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Journal of Chromatography A, 766 (1997) 286–291

JOURNAL OF CHROMATOGRAPHY A

Short communication

Capillary gas–liquid chromatography of acetate–methyl esters of bile acids

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Received 18 September 1996; revised 26 November 1996; accepted 28 November 1996

Abstract

Gas–liquid chromatographic separations of acetate–methyl esters of several common bile acids with and without a hydroxyl group at C-6 are compared with those of the corresponding trimethylsilyl ether–methyl esters on a CP-Sil-5 CB capillary column. Unlike the trimethylsilyl ether derivatives, the retention indices of the corresponding acetates were greatly influenced by the number of hydroxyl groups in the ring system. Epimeric hydroxyl groups at carbons 6, 7 as well as 12 increased retention index of the acetate–methyl esters of the bile acids, the effect of the 7β-hydroxyl group being most prominent. The 6β-acetoxyl group increased the retention index more than the 6α-acetoxy group and contrary to the trimethylsilyl ether derivatives, a 6β,7β-diacetoxy group showed larger increase in the retention index than the corresponding 6α,7β-diacetoxy group. The acetate derivatives of bile acid–methyl esters show larger retention times and reduced sensitivity than the corresponding trimethylsilyl ether derivatives. However, gas chromatography of bile acid acetate–methyl esters can be very useful for the characterization of bile acids and for bile acid analysis in the rat where muricholic acids and hyodeoxycholic acid are in abundance, since these bile acids are difficult to resolve from each other and from other common bile acids as the trimethylsilyl ether derivatives.

Keywords: Derivatization, GC; Bile acids; Muricholic acids

1. Introduction

Since the introduction of capillary columns for gas–liquid chromatography (GLC), bile acids have been routinely analyzed as the trimethylsilyl (TMS) ether–methyl ester derivatives apparently because of the ease of preparation and good resolution of bile acids on different capillary columns. Although the TMS ether–methyl esters of most of the common bile acids are well resolved [1,2], several bile acids pose difficulty in resolution when present together. This is specially the case for rat fecal and biliary bile acids where chenodeoxycholic, cholic, hyodeoxycholic and α-muricholic acids are present [3] and need to be resolved from each other. The conventional gas chromatographic methods do not completely resolve all these compounds in a mixture [2]. We were faced with this paradox in the course of our studies on the effect of cholic acid on rat biliary bile acid pattern. In our attempt to find other alternatives, we found that even though the *tert*-butyldimethyl-

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PII S0021-9673(96)01016-3

silyl ether–methyl ester derivatives of bile acids were better resolved than the corresponding TMS ether derivatives, the highly increased retention times of the bile acids posed limitation to the use of this derivatization method. It has been recently shown that dimethylethylsilyl ether–methyl esters of bile acids could be preferable to the TMS ether–methyl esters [4,5]; however, we found that the acetate–methyl esters of chenodeoxycholic, cholic, hyodeoxycholic and α-muricholic acids were very well resolved from each other and could be easily quantitated by gas–liquid chromatography. Since bile acids hydroxylated at C-6 are present in several animal species [6,7] and are excreted in the urine of patients with hepatobiliary diseases [8–11], we have attempted the GLC resolution of the acetate–methyl esters of several common bile acids with and without hydroxyl group at C-6 and compared their GLC retention indices with those of the corresponding TMS ether–methyl esters. Since most of the bile acid derivatives studied are well resolved as acetate–methyl esters on the column employed, we hope that the method will be useful for characterization of bile acids in biological fluids particularly where 6-hydroxy bile acids are suspected.

2. Experimental

Cholic, chenodeoxycholic, deoxycholic, lithocholic and 3α,6β-dihydroxy-5β-cholanoic acids were purchased from Steraloids, (Wilton, NH, USA). Hyodeoxycholic acid and hyocholic acid were from Canada Packers (Toronto, Canada). Ursodeoxycholic and ursocholic acids were gifts from Tokyo Tanabe, Japan. α-, β- and ω-muricholic acids were synthesized from chenodeoxycholic acid and the tetrahydroxy bile acids, 3α,6α,7α,12α-tetrahydroxy-, 3α,6β,7α,12α-tetrahydroxy- and 3α,6α,7β,12α-tetrahydroxy-5β-cholanoic acids were prepared from cholic acid and 3α,6β,7β,12β-tetrahydroxy-5β-cholanoic acid was prepared from ursocholic acid following literature methods [12–15]. Methyl esters of the bile acids were prepared by addition of 0.5–1 ml of 3% anhydrous methanolic hydrochloric acid (Aldrich, 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 N_2 and the methyl ester was crystallized from either pure methanol or 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. Pyridine and acetic anhydride were purchased from Aldrich and Sil-prep (hexamethyldisilazane–trimethylchlorosilane–pyridine, 3:1:9) used for preparation of TMS ether derivatives of the bile acid metyl esters was purchased from Alltech Associates (Deerfield, IL, USA).

2.1. Gas–liquid chromatography

A Hewlett-Packard model 5890A 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-5 CB (stationary phase, 100% dimethylsiloxane) capillary column (25 m×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, oven temperature was kept at 100°C for 2 min, then programmed at a rate of 35 C°/min to a final temperature of 278°C for analysis of the TMS ether derivatives [2] and 287°C for analysis of the acetate derivatives.

2.2. Derivatization

Acetates of bile acid methyl esters: The bile acid methyl ester (5–10 μg) was treated with 50 μl of each of anhydrous pyridine and distilled acetic anhydride and the reaction mixture was heated overnight at 45°C followed by evaporation to dryness at 45°C under a stream of nitrogen. The acetyl derivative formed was taken in 100 μl of hexane and 1–2 μl was injected without prior cleanup into the GLC column simultaneously with 5α-cholestane used as the internal standard.

2.3. TMS ethers of bile acid methyl esters

The bile acid methyl ester (5–10 μg) was reacted with 100 μl of Sil-Prep for 30 min at 55°C. Solvents

were evaporated at 55°C under N_2 and the TMS ether derivative formed was taken in 100 μl of hexane. One μl was injected into the GLC column simultaneously with 5α-cholestane. The retention times of the various bile acids (RRT) were calculated relative to that of 5α-cholestane. Also, the retention index values (Kováts) for the derivatized bile acids were determined by comparison with the retention times of C_{29}–C_{37} *n*-alkanes [16].

2.4. Isolation of rat fecal bile acids

Lyophilized rat faeces (50 μg) was repeatedly extracted with 1% ammoniacal methanol in screw capped tubes (4 ml×4; 1 h, at 70°C) and methanol was decanted and pooled. After filtration through filter paper, the combined methanol extract was evaporated to dryness under reduced pressure at 45°C. The residue was resuspended in 1 ml of 4 *M* sodium hydroxide and heated in an autoclave at 110°C for 4 h. After cooling, 1 ml of water was added and neutral compounds were extracted with hexane (3 ml×4). The aqueous solution was then acidified with 50% hydrochloric acid to pH 1 and bile acids were extracted with ethyl acetate (3 ml× 4), the ethyl acetate extract was washed with water to neutrality and evaporated to dryness [17]. Bile acids were methylated with methanolichydrochloric acid and aliquots were used to make the trimethylsilyl ethers and acetate derivatives for injection into the gas chromatograph without prior cleanup.

3. Results and discussion

In the course of our studies on biliary and fecal bile acids in the rat, we could not completely resolve and quantitate the TMS ethers of the methyl esters of hyodeoxycholic acid and α-muricholic acid from those of cholic acid and chenodeoxycholic acid on CP-Sil-5 CB capillary GLC column that was routinely employed for GLC purposes. To devise an alternative method for resolution of these compounds, we examined the GLC behaviour of their acetate–methyl esters. GLC of bile acid acetate-methyl esters has been well documented on packed columns [17–20] as well as capillary columns [21,22]. However, acetates are less volatile than the corresponding TMS ethers, therefore, higher column temperatures are needed for chromatography. But, the acetates have a distinct advantage that these derivatives are highly stable, can be reused and the parent bile acids can usually be reisolated after alkaline hydrolysis [19]. We found that the acetate-methyl esters of all four bile acids in question showed a baseline separation from each other when analyzed as the acetate–methyl esters and could be easily quantitated. Encouraged by this, we subjected the acetate–methyl esters of several bile acids and their 6-hydroxylated derivatives to gas chromatography to see if this mode of derivatization is comparable to trimethylsilylation. The retention indices (Kováts values) of the various bile acid acetate–methyl esters are given in Table 1 and those with an additional hydroxyl group at C-6 are given in Table 2. Retention indices of all bile acid derivatives were highly reproducible and for amounts of bile acids ranging from 0.02 μg to 0.2 μg injected onto the column, the detector response, as shown by the integrator, was linear.

As seen from Table 1, most bile acid acetate–methyl esters with epimeric acetate groups at C-3, C-7 and C-12 are well resolved from each other. Acetate groups at both C-7 and C-12 increase the retention index of lithocholic acid or isolithocholic acid, the effect being most pronounced with the 7β-acetate group. This is in parallel with the effect seen for the TMS ether derivatives of these bile acids. However, unlike for the TMS ethers [2], the acetate groups at C-7 and C-12 show an additive effect, so that the acetate methyl ester of cholic acid is eluted substantially later than that of chenodeoxycholic acid (Table 1). Bile acids with 12β-acetate are eluted later than the corresponding 12α-acetoxy compounds whereas the 12β-TMS compounds are generally eluted earlier than the 12α-TMS compounds [2] (Table 1). Thus knowledge of the retention time for the acetate and TMS ether can help establish configuration of the hydroxyl group at C-12 in the bile acid.

As in case of the TMS derivatives, 6α- and 6β-acetate groups increased the retention index of a bile acid, but unlike the TMS ethers, where the axial 6α-TMS group exerts an additive effect on the retention index of the bile acid with either a 7α- or 7β-TMS group but the equatorial 6β-TMS group does not, both 6α- and 6β-acetate groups showed

Table 1
GLC retention indices of trimethylsilyl ether–methyl esters and acetate–methyl esters of bile acids on CP-Sil-5 CB capillary column

5β-Cholanoic acid	Relative retention time		Retention index	
	TMS	Acetate	TMS	Acetate
3α-Hydroxy- (lithocholic acid)	1.340	1.433	3187	3179
3α-Hydroxy-Δ^6- (Δ^6-lithocholic acid)	1.292	1.359	3157	3136
3α-Hydroxy-Δ^7- (Δ^7-lithocholic acid)	1.340	1.430	3186	3177
3β-Hydroxy- (isolithocholic acid)	1.340	1.405	3187	3162
3α,7α-Dihydroxy- (chenodeoxycholic acid)	1.494	1.717	3270	3316
3α,7β-Dihydroxy- (ursodeoxycholic acid)	1.567	1.946	3306	3405
3β,7α-Dihydroxy- (isochenodeoxycholic acid)	1.420	1.724	3232	3319
3β,7β-Dihydroxy- (isoursodeoxycholic acid)	1.589	1.947	3316	3405
3α,12α-Dihydroxy- (deoxycholic acid)	1.451	1.589	3247	3260
3α,12β-Dihydroxy-	1.402	1.750	3221	3331
3α,7α,12α-Trihydroxy- (cholic acid)	1.528	1.785	3288	3344
3α,7β,12α-Trihydroxy- (ursocholic acid)	1.625	2.132	3332	3464
3α,7α,12β-Trihydroxy-	1.490	2.094	3268	3453
3α,7β,12β-Trihydroxy-	1.623	2.367	3313	3531

Retention times are expressed relative to that of 5α-cholestane. Retention time of 5α-cholestane was 13.671 min when the final temperature was 278°C and 12.665 min when the final temperature was 287°C. Retention indices (Kováts values) were determined by previous injection of a C_{31}–C_{37} hydrocarbon mixture under identical GLC conditions. The retention times of the various *n*-alkanes were as follows: C_{31}, 16.485 min; C_{32}, 18.606 min; C_{33}, 21.235 min; C_{34}, 24.496 min; C_{35}, 28.543 min; C_{36}, 33.562 min and C_{37}, 39.788 min.

Table 2
GLC retention indices of trimethylsilyl ether–methyl esters and acetate–methyl esters of 6-hydroxylated bile acids on CP-Sil-5 CB capillary column

5β-Cholanoic acid	Relative retention time		Retention index	
	TMS	Acetate	TMS	Acetate
3α,6α-Dihydroxy- (hyodeoxycholic acid)	1.519	1.926	3282	3397
3α,6β-Dihydroxy- (murocholic acid)	1.490	1.945	3268	3405
3α,6α,7α-Trihydroxy- (hyocholic acid)	1.687	2.091	3361	3452
3α,6β,7α-Trihydroxy- (α-muricholic acid)	1.496	2.157	3270	3471
3α,6α,7β-Trihydroxy- (ω-muricholic acid)	2.033	2.404	3483	3539
3α,6β,7β-Trihydroxy- (β-muricholic acid)	1.725	2.530	3374	3572
3α,6α,7α,12α-Tetrahydroxy-	1.709	2.164	3368	3474
3α,6β,7α,12α-Tetrahydroxy-	1.518	2.256	3282	3501
3α,6β,7β,12α-Tetrahydroxy-	1.635	2.720	3335	3615
3α,6β,7β,12β-Tetrahydroxy-	1.745	3.121	3382	3696

Retention times are expressed relative to that of 5α-cholestane. Relative retention times and retention indices were measured as described in Table 1.

similar additive effect on the retention time of the bile acid (Table 2). Further effect of a TMS group at 12α-position is generally small but acetate at 12α- or 12β-position increased the retention time of the bile acid even in the presence of a 6-hydroxyl group so that the tetrahydroxy compound 3α, 6β, 7β, 12β-tetrahydroxy-5β-cholanoic acid was eluted at 39.531 min (Table 2).

In Fig. 1 are shown the GLC chromatograms of several naturally occurring bile acids hydroxylated at C-3, 6, 7 and 12 that are commonly found in humans and in the rat. As can be seen, resolution between various bile acids when present together is much better for the acetates (Fig. 1B) than for the TMS ether derivatives (Fig. 1A). The GLC chromatograms of the TMS ether–methyl esters and acetate–methyl esters of the fecal bile acids in a Fisher 344 rat after 0.4% cholic acid feeding for 4 weeks are shown in Fig. 2A and Fig. 2B. Chenodeoxy-cholic, α-muricholic, cholic and hyodeoxycholic acids were all completely resolved when injected as the acetate-methyl esters (Fig. 2B) and a GLC peak with the retention index of hyodeoxycholic acid was observed (structure was confirmed by comparison of mass spectral fragmentation pattern with that of an authentic standard). When injected as the TMS ether-methyl esters, these four compounds appeared as two unresolved peaks (Fig. 2A). Thus, hyodeoxycholic acid was not identified in this bile sample when the bile acids were injected as the TMS ether-methyl ester derivatives but was clearly identified when injected as the acetate–methyl esters.

Effect of the length of side chain on retention index of bile acid acetate–methyl esters is shown in Table 3. Similar effects were seen for both the TMS ether derivatives and the acetates of the bile acid

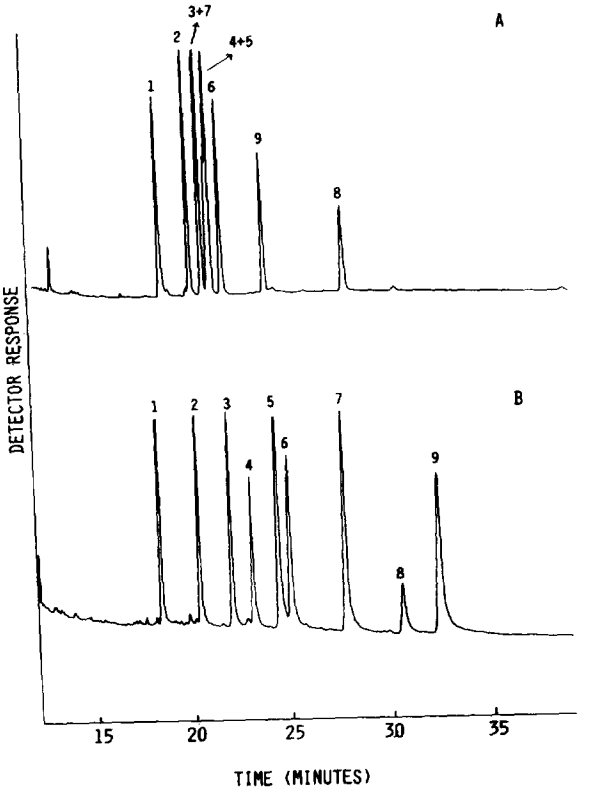

Fig. 1. GLC chromatogram of bile acids: A, trimethylsilyl ether–methyl esters; B, acetate–methyl esters. Peak identification, derivatives of: 1=3α-hydroxy-5β-cholanoic acid; 2=3α,12α-dihydroxy-5β-cholanoic acid; 3=3α,7α-dihydroxy-5β-cholanoic acid; 4=3α,7α,12α-trihydroxy-5β-cholanoic acid; 5=3α,6α-dihydroxy-5β-cholanoic acid; 6=3α,7β-dihydroxy-5β-cholanoic acid; 7=3α,6β,7α-trihydroxy-5β-cholanoic acid; 8=3α,6α,7β-trihydroxy-5β-cholanoic acid; 9=3α,6β,7β-trihydroxy-5β-cholanoic acid.

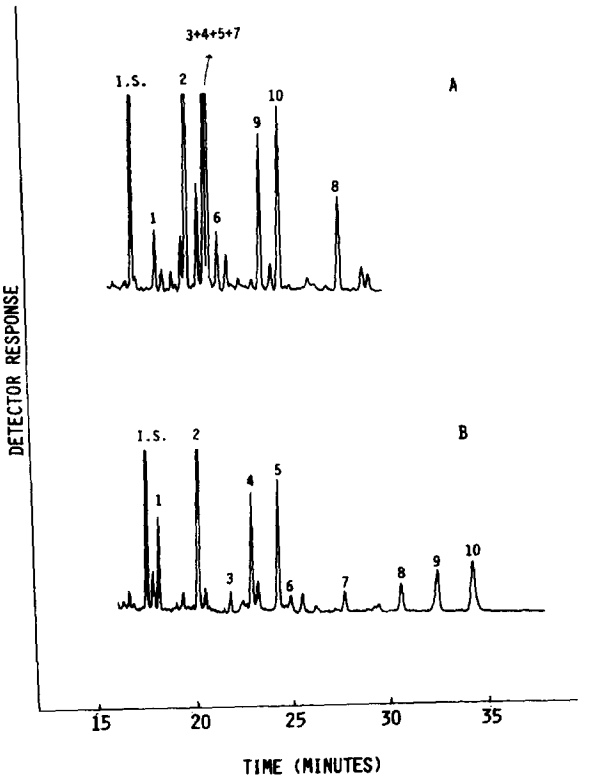

Fig. 2. GLC chromatogram of fecal bile acids in Fisher 344 rat after feeding 0.4% cholic acid: A, trimethylsilyl ether–methyl esters of the bile acids; B, acetate–methyl esters of the bile acids. Peak identification: peaks 1–9, same as in Fig. 1; peak 10=Δ^{22}-3α,6β,7β-trihydroxy-5β-cholanoic acid.

esters. Thus, both derivatives of nor-bile acids were eluted earlier whereas for bile acids with increased chain length, like the C_{25}- and C_{27}-bile acids, the retention indices increased in the same manner (Table 3).

In summary, we have shown that the acetate derivatives of bile acid methyl esters are generally better resolved on capillary GLC than the TMS ethers and may prove valuable for positive characterization of bile acids in an unknown sample. Further, acetates are stable derivatives and can be stored for long periods of time. In this way, a sample can also be used at a later time and stock solutions of reference standards can be stored for routine analysis. On the other hand, we found that the peak heights for the bile acid acetate–methyl esters were reduced on increasing the number of acetoxyl groups, so that the sensitivity was reduced with tri- and tetraacetoxy compounds. Nevertheless, we believe that acetates are a good alternative for the TMS ethers for capillary GLC, when better resolution or a more complete identification of the bile acid is required.

Acknowledgments

This work was supported by US Public Health Service grant HL-17818, a grant from the Veterans Affairs Research Service, Washington, DC. and National Cancer Institute grant CA-41108. The con-

Table 3
GLC retention indices of trimethylsilyl ether–methyl esters and acetate–methyl esters of bile acids with C_4-, C_6- and C_8-side chain on CP-Sil-5 CB capillary column

5β-Cholanoic acid	Relative retention time		Retention index	
	TMS	Acetate	TMS	Acetate
23-Nor-3α,7α-dihydroxy-(norchenodeoxycholic acid)	1.283	1.451	3151	3189
23-Nor-3α,12α-dihydroxy-(nordeoxycholic acid)	1.256	1.390	3134	3155
23-Nor-3α,7β-dihydroxy-(norursodeoxycholic acid)	1.335	1.635	3183	3281
23-Nor-3α,7α,12α-trihydroxy-(norcholic acid)	1.336	1.539	3185	3235
25-Homo-3α,7α-dihydroxy-(homochenodeoxycholic acid)	1.707	1.958	3366	3408
25-Homo-3α,7β-dihydroxy-(homoursodeoxycholic acid)	1.803	2.262	3405	3503
25-Homo-3α,7α,12α-trihydroxy-(homocholic acid)	1.752	2.038	3384	3435
3α,7α,12α-Trihydroxy-5β-cholestanoic acid	2.104	2.475	3504	3558

Retention times are expressed relative to that of 5α-cholestane. Relative retention times and retention indices were measured as described in Table 1.

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