Separation of mono-, di-, and trihydroxy stereoisomers of bile acids by capillary gas–liquid chromatography

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Summary Capillary gas–liquid chromatographic separation was studied for the complete set of the 26 theoretically possible isomers of mono-, di-, and trihydroxylated 5β-cholanic acids, which differ from one another in the number, position, and configuration of hydroxyl groups at C-3, C-7, and/or C-12 in the nucleus, as well as for some of their related acids. The bile acid samples were chromatographed as their methyl ester-trimethylsilyl (TMSi) ether derivatives and analyzed on three capillary columns coated with nonpolar OV-1, slightly polar OV-17, and polar SP-2340 as liquid phases. The retention times on capillary gas–liquid chromatography (GLC) responded dramatically to the minor structural differences, and almost complete separation of the positional and stereochemical isomers was achieved by the combined use of SP-2340 and OV-17 (or OV-1) capillary columns.—**Iida, T., F. C. Chang, T. Matsumoto, and T. Tamura.** Separation of mono-, di-, and trihydroxy stereoisomers of bile acids by capillary gas–liquid chromatography. *J. Lipid Res.* 1983. **24:** 211–215.

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A combination of gas–liquid chromatography (GLC) and mass spectrometry is well recognized as one of the most powerful tools for separating and identifying the individual components in a mixture of bile acids obtained in metabolic studies. Concerning the GLC of bile acids, a large number of studies have been concerned with the relationship between the structures and the retention times on various liquid phases as well as with the use of suitable derivatives such as acetates, trimethylsilyl (TMSi) ethers, or trifluoroacetates (1–4). Although those data are of special value in structural elucidations, the studies were almost exclusively carried out by using conventional packed columns which often render incomplete resolution of closely related bile acids, particularly of stereoisomers (5, 6).

Several groups of workers have recently demonstrated excellent separation of a mixture of some common bile acids, i.e., lithocholic, chenodeoxycholic, ursodeoxycholic, deoxycholic, and cholic acids, using glass capillary columns coated with OV-101 (7), SE-30 (8), PEG-20000 (9–11), or PEG-HT (12) as liquid phases. In addition, recent successful use of a new polar thermostable phase, SP-2340, for separation of C-24 epimeric 24-alkyl sterols (13), geometrical isomers of fatty acids (14), and diastereoisomers of tocopherols (15) prompted us to examine this phase in the analysis of bile acids.

As a result of work on a program of synthesis of potential bile acid metabolites (16–20), the complete set of 26 hydroxylated 5β-cholanic acids having one to three hydroxyl groups at positions C-3, C-7, and/or C-12 (including known, known but uncommon, and new acids) is now available. We report here capillary GLC analyses of the 26 acids, as well as of some related acids, as their methyl ester-TMSi ether derivatives, on three columns of differing liquid phase polarities.

MATERIALS AND METHODS

Bile acid samples

The source of bile acid samples used in this study was as follows: lithocholic, chenodeoxycholic, ursodeoxycholic, deoxycholic, cholic, hyocholic, and hyodesoxycholic acids were available from commercial sources;

TABLE 1. Gas chromatographic operating parameters for the three capillary columns used

	OV-1	OV-17	SP-2340
Column size (m × 0.28 mm i.d.)	30	30	50
Column temperature (°C)	245	245	210
Injection temperature (°C)	290	290	270
Carrier gas (N_2) flow rate (ml/min)	1.7	1.6	1.0
Splitting ratio	1:25	1:40	1:45
Scavenger gas (N_2) flow rate (ml/min)	50	50	50
Absolute retention time of methyl deoxycholate TMSi ether (min)[a]	29.4	28.2	38.0
Absolute retention time of n-hexatriacontane (C_{36}) (min)	31.7	32.1	20.6
Number of theoretical plates (N)[b]	44000	39000	24000
McReynolds' constant ($\Sigma\bar{1}$)	222	884	3678

[a] Internal reference standard.
[b] As measured on the methyl deoxycholate TMSi ether peak.

Abbreviations: GLC, gas–liquid chromatography; TMSi, trimethylsilyl; RRT, relative retention time(s); MU, methylene unit(s).

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the preparations of 3β-, 12α- and 12β-monohydroxy, 3β,12α-, 3α,12β- and 3β,12β-dihydroxy, and 3α,7α,12β-,3β,7α,12α- and 3β,7α,12β-trihydroxy 5β-cholanic acids have previously been reported (16, 17); remaining acids were synthesized recently in our laboratory (18–20). The methyl esters prepared by the diazomethane method were of satisfactory purity according to GLC, high-performance liquid chromatography, and thin-layer chromatography analyses. The methyl esters were then converted to their complete TMSi ether derivatives (1) for capillary GLC determinations. The derivatization was made by adding ca. 1 ml of TMS-HT (a mixture of pyridine–hexamethyldisilazane–trimethylchlorosilane 3:2:1; Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan) to ca. 1 mg of the methyl esters. After 30 min at room temperature, 0.1–0.3 μl of supernatant was injected simultaneously into the GLC with an internal reference standard.

Gas–liquid chromatography

A Shimadzu GC-7A gas chromatograph equipped with a flame ionization detector was used isothermally. The chromatograph was fitted with a support-coated open tubular type of glass capillary column coated with three different polarities of liquid phases: OV-1 (100% dimethyl silicone), OV-17 (50% phenyl methyl silicone),

TABLE 2. Retention data for the TMSi ethers of hydroxylated methyl 5β-cholanates on three capillary columns[a]

Hydroxy Substituent	OV-1			OV-17			SP-2340		
	RRT	MU	ΔMU	RRT	MU	ΔMU	RRT	MU	ΔMU
None	0.42	32.6		0.50	33.1		0.93	38.05	
Monohydroxyl									
3α[b]	0.85	35.1	2.5	0.93	35.25	2.15	1.42	39.5	1.45
3β	0.81	34.95	2.35	0.86	34.95	1.85	1.08	38.55	0.5
7α	0.59	33.75	1.15	0.59	33.6	0.5	0.73	37.15	−0.9
7β	0.70	34.65	2.05	0.73	34.4	1.3	1.02	38.3	0.25
12α	0.56	33.6	1.0	0.59	33.6	0.5	0.73	37.15	−0.9
12β	0.53	33.45	0.85	0.52	33.2	0.1	0.64	36.65	−1.4
Dihydroxyl									
3α,7α[c]	1.07	36.0	3.4	1.03	35.6	2.5	1.06	38.45	0.4
3β,7α	0.96	35.5	2.9	0.88	35.05	1.95	0.80	37.4	−0.65
3α,7β[d]	1.23	36.5	3.9	1.17	36.1	3.0	1.44	39.55	1.5
3β,7β	1.23	36.5	3.9	1.12	35.9	2.8	1.18	38.85	0.8
3α,12α[e]	1.00	35.7	3.1	1.00	35.5	2.4	1.00	38.25	0.2
3β,12α	0.99	35.65	3.05	0.91	35.15	2.05	0.86	37.75	−0.3
3α,12β	0.96	35.5	2.9	0.88	35.05	1.95	1.00	38.25	0.2
3β,12β	0.99	35.65	3.05	0.84	34.9	1.8	0.82	37.55	−0.5
7α,12α	0.68	34.3	1.7	0.59	33.6	0.5	0.53	35.9	−2.15
7β,12α	0.85	35.1	2.5	0.79	34.7	1.6	0.76	37.25	−0.8
7α,12β	0.68	34.3	1.7	0.59	33.6	0.5	0.53	35.9	−2.15
7β,12β	0.87	35.2	2.6	0.73	34.4	1.3	0.73	37.15	−0.9
3α,6α[f]	1.14	36.2	3.6	1.10	35.85	2.75	1.33	39.3	1.25
Trihydroxyl									
3α,7α,12α[g]	1.09	36.05	3.45	0.92	35.2	2.1	0.92	37.9	−0.15
3α,7α,12β	1.09	36.05	3.45	0.92	35.2	2.1	1.02	38.3	0.25
3α,7β,12α	1.31	36.75	4.15	1.17	36.1	3.0	1.22	39.0	0.95
3β,7α,12α	1.00	35.7	3.1	0.80	34.75	1.65	0.81	37.45	−0.6
3α,7β,12β	1.34	36.8	4.2	1.05	35.7	2.6	1.28	39.15	1.1
3β,7α,12β	1.06	35.95	3.35	0.80	34.75	1.65	0.83	37.6	−0.45
3β,7β,12α	1.31	36.75	4.15	1.07	35.75	2.65	1.14	38.7	0.65
3β,7β,12β	1.43	37.1	4.5	1.04	35.65	2.55	1.15	38.75	0.7
3α,6α,7α[h]	1.39	36.95	4.35	1.14	36.0	2.9	1.21	38.95	0.9

[a] Operating parameters for the capillary GLC data obtained are listed in Table 1: RRT, relative retention times; MU, methylene units; ΔMU, methylene unit contributions for hydroxyl groups.
[b] Methyl lithocholate.
[c] Methyl chenodeoxycholate.
[d] Methyl ursodeoxycholate.
[e] Methyl deoxycholate.
[f] Methyl hyodesoxycholate.
[g] Methyl cholate.
[h] Methyl hyocholate.

and SP-2340 (75% cyanopropyl silicone). These columns were purchased from Nihon Chromato Works Ltd. (Tokyo, Japan). Nitrogen was used as carrier gas and standard chart speed was 2.5 mm/min. Four to five analyses were made on each sample to establish accurate retention times. Relative retention time (RRT) was expressed relative to the TMSi ether of methyl deoxycholate, and methylene unit (MU) value (21, 22) was determined using n-tetratriacontane (C_{34}), n-hexatriacontane (C_{36}), and n-octatriacontane (C_{38}) as the standard hydrocarbons.

RESULTS AND DISCUSSION

Table 1 shows a complete description of the operating conditions under which the retention data were measured on three capillary columns. The table also contains the polarity of the liquid phases as determined by reported McReynolds constant ($\sum_1^5$) values (23).[2] Thus the polarity increases in the following order, OV-1, OV-17, and SP-2340. Nonselective OV-1 and slightly selective OV-17 phases are presently the most stable of the phases frequently used in columns for bile acid analysis, and they have been reported to give good resolution of bile acid mixtures as well as being good for GLC–mass spectral analysis (1, 3). SP-2340 with a reported upper limit of 275°C for a packed column[2] is a new liquid phase having strong polarity and thermostability.

The use of capillary columns coated with these liquid phases in the analysis of the hydroxylated methyl 5β-cholanate TMSi ethers usually gave sharp peaks with no tailing. For example, two compounds differing in RRT of 0.02 were clearly separated. The values shown in Table 1 were found to be optimal GLC conditions for each of the three columns so far examined.

Table 2 shows the retention data for the TMSi ethers of six monohydroxy, thirteen dihydroxy, and nine trihydroxy methyl 5β-cholanates. The data were expressed as the usual RRT and as the MU values, since the latter are more reproducible between laboratories, less dependent on operating temperature, and permit calculations of group contributions to retention time (21, 22).

The positional and stereochemical isomers of the TMSi ethers of six monohydroxy methyl 5β-cholanates having a hydroxyl function at position C-3, C-7, or C-12 are separated completely on OV-1 capillary column (**Fig. 1**), though OV-17 and SP-2340 columns failed to separate the 7α- and 12α-ol TMSi ethers. Of the four

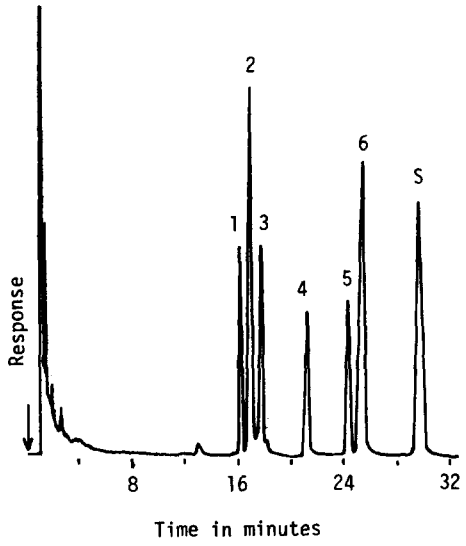

Fig. 1. Gas–liquid chromatographic separation of the methyl ester-trimethylsilyl ethers of monohydroxy bile acid mixtures on OV-1 glass capillary column: 12β-(1),12α-(2), 7α-(3), 7β-(4), 3β-(5), and 3α-(6) hydroxy methyl 5β-cholanate TMSi ethers and methyl deoxycholate TMSi ether (S). GLC conditions refer to Table 1.

stereoisomeric 3,7-diol TMSi ethers, 3α,7β and 3β,7β-diols are inseparable on OV-1 capillary column, but all four epimers are easily resolved on each of the other two columns. Similarly, OV-17 capillary GLC readily resolves the four stereoisomeric 3,12-diol TMSi ethers, though OV-1 and SP-2340 columns failed to completely separate the four epimers. The four stereoisomers of the 7,12-diol TMSi ethers elute before the 3,7- and 3,12-diol TMSi ethers mentioned above; the pair of 7α,12α- and 7α,12β-diol TMSi ethers is the sole inseparable epimeric pair on all the capillary columns examined. Mixtures of twelve 3,7-, 3,12-, and 7,12-diol TMSi ethers were well resolved by the combined use of OV-17 (or OV-1) and SP-2340 columns, and the eluting order from each column differed from the others (**Fig. 2**).

Of the three liquid phases tried, SP-2340 gave the best resolution of the eight stereoisomers of 3,7,12-trihydroxy methyl 5β-cholanate TMSi ethers (**Fig. 3**). The epimeric 3β,7β,12α- and 3β,7β,12β-triol TMSi ethers are only barely separated on SP-2340 column, but can be resolved completely on both OV-1 and OV-17 columns. However, OV-1 and OV-17 columns do not separate some of the other epimeric pairs.

Table 2 also contains the MU contributions for various hydroxyl functions to the parent ester (methyl 5β-cholanate), which are useful in predicting the structure of an unidentified bile acid. It should be noticed here that the observed MU values for di- and trihydroxy compounds do not usually conform with MU values calcu-

[2] Catalog from Nihon Chromato Works Ltd., No. 9, p. 22 (1982).

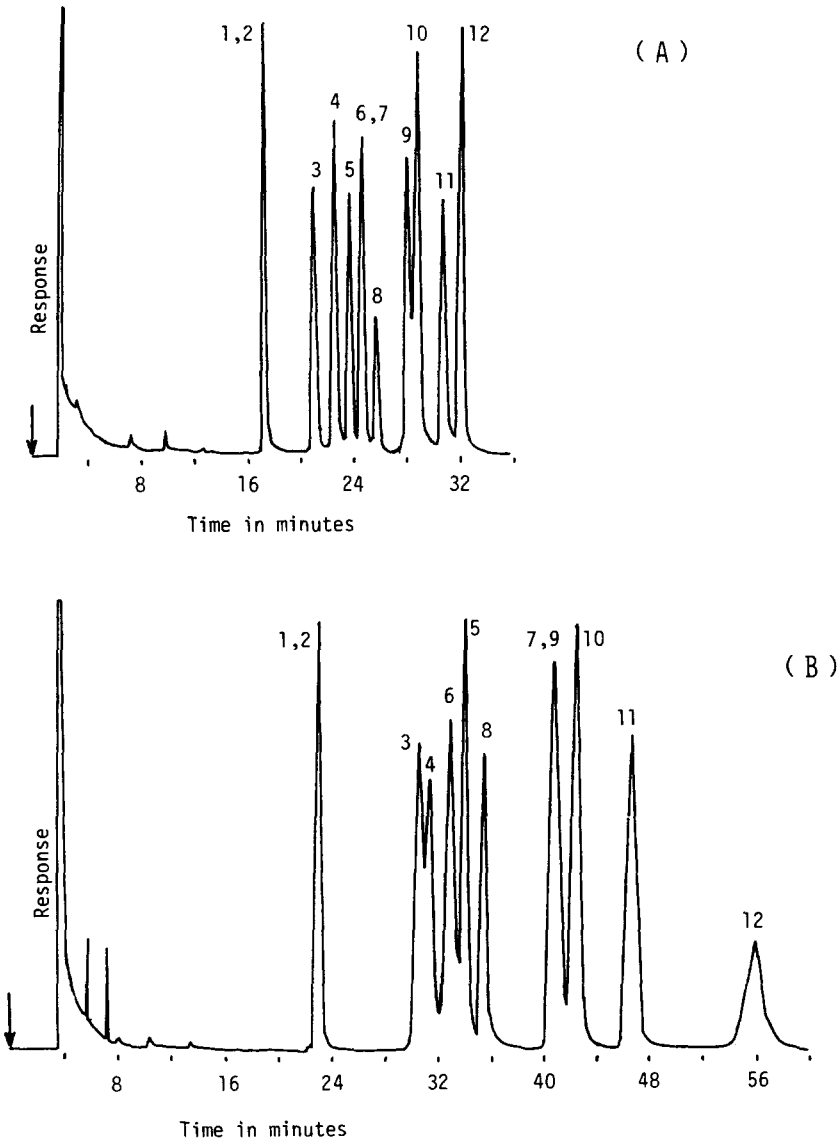

Fig. 2. Gas–liquid chromatographic separation of the methyl ester-trimethylsilyl ethers of dihydroxy bile acid mixtures on OV-17 (A) and SP-2340 (B) glass capillary columns: 7α,12α-(1), 7α,12β-(2), 7β,12β-(3), 7β,12α-(4), 3β,12β-(5), 3β,7α-(6), 3α,12β-(7), 3β,12α-(8), 3α,12α-(9, internal standard), 3α,7α-(10), 3β,7β-(11), and 3α,7β-(12) dihydroxy methyl 5β-cholanate TMSi ethers. GLC conditions refer to Table 1; the time of elution of compound 9 on SP-2340 differs from that mentioned in Table 1 since a different column was used in this separation.

lated by adding the contributions for individual hydroxyl groups to the parent ester. This discrepancy suggests that the retention times of di- and trihydroxy compounds are not predictable on the basis of simple factors, such as the relative effect of a single hydroxyl substituent of specific position and configuration, but are determined by the resultant effect of the complex relationships and interactions of the substituents with each other, with other parts of the molecules, and with the liquid phase used.

As mentioned above, although separation of bile acids is almost complete within any given group (e.g., trihydroxy group), separation of all bile acids is not complete even by capillary GLC (some groups of compounds overlap). However, this unfavorable limitation can be mostly overcome with the combined use of more than one type of capillary column having different polarities, such as the SP-2340 and OV-17 (or OV-1) pair. The final identification of an unknown bile acid metabolite would be made by co-injection with a reference com-

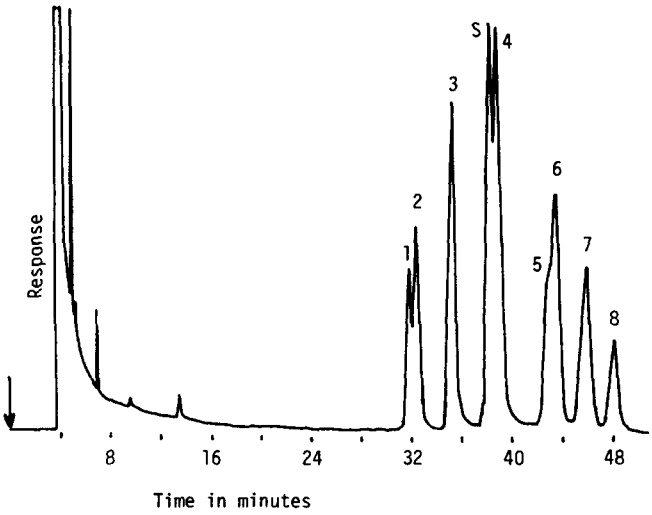

Fig. 3. Gas–liquid chromatographic separation of the methyl ester-trimethylsilyl ethers of trihydroxy bile acid mixtures on SP-2340 glass capillary column: 3β,7α,12α-(1), 3β,7α,12β-(2), 3α,7α,12α-(3), 3α,7α,12β-(4), 3β,7β,12α-(5), 3β,7β,12β-(6), 3α,7β,12α-(7), and 3α,7β,12β-(8) trihydroxy methyl 5β-cholanate TMSi ethers and methyl deoxycholate TMSi ether (S). GLC conditions refer to Table 1.

pound, and further confirmed by mass spectral comparison (24).

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