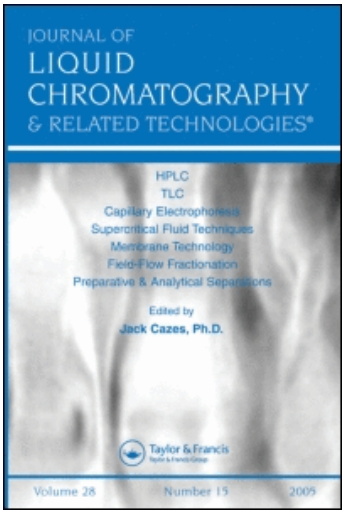

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Retention Behavior of Vitamin D N-Acetylglucosaminides During High-Performance Liquid Chromatography

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RETENTION BEHAVIOR OF VITAMIN D N-ACETYLGLUCOSAMINIDES DURING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The retention behavior of N-acetylglucosaminides of vitamin-D_2 and -D_3, and those of provitamin-D_2 and -D_3 are examined using reverse phase high performance liquid chromatography. Inclusion chromatography using cyclodextrin as the mobile phase additive is also used for this purpose. The addition of methyl-β-cyclodextrin to the mobile phase is effective in separating the pair of N-acetylglucosaminides of vitamin-D_2 and -D_3 or those of provitamin-D_2 and -D_3 .

INTRODUCTION

In the previous paper of this series, we clarified the retention behavior of vitamin D (D) and related compounds including the glucuronides or sulfates during high performance liquid chromatography (HPLC) and found that the inclusion chromatography using heptakis-(2, 6-di-*O*-methyl)-β-cyclodextrin (Me-β-CD) as the mobile phase additive is effective in separating the pair of D_2 (**1a**) and D_3 (**1c**) or related compounds.[1,2] Recently, the occurrence of bile acid N-acetylglucosaminide (*N*AG) in biological fluids[3] and the synthesis of

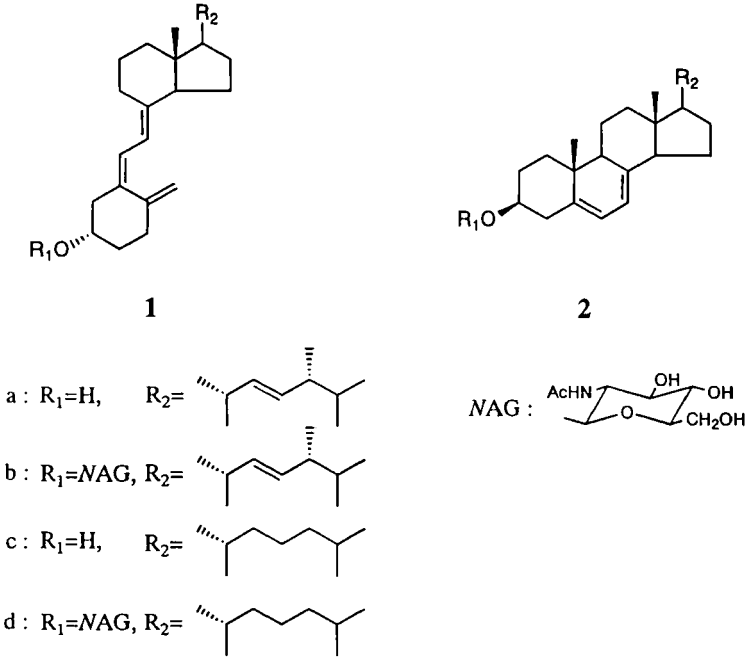

Figure 1. Structures of D, pro D and its *N*AGs

estrogen NAG as a reference compound for the determination of the compound in biological fluids[4] have been reported. These data prompted us to synthesize NAGs of D_2 (**1b**), D_3 (**1d**), pro D_2 (**2b**) and pro D_3 (**2d**) in order to examine their retention behavior during HPLC (Fig. 1).

MATERIALS AND METHODS

Materials

Me-β-CD was prepared and donated by Kao (Tokyo, Japan). D_2 (**1a**), D_3 (**1c**) and ergosterol (pro D_2, **2a**) were purchased from Tokyo Kasei Kogyo (Tokyo). 7-Dehydrocholesterol (pro D_3, **2c**) was obtained from Wako Pure Chemical Ind. (Osaka, Japan).

Preparation of N-Acetylglucosaminides of D and Pro D

The preparation of NAGs of D_2 (1b), D_3 (1d), pro D_2 (2b) and pro D_3 (2d) using respective pro D (2a,c) as a starting material was done in these laboratories and the details will be reported elsewhere in the near future. Some of the physical data are shown below. Proton nuclear magnetic resonance (^{1}HNMR) spectra were measured using a JEOL JNM-EX 270 (270 MHz) spectrometer (JEOL, Tokyo) and Me_4Si was used as the internal standard. Chemical shifts and *J*-values are given in ppm and Hz, respectively. The following abbreviations are used: s=singlet, d=doublet and m=multiplet. Fast atom bombardment mass (FABMS) spectra were measured using a JEOL JMS-DX 303 mass spectrometer. Pro D_2*N*AG (**2b**): ^{1}HNMR (CD_3OD-$CDCl_3$)δ: 2.03 (3H, s, CH_3CO-), 4.64 (1H, d, *J*=7.3 Hz, H-1'), 5.37-5.56 (2H, m, H-6, 7). FABMS *m/z* : 622 $(M+Na)^+$. D_2*N*AG (**1b**): FABMS *m/z* : 622 $(M+Na)^+$. pro D_3*N*AG (**2d**): ^{1}HNMR (CD_3OD-$CDCl_3$)δ: 4.62 (1H, d, *J*=7.6 Hz, H-1'), 5.38, 5.56 (1H each, m, H-6, 7). FABMS *m/z*: 610 $(M+Na)^+$. D_3*N*AG (**1d**): ^{1}HNMR (CD_3OD) δ: 1.89 (3H, s, CH_3CO-), 4.56 (1H, d, *J*=7.6 Hz, H-1'), 4.73 (1H, s, H-19E), 5.02 (1H, s, H-19Z), 6.04 (1H, d, *J*=11.2 Hz, H-7), 6.21(1H, d, *J*=11.2 Hz, H-6). FABMS *m/z* : 610 $(M+Na)^+$.

HPLC

HPLC was carried out using a TOSOH CCPD chromatograph (TOSOH, Tokyo) equipped with a JASCO UVIDEC- 100-II ultraviolet detector (UV) (JASCO, Tokyo). Reverse phase columns [Develosil ODS-5, 5 μm, 15 x 0.46 cm i.d. (Nomura, Seto, Japan), Inertsil ODS-2, 5 μm, 25 x 0.46 cm i.d. (GL Sciences, Tokyo), CAPCELL PAK C_{18}, 5 μm, 25 x 0.46 cm i.d. (SHISEIDO, Tokyo) and TSKgel Super-ODS, 2 μm, 10 x 0.46 cm i.d. (TOSOH)] were used under ambient conditions at a flow rate of 1 mL/min.

RESULTS AND DISCUSSION

Separation using Conventional Method

Initially, efforts were directed at the separation of the pair of *N*AGs of D_2 (**1b**) and D_3 (**1d**) and those of pro D_2 (**2b**) and pro D_3 (**2d**) during reverse phase HPLC. MeOH was superior to MeCN as an organic modifier during the separation of the D related compounds as previously reported.[2] The separation

Table 1

Separation of *N*AGs

	Column and Solvent System (t_R:min)				
Compounds	**1**	**2**	**3**	**4a**	**4b**
D_2*N*AG (**1b**)[1]	18.7	15.2	18.7	8.8	
D_3*N*AG (**1d**)	19.9	16.0	19.6	9.5	
Resolution (Rs)	0.88	0.74	1.21	1.77	
pro D_2*N*AG (**2b**)[2]	25.6				10.9
pro D_3*N*AG (**2d**)	27.3				11.9
Rs	1.04				1.78

1. Develosil ODS-5 [MeOH-H_2O (10:1)]. 2. Inertsil ODS-2 [MeOH-H_2O (10:1)]. 3. CAPCELL PAK C_{18} [MeOH-H_2O (10:1)]. 4a. TSKgel Super-ODS [MeOH-H_2O (8:1)] 4b. TSK gel Super ODS (MeOH-H_2O (10:1)], Detection: UV 1) 265 nm 2) 254 nm.

of D_2- and D_3-*N*AG (**1b,d**) is summarized in Table 1, in which TSKgel Super-ODS gave the best results with shortest t_R and greatest Rs as shown in Fig. 2a. The separation of pro D_2- and pro D_3-*N*AG (**2b,d** :Rs 1.78) was also done using this column (Table 1, Fig. 2b).

Separation using Inclusion Chromatography

We next applied the inclusion chromatography using Develosil ODS-5 and Me- β -CD as the column and the mobile phase additive, respectively, for the separation of the pair of these *N*AGs. Both pairs (**1b,d: 2b,d**) showed satisfactory results (Rs 3.44, 3.23 with the shorter t_R, respectively) with the addition of 5 mM of the host compound. The elution order of 1d and 2d became faster than that of **1b** and **2b** with the addition of the host compound, respectively (Table 1, Fig. 3a,b). These data are compatible with the previously obtained results.[1,2]

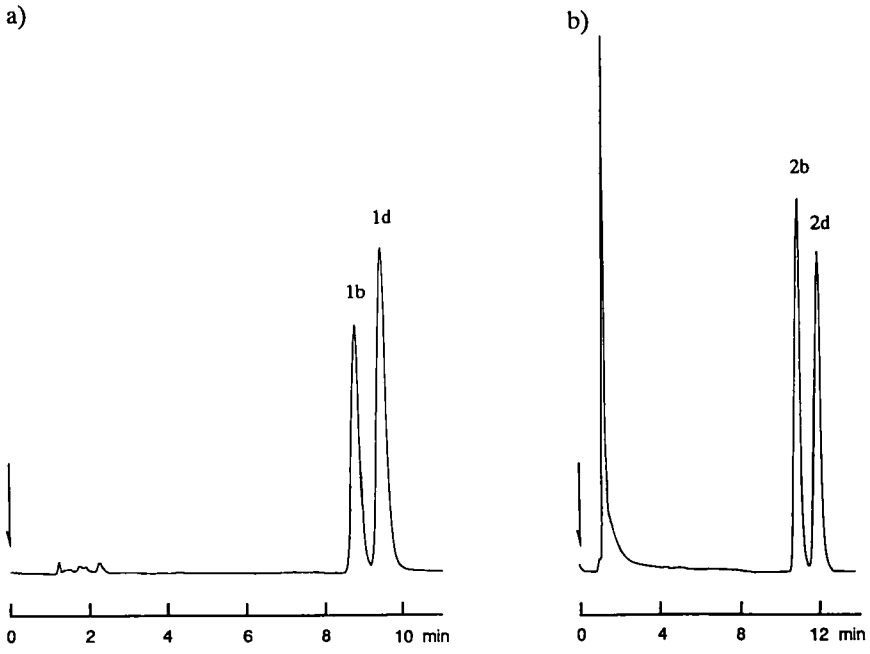

Figure 2. Separation using conventional method. Conditions: column, TSKgel Super-ODS; mobile phase, a) MeOH-H_2O (8:1) b) MeOH-H_2O (10:1); detection: UV a) 265 nm b) 254 nm.

All the above data showed that TSKgel Super-ODS gave the best results among the examined reverse phase columns. The inclusion chromatography using Me- β -CD as an additive is also effective for the separation of these compounds.

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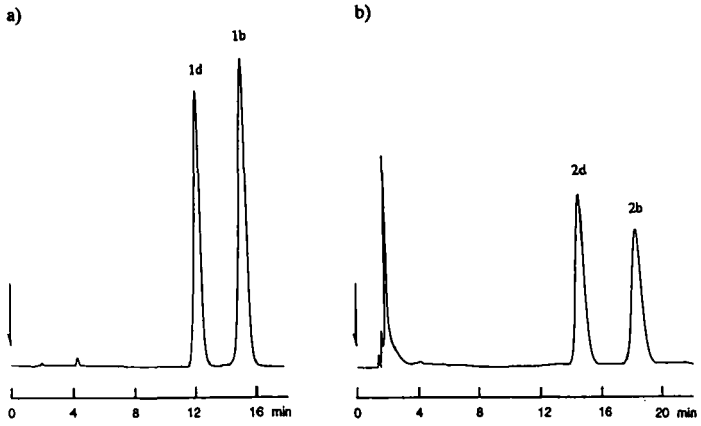

Figure 3. Separation using inclusion chromatography. Conditions: column, Develosil ODS-5; mobile phase, MeOH-H_2O (10:1) containing Me-β-CD (5 mM); detection: UV, a) 265 nm b) 254 nm.

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