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Preparative isolation of vitamin D_2 from previtamin D_2 by recycle high-performance liquid chromatography

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ABSTRACT

A preparative high-performance liquid chromatographic method, based on recycle chromatography, to separate vitamin D_2 (ergocalciferol) from previtamin D is described. The method provides efficient separation by means of a mixture of methanol, acetonitrile and hexane as eluent on a reversed-phase C_{18} column. Scale-up to a 2-in. diameter column resulted in the collection of 100% pure fractions based on UV detection at 265 nm. The total throughput and the economics of the purification were also optimized.

INTRODUCTION

The use of high-performance liquid chromatography (HPLC) to separate vitamin D_2 (ergocalciferol) from its precursors is well documented [1–8]. Reversed-phase chromatography of vitamin D_2 from tachysterol and vitamin D_3 (cholecalciferol) have been reviewed [5–8], but the resolution of the peaks was either partial or only one of the peaks could be isolated. A method for resolving these components in a crude vitamin D resin or crude extract is of value in the preparative isolation of one or more of these components.

Recycle chromatography allows a sample to be passed over a column several times without the need for re-injection of the sample or a long column. This results in the use of less solvent while still attaining high resolution and efficiency throughout the separation. In preparative chromatography this has an additional and very important advantage of maintaining a reasonable back-pressure as the column used need not be longer to allow for the added efficiency desired.

Recycle chromatography coupled to a more selective method of separating the vitamin D_2 and D_3 and tachysterol components would allow for high-purity fractions of these components to be collected for research or production use with minimum solvent use and better than average yield. The vitamin D_2 fraction was of particular interest in this work and a method for the recovery of 100% pure fractions of this vitamin from a crude vitamin D resin is described in this paper.

EXPERIMENTAL

Materials

Vitamin D resin was obtained from Vitamins, Inc. (Chicago, IL, USA). Vitamin D_2 and D_3 standards were obtained from Sigma (St. Louis, MO, USA). All of the solvents used for the separations were of HPLC grade from Burdick & Jackson (Muskegon, MI, USA).

Instrumentation

The preparative HPLC system used consisted of a VERSA Prep from VAREX (Burtonsville, MD, USA) complete with a variable-wavelength Linear Model 200 UV–VIS detector equipped with a 4.6-μl, 3-mm path-length flow cell. A 2.0-ml sample loop was fitted to the unit's Rheodyne Model 7125 injection valve. Also part of the VERSA Prep System was an IBM AT Type computer data acquisition, control and analysis system, a sixteen-port fraction collection system and recycle capability.

The analytical system used for fraction purity assessment was an HP 1050 Q HPLC System from Hewlett-Packard (Avondale, PA, USA), complete

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with their MS-DOS ChemStation Data acquisition, control and analysis system. An LDC/Milton Roy (Rochester, NY, USA) Spectromonitor D variable-wavelength detector was also used.

Chromatographic conditions

To avoid premature degradation of the pure vitamin standards, all samples and fractions were kept at less than 4°C, in the absence of oxygen and away from any light sources whenever possible. Fractions were analyzed as soon as collected to minimize errors in purity assessment.

All analytical separations were conducted using a 25 cm × 4.6 mm I.D. Zorbax PRO-10, C_{18} columns from DuPont (Wilmington, DE, USA). The flow-rate was set at 1.00 ml/min. A Rheodyne Model 7125 valve with a 20-μl loop was always overfilled 2.5 times for each injection. The UV detector was set at 265 nm except where noted otherwise.

The preparative separations were completed on the same type of material used in the analytical column above except that either a 25 cm × 1 or 2 in. I.D. column was used as needed. All of the preparative columns were packed in our laboratory. The flow-rate through the 1 in. I.D. column was preset at 25.0 ml/min and that through the 2 in. I.D. column at 90.0 ml/min. The 2.0-ml loop was always overfilled with sample 2.5 times prior to injection. UV detection at 300 nm, rather than 265 nm, was used to reduce peak saturation at the higher loads.

All chromatography was performed using a mobile phase consisting of methanol–acetonitrile–hexane (95:3:2, v/v/v). The solvents were premixed in advance and fully sparged with helium before use.

The vitamin D_2 standards were weighed and diluted into vials containing the mobile phase to cover a range of concentrations from 25 to 2500 ng/μl (or 200–20000 USP units, where $4 \cdot 10^6$ USP units = 1 g of vitamin D_2). All collected fractions and standards were diluted as needed and analyzed on the analytical system to determine their total peak area and purity. Linear regression performed on the calibration graph allowed quantification to be performed on the collected fractions.

RESULTS AND DISCUSSION

A modified reversed-phase method for separating a variety of fat-soluble vitamins was developed. The method provided for an almost baseline separation of vitamins D_2 and D_3. Because of the reproducibility and good resolution of the vitamin D_2 and D_3 peaks, this method was chosen for the preparative chromatography which follows.

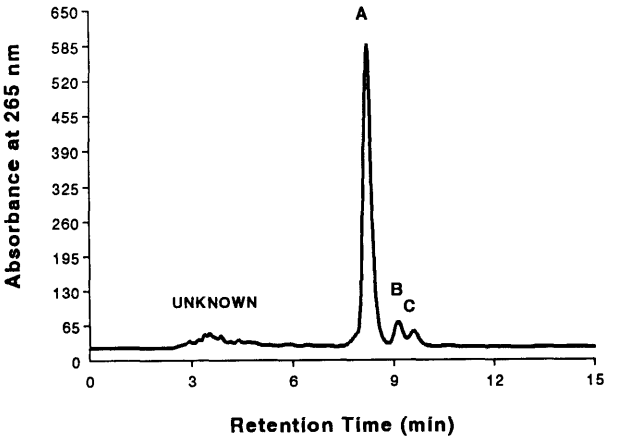

Fig. 1. Chromatogram of crude vitamin D resin at 265 nm obtained by using the system described. Components identified include (A) vitamin D_2 (ergocalciferol), (B) vitamin D_3 (cholecalciferol) and (C) tachysterol.

Seven pure vitamin D_2 solutions containing amounts between 25 and 2500 ng/μl were chromatographed on the analytical system to determine the equation of the calibration line. Table I gives the equation of the calculated line and the concentration *vs.* area responses obtained. Once determined, all subsequent fractions collected were analyzed in a similar fashion to determined their purity and total

TABLE I

RESULTS OF THE ANALYSIS OF VITAMIN D_2 STANDARDS

Linear regression (r^2 = 0.999); y = 0.0401x − 202.

Vitamin D_2 (ng/μl)	USP Units[a]	Peak-area units
25	200	416
50	400	755
100	800	1570
250	2000	3855
500	4000	7638
1250	10 000	18 984
2500	20 000	40 334

[a] $4 \cdot 10^6$ USP units = 1 g of vitamin D_2.

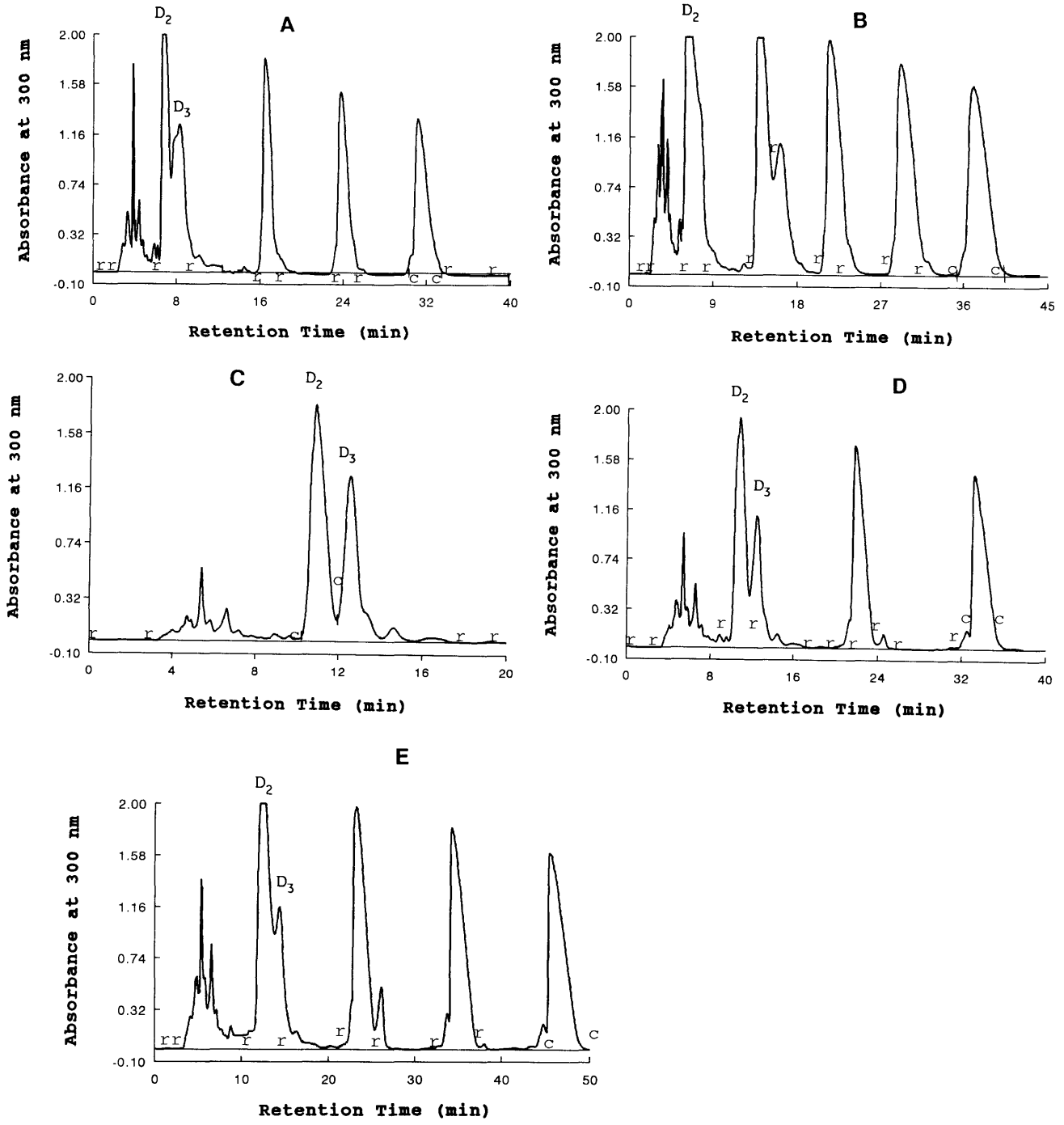

Fig. 2. Preparative chromatograms of vitamin D resin obtained on a Zorbax PRO-10 C_{18} column using methanol–acetonitrile–hexane as eluent. UV absorbance detection at 300 nm. (A) 1 in. I.D. column and 200-mg load; (B) same column as (A) but 400-mg load; (C) 2 in. I.D. column and 500-mg load; (D) same column as (C) but 1-g load; (E) same column as (C) but 2-g load. Letters r denote points at which recycle mode was started; c denotes point of collection.

area. All analytical determinations were performed at 265 nm.

A sample of the crude resin was analyzed using the same technique and found to contain 70% pure vitamin D_2 by percentage peak-area calculations. The chromatogram of the crude resin is shown in Fig. 1.

Crude resin dissolved in 2.00 ml of mobile phase containing either 200 or 400 mg of resin was injected onto the 25 cm × 1 in. I.D. column at a flow-rate of 25.0 ml/min. The UV–VIS detector was set at 300 nm. The resulting chromatograms are shown in Fig. 2A and B. These chromatograms show the crude resin being recycled and purified as the sample passes over the same column.

The use of recycle chromatography allowed the non-vitamin D_2-containing resin to exit to waste while the peak of interest was allowed to cycle back onto the column for further separation. The peak of interest was then collected when the chromatogram showed sufficient resolution for the peak to be collected at 100% purity.

The above procedure was repeated with larger sample loads on the 25 cm × 2 in. I.D. column. The vitamin D_2 was selectively recycled until a 100% pure peak could be fractioned out of the resulting material. The 2 in. I.D. column was run at a higher flow-rate of 90.0 ml/min and at a detection wavelength of 300 nm to reduce peak saturation. The resulting chromatograms are shown in Fig. 2C–E.

The fractions from all of the preparative runs were collected, measured for total volume and analyzed after appropriate dilutions on the analytical HPCL system. The total recovery of vitamin D_2 was between 60 and 100% on the two preparative columns. An example chromatogram of the analysis of the resulting pure preparative vitamin D_2 fraction collected is shown in Fig. 3 and confirms a 100% pure vitamin D_2 fraction.

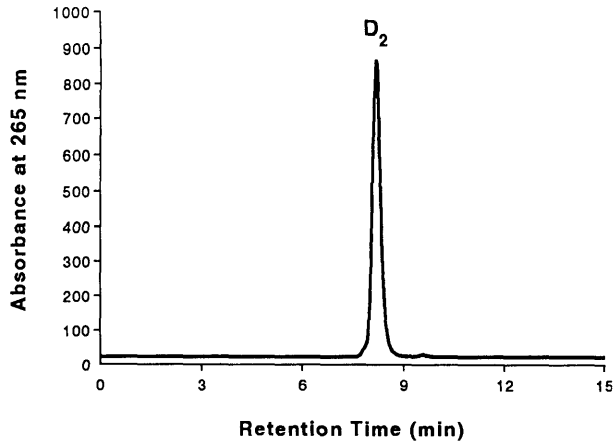

Fig. 3. Analytical chromatogram of vitamin D_2 on a Zorbax PRO-10 C_{18} column using methanol–acetonitrile–hexane as eluent. UV absorbance detection at 265 nm.

The recovery and throughput using recycle chromatography are listed in Table II and show peak throughputs of 6.6 mg/min of vitamin D_2 on the 1 in. I.D. column and 16.4 mg/min using the 2 in. I.D. column. These rates reflect the savings in solvent achieved by using the recycle technique.

CONCLUSIONS

The method described demonstrates the use and advantages of recycle chromatography in a preparative mode. The savings in solvent and in the other-

TABLE II

PREPARATIVE THROUGHPUT AND YIELD OF VITAMIN D_2 BY RECYCLE CHROMATOGRAPHY

Column I.D. (in.)	Raw resin injected (mg)	Vitamin D_2 collected (total) (mg)	Potential vitamin D_2 possible (mg)	Recovery (%)	Throughput (mg/min)
1	200	119	140	85	6.6
1	400	251	280	90	6.6
2	500	(356)	350	> 100	8.9
2	1000	515	700	74	11.7
2	2000	834	1400	60	16.4

wise use of a longer, higher back-pressure column are obvious. The gain in efficiency from recycling the peak is equivalent to using a much longer column. Recycling the peak and not the other resin components allowed an easy recovery of the vitamin D_2 by simply collecting it once it was very pure. All of this was done on a column which normally would not have the efficiency alone to separate the peak on the first pass without a loss of either material or purity.

This method proved useful in the separation and purification of vitamin D_2 from its pre-vitamin components. This same technique could be used to separate other fat-soluble vitamins in a similar manner.

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