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SEPARATION OF VITAMIN D_3 METABOLITES AND THEIR ANALOGUES BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY*

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SUMMARY

The separation of vitamin D_3 metabolites and their analogues by high-pressure liquid chromatography was investigated using a Zorbax SIL column. Each metabolite can be separated effectively with a solvent consisting of 2% methanol–methylene chloride or gradient elution from 0.02% to 6% methanol–methylene chloride. The C-24 epimers of 1α,24-dihydroxy- and 1α,24,25-trihydroxy-vitamin D_3 are resolved as their free forms, while the epimers of 24-hydroxy- and 24,25-dihydroxy-vitamin D_3 can be separated as their trimethylsilyl derivatives.

INTRODUCTION

During the past decade, it has been well established, mainly by DeLuca and co-workers, that vitamin D_3 (D_3) must first be metabolized in the liver to 25-hydroxy-vitamin D_3[25-(OH)-D_3] and subsequently in the kidney to 1α,25-dihydroxy-vitamin D_3 [1α,25-$(OH)_2$-D_3] in order to carry out its well known functions involving the stimulation of intestinal calcium transport, bone calcium mobilization and calcification of bone. Other D_3 metabolites, 24,25- and 25,26-dihydroxy-vitamin D_3 [24,25- and 25,26-$(OH)_2$-D_3] and 1α,24,25-trihydroxy-vitamin D_3 [1α,24,25-$(OH)_3$-D_3], have also been isolated and their structures and biological activities determined by DeLuca[1]. However, the biological significance and the stereochemistry of the 24-hydroxyl group of these compounds have not been clarified. During the course of our studies of the synthesis of the D_3 metabolites and on the determination of the configuration of the 24-hydroxyl group, it became necessary to develop a separation method that has high resolution and sensitive detection system. A study was therefore carried out of the separation of D_3 metabolites and their analogues by high-pressure liquid chromatography (HPLC). The compounds studied are shown in the structure below.

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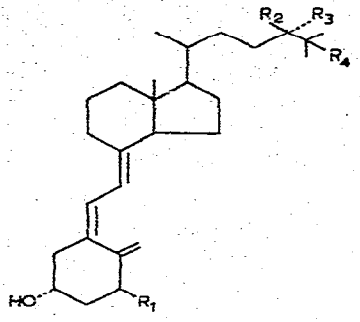

	R_1	R_2	R_3	R_4
D_3	H	H	H	H
1α-(OH)-D_3	OH	H	H	H
24*R*-(OH)-D_3	H	OH	H	H
24*S*-(OH)-D_3	H	H	OH	H
25-(OH)-D_3	H	H	H	OH
1α,24*R*-$(OH)_2$-D_3	OH	OH	H	H
1α,24*S*-$(OH)_2$-D_3	OH	H	OH	H
1α,25-$(OH)_2$-D_3	OH	H	H	OH
24*R*,25-$(OH)_2$-D_3	H	OH	H	OH
24*S*,25-$(OH)_2$-D_3	H	H	OH	OH
1α,24*R*,25-$(OH)_3$-D_3	OH	OH	H	OH
1α,24*S*,25-$(OH)_3$-D_3	OH	H	OH	OH

TABLE I

RETENTION TIMES OF VITAMIN D_3 METABOLITES AND THEIR ANALOGUES IN HIGH PRESSURE LIQUID CHROMATOGRAPHY ON ZORBAX SIL

Compound	*Retention time (min)*	*Solvent*	*Pressure* (kg/cm²)*
D_3	3.7	Gradient elution:	93
24*R*-(OH)-D_3	10.1	0.02% MeOH–	
24*S*-(OH)-D_3	10.1	CH_2Cl_2, gradient rate	
25-(OH)-D_3	10.7	0.3%/min, to 6% MeOH–	
1α-(OH)-D_3	12.2	CH_2Cl_2	
24*R*,25-$(OH)_2$-D_3	13.4		
24*S*,25-$(OH)_2$-D_3	13.4		
1α,24*R*-$(OH)_2$-D_3	15.4		
1α,24*S*-$(OH)_2$-D_3	15.6		
1α,25-(OH)-D_3	16.9		
1α,24*R*,25-$(OH)_3$-D_3	21.0		
1α,24*S*,25-$(OH)_3$-D_3	21.2		
D_3	2.3	2% MeOH–CH_2Cl_2	90
24*R*-(OH)-D_3	3.0		
24*S*-(OH)-D_3	3.0		
25-(OH)-D_3	3.8		
1α-(OH)-D_3	6.2		
24*R*,25-$(OH)_2$-D_3	7.7		
24*S*,25-$(OH)_2$-D_3	7.7		
1α,24*R*-$(OH)_2$-D_3	13.1		
1α,24*S*-$(OH)_2$-D_3	13.9		
1α,25-$(OH)_2$-D_3	19.2		
24*R*-(OH)-D_3 di-TMS	13.8	2% CH_2Cl_2–*n*-hexane	93
24*S*-(OH)-D_3 di-TMS	12.1		
24*R*,25-$(OH)_2$-D_3 tri-TMS	21.2	2% CH_2Cl_2–*n*-hexane	83
24*S*,25-$(OH)_2$-D_3 tri-TMS	18.4		
1α,24*R*-$(OH)_2$-D_3	16.9	1.5% MeOH–*n*-hexane	92
1α,24*S*-$(OH)_2$-D_3	18.0		
1α,24*R*,25-$(OH)_3$-D_3	12.5	3.5% MeOH–CH_2Cl_2	90
1α,24*S*,25-$(OH)_3$-D_3	13.2		

* Flow-rate 0.4–0.42 ml/min.

EXPERIMENTAL

D_3 derivatives

All of the samples of D_3 metabolites and their analogues were synthetic compounds: 25-(OH)- (ref. 2), 1α-(OH)- (refs. 3 and 4), 24*R*-(OH)- (ref. 5), 24*S*-(OH)- (ref. 5), 1α,25-$(OH)_2$- (refs. 6 and 7), 1α,24*R*-$(OH)_2$- (ref. 8), 1α,24*S*-$(OH)_2$- (ref. 8), 24*R*,25-$(OH)_2$- (refs. 9 and 10), 24*S*,25-$(OH)_2$- (refs. 9 and 10), 1α,24*R*,25-$(OH)_3$- (ref. 11), and 1α,24*S*,25-$(OH)_3$-D_3 (ref. 11).

Trimethylsilyl (TMS) derivatives

In a small PTFE-capped tube, 10 μg of hydroxy-D_3 were dissolved in 50 μl of dry *n*-hexane plus 10 μl of trimethylsilylimidazole. After heating at 70° for 10 min, or at 50° for 20 min, 0.3 ml of water was added, the mixture was extracted with *n*-hexane and the extract was injected into the chromatograph. The TMS group was removed by treating the TMS ether (10 μg) with 0.1 ml of 1% potassium hydroxide in methanol at room temperature for 2 h. The free D_3 was extracted with ethyl acetate.

Instrument

A Shimadzu–DuPont Model 830 liquid chromatograph equipped with a UV detector (254 nm) and a gradient system was used, with a Zorbax SIL column, 25 cm × 2.1 mm I.D. The separation conditions are given in Table I.

RESULTS AND DISCUSSION

Separation of D_3 metabolites and their analogues

In comparison with silicic acid or Sephadex column chromatography, which have been employed for the separation of the metabolites[12], a higher resolution was obtained by HPLC. After testing several types of column, we found that Zorbax SIL exhibited the best resolution for this purpose.

D_3, 25-(OH)-D_3, 24,25-$(OH)_2$-D_3 and 1α,25-$(OH)_2$-D_3 can easily be separated from each other with a solvent consisting of 2% methanol–methylene chloride (Fig. 1). By gradient elution with a mobile phase of 0.02–6% methanol in methylene chloride, all of the metabolites so far isolated and their analogues, 1α-(OH)-, 24-(OH)- and 1α,24-$(OH)_2$-D_3, can be separated, as shown in Fig. 2.

These results illustrate the usefulness of HPLC for the metabolic studies of D_3 and its hydroxy analogues, which has also been reported by Jones and DeLuca[13].

Separation of the epimers of the 24-hydroxyl group

In previous papers[9,10], the synthesis of 24*R*- and 24*S*-24,25-$(OH)_2$-D_3 was reported. For the identification of the metabolite 24,25-$(OH)_2$-D_3 isolated by Holick *et al.*[14], it was necessary to find a method for separating the epimers. As shown in Figs. 1 and 2 and Table I, the 24*R* and 24*S* isomers of 24-(OH)- and 24,25-$(OH)_2$-D_3 exhibited identical retention times but of 1α,24-$(OH)_2$- and 1α,24,25-$(OH)_3$-D_3 had slightly different retention times. It was found that the TMS derivatives of the 24-epimers of 24,25-$(OH)_2$-D_3 could be separated using the solvent methylene chloride–*n*-hexane (Fig. 3). The TMS derivative was also resolved by TLC using silica gel. This

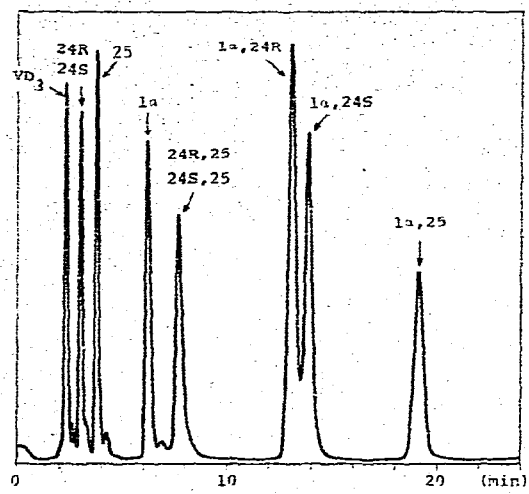

Fig. 1. Separation of vitamin D_3 (VD_3) metabolites and their analogues on Zorbax SIL with 2% methanol–methylene chloride.

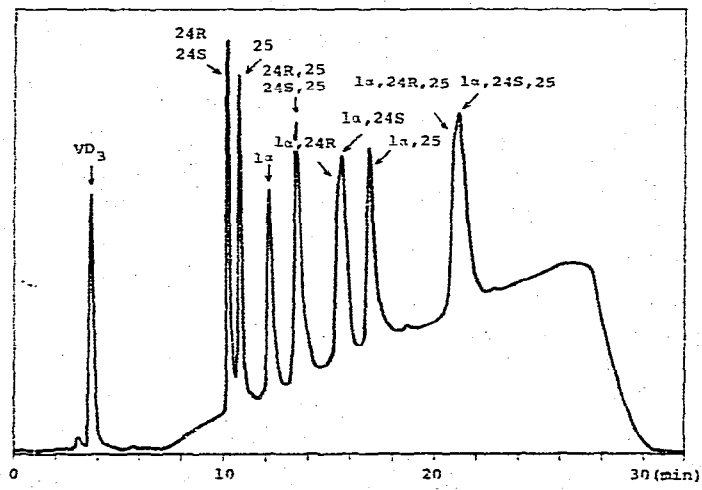

Fig. 2. Separation of vitamin D_3 (VD_3) metabolites and their analogues on Zorbax SIL with gradient elution from 0.02 to 6% methanol in methylene chloride.

derivative was selected because of its easy derivatization and removal. By this method, the configuration of the 24-hydroxyl group of natural 24,25-$(OH)_2$-D_3 was determined to be *R*, using co-chromatography with the ^{3}H-labelled compound obtained biologically[15].

The epimers of 24-(OH)-D_3 can be also separated as their TMS derivatives (Table I). This method was applied to the separation of *R* and *S* epimers of [24-^{3}H]-24-(OH)-D_3 derived from 24-oxo-D_3 by sodium [^{3}H] borohydride reduction[16].

On the contrary, TMS derivatives of 24-epimers of 1α,24-$(OH)_2$-D_3 and 1α,24,25-$(OH)_3$-D_3 had the same retention time, but those epimers could be separated as their free forms as shown in Fig. 4 and Table I. The 24*R* isomer was eluted faster than the 24*S* isomer, while with the TMS derivatives of 24-(OH)- and 24,25-$(OH)_2$-D_3

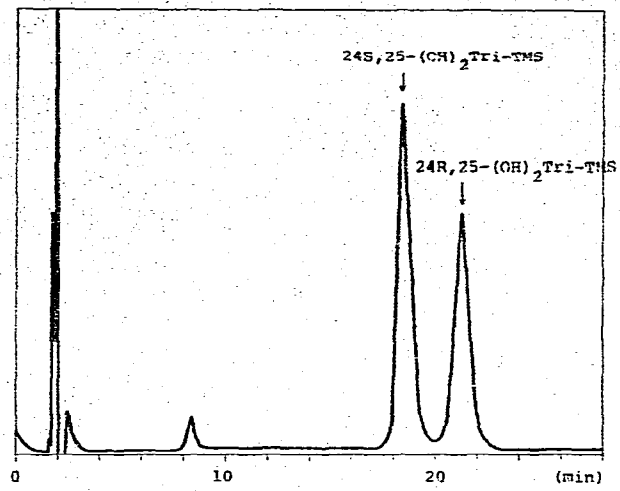

Fig. 3. Separation of 24*R*,25-$(OH)_2$-D_3 tri-TMS and 24*S*,25-$(OH)_2$-D_3 tri-TMS on Zorbax SIL with 2% methylene chloride–*n*-hexane.

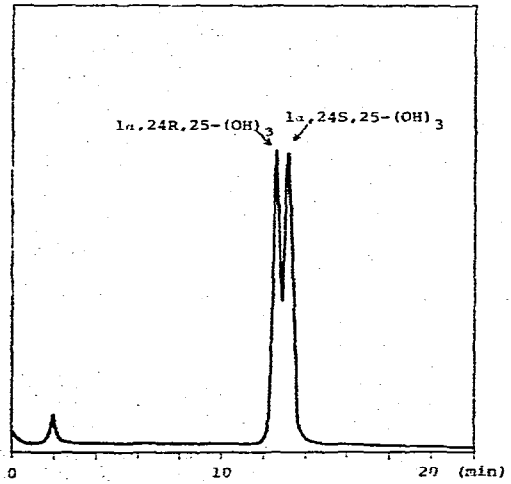

Fig. 4. Separation of 1α,24*R*,25-$(OH)_3$-D_3 and 1α,24*S*,25-$(OH)_3$-D_3 on Zorbax SIL with 3.5% methanol–methylene chloride.

the reverse elution order was observed. It is interesting to note the effect of the 1α-hydroxyl group on the separation of the stereoisomers at the side-chain of steroids.

For the synthesis of vitamin D_3 analogues, usually the hydroxycholesterols are converted into the D_3 form via a 5,7-diene system. In the synthetic route to the 24*R* and 24*S* isomers of 24-hydroxy-D_3 analogues, the C-24 epimers could also be separated at the stage of the formation of the hydroxycholesterol derivatives. We found that the 24-epimers of the Δ^5-compound can be resolved more easily than that of the corresponding D_3 form. Thus, the 24-epimers of 24-hydroxycholesterol dibenzoate[5], 24,25-dihydroxycholesterol 3,24-dibenzoate 25-TMS ether[9,10], 1α,24,25-trihydroxycholesterol 1,3,24-tribenzoate 25-TMS ether[11] and 1α,24-dihydroxycholesterol 3,24-

dibenzoate[8] could be separated on a silica gel column, and their absolute configurations at the C-24 position were determined[9–11,17]. In all of these derivatives, the *R* isomer is less polar than the *S* isomer.

Although gas chromatography can be used for the identification of D_3 metabolites[18], HPLC is much more useful because it gives sharper peaks and a higher resolution and also can be used for preparative purposes.

These results demonstrate that HPLC is very useful for the identification and determination of D_3 metabolites and their analogues and especially for metabolic studies using labelled compounds.

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