each laboratory to recalculate these factors under individual experimental conditions. Indeed, the relative peak heights of vitamin D and pre-vitamin D are not only dependent on their relative UV absorbance at 254 nm but also on the peak width, which is in turn closely dependent on the relative retention times in an isocratic elution process. Provided that the retention times are constant, the calculation of the pre-vitamin D response factor is not required for further assays.

In order to obtain the best correlation between the HPLC results and the actual potential content of vitamin D, a bioactivity factor is assigned to the pre-vitamin D content found by the HPLC method.

The values of the relative standard deviation are in the range accepted for classical determinations by liquid chromatography. Without internal standard, they are less than 1.5%, even with complex multivitamin formulations: oily solutions and oily multivitamin solutions, 0.5%; resins, 2.0%; dry concentrates, 1.2%; multivitamin capsules, 0.5–2.0%; tablets, 1.2%; aqueous solutions, 1.2%.

Special attention has been paid to the choice of solvent petroleum (light instead of pentane, hexane or isooctane; no pretreatment of the solvents) and to the reduction of analysis time (extraction, 15–45 min; HPLC separation, 10 min when tachysterol content is negligible).

ACKNOWLEDGEMENTS

We thank Dr. M. P. Rappoldt (Philips-Duphar) for his generous gift of tachysterol$_3$-3,5-dinitro-4-methylbenzoate.

REFERENCES

1 G. J. Krol, C. A., Mannan, F. Q. Gemmill, Jr., G. E. Hicks and B. T. Kho, *J. Chromatogr.*, 74 (1972) 43.
2 R. C. Williams, J. A. Schmit and R. A. Henry, *J. Chromatogr. Sci.*, 10 (1972) 494.
3 D. F. Tomkins and R. J. Tscherne, *Anal. Chem.*, 46 (1974) 1602.
4 H. Steuerle, *J. Chromatogr.*, 115 (1975) 447.
5 H. Hofsass, A. Grant, N. J. Alicino and S. B. Greenbaum, *J. Ass. Offic. Anal. Chem.*, 59 (1976) 251.
6 K. Tsukida, A. Kodama and K. Saiki, *J. Nutr. Sci. Vitaminol.*, 22 (1976) 15.
7 K. A. Tartivita, J. P. Sciarello and B. C. Rudy, *J. Pharm. Sci.*, 65 (1976) 1024.
8 F. Boomsma, H. J. C. Jacobs, E. Havinga and A. van der Gen, *Rec. J. Royal Netherlands Chem. Soc.*, 96 (1977) 104.
9 H. J. C. Jacobs, F. Boomsma, E. Havinga and A. van der Gen, *Rec. J. Royal Netherlands Chem. Soc.*, 96 (1977) 113.
10 K. H. Hanewald, M. P. Rappoldt and J. R. Roborgh, *Rec. Trav. Chim. Pays-Bas*, 80 (1961) 1003.
11 J. A. Keverling Buisman, K. H. Hanewald, F. J. Mulder, J. R. Roborgh and K. J. Keuning, *J. Pharm. Sci.*, 57 (1968) 1326.
12 W. H. C. Shaw and J. P. Jefferies, *Analyst (London)*, 82 (1957) 8.
13 H. R. Bollinger, in E. Stahl (Editor), *Thin-Layer Chromatography*, Academic Press, New York, 1965, p. 225.
14 H. Berkemeier and K. Pfordte, *Naturwiss.*, 50 (1963) 647.