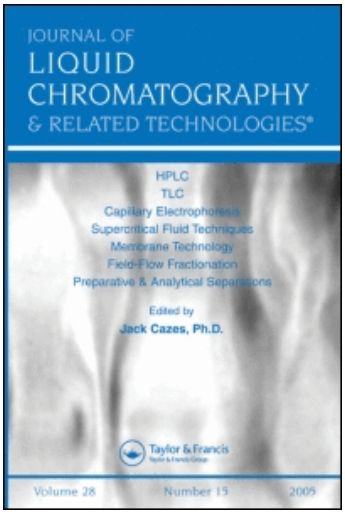

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COMPARISON OF HPLC SEPARATION OF VITAMIN D_3 METABOLITES AND THEIR ISOTACHYSTEROL$_3$ DERIVATIVES

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ABSTRACT

Separation of vitamin D_3, its four metabolites and their corresponding isotachysterol$_3$ derivatives was studied using four HPLC systems: two reverse phase columns, a silica column and a cyanopropyl silica column. Most of them gave a good separation between the compounds studied, although both reverse phase systems were less efficient in the separation of dihydroxylated vitamin D_3 metabolites and isotachysterol compounds. Isotachysterol$_3$ derivatives behaved analogously to their vitamin D_3 counterparts on all four systems, but their retention times were different. This indicates that chemical derivatization to isotachysterols can be used as a part of chromatographic identification of unknown vitamin D_3 metabolites as well as in the determination of major vitamin D_3 compounds in biological samples.

INTRODUCTION

Vitamin D_3 is converted <u>in vivo</u> or <u>in vitro</u> to over 20 polar metabolites with hydroxyl or keto groups in their side chain or

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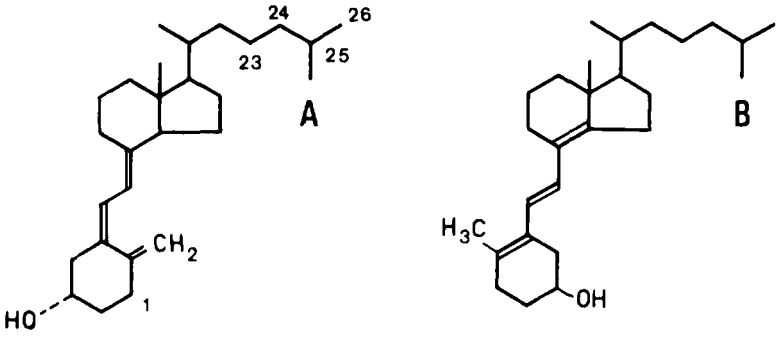

Figure 1. Structures of vitamin D_3 (A) and isotachysterol$_3$ (B) and the numbering of the metabolically active carbons of vitamin D_3.

carbon-1 (see Figure 1)(1). Due to the small concentrations of vitamin D_3 compounds in biological samples as well as to the presence of several metabolites in a single sample, one or more HPLC steps are required for their purification, identification or determination (2,3). Some metabolites can comigrate through several HPLC systems (3), which indicates that unknown vitamin D_3 metabolites cannot always be identified on the basis of cochromatography with authentic standards. When sufficient amounts of purified metabolites are available, they can be subjected to mass spectrometry for structure determination. This method, however, is not available to all investigators wishing to study the metabolism of vitamin D_3, and it is not suitable when radioactive compounds are to identified.

Vitamin D_3 and its metabolites with modifications in the side chain of the molecule can be chemically isomerised to corresponding isotachysterol$_3$ derivatives (Figure 1). In this reaction, the double bond system of the secosteroid nucleus is rearranged and the A ring of the molecule rotated to expose the hydroxyl

group of carbon-1 differently. Such changes alter both the spectral properties and the polarity of the molecule (4). The present work examined the effects of isotachysterol$_3$ derivatization on the HPLC behaviour of five vitamin D_3 compounds.

MATERIALS AND METHODS (10)

Vitamin D_3 was purchased from Serva Feinbiochemica. 25(OH)D_3 was a gift from The Upjohn Co. 24,25(OH)$_2D_3$, 25,26(OH)$_2D_3$ and 25(OH)D_3-lactone were donated by F. Hoffmann - La Roche & Co. 25(OH)D_3-lactone was purified by HPLC before use. Standards were initially dissolved in ethanol and the concentrations of the solutions were determined spectrophotometrically. 30 - 60 ng of each compound was used for injections.

Organic solvents (HPLC grade) were from Merck, Rathburn and Orion; they were used as received. HPLC grade water was obtained from a Millipore Milli-Q system.

The HPLC system consisted of a Perkin-Elmer Series I pump, a LC-75 variable wavelength detector, and a Rheodyne Model 7125 injector. The chromatograms were recorded using a Shimedzu C-R1B integrator. Detection wavelengths were 265 nm for vitamin D_3 compounds and 301 nm for their isotachysterol$_3$ derivatives.

The columns and mobile phases were as follows:

A. Vydac C18 (The Separations Group), 5 μm, 25 cm x 4.5 mm I.D.; acetonitrile-methanol (1:1), 1 ml/min

B. Waters Z-module with a Radial-Pak C18 cartridge, 10 μm, 10 cm x 8 mm I.D.; methanol-water (9:1), 2 ml/min

C. Zorbax-Sil Golden Series (Du Pont), 3 μm, 8 cm x 6.2 mm I.D.; hexane isopropanol (23:2), 1.5 ml/min

D. Zorbax-CN (Du Pont), 6 μm, 25 cm x 4.6 mm I.D.; hexane-isopropanol-methanol (96:8:1), 1.5 ml/min

Vitamin D_3 and its metabolites were isomerised to isotachysterols by treatment with hydrochloric acid gas in chloroform (5). No carrier was added when all metabolites were isomerised simultaneously.

RESULTS AND DISCUSSION

HPLC chromatograms of the vitamin D_3 compounds from the four systems are shown in Figures 2A-5A, and those of their ITS_3 derivatives in Figures 2B-5B. The retention times of all compounds are given in Table I.

Very good recoveries for all isotachysterols were obtained from the HCl-catalysed isomerization reaction, and no traces of unisomerized vitamin D_3 compounds could be observed when the eluant was monitored at 301 nm. ITS_3 derivatives can be formed by several methods, but the HCl procedure is the most efficient of them (5).

The separation of vitamin D_3 and $25(OH)D_3$ from other vitamin D_3 metabolites usually presents no difficulties (2,3,6), and it was easily performed in the present study; similar results were also observed here for ITS_3 and $25(OH)ITS_3$ (Table I). Hydroxyl groups increase significantly the polarity of vitamin D_3 metabolites, but there seems to be only a small difference in polarity, when two hydroxyls are present in adjacent carbons, as in the case of $24,25(OH)_2D_3$ and $25,26(OH)_2D_3$. Therefore, these two metabolites could not be well separated by either reverse phase system (Figures 2 and 3), and $24,25(OH)_2ITS_3$ and $25,26(OH)_2ITS_3$ only on reverse phase system B. Better separations were observed on straight phase systems.

$25(OH)D_3$-lactone and $24,25(OH)_2D_3$ tend to comigrate on silica columns with hexane-isopropanol as the mobile phase, but they can be separated using dichloromethane-based mobile phases

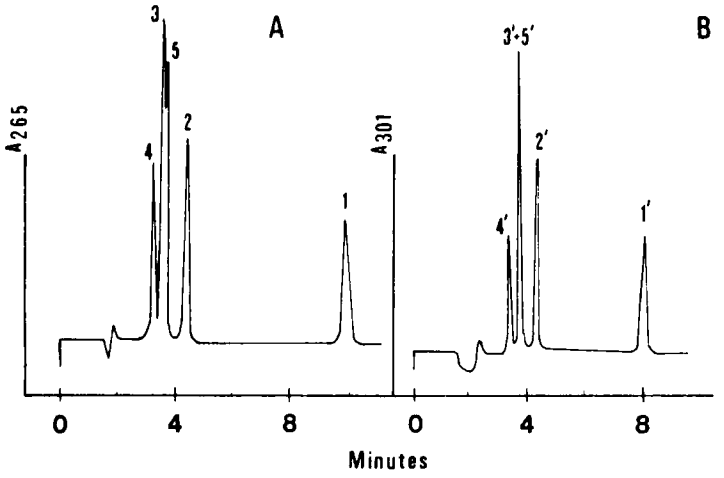

Figure 2. Chromatogram of vitamin D_3 compounds (A) and their isotachysterol$_3$ derivatives (B) on HPLC system A (Vydac C18, acetonitrile-methanol (1:1)). 1) vitamin D_3, 2) 25(OH)D_3, 3) 24,25(OH)$_2D_3$, 4) 25(OH)D_3-lactone, 5) 25,26(OH)$_2D_3$. 1') ITS$_3$, 2') 25(OH)ITS$_3$, 3') 24,25-(OH)$_2$ITS$_3$, 4') 25(OH)ITS$_3$-lactone, 5') 25,26(OH)$_2$ITS$_3$.

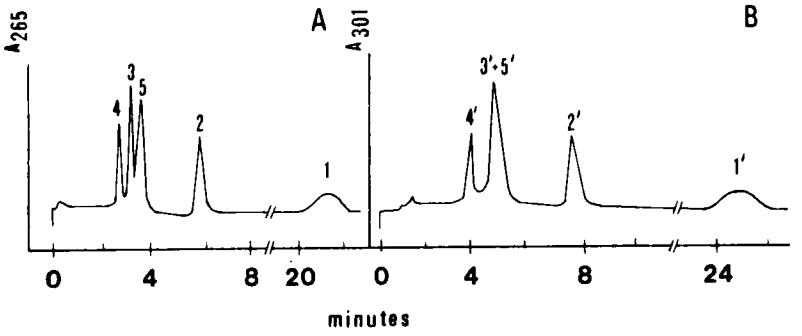

Figure 3. Chromatogram of vitamin D_3 compounds (A) and their isotachysterol$_3$ derivatives on HPLC system B (Z-module with Radial-Pak, methanol-water (9:1)). Peak identification as in Figure 2.

or by reverse phase HPLC (3). In our hands, however, the 3-μm particle-packed Zorbax-Sil column of system C gave an efficient separation of these two metabolites, this being further enhanced after isomerization to 25(OH)ITS_3-lactone and 24,25$(OH)_2ITS_3$ (Figure 4). On system D, the cyanopropyl silica column had a strong affinity for both 25(OH)D_3-lactone and 25(OH)ITS_3-lactone, which eluted later than the more polar 25,26$(OH)_2D_3$ and 25,26-$(OH)_2D_3$ and 25,26(OH)ITS_3 (Figure 5). The separation of 24,25-$(OH)_2D_3$ from 25(OH)D_3-lactone using a cyanobonded phase packing has also recently been described (7). Isomerization had on system D only a slight effect on the retention of other metabolites than lactone. The elution order of the vitamin D_3 compounds did not change after isomerization on any of the four HPLC systems (Table I).

The present work is the first one to desribe the isomerization and HPLC behaviour of 25,26(OH)ITS_3 and 25(OH)ITS_3-lactone. These derivatives migrated on an analogous manner to their vitamin D_3 counterparts on all four systems. In earlier HPLC studies of ITS_3, 25(OH)ITS_3 and 24,25$(OH)_2ITS_3$, only straight phase HPLC was used (4,8). 25(OH)ITS_3 and 24,25$(OH)_2ITS_3$ had longer retention times than 25(OH)D_3 and 24,25$(OH)_2D_3$, respectively, while that of ITS_3 was similar or shorter than that of vitamin D_3. In this study, all isotachysterol$_3$ derivatives except ITS_3 on system A also had longer retention times on reverse phase systems, which suggests that the rearrangement of the double bonds in the isomerization reaction counteracts the better exposure of the hydroxyl group of carbon-3 and thus increases the affinity of ITS_3 derivatives to the organic phase of C18 column packings.

No other compounds than vitamin D_3 (and its synthetic analog, vitamin D_2) and their side-chain modified metabolites are known to undergo a similar isomerization reaction upon treatment with HCl gas to yield isomers with altered chromatographic and spectral properties. The specificity of this reaction has been

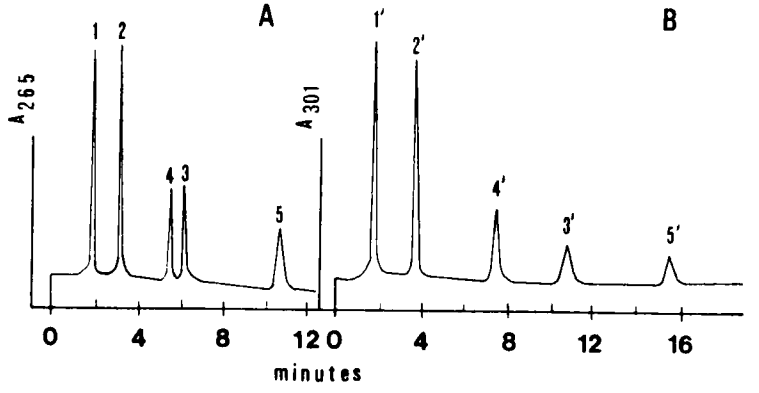

Figure 4. Chromatogram of vitamin D_3 compounds (A) and their isotachysterol derivatives (B) on HPLC system C (Zorbax-Sil, hexane-isopropanol (23:2)). Peak identification as in Figure 2.

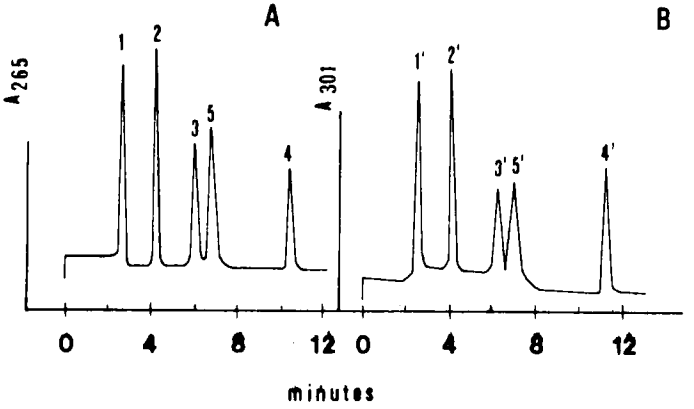

Figure 5. Chromatogram of vitamin D_3 compounds (A) and their isotachysterol$_3$ derivatives (B) on HPLC system D (Zorbax-CN, hexane-isopropanol-methanol (96:8:1)). Peak identification as in Figure 2.

Table I. Retention times (min.) of the vitamin D_3 compounds and their isotachysterol$_3$ derivatives.

	HPLC system			
	A	B	C	D
vitamin D_3	9.75	21.50	1.92	2.70
ITS_3	8.08	25.25	1.75	2.67
$25(OH)D_3$	4.42	5.96	3.25	4.28
$25(OH)ITS_3$	4.42	5.67	3.72	4.28
$24,25(OH)_2D_3$	3.50	3.58	6.25	6.00
$24,25(OH)_2ITS_3$	3.83	5.00	10.67	6.25
$25(OH)D_3$-lactone	3.15	3.20	5.55	10.50
$25(OH)ITS_3$-lactone	3.50	4.12	7.33	11.33
$25,26(OH)_2D_3$	3.62	3.92	10.67	6.83
$25,26(OH)_2ITS_3$	3.83	5.17	15.50	7.08

utilised in an assay of serum $25(OH)D_3$ (and $25(OH)D_2$): conversion to $25(OH)ITS_3$ enhanced the sensitivity of the method and provided a better separation from interfering sample constituents (8). Our results suggest that also vitamin D_3 and $24,25(OH)_2D_3$, which may be present in serum in concentrations large enough to be measured by HPLC, could be specifically determined as ITS_3 and $24,25(OH)ITS_3$ by both reverse and straight phase HPLC (systems A and C).

It has been recommended that chemical derivatization to forms with different chromatographic properties should be a part of the identification of unknown steroids by chromatographic means (9). According to our data, this principle can be well applied to vitamin D_3 metabolites, as their ITS_3 derivatives all had different mobilities on at least three out of four HPLC sys-

tems. In our laboratory, the procedures described here are currently being used in the identification of vitamin D_3 metabolites from in vitro incubations.

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10. Abbreviations: 25(OH)D_3, 25-hydroxyvitamin D_3; 24,25(OH)$_2D_3$, 24,25-dihydroxyvitamin D_3; 25,26(OH)$_2D_3$, 25,26-dihydroxyvitamin D_3; 25(OH)D_3-lactone, 25-hydroxyvitamin D_3-26,23-lactone; ITS_3, isotachysterol$_3$. 25(OH)ITS_3, 24,25(OH)$_2ITS_3$, 25,26(OH)$_2$-ITS_3, 25(OH)ITS_3-lactone, isotachysterol derivatives of the above vitamin D_3 metabolites in respective order.