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HIGH-EFFICIENCY LIQUID CHROMATOGRAPHIC SEPARATION OF VITAMIN D FROM PRECALCIFEROL

G. J. KROL, C. A. MANNAN, F. Q. GEMMILL, JR., G. E. HICKS AND B. T. KHO
Ayerst Laboratories Inc., Rouses Point, N.Y. 12979 (U.S.A.)
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SUMMARY

Vitamin D was separated from precalciferol within 1.2 min with a high-efficiency adsorptive liquid chromatographic support (Vydac) and a low viscosity solvent. The efficiency of the chromatographic system was studied as a function of solvent viscosity and flow-rate. In agreement with Van Deemter's and GIDDINGS' equations for the theoretical plate height, decreasing flow-rate and solvent viscosity enhanced the efficiency of the column. However, in the presence of low viscosity solvents, the observed relationship between plate height and flow-rate could be interpreted best by GIDDINGS' equation for eddy current and mass transfer "coupling". The chromatographic system was applied to (1) separation of vitamin A acetate, palmitate, aldehyde, alcohol, and vitamin E from vitamin D and (2) a study of cholecalciferol isomerization. The observed isomerization equilibrium constants are in agreement with previously published results.

INTRODUCTION

Quantitative analysis of vitamin D (calciferol)* was approached by thin-layer[1,2], liquid[3–5], and gas[6,7] chromatography. Calciferol was separated from precalciferol by thin-layer chromatography[2] (TLC) and by low-efficiency liquid chromatography (LC) on derivatized Sephadex LH-20 support, as described in this communication. However, the above procedures are either indirect and complex or relatively slow. Thus, there is still a need for a fast, simple and direct chromatographic separation of vitamin D from its isomers (*e.g.* precalciferols) and other lipophilic vitamins.

The advent of high-efficiency liquid chromatographic instrumentation and column supports offered a new approach to the separation of calciferol from precalciferol. A new adsorptive high-efficiency support (Vydac) was recently introduced at the Pittsburgh Analytical Conference[8]. We have studied the efficiency of Vydac support as a function of solvent viscosity and flow-rate. Using low viscosity solvents, baseline separation of calciferol from precalciferol was obtained even at exceptionally high flow-rates (5.7 cm/sec). The minimum time necessary for such separation was 1.2 min. Equivalent separation of calciferol from precalciferol on a derivatized Sephadex LH-20 column required more than 1 h.

* Vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol).

The chromatographic system described in this communication was applied to analysis of the calciferol–precalciferol equilibrium at several temperatures. The observed equilibrium constants are in agreement with the previously reported results[2] based on quantitative TLC. The Vydac column was also found suitable for separation of vitamin D from other lipophilic vitamins.

EXPERIMENTAL

Materials

Vitamin A acetate (all-*trans*-retinyl acetate), vitamin A alcohol (all-*trans*-retinol), vitamin A aldehyde (all-*trans*-retinal), vitamin D_2 (ergocalciferol), vitamin D_3 (cholecalciferol), and vitamin E (*d*-α-tocopherol) were obtained from Eastman Organic Chemicals, Rochester, N.Y. Freshly prepared stock 2,2,4-trimethylpentane (isooctane) solutions of above compounds were stored in the refrigerator and diluted with appropriate chromatographic solvent prior to use.

Spectrographic or reagent grade cyclohexane, heptane, hexane, isooctane, and pentane were checked for UV absorbance at 254 nm. If the UV absorbance of the solvent at 254 nm was greater than 0.05 absorbance units (measured in a 1-cm cell), the solvent was purified by distillation or fuming sulfuric acid–potassium permanganate–sodium carbonate treatment, followed by distillation of the organic phase.

Apparatus

High-efficiency column. Chromatographic support Vydac™ and a 1 m × 2 mm I.D. stainless-steel column with inlet and outlet fritted discs were obtained from Applied Science Laboratories, Inc., State College, Pa. The column was packed by the modified tap-fill procedure[9]. Prior to packing, the Vydac™ support was sieved through a 325-mesh screen.

Hydroxyalkoxypropyl–Sephadex column. Sephadex LH-20 (obtained from Pharmacia Fine Chemicals Inc., Piscataway, N.J.), was treated as described in the previous publications[10,11]. The hydroxyalkoxypropyl–Sephadex (HAPS) gel was equilibrated with the solvent and packed in the column as described previously[12]. Kontes glass chromaflex columns 25 cm × 1.2 cm (I.D.) which were obtained from Kontes, Vineland, N.J., were used with the HAPS support.

Pumps and detectors. A DuPont 820 liquid chromatograph with 254-nm UV detector (E. I. DuPont De Nemours and Co., Instrument Products Division, Wilmington, Del.) was used for the high-efficiency chromatography. However, the low flow-rate elutions which involved pressures below 100 p.s.i. were carried out with either a DuPont 820 or a Varian Aerograph™ constant pressure pump. A Honeywell Model 19 recorder, equipped with a disc integrator was used for quantitation of chromatographic peaks.

The elution of the HAPS column was monitored by a Beckman DB recording spectrophotometer–flow cell system (Beckman Instruments Inc., Fullerton, Calif.).

Chromatographic procedure

Samples were injected into the high-efficiency column with either a Hamilton or Precision Sampling Corp. 10-μl syringe. At pressures below 100 p.s.i., on-stream injections were suitable; above 100 p.s.i., stop-flow injections were practiced. With

the exception of chromatography with the pentane solvent system, the injected sample was dissolved in the chromatographic solvent. Due to the exceptionally low boiling point of pentane, hexane was used as sample diluent in the pentane system.

A 250-μg sample of vitamin D dissolved in 1 ml of the chromatographic solvent was applied to the HAPS column. The column was eluted with the aid of gravity or constant volume piston pump. The elution profile was measured at 265-nm wavelength with a Beckman DB spectrophotometer and 1-cm flow-cell (0.5 ml dead volume).

The vitamin D isomerization

The vitamin D samples were dissolved in isooctane at approximately 1 mg/ml concentration and stored in low-actinic glass vials, sealed tightly with a PTFE-lined cap. The samples were stored in constant temperature ($\pm$ 1) baths until isomerization reached equilibrium. The time necessary to reach equilibrium was obtained from previously published data[13]. After incubation, the isooctane solutions were diluted with chromatographic solvent (with exception of the pentane solvent where hexane was used as diluent) to approximately 50 μg per ml and 6 μl of solution were injected into the high-efficiency column.

Quantitation of the chromatographic peaks was based on disc integration. However, since the absorptivity of precalciferol at 254 nm is considerably lower than the absorptivity of calciferol, the area of the precalciferol peak was corrected for the difference. The correction factor was determined by two independent procedures. One procedure was based on determination of the area of a precalciferol peak formed by isomerization of a known amount of calciferol. Since the area of the calciferol peak was calibrated with a reference sample, the relative areas of the calciferol and precalciferol peaks at 254 nm can be determined from the decrease in the size of calciferol peak and corresponding increase in the size of precalciferol peak. No side reaction was observed. This ratio was found to be 2.45 $\pm$ 0.05.

An alternate procedure was based on semi-preparative chromatography of a known mixture of the two isomers on the HAPS column. The isolated chromatographic fractions were scanned spectrophotometrically and the absorbance ratio at 254 nm was determined from the concentration of the two isomers. The time necessary for this analysis was not sufficient to reverse appreciably the isomerization equilibrium.

RESULTS AND DISCUSSION

Figs. 1 and 2 illustrate separation of cholecalciferol from precholecalciferol on the Vydac column with low viscosity (pentane) solvent system at 0.15 ml/min and 5.2 ml/min flow-rates, respectively. Fig. 1 also depicts the separation of cholecalciferol from vitamin A palmitate, acetate, aldehyde, alcohol and vitamin E. However, even at the low flow-rate cholecalciferol could not be separated from ergocalciferol.

The high flow-rate separation still yielded essentially complete resolution (R_s = 1.1) of cholecalciferol (or ergocalciferol) from precalciferol within 1.2 min. An equivalent separation of cholecalciferol from precalciferol with cyclohexane (our most viscous solvent) required 8 min.

The dependence of column efficiency on flow-rate and solvent viscosity is

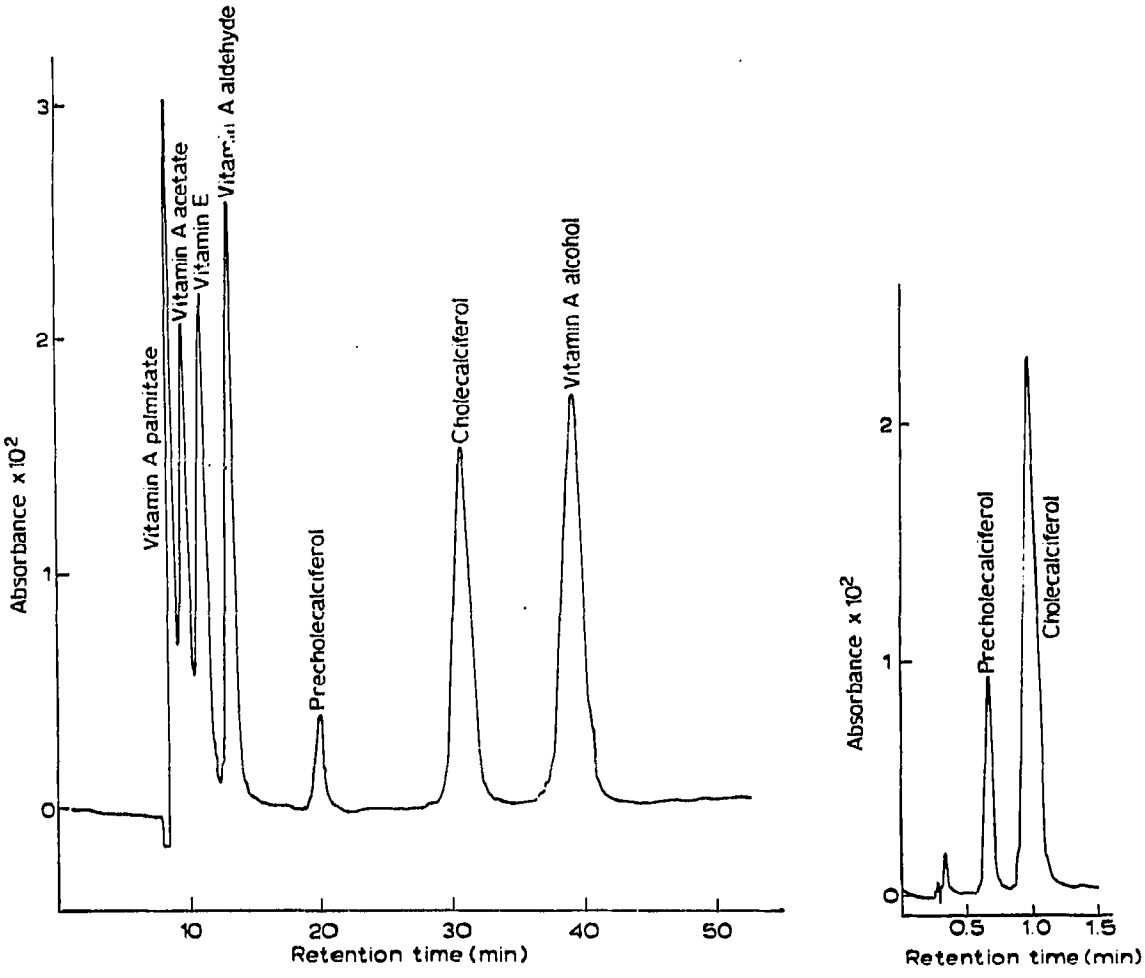

Fig. 1. Chromatogram of a mixture of vitamins separated on the Vydac (1 m × 2 mm I.D.) column with pentane–tetrahydrofuran (97.5:2.5) solvent at 0.15 ml/min flow-rate (0.15 ml/min = 0.19 cm/sec.; the cm/sec flow-rate was obtained from the unretained peak). Detector and chromatograph are described in the text.

Fig. 2. Chromatogram of cholecalciferol and precholecalciferol mixture separated on the Vydac (1 m × 2 mm I.D.) column with pentane–tetrahydrofuran (97.5:2.5) solvent at 5.2 ml/min flow-rate (5.2 ml/min = 5.7 cm/sec).

summarized graphically in Fig. 3. The viscosity of the solvents used in this study ranged from 0.2 to 1.0 cP (see Fig. 4). The observed relationship between the height of the theoretical plate (H) and flow-rate (v) indicates that (1) H increases with increasing viscosity of the solvent and (2) the relationship between H and v becomes more linear in the presence of higher viscosity solvents. A linear relationship between H and v has been previously observed and described by a modified form of the Van Deemter equation[14]. However, in this and other published studies[15] where the chromatographic system involved a low viscosity solvent and a microsphere particle support, the relationship between H and v was found to be non-linear. A non-linear relationship between H and v has been predicted and explained by GIDDINGS[16] in terms of eddy diffusion "coupling" with mass transfer. The fact that there is more evidence for the apparent "coupling" of eddy diffusion with mass transfer in the presence of lower viscosity solvents is also consistent with GIDDINGS' interpretation

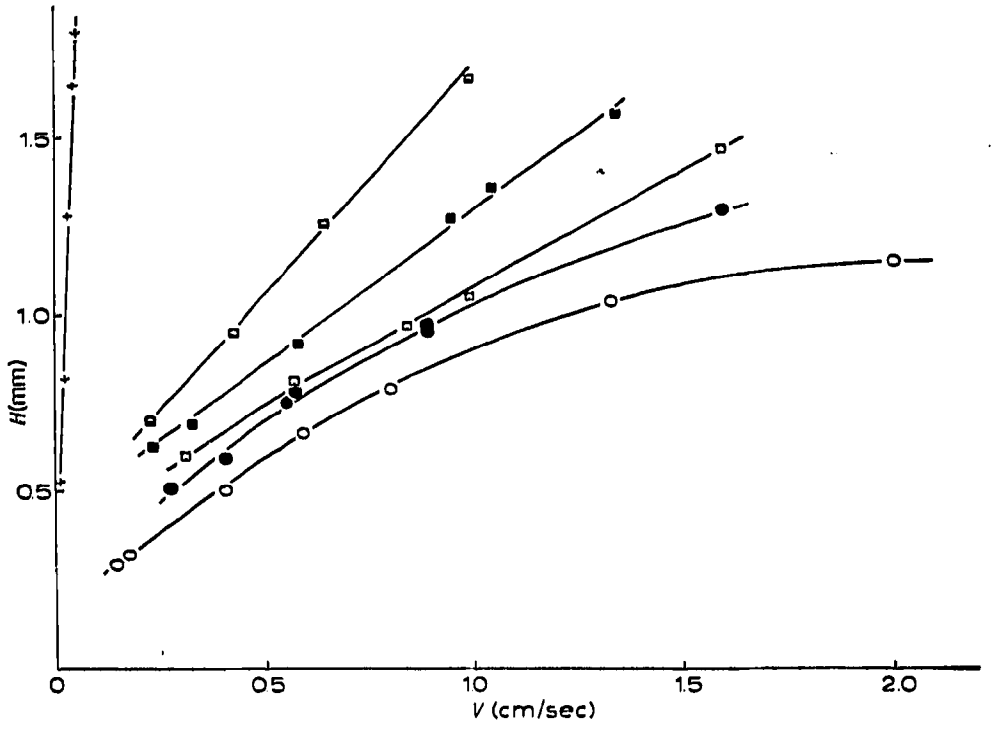

Fig. 3. H (height equivalent to a theoretical plate in mm) *vs.* v (solvent flow-rate in cm/sec) plots for the Vydac column chromatographed with hydrocarbon–tetrahydrofuran (97.5 : 2.5) solvents and HAPS column chromatographed with heptane–carbon tetrachloride (99 : 1) solvent. Vydac column solvents: ○—○, pentane–tetrahydrofuran; ●—●, hexane–tetrahydrofuran; □—□, heptane–tetrahydrofuran; ■—■ isooctane–tetrahydrofuran; ⬓—⬓, cyclohexane–tetrahydrofuran. HAPS column solvent: +—+, heptane–carbon tetrachloride.

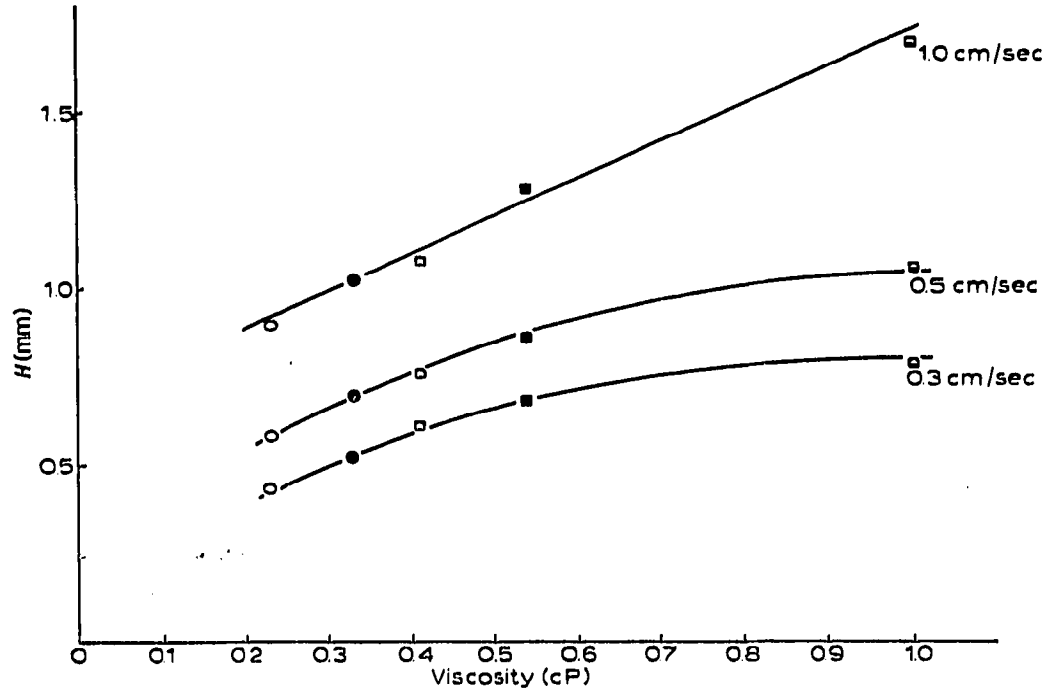

Fig. 4. H (height equivalent to a theoretical plate in mm) *vs.* viscosity (in centipoise units) plots, at specified flow-rates, for the Vydac column chromatographed with hydrocarbon–tetrahydrofuran (97.5 : 2.5) solvents. Vydac column solvents: ○—○, pentane–tetrahydrofuran; ●—●, hexane–tetrahydrofuran; □—□, heptane–tetrahydrofuran; ■—■, isooctane–tetrahydrofuran; ⬓—⬓, cyclohexane–tetrahydrofuran.

of zone spreading. In the presence of low viscosity solvent, lateral diffusion of solute is facilitated, which leads to "coupling" of mass transfer with eddy diffusion and subsequently a non-linear relationship between H and v. An alternative explanation may be that since the relative contribution of the mass transfer term to H tends to increase with increasing viscosity (decreasing diffusion coefficient), and H is linearly proportional to mass transfer[17], increasing viscosity of the solvent leads to a more linear relationship between H and v.

Fig. 4 presents the relationship between the viscosity of the solvent and H, observed at constant flow-rate. It is interesting to note that the relationship between viscosity and flow-rate becomes more linear with increasing flow-rate. This observation implies that at higher flow-rates, diffusion, being proportional to viscosity, is the rate determining step even for low viscosity solvents.

Due to the considerably larger particle size and the strong dependence of H on the flow-rate (see Fig. 3), the HAPS column is intrinsically less efficient than the Vydac column. However, the HAPS column also separates cholecalciferol from precholecalciferol (see Fig. 5) and is rather useful for preparative and clean-up purposes separation of vitamin D from large excess of vitamin A).

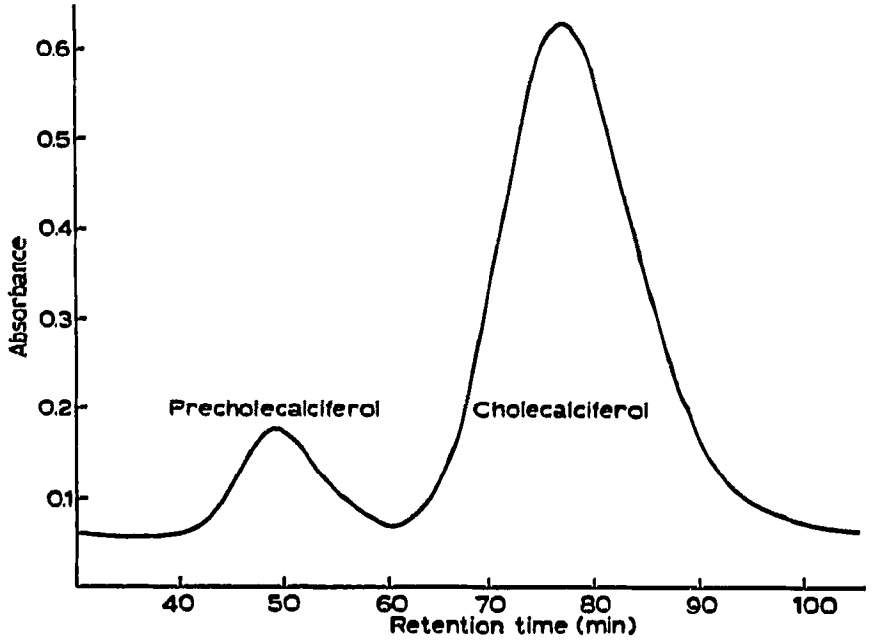

Fig. 5. Chromatogram of cholecalciferol and precholecalciferol mixture separated on the HAPS (20 cm × 1.2 cm I.D.) column with heptane–carbon tetrachloride (99:1) solvent. Column was monitored at 265 nm with Beckman DB spectrophotometer.

Table I compares the previously reported isomerization equilibrium ratios[2] to the ratios which were observed in this study. The previously reported results were obtained with a quantitative TLC procedure. However, in spite of the difference in methodology, the observed agreement between the two sets of ratios is essentially within experimental error. Possibility of oxidation of vitamin D during thin-layer analysis was circumvented by addition of antioxidants[2]. Antioxidants were found unnecessary in the liquid chromatography procedures reported here.

A quantitative chromatographic procedure based on HAPS and Vydac columns is now being applied to analysis of pharmaceutical vitamin formulations. A simple

TABLE I

VITAMIN D ISOMERIZATION EQUILIBRIUM RATIO AS A FUNCTION OF TEMPERATURE

Equilibrium ratio = calciferol/precalciferol concentration at equilibrium.

Method of analysis	*Equilibrium ratio*		
	60°	*100°*	*120°*
TLC[a]	5.2	2.6	1.8
LC	4.9 ± 0.2	2.5 ± 0.1	1.8 ± 0.1

[a] Values from ref. 2.

extraction which does not involve derivatization or saponification (thus avoiding isomerization of vitamin D) is being investigated. The high-efficiency chromatographic system involves relatively low pressures (100 p.s.i.) and the Vydac column has been used for almost two months with no apparent loss of efficiency. However, precautions were taken to avoid exposure of Vydac support to water and humid air during column packing and analysis.

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