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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY DETERMINATION OF THREE HYDROXYVITAMINS D_3 IN PHARMACEUTICAL FORMULATIONS

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SUMMARY

Both partition chromatography on octadecyl bonded phase and adsorption chromatography on silica gel have been used to study (i) the separation of 1α-hydroxyvitamin D_3, 25-hydroxyvitamin D_3 and 1α,25-dihydroxyvitamin D_3, (ii) the separation of these hydroxyvitamins D_3 from their corresponding prehydroxyvitamins D_3, and (iii) the separation and the quantitative determination of hydroxyvitamins D_3 and prehydroxyvitamins D_3 in pharmaceutical formulations.

The separate determination of hydroxyvitamins D_3 and prehydroxyvitamins D_3 was found to be possible only on silica gel.

Conversion factors for prehydroxyvitamins D_3 and hydroxyvitamins D_3 were calculated from measurements of UV absorption at 254 nm after thermal isomerization of the hydroxyvitamins D_3.

INTRODUCTION

Vitamin D_3 is metabolized to hydroxylated products that show high and specific biological activity; these metabolites are chemically synthesized from cholesterol or cholesterol derivatives for use in pharmaceutical formulations. Because of their high activity, the concentrations of these metabolites in these preparations is often very low and a specific and sensitive method is essential for their determination.

The temperature-dependent equilibrium between vitamin D and previtamin D in solution is well known. A similar reversible isomerization occurs between hydroxyvitamins D_3 [(COH)-D_3] and the corresponding prehydroxyvitamins D_3. Furthermore, in some chemical syntheses, the prehydroxyvitamin D_3 is the direct precursor of the hydroxyvitamin D_3[1].

The separation and the determination of the prehydroxyvitamins D_3 are thus often necessary; only an analytical method that does not require the sample to be heated during extraction and/or analysis will preserve the initial concentration of hydroxyvitamin D_3 and of prehydroxyvitamin D_3.

For all these requirements, high-performance liquid chromatography (HPLC)

TABLE I

HPLC CONDITIONS

Separated compounds	*Column*	*Mobile phase*	*Flow-rate (ml/min)*	*Approximate resulting pressure (p.s.i.)*	*Chart speed (cm/min)*	*Retention time (min)*	*Figure*
A. Separation of 1α-OH-D_3, 25-OH-D_3 and 1α,25-$(OH)_2$-D_3							
1α-OH-D_3,25-OH-D_3 and 1α,25-$(OH)_2$-D_3	LiChrosorb Si 60	Light petroleum (b.p., 60–80°)-dichloroethane-tetrahydrofuran-*n*-propanol (50:25:24:5, v/v)	1.0	900	1.0	25-OH-D_3 : 4.2 1α-OH-D_3 : 6.9 1α,25-$(OH)_2$-D_3 : 10.0	1
	μBondapak C_{18}	Ethanol 94%-water (8:2, v/v)	1.5	2500	0.5	1α,25-$(OH)_2$-D_3 : 3.4 25-OH-D_3 : 4.8 1α-OH-D_3 : 9.0	2
B. Separation of hydroxyvitamins D_3, from their respective prehydroxyvitamins							
1α-OH-D_3 and pre-1α-OH-D_3	LiChrosorb Si 60	Light petroleum (b.p. 60–80°)-dichloroethane-tetrahydrofuran-*n*-propanol (50:25:24:5, v/v)	1.5	1200	1.0	1α-OH-D_3 : 4.7 pre-1α-OH-D_3 : 5.7	3
25-OH-D_3 and pre-25-OH-D_3	LiChrosorb Si 60	Light petroleum (b.p. 60–80°)-dichloroethane-tetrahydrofuran-*n*-propanol (40:5:15:0.6, v/v)	1.0	800	1.0	pre-25-OH-D_3 : 7.1 25-OH-D_3 : 7.6	4
1α,25-$(OH)_2$-D_3 and pre-1α,25-$(OH)_2$-D_3	LiChrosorb Si 60	Light petroleum (b.p. 60–80°)-dichloroethane-tetrahydrofuran-*n*-propanol (50:25:24:5, v/v)	1.5	1200	0.5	1α,25-$(OH)_2$-D_3 : 6.7 pre-1α,25-$(OH)_2$-D_3 : 9.0	5
C. Quantitative determination in pharmaceutical formulations							
1α-OH-D_3 (encapsulated oily solution)	LiChrosorb Si 60	Light petroleum (b.p. 60–80°)-dichloroethane-tetrahydrofuran-*n*-propanol (50:25:24:5, v/v)	1.0	900	1.0	1α-OH-D_3 : 4.5 pre-1α-OH-D_3 : 5.6	6
25-OH-D_3 (solution in propyleneglycol)	LiChrosorb Si 60	Light petroleum (b.p. 60–80°)-dichloroethane-tetrahydrofuran-*n*-propanol (40:5:15:0.6, v/v)	1.0	800	1.0	pre-25-OH-D_3 : 7.1 25-OH-D_3 : 7.6	4
	μBondapak C_{18}	Ethanol 94%-water (8:2, v/v)	1.5	2500	0.5	unresolved (25-OH-D_3 + pre-25-OH-D_3) : 4.5	7
1α,25-$(OH)_2$-D_3 (encapsulated oily solution)	LiChrosorb Si 60	Light petroleum (b.p. 60–80°)-dichloroethane-tetrahydrofuran-*n*-propanol (50:25:24:5, v/v)	1.5	1200	0.5	1α,25-$(OH)_2$-D_3 : 6.8 pre-1α,25-$(OH)_2$-D_3 : 9.1	8
	μBondapak C_{18}	Ethanol 94%-water (7:3, v/v)	1.5	2500	0.5	unresolved (1α,25-$(OH)_2$-D_3 + pre-1α,25-$(OH)_2$-D_3) : 5.1	9

is the most suitable technique. Several methods have been described for the HPLC determination of hydroxyvitamins D (without prehydroxyvitamin D determination); for example, 25-OH-D in food[2] and in biological material[3-9]; 25-OH-D, 24,25-$(OH)_2$-D, 1α-OH-D, 1α,25-$(OH)_2$-D and 25,26-$(OH)_2$-D in biological samples[10-13].

This paper describes a method for the determination of hydroxyvitamins D_3 and prehydroxyvitamins D_3 (1α-OH-D_3, 25-OH-D_3 and 1α,25-$(OH)_2$-D_3) in commercial pharmaceutical preparations. The described HPLC method could also be applied for the determination of these hydroxyvitamins D_3 after extraction from biological fluids.

EXPERIMENTAL

Equipment

A high-performance liquid chromatograph (Model 6000A solvent delivery system) operating at room temperature, equipped with an UV absorption detector (Model 440) at 254 nm, a septumless injector (Model U6K) were all supplied by Waters Assoc. (Milford, Mass., U.S.A.). A BD41 Kipp & Zonen (Emmen, The Netherlands) recorder and a Varian (Palo Alto, Calif., U.S.A.) integrator Aerograph® Model CDS 111 were used. A 25-μl syringe (Type 802RN-84816) was supplied by Hamilton (Reno, Nev., U.S.A.). Solvent degassing was performed in a Bransonic Ultrasonic cleaner (Model 32, 50-55) supplied by Branson (Stamford, Conn., U.S.A.). Amber glassware (stoppered volumetric flasks and vials) was used.

Columns

For adsorption chromatography, a 250 mm × 4 mm I.D. stainless steel column Hibar® prepacked with LiChrosorb Si 60 (mean particle size 5 μm) was supplied by Merck (Darmstadt, G.F.R.).

For partition chromatography, a 300 mm × 3.9 mm I.D. stainless-steel column prepacked with μBondapak C_{18} (mean particle size 10 μm) was supplied by Waters Assoc.

Solvents

All solvents were analytical grade (except tetrahydrofuran) and anhydrous (except ethanol). Light petroleum (b.p. 60–80°, Merck) was distilled twice. Tetrahydrofuran was of spectroscopic grade or for "high-performance liquid chromatography" (Uvasol or Lichrosolv, Merck). Water for HPLC was obtained by distillation of deionized water with a Quickfitt apparatus (Staffordshire, Great Britain).

Just before analysis, 2,6-di-*tert*-butyl-4-methylphenol (BHT) (0.02%) was added to all solvents used for dissolution and/or dilutions of the samples and the standards.

Solvent systems for chromatography on silica gel were made by mixing various proportions of light petroleum (b.p. 60–80°), 1,2-dichloroethane, tetrahydrofuran and -propanol (Table I). Solvent systems for chromatography on octadecyl reversed phase were made by mixing various proportions of ethanol (94%) and water (Table I).

Reagents

BHT was supplied by Aldrich (Milwaukee, Wisc., U.S.A.), 1α,25-dihydroxy-

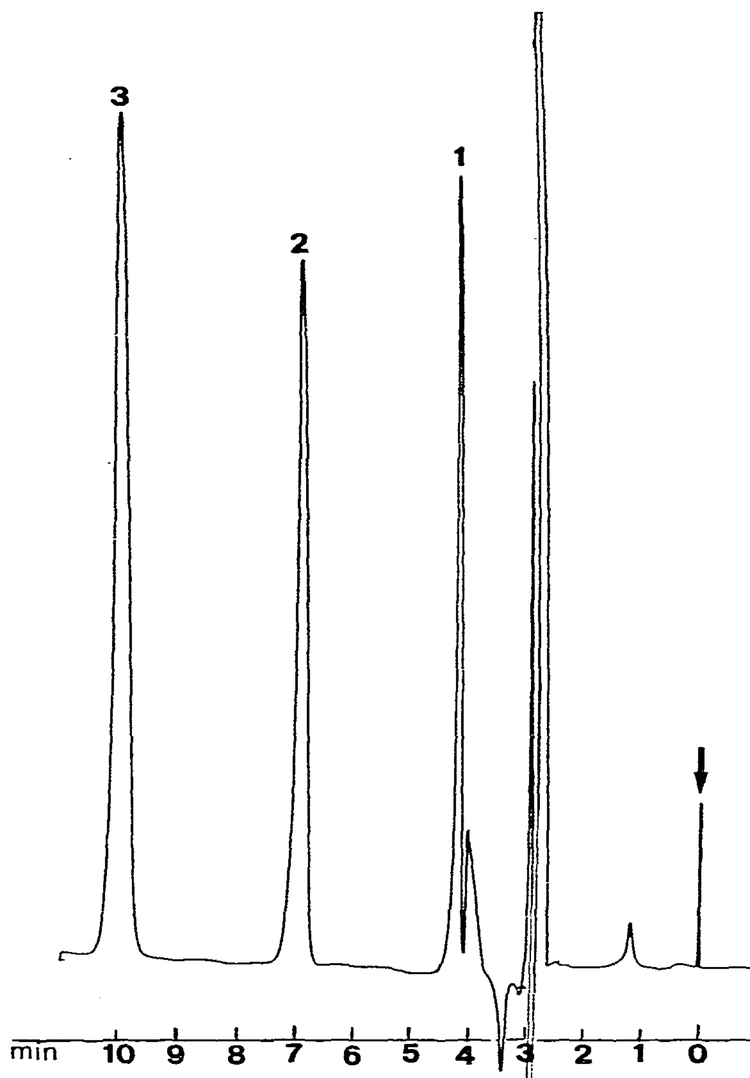

Fig. 1. HPLC separation of 25-hydroxyvitamin D_3 (1), 1α-hydroxyvitamin-D_3 (2) and 1α,25-dihydroxyvitamin D_3 (3) on silica gel (Table I).

vitamin D_3 by Roche (Basel, Switzerland), 1α-hydroxyvitamin D_3 by Leo Laboratories (Hayes, Great Britain) and 25-hydroxyvitamin D_3 by Roussel (Paris, France). Prehydroxyvitamins D_3 were prepared by thermal isomerization of the corresponding hydroxyvitamins D_3.

Hydroxyvitamins D_3 standard solutions

For partition chromatography, hydroxyvitamins D_3 were dissolved in ethanol. For adsorption chromatography, they were dissolved in pure tetrahydrofuran, then diluted with isooctane in the following ratios: tetrahydrofuran–isooctane (1:99) for 1α-OH-D_3 and 25-OH-D_3, and 1:9 for 1α,25-$(OH)_2$-D_3.

The required concentrations and dilutions were calculated from the sample concentration after an eventual dilution.

Determination of prehydroxyvitamins D_3 conversion factors

About 1 mg of pure substance was dissolved in *ca.* 12 ml of a suitable solvent. 5 ml of this initial solution were stored at −18° ("non-isomerized solution").

Isomerization was performed by reflux of 5 ml of the initial solution, in a steam bath (100°) and in darkness. After heating, the solution was abruptly cooled in an ice bath ("isomerized solution"). Just before the HPLC analysis on the silica column, both solutions were allowed to warm to room temperature and diluted to 10 ml.

The peak areas of hydroxyvitamin D_3 and prehydroxyvitamin D_3 were integrated. The conversion factor for prehydroxyvitamin D_3 is given by the peak area of hydroxyvitamin D_3 in the "non-isomerized solution" minus the peak area of hydroxyvitamin D_3 in the "isomerized solution", divided by the peak area of prehydroxyvitamin D_3 in the "isomerized solution".

The values of these conversion factors and the ratio of hydroxyvitamin D_3 to prehydroxyvitamin D_3, as functions of isomerization temperatures and heating times are listed in Table II.

TABLE II

CALCULATED CONVERSION FACTOR (FOR PEAK AREA), ISOMERIZATION CONDITIONS AND RATIO OF HYDROXYVITAMIN D_3 AND PREHYDROXYVITAMIN D_3 AFTER ISOMERIZATION

Substance	*Conversion factor**	*Isomerization conditions*			*Ratio (%) of*	
		Solvent	*Heating temp. (°C)*	*Heating time (min)*	*Vitamin*	*Previtamin*
1α-OH-D_3	2.15	Isooctane –THF (1:99)	95	90	79.1	20.9
25-OH-D_3	1.82	Propylene glycol	100	60	78.2	21.8
1α,25-$(OH)_2$-D_3	1.61	Isooctane –THF (5.3:0.7)	85	100	84.8	15.2
Vitamin D_3	2.45[17]	not reported	85	100**	77.0	23.0**
		not reported	95	50**	73.5	26.5**
		not reported	100	30	72.0	28.0[15]
		Isooctane	100	30	72.7	27.3[16]

* The peak area of prehydroxyvitamin D_3 or previtamin D_3 must be multiplied by this conversion factor to convert it into hydroxyvitamin D_3 or vitamin D_3.

** Extrapolation from earlier values[15].

Preparation of sample solutions

Pharmaceutical formulations contain only one of the three hydroxyvitamins D_3 investigated, dissolved in a suitable carrier with eventually an added antioxidant.

Pydroxyvitamins D_3. In the formulations of 1α,25-$(OH)_2$-D_3, the concentrations of the active principle were 0.50 and 0.25 μg for 160 mg of an oily carrier with two antioxidants. For reversed phase chromatography, the oil was diluted with absolute ethanol, to obtain a concentration of *ca.* 1 μg/ml. For adsorption chromatography, the oil was injected directly, without any dilution.

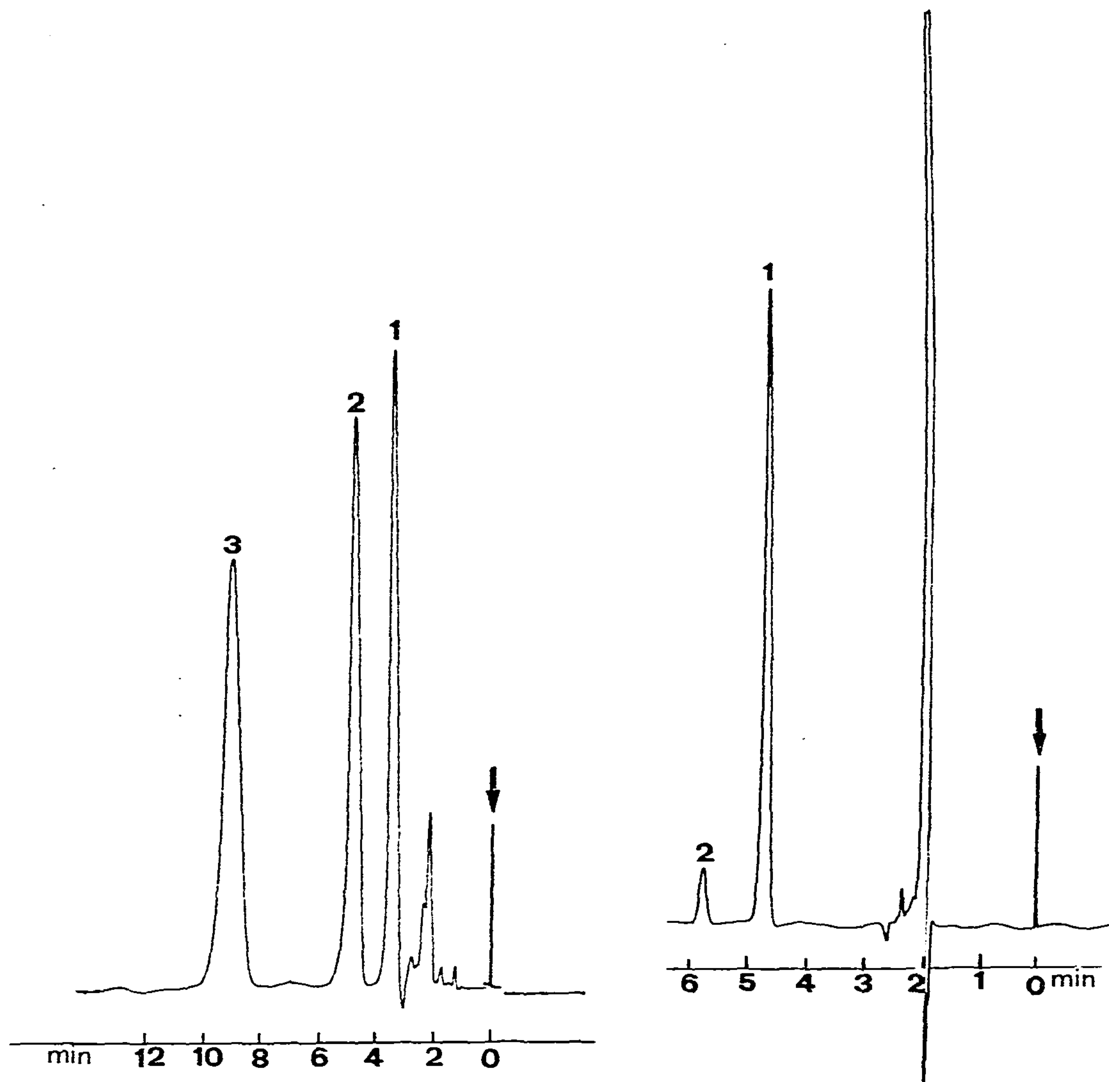

Fig. 2. HPLC separation of 1α,25-dihydroxyvitamin D_3 (1), 25-hydroxyvitamin D_3 (2) and 1α-hydroxyvitamin D_3 (3) on octadecyl bonded phase (Table I).

Fig. 3. HPLC separation of 1α-hydroxyvitamin D_3 (1) and pre-1α-hydroxyvitamin D_3 (2) on silica gel (Table I).

The commercial form of 25-OH-D_3 investigated was a solution of 15 mg of the active principle in 100 ml of propylene glycol. For partition chromatography, the solution was diluted with ethanol; for adsorption chromatography, it was diluted with tetrahydrofuran to obtain solutions containing *ca.* 25–50 μg/ml.

Capsules of 1α-OH-D_3 contained 1 μg in 100 mg of an oily carrier with an antioxidant. For adsorption chromatography, the solution was diluted with tetrahydrofuran to concentrations of *ca.* 2–3 μg/ml.

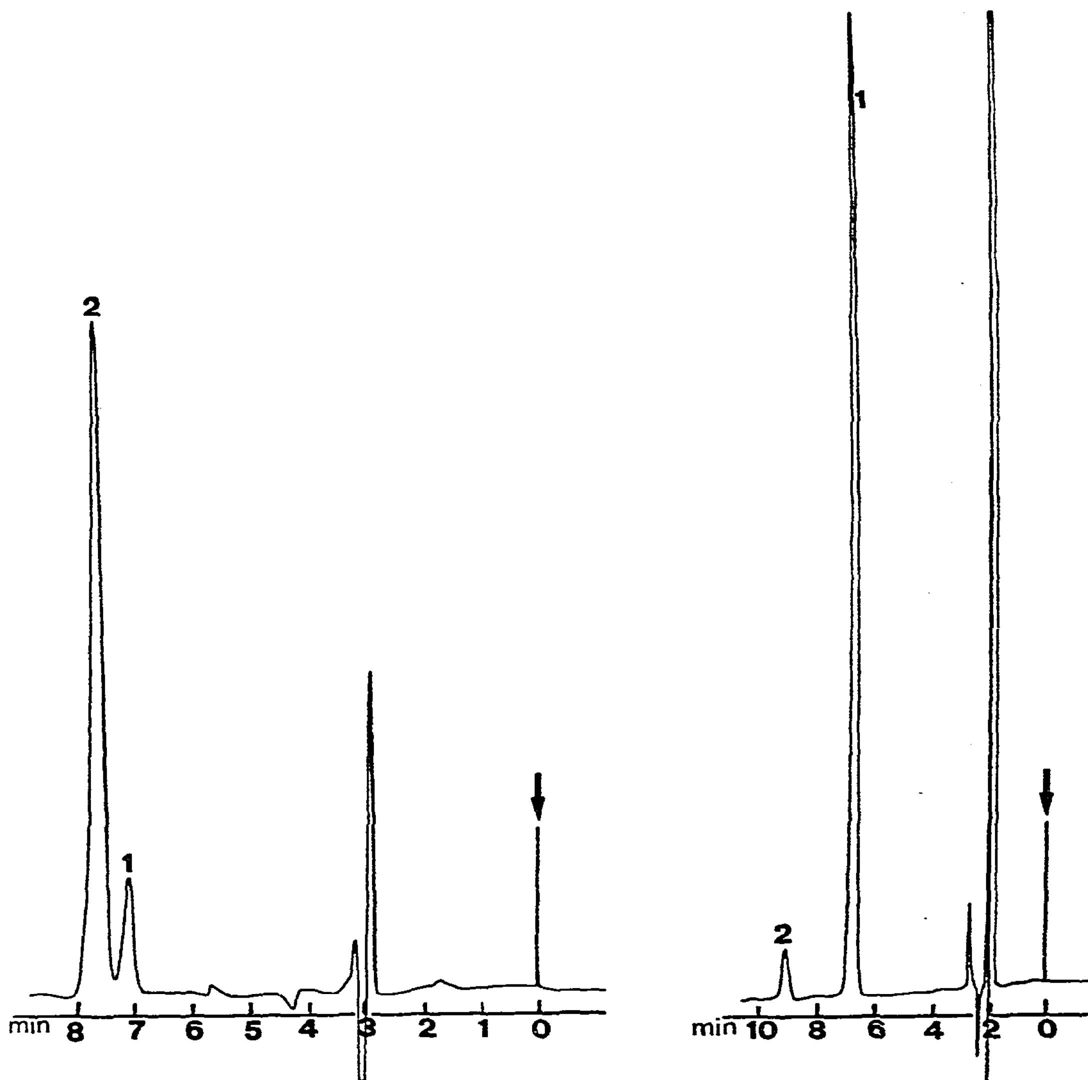

Fig. 4. HPLC separation of pre-25-hydroxyvitamin D_3 (1) and 25-hydroxyvitamin D_3 (2) in a pharmaceutical formulation on silica gel (Table I).

Fig. 5. HPLC separation of 1α,25-dihydroxyvitamin D_3 (1) and pre-1α,25-dihydroxyvitamin D_3 (2) on silica gel (Table I).

High-performance liquid chromatography

The HPLC columns were eluted at a constant flow-rate (1–2 ml/min; Table I) and were equilibrated before analysis, for 60 min (silica gel) or for 30 min (octadecyl bonded phase).

For quantitative determinations, the standard solution was injected until the peak area response was reproducible to less than 1 %. Standard and sample solutions were injected alternately.

When a column clean-up with ethanol (octadecyl column) or chloroform

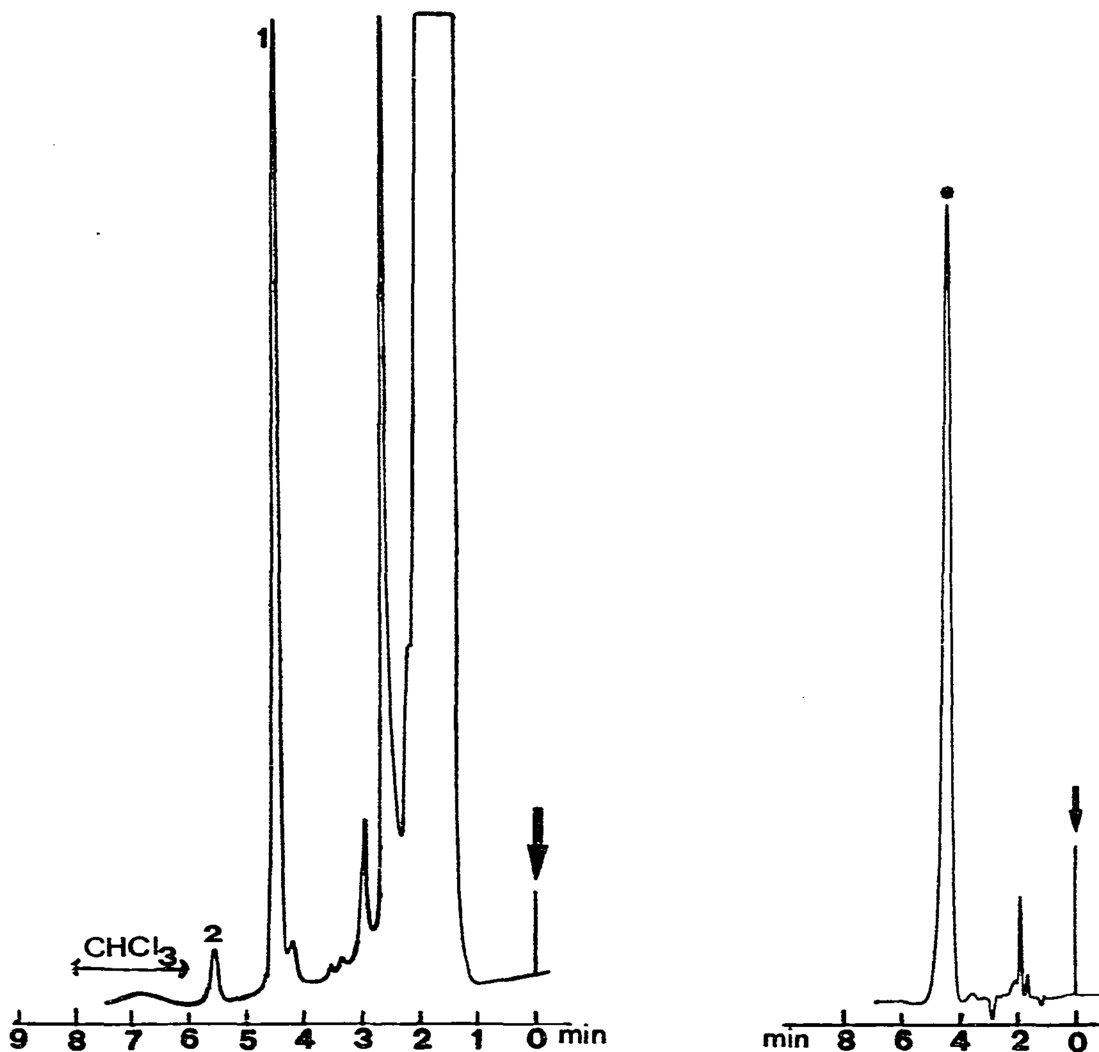

Fig. 6. HPLC separation of 1α-hydroxyvitamin D_3 (1) and pre-1α-hydroxyvitamin D_3 (2) in a pharmaceutical formulation on silica gel (Table I).

Fig. 7. High-performance liquid chromatogram of (25-hydroxyvitamin D_3 + pre-25-hydroxyvitamin D_3) (unresolved peaks*), in a pharmaceutical formulation, on octadecyl bonded phase (Table I).

(silica gel column) was required, the column was re-equilibrated with the mobile phase for 5 or 10 min, respectively, after return to the baseline.

Calculations

After separation of hydroxyvitamin D_3 from prehydroxyvitamin D_3 by chromatography on silica gel, the potential hydroxyvitamin D_3 content is calculated as follows. The integrated peak area of prehydroxyvitamin D_3 (corrected by the calculated conversion factor) is added to the integrated peak area of hydroxyvitamin D_3; thi

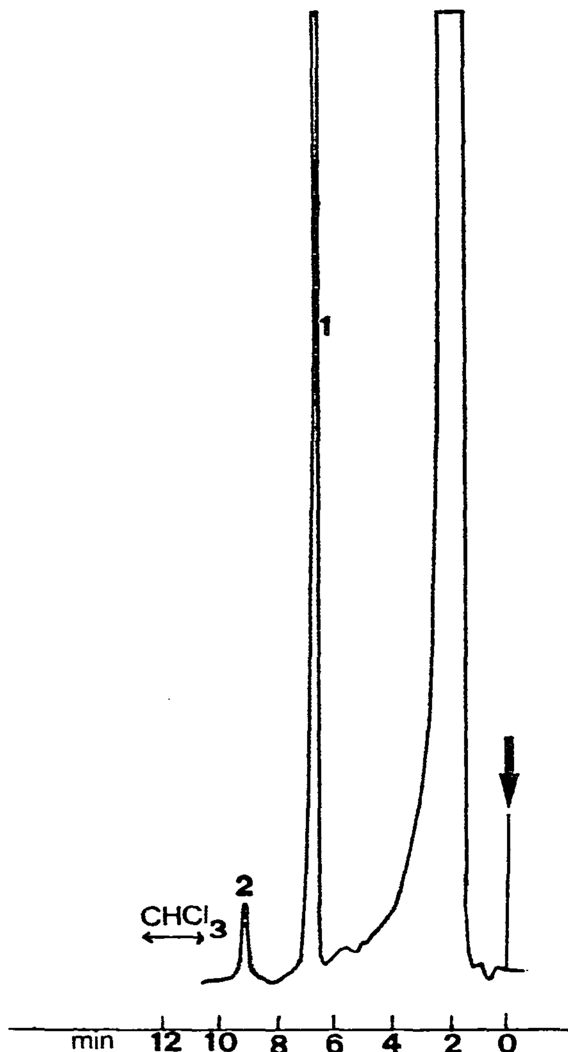

Fig. 8. HPLC separation of 1α,25-dihydroxyvitamin D_3 (1) and pre-1α-25-dihydroxyvitamin D_3 (2) in a pharmaceutical formulation on silica gel (Table I).

sum is compared with the integrated peak area of the standard solution injected immediately before or after the sample solution. The integrated peak area of prehydroxyvitamin D_3 should be corrected for a bioactivity factor, but this has not yet been done.

RESULTS AND DISCUSSION

Both partition chromatography on octadecyl bonded phase and adsorption chromatography on silica gel were used to study (i) the separation of 1α-OH-D_3, 25-OH-D_3 and 1α,25-$(OH)_2$-D_3, (ii) the separation of these hydroxyvitamins D_3 from their respective prehydroxyvitamins, (iii) the separation of hydroxyvitamins D_3 and prehydroxyvitamins D_3 in pharmaceutical formulations.

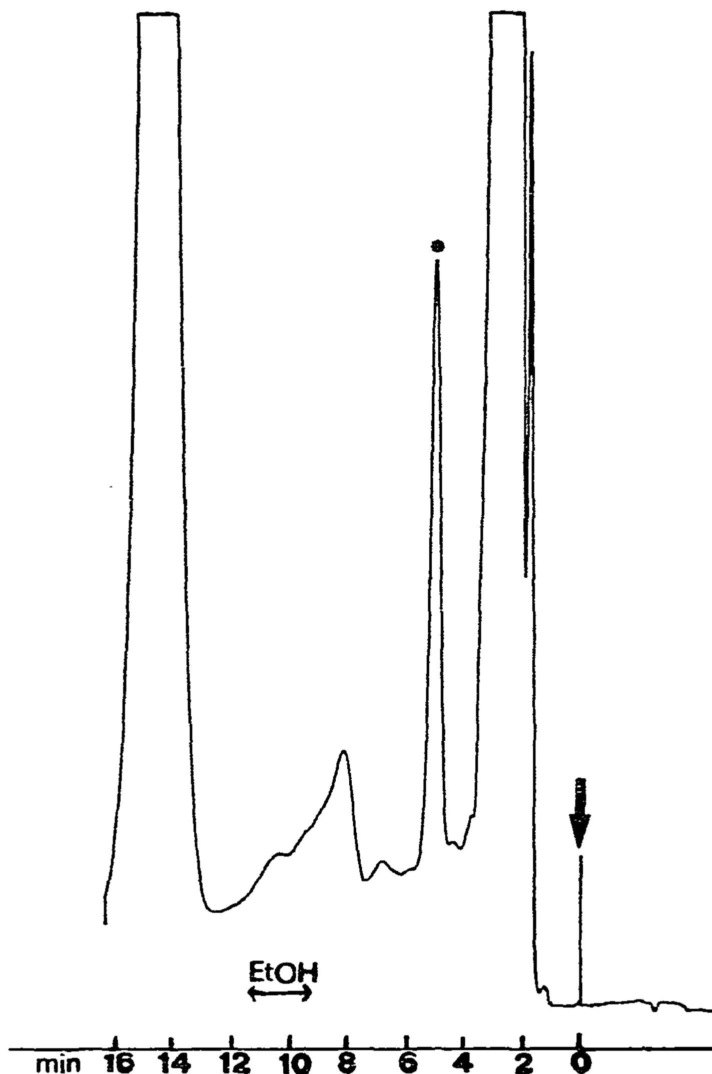

Fig. 9. High-performance liquid chromatogram of (1α,25-dihydroxyvitamin D_3 + pre-1α,25-dihydroxyvitamin D_3) (unresolved peaks *) in a pharmaceutical formulation on octadecyl bonded phase (Table I).

All chromatographic conditions are listed in Table I.

Hydroxyvitamins D_3 are separated from their corresponding prehydroxyvitamins D_3 on silica gel (Figs. 3–6 and 8) but they are not resolved with the solvent system ethanol –water used for reversed phase chromatography. This lack of resolution has also been observed for the pairs vitamins $D_{2/3}$/previtamins $D_{2/3}$. However De Vries *et al.*[14] have recently carried out this separation on octadecyl bonded phase with acetonitrile–propionitrile–water (79:15:6, v/v) as mobile phase.

If prehydroxyvitamin D_3 need not be determined separately or if it is not present (as shown by silica gel chromatography), then reversed-phase chromatography is more convenient than adsorption chromatography because of the shorter conditioning time and the greater stability.

With octadecyl bonded phase, the use of ethanol instead of methanol greatly improves the resolution. However, complete separation from the excipients was not achieved in the case of 1α-OH-D_3, which could be determined only on silica gel. When the oily solution is encapsulated, some of the constituents can dissolve in the oil and give rise to interferences in the HPLC chromatogram. With 25-OH-D_3 solution in propyleneglycol and with encapsulated 1α,25-$(OH)_2$-D_3 oily solution, partition chromatography was successfully applied (Figs. 7 and 9); with 1α,25-$(OH)_2$-D_3, ethanol was pumped through the column after each injection of the sample solution to elute impurities.

Silica gel chromatography was suitable for the determination of hydroxyvitamins D_3 and prehydroxyvitamins D_3 in the pharmaceutical formulations investigated (Figs. 4, 6 and 8); pre-25-OH-D_3 was eluted before 25-OH-D_3 (Fig. 4), in contrast to the other prehydroxyvitamins.

As previously described for reversed-phase chromatography, it was necessary to clean up the silica gel column with chloroform after each injection of a sample of 1α-OH-D_3 or 1α,25-$(OH)_2$-D_3.

In order to calculate the potential hydroxyvitamin D_3 content, the conversion factors of prehydroxyvitamins D_3 into their corresponding hydroxyvitamins D_3 (UV at 254 nm) were determinated. The calculations were carried out using the integrated peak areas (Table II) to obtain factors independent of the chromatographic conditions.

The relative concentrations of hydroxyvitamins D_3 and prehydroxyvitamins D_3 differed from the previously reported relative concentrations of vitamin D and previtamin D at given temperatures[15,16] (Table II).

The response of the HPLC UV detector was linear within the range 1–100 μg/ml (25–50 μl sample; sensitivity limit 0.5 μg/ml).

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REFERENCES

1 T. Sato, H. Yamauchi, Y. Ogata, M. Tsujii, T. Kunii, K. Kagel, Sh. Toyoshima and T. Kobayashi, *Chem. Pharm. Bull.*, 26 (1978) 2933.
2 K. Th. Koshy and A. L. Van der Slik, *J. Agr. Food Chem.*, 27 (1979) 180.
3 J. A. Eisman, R. M. Shepard and H. F. De Luca, *Anal. Biochem.*, 80 (1977) 298.
4 K. T. Koshy and A. L. Van der Slik, *Anal. Lett.*, 10 (1977) 523.
5 T. J. Gilbertson and R. P. Stryd, *Clin. Chem.*, 23 (1977) 1700.
6 G. Jones, in A. W. Norman, K. Schaefer, J. W. Coburn, H. F. De Luca, D. Fraser, H. G. Grigoleit and D. von Herrath (Editors) *Proceedings 3rd Workshop Vitamin D: Biochemical Chemical and Clinical Aspects Related to Calcium Metabolism*, W. de Gruyter, Berlin, 1977, p. 515.
7 G. Jones, *Clin. Chem.*, 24 (1978) 287.
8 B. Nilsson, L. Tejler and J.-Fr. Dymling, paper presented at *International Liquid Chromatography Symposium II. Biological/Biomedical Applications of Liquid Chromagraphy*, Boston (Mass. U.S.A.), October 5–6, 1978.
9 R. P. Stryd and T. J. Gilbertson, *Clin. Chem.*, 24 (1978) 927.

10 Gl. Jones and H. F. De Luca, *J. Lipid Res.*, 16 (1975) 448.
11 P. W. Lambert, B. J. Syverson, C. D. Arnaud and T. C. Spelsberg, *J. Steroid Biochem.*, 8 (1977) 929.
12 A. E. Caldas, R. W. Gray and J. Lemann, Jr., *J. Lab. Clin. Med.*, 91 (1978) 840.
13 G. Jones, A. Rosenthal, D. Segev, Y. Mazur, F. Frolow, Y. Halfon, D. Rabinovich and Z. Shakked, *Biochemistry*, 18 (1979) 1094.
14 E. J. de Vries, J. Zeeman, R. J. E. Esser, B. Borstje and Fr. J. Mulder, *J. Ass. Offic. Anal. Chem.*, 62 (1979) 129.
15 J. A. Keverling Buisman, K. H. Hanewald, F. J. Mulder, J. R. Roborg, and K. J. Keuning, *J. Pharm. Sci.*, 57 (1968) 1326.
16 R. Vanhaelen-Fastré and M. Vanhaelen, *J. Chromatogr.*, 153 (1978) 219.
17 G. J. Krol, C. A. Mannan, F. Q. Gemmill, Jr., G. E. Hicks and B. T. Kho, *J. Chromatogr.*, 74 (1972) 43.