

Short communication

Capillary gas–liquid chromatography of 6-hydroxylated bile acids

Ashok K. Batta^{a,*}, Suresht K. Aggarwal^a, G. Stephen Tint^b, Manju Batta^b,
Gerald Salen^{a,b}

^aDepartment of Medicine, and Sammy Davis, Jr. Liver Institute, University of Medicine and Dentistry of New Jersey,
New Jersey Medical School, Newark, NJ 07103, USA

^bVeterans Administration Medical Center, East Orange, NJ 07019, USA

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Abstract

Gas–liquid chromatographic separation of several bile acids with a hydroxyl group at C-6 is described on two capillary columns, CP-Sil-19 CB and CP-Sil-5 CB. The gas–liquid chromatographic retention indices of bile acids with 6 α - and 6 β -hydroxyl groups are compared with those of bile acids without the C-6 hydroxyl group and the effect of the C-6 hydroxyl group on the retention indices of bile acids is determined. Both 6 α - and 6 β -hydroxyl groups increase the retention index of a bile acid. The retention indices of 6 α - or 6 β -hydroxyl derivatives of chenodeoxycholic acid were found to be higher than those of the corresponding 6-hydroxy derivatives of cholic acid on the CP-Sil-19 CB column but lower on the CP-Sil-5 CB column. Although all 6-hydroxylated derivatives of lithocholic, chenodeoxycholic and cholic acids were not completely resolved on either column alone, combining the two columns resulted in the complete separation of all these compounds.

1. Introduction

Bile acids hydroxylated at C-6 have been isolated from the bile of several animal species, e.g., α -, β - and ω -muricholic acids in rat [1] and hyodeoxycholic acid and hyocholic acid in the pig [2]¹. Substantial amounts of these compounds are excreted in the urine of patients with

hepatobiliary diseases (see Ref. [3], and references therein) and it is considered that hydroxylation at C-6 may facilitate hepatic clearance of excessive amounts of toxic endogenous bile acids during these diseases [4]. In order to make a sensible correlation between the presence of these compounds and the disease, it is important to have available analytical methods that can be

* Corresponding author.

¹ The following abbreviations were used: lithocholic acid, 3 α -hydroxy-5 β -cholanoic acid; chenodeoxycholic acid, 3 α ,7 α -dihydroxy-5 β -cholanoic acid; deoxycholic acid, 3 α ,12 α -dihydroxy-5 β -cholanoic acid; hyodeoxycholic acid, 3 α ,6 α -dihydroxy-5 β -cholanoic acid; cholic acid, 3 α ,7 α ,12 α -trihydroxy-5 β -cholanoic acid; hyocholic acid, 3 α ,6 α ,7 α -trihydroxy-5 β -cholanoic acid; α -muricholic acid, 3 α ,6 β ,7 α -trihydroxy-5 β -cholanoic acid; β -muricholic acid, 3 α ,6 β ,7 β -trihydroxy-5 β -cholanoic acid; ω -muricholic acid, 3 α ,6 α ,7 β -trihydroxy-5 β -cholanoic acid; GLC, gas–liquid chromatography; rrt, relative retention time; RI, retention index (Kovats value); TMS, trimethylsilyl.

used for the detection of the various 6-hydroxy bile acids from biological fluids. We report herein the gas-liquid chromatographic (GLC) characteristics of the trimethylsilyl (TMS) ethers of several 6 α - and 6 β -hydroxy bile acid methyl esters and their GLC retention indices are compared with those of the corresponding bile acid derivatives without a hydroxyl group at C-6. Since most of the bile acid derivatives studied are well resolved on the columns employed, we hope that the method will be useful for characterization of 6-hydroxy bile acids in biological fluids.

2. Experimental

Cholic acid, chenodeoxycholic acid, deoxycholic acid, lithocholic acid and 3 α ,6 β -dihydroxy-5 β -cholanoic acid were purchased from Steraloids (Wilton, NH, USA). Hyodeoxycholic acid and hyocholic acid were from Canada Packers (Toronto, Canada). Ursodeoxycholic acid (3 α ,7 β -dihydroxy-5 β -cholanoic acid) and ursocholic acid (3 α ,7 β ,12 α -trihydroxy-5 β -cholanoic acid) were gifts from Tokyo Tanabe, Japan. The 12 β -hydroxy bile acids were prepared via reduction of the corresponding 12-oxo derivatives with potassium/*tert.*-amyl alcohol [5]. The α -, β - and ω -muricholic acids were synthesized from chenodeoxycholic acid and 3 α ,6 α ,7 α ,12 α -, 3 α ,6 β ,7 α ,12 α -, 3 α ,6 β ,7 β ,12 α - and 3 α ,6 α ,7 β ,12 α -tetrahydroxy-5 β -cholanoic acids were synthesized from cholic acid following literature methods [6–8]. The 3 α ,6 β ,7 α ,12 β - and 3 α ,6 β ,7 β ,12 β -tetrahydroxy-5 β -cholanoic acids were synthesized from 3 α ,12 β -dihydroxy-5 β -chol-6-enoic acid following methods previously published [9]. Methyl esters of the bile acids were prepared by addition of 0.5–1 ml of 3% anhydrous methanolic hydrochloric acid (Aldrich Chemical Co., Milwaukee, WI, USA) to 5–20 mg of the respective bile acid and keeping at room temperature for 2 h. Solvent was then evaporated at 55°C under N₂ and the methyl ester was crystallized from either pure methanol or from aqueous methanol. All compounds were

> 98% pure as judged by gas-liquid chromatography and all synthesized compounds exhibited mass spectral fragmentation patterns compatible with their structures. Sil-Prep (hexamethyldisilazane-trimethylchlorosilane-pyridine, 3:1:9) was used for preparation of trimethylsilyl ether derivatives of the bile acids and was purchased from Alltech Associates (Deerfield, IL, USA).

2.1. Gas-liquid chromatography

A Hewlett-Packard Model 5880A gas chromatograph equipped with a flame ionization detector and an injector with a split/splitless device for capillary columns was used for all separations. The chromatographic column consisted of a chemically bonded fused-silica CP-Sil-19 CB (stationary phase, 85% dimethyl, 7% cyanopropyl, 7% phenyl and 1% vinylsiloxane) or CP-Sil-5 CB (stationary phase, 100% dimethylsiloxane) capillary column (25 m \times 0.22 mm I.D.) (Chrompack, Raritan, NJ, USA) and helium was used as the carrier gas. The GLC operating conditions were as follows. Injector and detector temperatures were 260°C and 290°C, respectively. After injection, the oven temperature was kept at 100°C for 2 min, then programmed at a rate of 35°C/min to a final temperature of 265°C when using a CP-Sil-19 CB column and 278°C when using a CP-Sil-5 CB column [10].

2.2. Derivatization

The bile acid methyl esters (5–10 μ g) were reacted with 100 μ l of Sil-Prep for 20 min at 55°C. Solvents were evaporated at 55°C under N₂ and the trimethylsilyl (TMS) ether derivatives formed were redissolved in 100 μ l of hexane. One μ l was injected onto the GLC column simultaneously with 5 α -cholestane, the internal standard. The retention times of the various bile acids (RRT) were calculated relative to that of 5 α -cholestane. Also, the retention index values (Kovats) for the derivatized bile acids were determined by comparison with the retention times of C₂₉–C₃₇ *n*-alkanes [11].

3. Results and discussion

Both 6 α - and 6 β -hydroxylated bile acids are native to several animal species and are excreted in substantial amounts in patients with cholestatic liver disease. It is important to have a reliable GLC method for the detection and quantitation of 6 α - and 6 β -hydroxylated bile acids in the presence of the endogenous bile acids found in biological fluids and for the metabolic studies of these 6-hydroxylated bile acids. We have examined the GLC behavior of the methyl ester-TMS ethers of several common bile acids with epimeric hydroxyl groups at C-7 and C-12 on a relatively less polar CP-Sil-5 CB capillary column and a more polar CP-Sil-19 CB column and compared the effect of an added 6 α - or 6 β -trimethylsilyloxy group. Retention times of all bile acid derivatives were highly reproducible on both columns. For amounts of bile acids ranging from 5 to 100 ng injected onto each column, the detector response, as shown by the integrator, was linear. Furthermore, the peak

areas of the various compounds were similar to that obtained for 5 α -cholestane when similar amounts were injected onto the column.

Table 1 lists the retention indices of the TMS ethers of various bile acid methyl esters epimeric at C-7 and C-12 on CP-Sil-5 CB and CP-Sil-19 CB columns. As can be seen, derivatives of 12 β -hydroxy bile acids have lower retention indices than those of the corresponding 12 α -hydroxy epimers on both columns. In contrast, the 7 β -hydroxy epimers were eluted significantly later than their 7 α -hydroxy counterparts, the more polar CP-Sil-19 CB column showing greater resolutions between the corresponding pairs. A TMS group at C-7 or C-12 significantly increases the retention index of a 3 α -trimethylsilyloxy bile acid methyl ester, the effect being most pronounced with the 7 β -TMS substituent and only minor with a 12 β -TMS substituent. However, when occurring together in the same compound, these groups do not show an additive effect. In fact, on the more polar column, bile acid derivatives with 7 α ,12 α -dihydroxy groups

Table 1

GLC retention indices of methyl ester-trimethylsilyl ethers of bile acids epimeric at C-7 and C-12 on CP-Sil-19 CB and CP-Sil-5 CB capillary columns

5 β -Cholanoic acid	Relative retention time		Retention index	
	CP-Sil-19	CP-Sil-5	CP-Sil-19 ^a	CP-Sil-5 ^b
3 α -Hydroxy-	1.59	1.34	3339	3157
3 α ,7 α -Dihydroxy-	1.73	1.49	3397	3244
3 α ,7 β -Dihydroxy-	1.85	1.57	3439	3279
3 α ,12 α -Dihydroxy-	1.67	1.45	3373	3221
3 α ,12 β -Dihydroxy-	1.60	1.39	3345	3191
3 α ,7 α ,12 α -Trihydroxy-	1.69	1.53	3381	3261
3 α ,7 β ,12 α -Trihydroxy-	1.85	1.62	3440	3303
3 α ,7 α ,12 β -Trihydroxy-	1.66	1.49	3370	3240
3 α ,7 β ,12 β -Trihydroxy-	1.85	1.62	3438	3305

Retention times are expressed relative to that of 5 α -cholestane. Retention time of 5 α -cholestane was 11.65 min on CP-Sil-19 CB column and 13.20 min on CP-Sil-5 CB column. Retention indices (Kovats values) were determined by previous injection of a hydrocarbon mixture C₃₁–C₃₇ under identical GLC conditions.

^a The retention times of the various *n*-alkanes on CP-Sil-19 CB column were as follows: C₃₁, 13.52 min; C₃₂, 15.26 min; C₃₃, 17.46 min; C₃₄, 20.22 min; C₃₅, 23.71 min; C₃₆, 28.12 min; and C₃₇, 33.67 min.

^b The retention times of the various *n*-alkanes on CP-Sil-5 CB column were as follows: C₃₁, 16.49 min; C₃₂, 18.61 min; C₃₃, 21.24 min; C₃₄, 24.50 min; C₃₅, 28.54 min; C₃₆, 33.56 min; and C₃₇, 39.79 min.

Table 2

GLC retention indices of methyl ester-trimethylsilyl ethers of 6-hydroxylated bile acids on CP-Sil-19 CB and CP-Sil-5 CB capillary columns

5 β -Cholanoic acid	Relative retention time		Retention index	
	CP-Sil-19	CP-Sil-5	CP-Sil-19	CP-Sil-5
3 α ,6 α -Dihydroxy-	1.80	1.52	3422	3256
3 α ,6 β -Dihydroxy-	1.71	1.49	3389	3242
3 α ,6 α ,7 α -Trihydroxy-	1.94	1.69	3471	3336
3 α ,6 β ,7 α -Trihydroxy-	1.64	1.50	3359	3246
3 α ,6 α ,7 β -Trihydroxy-	2.44	2.01	3608	3453
3 α ,6 β ,7 β -Trihydroxy-	1.99	1.72	3486	3348
3 α ,6 α ,7 α ,12 α -Tetrahydroxy-	1.86	1.70	3445	3340
3 α ,6 β ,7 α ,12 α -Tetrahydroxy-	1.58	1.52	3338	3254
3 α ,6 α ,7 β ,12 α -Tetrahydroxy-	2.29	1.99	3570	3447
3 α ,6 β ,7 β ,12 α -Tetrahydroxy-	1.93	1.63	3468	3310
3 α ,6 β ,7 α ,12 β -Tetrahydroxy-	1.59	1.49	3342	3243
3 α ,6 β ,7 β ,12 β -Tetrahydroxy-	1.93	1.74	3467	3356

Retention times are expressed relative to that of 5 α -cholestane. Retention indices (Kovats values) were determined as described above. Retention times of 5 α -cholestane and C₃₁ to C₃₇ hydrocarbons in both columns are shown in Table 1.

are consistently eluted before those of the corresponding bile acids with a 7 α -hydroxy group (Tables 1 and 2). This seems to be a general phenomenon since a similar relationship has been observed for bile alcohols with hydroxyl groups at the 7 α - and 12 α -positions where the TMS ethers of bile alcohols with a 7 α ,12 α -dihydroxy structure were always eluted before the TMS ethers of the corresponding bile alcohols with only a 7 α -hydroxyl group on the more polar CP-Sil-19 CB column and later on the less polar CP-Sil-5 CB column [12].

Both 6 α - and 6 β -TMS groups increase the retention index of a bile acid. However, their effect is greatly influenced by the presence of the vicinal TMS group at C-7 (Tables 2 and 3). Thus, while the axial 6 α -TMS group exerts an additive effect on the retention index of the bile acid with either a 7 α - or 7 β -TMS group, the effect of an equatorial 6 β -TMS group is greatly suppressed (e.g., hyocholic acid and ω -muricholic acid vs. α - and β -muricholic acids; Tables 2 and 3). Further, the effect of a TMS group at the 12 α -position is generally small and compounds with a 12 α -TMS group had shorter retention indices than the corresponding compounds with-

out the 12 α -TMS group when chromatographed on the CP-Sil-19 CB column (Table 2) as observed for bile acids or bile alcohols without the hydroxyl group at C-6. However, on the less polar CP-Sil-5 CB column, though the two tetrahydroxy compounds with a 7 α ,12 α -grouping eluted from the column later than α -muricholic acid and hyocholic acid as one would expect, the other two compounds with a 7 β ,12 α -grouping in fact eluted before β - and ω -muricholic acids.

Although the methyl ester-TMS ethers of hyocholic acid and the muricholic acids and their 12 α -hydroxy derivatives can be well resolved on both columns, overlap occurs when compounds of the two series are co-chromatographed. A GLC chromatogram of all eight epimers is shown in Fig. 1. As seen from the figure, resolutions between the various pairs are generally better on the CP-Sil-19 CB column and the methyl ester-TMS ethers of hyocholic acid and ω -muricholic acid that do not show baseline resolution from the corresponding 12 α -hydroxy compounds on the CP-Sil-5 CB column are completely resolved on the CP-Sil-19 CB column. On the other hand, the methyl ester-TMS ether of hyocholic acid could not be resolved from the corresponding

Table 3

Comparative effects of TMS groups at C-6, C-7 and C-12 on the retention index of methyl ester-TMS ether of lithocholic acid on CP-Sil-5 CB and CP-Sil-19 CB columns

Trimethylsilyloxy substituent	Δ RI value	
	CP-Sil-19 CB	CP-Sil-5 CB
6 α	83	99
6 β	50	85
7 α	58	87
7 β	100	122
12 α	36	64
12 β	6	34
7 α ,12 α	43	96
7 β ,12 α	102	146
7 α ,12 β	31	83
7 β ,12 β	99	148
6 α ,7 α	132	179
6 β ,7 α	20	89
6 α ,7 β	269	296
6 β ,7 β	147	191
6 α ,7 α ,12 α	106	185
6 β ,7 α ,12 α	-1	97
6 α ,7 β ,12 α	231	290
6 β ,7 β ,12 α	129	153
6 β ,7 α ,12 β	3	86
6 β ,7 β ,12 β	128	199

The Δ RI values for the epimeric hydroxyl groups were calculated by subtracting the retention index for the TMS ether of methyl lithocholate from that of the methyl ester-TMS ether of the given bile acid.

derivative of 3 α ,6 β ,7 β ,12 α -tetrahydroxy-5 β -cholanoic acid on CP-Sil-19 CB column. However, the two compounds were well resolved on the CP-Sil-5 CB column. Thus, a combination of the two columns is necessary for complete resolution of all eight compounds.

The application of retention indices on polar and non-polar columns has recently been demonstrated by Iida et al. [13] for several bile acid methyl ester-TMS ethers. An important advantage of using our capillary columns is that the retention indices for compounds with chenodeoxycholic acid ring hydroxylations are consistently higher than those for bile acids with cholic acid ring hydroxylations on the more polar CP-Sil-19 CB column and they are consistently lower on the less polar CP-Sil-5 CB column. This was found to be true even when a 6 α - or 6 β -hydroxyl group was also present with the excep-

tion of the 7 β -hydroxy compounds, β - and ω -muricholic acids and their 12 α -hydroxy derivatives, 3 α ,6 α ,7 β ,12 α -tetrahydroxy-5 β -cholanoic acid and 3 α ,6 β ,7 β ,12 α -tetrahydroxy-5 β -cholanoic acid, on the CP-Sil-5 CB column (Table 2). We hope that the GLC method presented here will be useful for characterization of new 6-hydroxylated bile acids and for the routine analysis of serum and urinary bile acids in patients with cholestatic liver diseases, where gas-liquid chromatography-mass spectrometry may be the only means of characterization.

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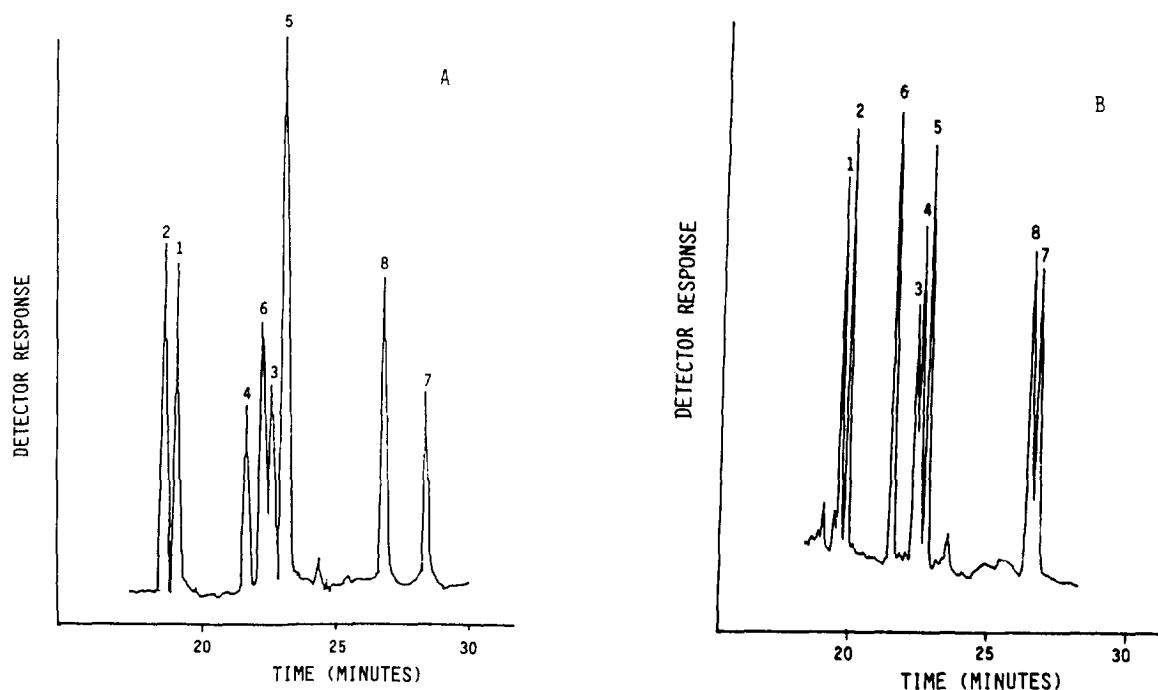


Fig. 1. GLC chromatogram of the methyl ester-TMS ether derivatives of 6-hydroxy bile acids. (A) CP-Sil-19 CB column; (B) CP-Sil-5 CB column. Peak identification, methyl ester-TMS ether of: 1 = $3\alpha,6\beta,7\alpha$ -trihydroxy- 5β -cholanoic acid; 2 = $3\alpha,6\beta,7\alpha,12\alpha$ -tetrahydroxy- 5β -cholanoic acid; 3 = $3\alpha,6\alpha,7\alpha$ -trihydroxy- 5β -cholanoic acid; 4 = $3\alpha,6\alpha,7\alpha,12\alpha$ -tetrahydroxy- 5β -cholanoic acid; 5 = $3\alpha,6\beta,7\beta$ -trihydroxy- 5β -cholanoic acid; 6 = $3\alpha,6\beta,7\beta,12\alpha$ -tetrahydroxy- 5β -cholanoic acid; 7 = $3\alpha,6\alpha,7\beta$ -trihydroxy- 5β -cholanoic acid; 8 = $3\alpha,6\alpha,7\beta,12\alpha$ -tetrahydroxy- 5β -cholanoic acid; I.S. = internal standard (5α -cholestane).

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