Capillary gas–liquid chromatographic separation of bile alcohols

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Summary Gas–liquid chromatographic separation of C_{23}, C_{24}, C_{25}, C_{26}, and C_{27} bile alcohols with either 3α,7α-dihydroxylated or 3α,7α,12α-trihydroxylated ring system on two capillary columns, CP-Sil-19 CB and CP-Sil-5 CB, is described. Bile alcohols with two ring hydroxyl groups at 3α- and 7α-positions consistently showed larger retention times on CP-Sil-19 CB columns and smaller retention times on CP-Sil-5 CB columns than the corresponding bile alcohols with three ring hydroxyl groups at 3α-, 7α-, and 12α-positions. Resolutions of all bile alcohols were better on CP-Sil-19 CB columns; however, we hope that the gas–liquid chromatographic characteristics on the two columns will be useful for better identification of bile alcohols in biological fluids.—**Batta, A. K., S. K. Aggarwal, R. Mirchandani, S. Shefer, and G. Salen.** Capillary gas–liquid chromatographic separation of bile alcohols. *J. Lipid Res.* 1992. **33:** 1403–1407.

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Bile alcohols are considered to be the evolutionary predecessors of bile acids (1, 2). These polyhydroxylated steroids were thought to be native to bile of primitive vertebrates only (3); however, several reports of their occurrence in higher animals have recently appeared (4–9). Bile alcohols have also been shown to be obligate intermediates in the biosynthesis of primary bile acids, chenodeoxycholic acid and cholic acid, and accumulate in patients with defective bile acid synthesis, cerebrotendinous xanthomatosis (CTX). Thus, Salen, Shefer, and Berginer (10) reported the presence of large amounts of C_{27}-bile alcohols hydroxylated at C-25 in the bile, urine, and feces of patients with CTX. More recently, Huighebaert et al. (11) reported excretion of several C_{26}- and C_{27}-bile alcohols in the urine of patients with primary biliary cirrhosis while Kibe et al. (9) reported C_{25}- and C_{26}-bile alcohols in the bile of a patient with cholestasis due to gallstone in a common bile duct. Urinary excretion of 27-nor-5β-cholestane-3α,7α,12α,24,25-pentol has been reported in healthy subjects (12) and in increased amounts in patients with cirrhosis (13–15). These bile alcohols have nuclear hydroxyl groups present in cholic acid (3α,7α,12α-trihydroxy-) and only a few bile alcohols with hydroxyl groups present in chenodeoxycholic acid nucleus (3α,7α-dihydroxy-) have so far been reported (16, 17). Gas–liquid chromatography has been generally used for analysis of bile alcohols (9, 15–19), but a systematic study of the gas–liquid chromatography of bile alcohols is lacking (20). In this paper, we describe a capillary gas–liquid chromatographic separation of a number of C_{23}-, C_{24}-, C_{25}-, C_{26}-, and C_{27}-bile alcohols with hydroxyl groups at 3α,7α- and 3α,7α,12α- in the ring system. We hope that the method will facilitate identification of new bile alcohols with the cholic acid and chenodeoxycholic acid nucleus.

EXPERIMENTAL

Materials

The bile alcohols 24-nor-5β-cholane-3α,7α,23-triol and 24-nor-5β-cholane-3α,7α,12α,23-tetrol were prepared by reduction of nor-chenodeoxycholic acid and nor-cholic acid (21) with diborane/tetrahydrofuran (22). The corresponding C_{24}-bile alcohols were similarly prepared starting with chenodeoxycholic acid and cholic acid, respectively, and the C_{25}-bile alcohols were prepared by reduction of homochenodeoxycholic acid and homocholic acid (23), respectively. All other bile alcohols were prepared following literature methods (24–28). All synthesized compounds were > 98% pure as judged by thin-layer chromatography and gas–liquid chromatography (GLC) and their mass spectral fragmentation patterns were completely compatible with their structures.

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 × 0.22 mm I.D.) (Chrompack, Inc., Raritan, NJ) 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, oven temperature was kept at 100°C for 2 min, then

Abbreviations: chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid; cholic acid, 3α,7α,12α-trihydroxy-5β-cholanoic acid; nor-chenodeoxycholic acid, 24-nor-3α,7α-dihydroxy-5β-cholanoic acid; nor-cholic acid, 24-nor-3α,7α,12α-trihydroxy-5β-cholanoic acid; homochenodeoxycholic acid, 3α,7α-dihydroxy-5β-homocholanoic acid; homocholic acid, 3α,7α,12α-trihydroxy-5β-homocholanoic acid; GLC, gas–liquid chromatography; TLC, thin-layer chromatography; rrt, relative retention time; TMS, trimethylsilyl; CTX, cerebrotendinous xanthomatosis.

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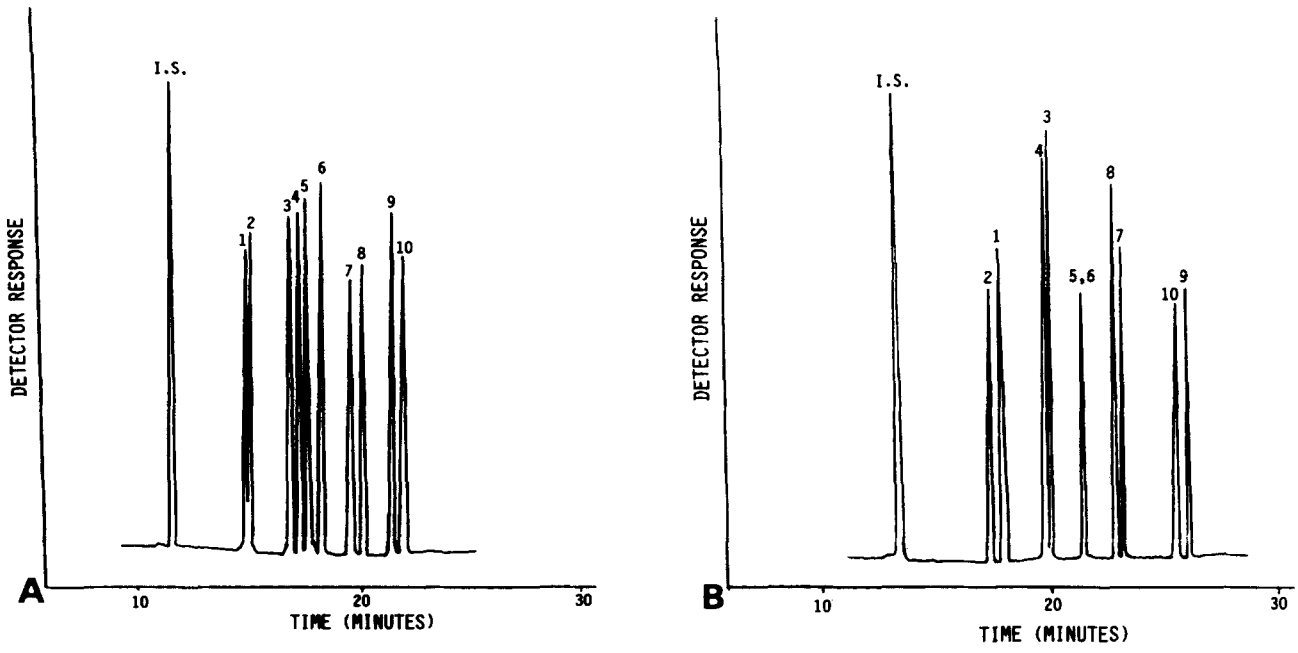

Fig. 1 Gas–liquid chromatogram of the trimethylsilyl ether derivatives of C_{23}, C_{24}, C_{25}, C_{26}, and C_{27} bile alcohols. A: CP-Sil-19 CB column; B: CP-Sil-5 CB column. Peak identification, trimethylsilyl ether of: 1, 24-nor-5β-cholane-3α,7α,12α,23-tetrol; 2, 24-nor-5β-cholane-3α,7α,23-triol; 3, 5β-cholane-3α,7α,12α,24-tetrol; 4, 5β-cholane-3α7α,24-triol; 5, 24-nor-5β-cholestane-3α,7α,12α,25-tetrol; 6, 24-nor-5β-cholestane-3α,7α,25-triol; 7, 5β-homocholane-3α,7α,12α,25-tetrol; 8, 5β-homocholane-3α,7α,25-triol; 9, 5β-cholestane-3α,7α,12α,25-tetrol; 10, 5β-cholestane-3α,7α,25-triol; I.S., internal standard (5α-cholestane).

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 (29).

Derivatization

The bile alcohol (5–10 μg) was reacted with 100μl of Sil-Prep (hexamethyldisilazane–trimethylchlorosilane–pyridine 3:1:9; Alltech Associates, Inc., Deerfield, IL) for 20 min at 55°C. Solvents were evaporated at 55°C under N_2 and the trimethylsilyl (TMS) ether derivative formed was taken up in 100 μl of hexane. One μl was injected into the GLC column simultaneously with 5α-cholestane, the internal standard. The retention times of the various bile alcohols (rrt) were calculated relative to that of 5α-cholestane. Also, the retention index values (Kovats) for the derivatized bile alcohols were determined by comparison with the retention times of C_{29}–C_{37} n-alkanes (30).

RESULTS AND DISCUSSION

Bile alcohols with 4–8 carbons in the side chain and either 3α,7α-dihydroxy groups (chenodeoxycholic acid derivatives) or 3α,7α,12α-trihydroxy groups (cholic acid derivatives) in the ring system were chromatographed as their trimethylsilyl ether derivatives on two different capillary columns, CP-Sil-19 CB and a slightly less polar column, CP-Sil-5 CB. It was found that all ten bile alcohols studies were well resolved when chromatographed on the CP-Sil-19 CB column (**Fig. 1A**) but 24-nor-5β-cholestane-3α,7α,25-triol showed almost the same retention time as 24-nor-5β-cholestane-3α,7α,12α,25-tetrol on the CP-Sil-5 CB column (Fig. 1B). Retention times were highly reproducible on both columns and for amounts of bile alcohols ranging from 5 ng to 100 ng injected onto each column, the detector response, as shown by the integrator, was linear. Further, the recoveries of the various compounds were similar to that of 5α-cholestane and the correction factors for all compounds corresponding to 5α-cholestane were 0.95–1.1 on both columns.

We had previously used CP-Sil-5 CB capillary column for gas–liquid chromatography of common bile acids, and we had observed that the rrt of the methyl ester trimethylsilyl ether of chenodeoxycholic acid was less than the corresponding derivative of cholic acid (29). As seen from **Table 1**, bile alcohols show a similar behavior: compounds with the 3α,7α-dihydroxy system (chenodeoxycholic acid) have smaller rrt values and retention indices

TABLE 1. GLC retention times of the trimethylsilyl ethers of bile alcohols

Bile alcohol	Relative Retention Times[a]		Retention Indices (Kovats)	
	CP-Sil-19	CP-Sil-5	CP-Sil-19[b]	CP-Sil-5[c]
24-Nor-5β-cholane-3α,7α,23-triol	1.32[d]	1.32	3199	3146
24-Nor-5β-cholane-3α,7α,12α,23-tetrol	1.29[d]	1.35	3186	3166
5β-Cholane-3α,7α,24-triol	1.49	1.51[d]	3298	3251
5β-Cholane-3α,7α,12α,24-tetrol	1.45	1.53[d]	3278	3260
5β-Homocholane-3α,7α,25-triol	1.75	1.75	3405	3360
5β-Homocholane-3α,7α,12α,25-tetrol	1.70	1.78	3385	3370
24-Nor-5β-cholestane-3α,7α,25-triol	1.59	1.64[e]	3339	3311
24-Nor-5β-cholestane-3α,7α,12α,25-tetrol	1.53	1.64[e]	3314	3311
5β-Cholestane-3α,7α-diol	1.33	1.37[f]	3212	3174
5β-Cholestane-3α,7α,12α-triol	1.30	1.39[f]	3195	3187
5β-Cholestane-3α,7α,25-triol	1.92	1.96	3462	3436
5β-Cholestane-3α,7α,12α,25-tetrol	1.87	2.01	3447	3451
5β-Cholestane-3α,7α,26-triol	2.13	2.13[d]	3526	3491
5β-Cholestane-3α,7α,12α,26-tetrol	2.04	2.15[d]	3502	3496
5β-Cholestane-3α,7α,24R,25-tetrol	2.52	2.61[d]	3624	3615
5β-Cholestane-3α,7α,12α,24R,25-pentol	2.40	2.63[d]	3597	3619
5β-Cholestane-3α,7α,24S,25-tetrol	2.57	2.66[e]	3635	3628
5β-Cholestane-3α,7α,12α,24S,25-pentol	2.44	2.66[e]	3607	3627

Fused silica CP-Sil-19 CB and CP-Sil-5 CB capillary columns (20 M × 0.22 mm) were used for GLC. Chromatographic conditions were as described in the Experimental section.

[a]Retention times of the trimethyl ether derivatives of the bile alcohols are given relative to that of 5α-cholestane. Retention time of 5α-cholestane was 11.65 min on CP-Sil-19 CB column and 13.20 min on CP-Sil-5 CB column.

[b]Kovats values determined by previous injection of a hydrocarbon mixture C_{31}–C_{37} under GC conditions identical to those employed when CP-Sil-19 CB column was used, and comparison of retention times. The retention times of the various n-alkanes were as follows: C_{31}, 13.52 min; C_{32}, 15.26 min; C_{33}, 17.46 min; C_{34}, 20.22 min; C_{35}, 23.71 min; C_{36}, 28.12 min; and C_{37}, 33.67 min.

[c]Kovats values determined by previous injection of a hydrocarbon mixture C_{31}–C_{37} under GC conditions identical to those employed when CP-Sil-5 CB column was used, and comparison of retention times. The retention times of the various n-alkanes were as follows: C_{31}, 16.49 min; C_{32}, 18.61 min; C_{33}, 21.24 min; C_{34}, 24.50 min; C_{35}, 28.54 min; C_{36}, 33.56 min and C_{37}, 39.79 min.

[d]The GC peaks were separated from each other, but did not show base-line resolution.

[e]The peaks did not separate from each other.

[f]The peaks showed base-line resolution.

than the corresponding derivatives with 3α,7α,12α-trihydroxy system (cholic acid). On the other hand, the retention indices of the compounds with 3α,7α,12α-trihydroxy groups in the ring system were consistently lower than those of the corresponding compounds with a 3α,7α-dihydroxy system when chromatographed on the more polar CP-Sil-19 CB column as observed by Koopman et al. (31) for some derivatives of chenodeoxycholic acid and cholic acid.

The bile alcohol profile in the urine of patients with CTX is highly complex (18), and in **Fig. 2** we have compared the urinary bile alcohols in a CTX patient on both columns. The presence of 5β-cholestane-3α,7α,12α,25-tetrol, 5β-cholestane-3α,7α,12α,24R,25-pentol and 5β-cholestane-3α,7α,12α,24S,25-pentol was demonstrated from their retention indices in the two columns. Also, two isomers of 5β-cholestane-3α,7α,12α,23,25-pentol and a 5β-cholestane-3α,7α,12α,24,25,26-hexol were identified by gas–liquid chromatography–mass spectrometry. The presence of bile alcohols with chenodeoxycholic acid ring structure could not be confirmed on the CP-Sil-5 CB column, since, the trimethylsilyl ethers of 5β-cholestane-3α,7α,12α,24S,25-pentol and 5β-cholestane-3α,7α,24S,25-tetrol are inseparable (Table 1). However, these compounds are separated on the CP-Sil-19 CB column, and when the derivatized urinary bile alcohols in the CTX patient were chromatographed on this column, no peaks due to the derivatized bile alcohols with 3α,7α-dihydroxy system were observed, thus confirming the absence of bile alcohols with chenodeoxycholic acid ring system in this disease.

An important advantage of using the polar and nonpolar capillary columns is that the retention times for compounds with chenodeoxycholic acid ring hydroxyla-

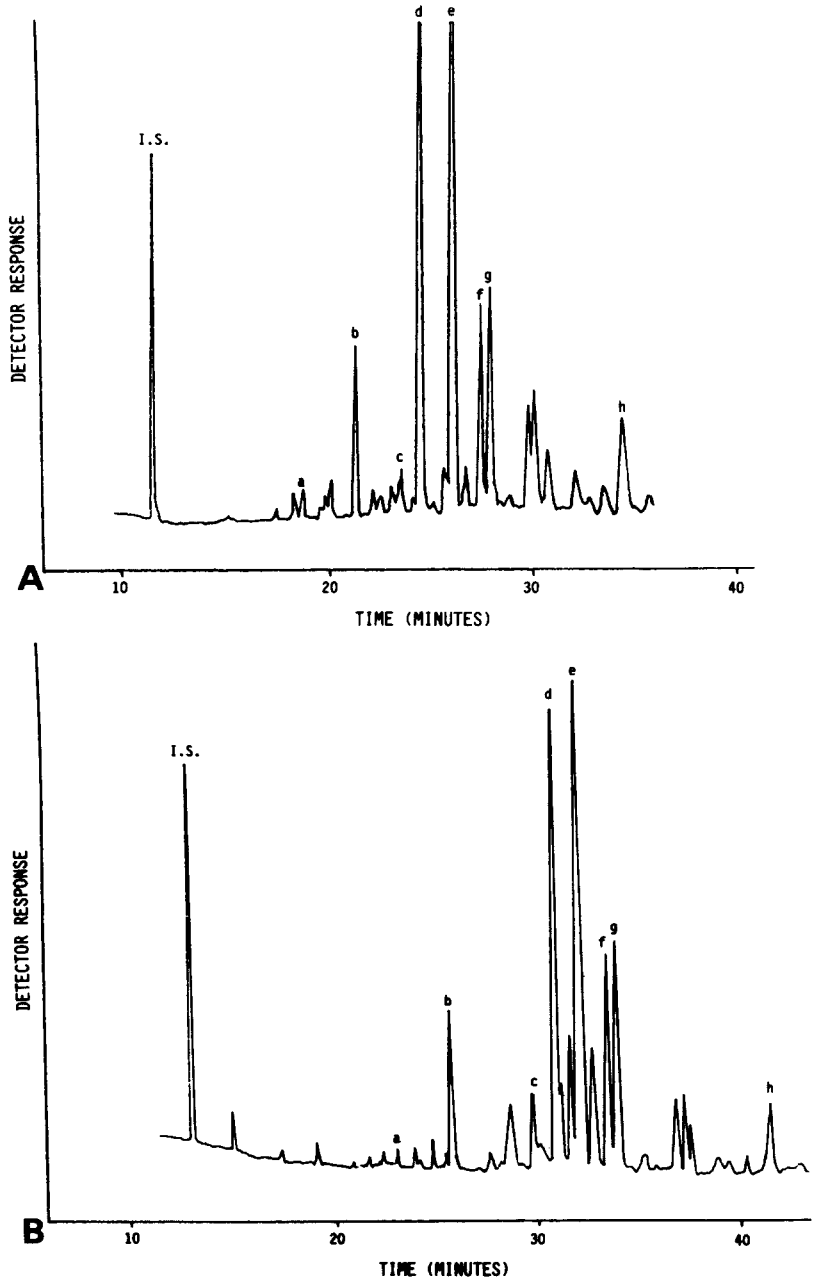

Fig. 2. Gas–liquid chromatogram of the trimethylsilyl ether derivatives of bile alcohols in the urine of a patient with cerebrotendinous xanthomatosis. A: CP-Sil-19 CB column; B: CP-Sil-5 CB column. Peak identification, trimethylsilyl ether of: a, 5β-cholestane-3α,7α,12α,23-tetrol; b, 5β-cholestane-3α,7α,12α,25-tetrol; c, 5β-cholestane-3α,7α,12α,22,25-pentol; d, 5β-cholestane-3α,7α,12α,23,25-pentol; e, 5β-cholestane-3α,7α,12α,23,25-pentol (isomer of d); f, 5β-cholestane-3α,7α,12α,24R,25,pentol; g, 5β-cholestane-3α,7α,12α,24S,25-pentol; h, 5β-cholestane-3α,7α,12α,24,25,26-hexol; I.S., internal standard (5α-cholestane).

tions are consistently higher than those for bile alcohols with cholic acid ring hydroxylations on the more polar CP-Sil-19 CB and they are consistently lower on the less polar CP-Sil-5 CB column. The application of such a combination of polar and nonpolar columns has recently been demonstrated by Iida et al. (32) for a number of bile acids. Such measurements may help in the routine analysis of serum bile alcohols in patients with liver disease, where very small amounts of samples are available and gas–liquid chromatography is the only means of characterization.

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