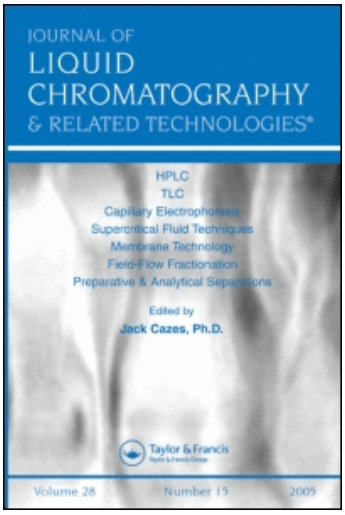

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Retention Behavior of Vitamin D and Related Compounds During High-Performance Liquid Chromatography

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RETENTION BEHAVIOR OF VITAMIN D AND RELATED COMPOUNDS DURING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The retention behavior of vitamin D_2-D_5 and provitamin D_2-D_5 are examined using high-performance liquid chromatography. Inclusion chromatography using cyclodextrin as the mobile phase additive in reversed-phase high-performance liquid chromatography is also used for this purpose. Reversed-phase high-performance liquid chromatography is more effective than normal-phase high-performance liquid chromatography in separating these analogs. The addition of methyl-β-cyclodextrin to the mobile phase is effective in separating the pair of vitamin-D_2 and -D_3 or provitamin-D_2 and -D_3. The separation of the pair of stereoisomeric Cookson-type derivatives of vitamin-D_2 or -D_3 was also examined and found that normal-phase high-performance liquid chromatography is effective for this purpose.

INTRODUCTION

In the previous paper of this series, we clarified the retention behavior of conjugated metabolites of vitamin D (D) and related compounds during high-performance liquid chromatography (HPLC) and found that the inclusion chromatography using cyclodextrin (CD) as a mobile phase additive is effective in separating the pairs of fluorescent derivatives of the D_3 and D_2 conjugates or related compounds [1]. Naturally obtained D includes D_2-D_7 , which can be differentiated from one another by the side chain structure of the steroid moiety. Although the separation of sterols by HPLC has been done [2], that of D has not been precisely examined. These data prompted us to examine the retention behavior of D and related compounds during HPLC.

MATERIALS AND METHODS

Materials

Heptakis-(2, 6-di-O-methyl)- β -cyclodextrin (Me- β -CD) was prepared and donated by Kao (Tokyo, Japan). The other CDs (α, β and γ) were donated by Nihon Shokuhin Kako (Tokyo). D_2, D_3 and ergosterol (pro D_2) were purchased from Tokyo Kasei Kogyo (Tokyo). 7-Dehydrocholesterol (pro D_3) was obtained from Wako Pure Chemical Ind. (Osaka, Japan). D_4 and provitamin D_4 (pro D_4) were kindly donated by Dr. Tachibana (Nisshin Flour Milling Co., Saitama, Japan). D_5 and pro D_5 were prepared from β-sitosterol (Funakoshi, Tokyo) in this laboratory in the usual way [3]. The fluorescent Cookson-type reagent, 4-[4-(6-methoxy-2-benzoxazolyl)phenyl]-1, 2, 4-triazoline-3, 5-dione (MBOTAD), was synthesized in this laboratory as previously described [4]. Preparative thin-layer chromatography (prep. TLC) was done with pre-coated TLC (silica gel HF_{254}, 20 x 20 cm, 0.5 mm: E. Merck AG, Damstadt, Germany).

Apparatus

HPLC was carried out using a Shimadzu LC-6A chromatograph (Shimadzu, Kyoto, Japan) equipped with a Shimadzu SPD-6AV ultraviolet detector (UV). Reversed- (YMC • GEL $C_{8,}$ 5 μm, 15 cm x 0.46 cm i.d.)(YMC, Kyoto) and normal- (Develosil 60-5, 5 μm, 25 cm x 0.46 cm i.d.)(Nomura Chem., Seto, Japan) phase columns were used under ambient conditions at a flow rate of 1 ml/min. ^{1}HNMR spectra were measured with a JNM-EX 270 (270 MHz) spectrometer. $CDCl_3$ was used as the solvent with Me_4Si as internal standard. Chemical shifts and *J*-values are given in ppm and Hz, respectively. The following abbreviations are used: d=doublet and m=multiplet.

Preparation of MBOTAD Adducts of D_2 and D_3

Derivatization of D_2 or D_3 (each *ca.* 52 μmol) with MBOTAD (*ca.* 82 μmol) was done according to a previously described procedure [4]. The reaction mixture was submitted to prep. TLC using $CHCl_3$-AcOEt (2:1) as the developing solvent and the following zones were extracted with AcOEt: D_2, ^2Rf 0.24 (yield 55.9%), 0.36 (8.8%); D_3, ^2Rf 0.24 (86.1%), 0.19 (12.0%).

RESULTS AND DISCUSSION

Although D includes D_2-D_7 , we have examined the retention behavior of D_2-D_5 and pro D_2-D_5, which are available during HPLC. These can be differentiated from one another by the side chain structure of the steroid moiety as shown in Fig. 1.

Separation of D_2-D_5 or pro D_2-D_5

Initially, efforts were directed at the separation of D_2-D_5 during normal-phase HPLC. Although two solvent systems [CH_2Cl_2-MeOH (200:1), CH_2Cl_2-isopropanol (120:1)] were examined, D_2-D_5 were eluted at the same retention times (t_R 10.00, 9.19 min, respectively).

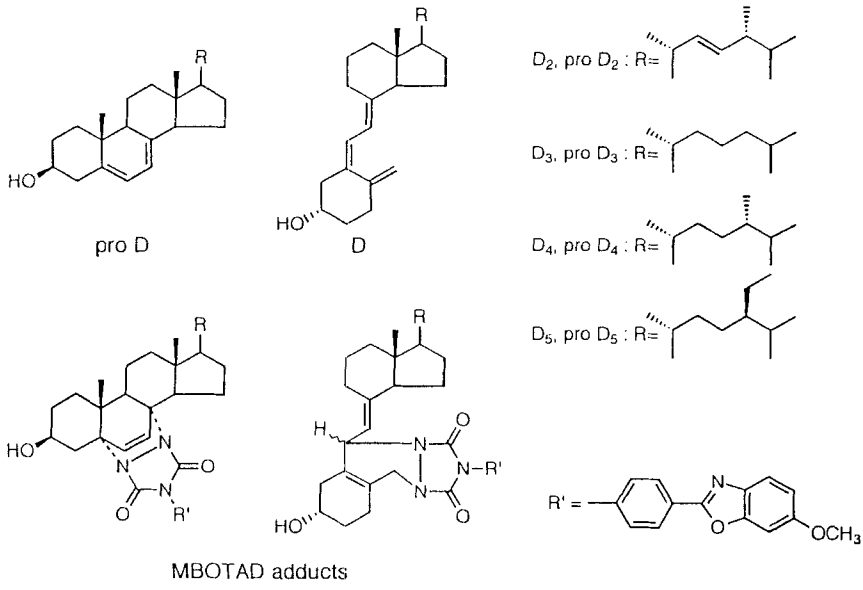

Figure 1. Structures of pro D, D and its MBOTAD adducts

The separation of D_2-D_5 by reversed-phase HPLC using an octylsilyl column and MeOH or MeCN as an organic modifier has been attempted. MeOH was superior to MeCN as an organic modifier, the latter gave an unsymmetrical peak, but D_2 and D_3 could not be separated by both organic modifiers (Fig. 2a). These data prompted us to use inclusion chromatography using CD as the mobile phase additive for this separation. Although the high concentration of Me-β-CD as the host compound (7 mM) was necessary, a satisfactory result was obtained as shown in Fig. 2b. The other CDs (α- and γ-CD) were ineffective while β-CD could not be used at the more than 1 mM because of its sparing solubility in the mobile phase. The former phenomenon can be explained by the cavity size of the host compounds; α-CD was too small and that of γ-CD was too big to include the steroid.

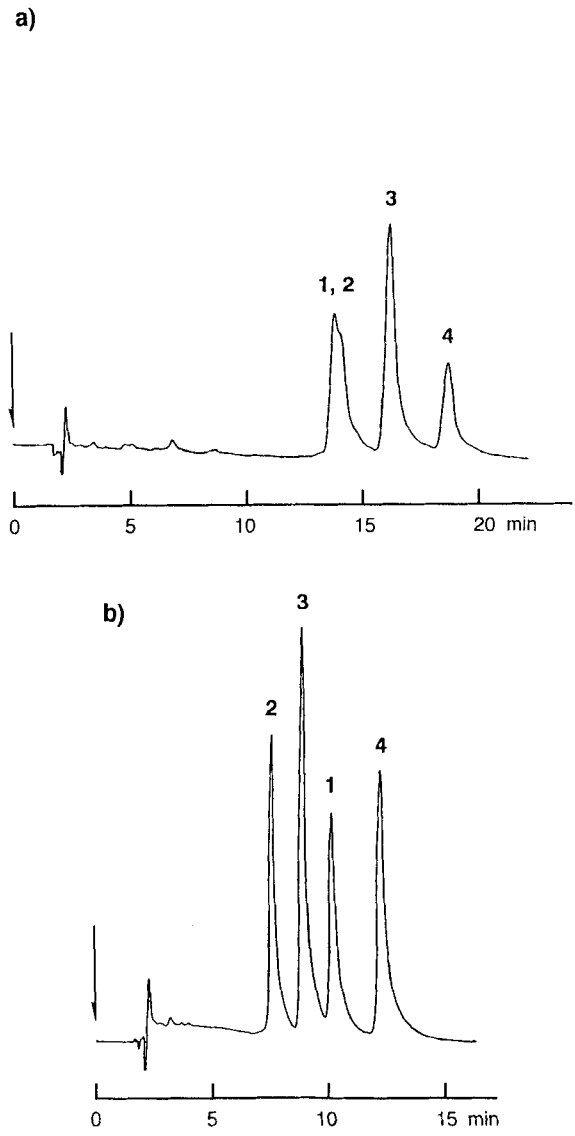

Figure 2. Separation of D_2-D_5

Conditions: mobile phase, MeOH-H_2O (9:1) containing Me-β-CD, a) 0 mM b) 7 mM ; detection, UV 265 nm.

1: D_2 2: D_3 3: D_4 4: D_5

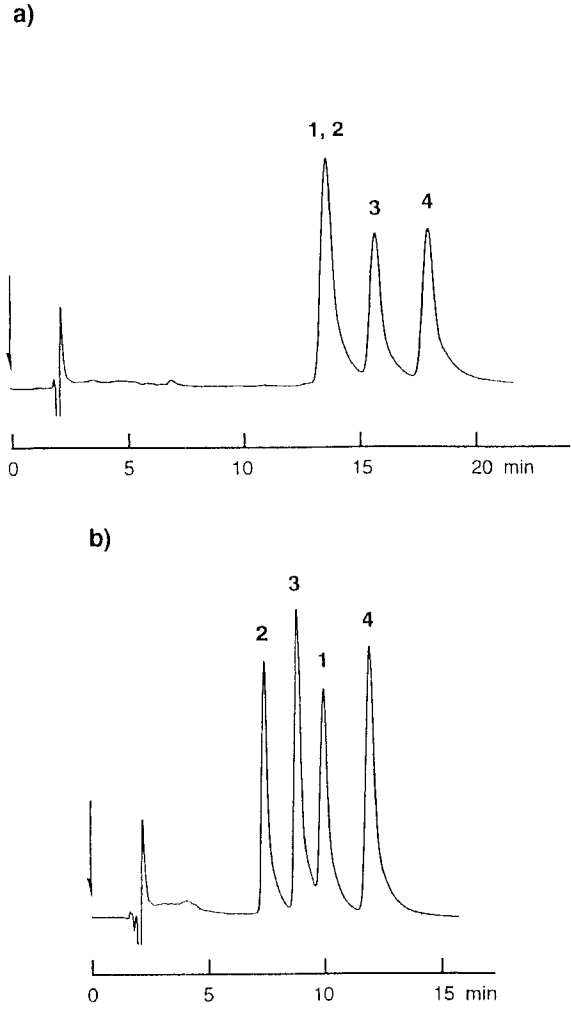

Figure 3. Separation of pro D_2-D_5

Conditions: mobile phase, MeOH-H_2O (9:1) containing Me-β-CD, a) 0 mM b) 7 mM ; detection, UV 280 nm.

1: pro D_2 2: pro D_3 3: pro D_4 4: pro D_5

Table 1. ^{1}HNMR spectra data and separation of stereoisomeric MBOTAD adducts of D_2 or D_3

Adduct		^{1}HNMR H-4 α	^{1}HNMR H-3 α	HPLC[a] t_R (min)	HPLC[a] Resolution (6*R* vs. 6*S*)
D_2	6*R*	2.51, d (15.6)[b]	4.03, m	16.35	
	6*S*	-[c]	4.13, m	11.14	8.17
D_3	6*R*	2.44, d (15.8)	4.14, m	16.46	
	6*S*	-	4.14, m	11.26	8.16

a) Conditions: CH_2Cl_2-MeOH (60:1), UV (280 nm)

b) *J*-value

c) Superimposed with other methylene protons

Almost the same phenomena as that previously described were observed during the separation of pro D_2-D_5. A satisfactory separation has been obtained by the addition of Me-β-CD (7 mM) to the mobile phase as shown in Fig. 3a,b.

Separation of stereoisomeric MBOTAD adducts of D_2 or D_3

It is well known that the derivatization of D with the Cookson-type reagent produces two stereoisomeric adducts, β - and α -side attacked, in a ratio of approximately 6:1; this is helpful in identifying the desired compounds in biological fluids [5]. Although we have developed the highly sensitive fluorescent Cookson-type reagent, MBOTAD, the stereochemistry of the adducts with D has not been clarified (Fig. 1). Based on the ^{1}HNMR spectra (Table 1) of adducts of D_2 and D_3, the obtained main and minor compounds are elucidated as 6*S* and 6*R* compounds, respectively [4]. These stereoisomers were clearly separated by normal phase HPLC as shown in Table 1. The addition of Me-β-CD to the mobile phase of the reversed-phase HPLC improved the separation of these

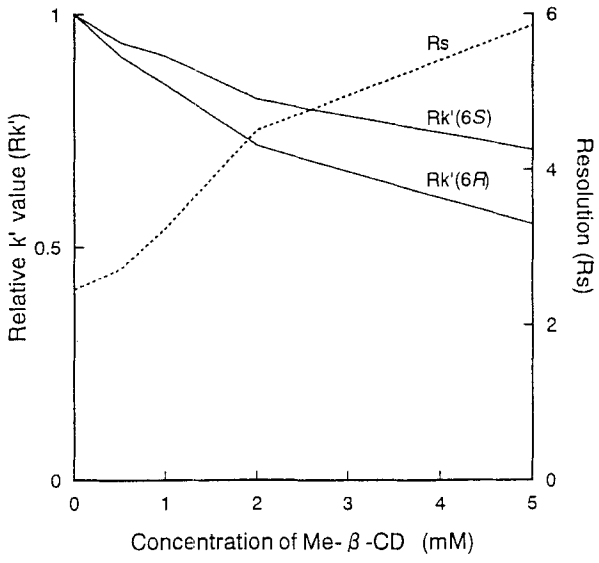

Figure 4. Effect of Me-β-CD on the resolution of the stereoisomeric MBOTAD adducts of D_3
Conditions: mobile phase, MeCN-H_2O (5:1) containing Me-β-CD as indicated; detection, UV 280nm.

stereoisomeric pairs as shown in Fig. 4. The other CDs were not very effective in the separation of the MBOTAD adducts of conjugated D [1].

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