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Short communication

Capillary gas chromatographic analysis of serum bile acids as the *n*-butyl ester–trimethylsilyl ether derivatives

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Abstract

Gas chromatographic separations of *n*-butyl ester-trimethylsilyl ether derivatives of several common bile acids were compared with those of the corresponding methyl ester-trimethylsilyl ether derivatives on a CP-Sil-5 CB capillary column. Both types of derivatives were similarly resolved from each other. However, the *n*-butyl ester-trimethylsilyl ether derivatives of the bile acids showed longer retention times than the corresponding methyl ester-trimethylsilyl ethers and unlike the methyl ester-trimethylsilyl ether derivatives, were completely resolved from and eluted later than the trimethylsilyl ethers of common plasma sterols including sitosterol. A simplified method of plasma work-up for quantitation of bile acids and application of the above method in quantification of plasma bile acids in humans is described. © 1998 Elsevier Science B.V.

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1. Introduction

Since the introduction of capillary columns for gas chromatography (GC), bile acids have been routinely analyzed as the methyl ester-trimethylsilyl (TMS) ether derivatives apparently because of the ease of preparation and good resolutions on different capillary columns. Although the methyl ester-TMS ethers of most common bile acids are well resolved from each other [1,2], overlaps of cholesterol with lithocholic acid may occur during plasma bile acid analysis. To avoid this interference due to plasma sterols, we needed to derivatize bile acids so that even lithocholic acid (3α-hydroxy-5β-cholanoic acid), with the least retention time of the common C_{24}-bile acids as the methyl ester-TMS ether, would elute sufficiently later than the sterols. Derivatization of the hydroxyl groups in the bile acids to form acetates, methyl ethers or dimethylethylsilyl ethers [1,3–7] increased retention times of both sterols and bile acids. To increase the retention times of bile acids, Tsaconas et al. [8] prepared their isobutyl esters, since esterification of bile acids with homologs of methanol increased the retention times of their TMS ethers [9], and they were able to resolve the isobutyl ester-TMS ether of lithocholic

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acid from the TMS ethers of cholesterol and certain other sterols [8]. However, we found that although the isobutyl esters were eluted later than cholesterol on the CP-Sil-5 CB capillary column, sitosterol, an important sterol in plasma from patients with the lipid storage disease, sitosterolemia [10], showed longer retention time than lithocholic acid. Furthermore, nor-deoxycholic acid (3α,12α-dihydroxy-24-nor-5β-cholan-23-oic acid) and nor-cholic acid (3α,7α,12α-trihydroxy-24-nor-5β-cholan-23-oic acid), that were routinely used as internal recovery standards, were also eluted in the same region as the sterols. We tested esters of bile acids with other short-chain alcohols and found that the *n*-butyl ester-TMS ether derivatives of lithocholic acid, deoxycholic acid (3α,12α-dihydroxy-5β-cholanoic acid), chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholanoic acid), cholic acid (3α,7α,12α-trihydroxy-5β-cholanoic acid), ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholanoic acid) and ursocholic acid (3α,7β,12α-trihydroxy-5β-cholanoic acid) were all resolved from each other and from both cholesterol and sitosterol and the method could be adapted to determine plasma bile acid composition with no possible contamination due to plasma sterols.

2. Experimental

2.1. Reagents and chemicals

Cholic, chenodeoxycholic, deoxycholic and lithocholic acids were purchased from Steraloids (Wilton, NH, USA). Ursodeoxycholic and ursocholic acids were gifts from Tokyo Tanabe, Japan. Nor-deoxycholic acid and nor-cholic acid were prepared according to the method of Schteingart and Hofmann [11]. Methyl esters of the bile acids were prepared by addition of 100 μl of 3% anhydrous methanolic hydrochloric acid (Aldrich, Milwaukee, WI, USA) to 5–20 μg of the respective bile acid and keeping at room temperature for 2 h followed by evaporation of solvent at 55°C under N_2. The *n*-butyl esters were prepared by addition of 100 μl of *n*-butanol to 5–20 μg of the bile acid followed by addition of 20 μl of a 40% solution of hydrogen chloride in dioxane. The reaction mixtures were heated at 60°C for 4 h followed by overnight at room temperature and then solvents were evaporated at 60°C under N_2. All compounds were >98% pure as judged by GC of the TMS derivatives and exhibited mass spectral fragmentation patterns compatible with their structures. Sil-Prep [hexamethyldisilazane–trimethylchlorosilane–pyridine (3:1:9)] used for preparation of TMS ether derivatives of the bile acid esters was purchased from Alltech Associates (Deerfield, IL, USA).

2.2. Gas chromatography

A Hewlett-Packard Model 6890 gas chromatograph equipped with a flame ionization detection system 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., df 0.25 μm) (Chrompack, Raritan, NJ, USA) and helium was used as the carrier gas. The GC 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 [12].

2.3. Trimethylsilylation

The esterified bile acid (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 and 1 μl was injected into the GC column. The retention times of the various bile acids were calculated relative to that of nor-deoxycholic acid.

2.4. Isolation of plasma bile acids

To plasma (1 ml) were added nor-cholic acid (10 μg in 100 μl methanol), acetate buffer pH, 5.6 (2 ml), 1.86% EDTA (1 ml), 0.87% mercaptoethanol (1 ml) and 0.1 mg cholylglycine hydrolase and 0.1 mg β-glucuronidase (suspended together in 1 ml acetate buffer, pH 5.6) and the resulting suspension was incubated at 37°C for 18 h [12]. The reaction mixture was then passed through a prewashed C_{18} reversed-phase Sep-Pak cartridge [13] and the liberated bile

acids were eluted with 5 ml acetone. After evaporation of acetone, 100 μl of *n*-butanol and 20 μl of 40% solution of hydrogen chloride in dioxane were added and the contents were heated at 60°C for 4 h and then kept overnight at room temperature. After evaporation of solvents at 60°C, the esterified bile acids were subjected to trimethylsilylation. The TMS ether derivative formed was taken in 100 μl of hexane and 5 μl was injected in splitless mode into the GC column.

3. Results and discussion

In course of our studies on plasma bile acids in patients, we found occasional samples where cholesterol was not completely extracted out from the bile acid fraction during routine extraction procedures. Since, the GC retention times of the TMS derivative of cholesterol and that of methyl lithocholate are not greatly different on CP-Sil-5 CB capillary column (Table 1), lithocholic acid was overestimated in approximately 5% of plasma samples. Although cholesterol was well resolved from the isobutyl ester-TMS ethers of bile acids, sitosterol and campesterol, two of the major fecal sterols and present in significant amounts in plasma of patients with sitosterolemia [10], still interfered. Child et al. [3] prepared the *n*-butyl ester-acetate derivatives and were able to separate several fatty acids, sterols and bile acids in feces. However, since the acetate derivatives show decreased peak heights and therefore increased lower limits of detection for the trihydroxy bile acids [5], the method is not quite suitable for quantitation of bile acids in plasma, where the concentrations of bile acids are very low and the trihydroxy bile acid, cholic acid, is the major plasma bile acid. We have devised a gas chromatographic method where bile acids are converted into their *n*-butyl ester-TMS ether derivatives and found that the *n*-butyl ester-TMS ether of lithocholic acid showed significantly longer retention time than the TMS ethers of cholesterol and sitosterol. Therefore, even if sterols are not completely extracted out, being well resolved from bile acids on GC, do not interfere in the quantitation of plasma bile acids. Furthermore, the *n*-butyl ester-TMS ether derivative of nor-cholic acid was also eluted later than the abovementioned sterols and could be safely used as internal recovery standard.

Table 1
GC retention times of TMS ether-methyl esters and *n*-butyl esters of bile acids on CP-Sil-5 CB capillary column

	Relative retention time	
TMS ether of	Methyl ester	*n*-Butyl ester
Lithocholic acid	1.063	1.107
Chenodeoxycholic acid	1.179	1.241
Ursodeoxycholic acid	1.235	1.275
Deoxycholic acid	1.147	1.198
Cholic acid	1.205	1.257
Ursocholic acid	1.273	1.336

Retention times are expressed relative to that of nor-deoxycholic acid used as internal standard. Retention times of the methyl ester-TMS ethers of nor-deoxycholic acid and nor-cholic acid were 15.214 min and 16.005 min, respectively, while those of their *n*-butyl ester-TMS ethers were 20.885 min and 22.360 min, respectively. Retention times of TMS ethers of cholesterol and sitosterol under these GC conditions were 16.474 min and 20.916 min, respectively.

The GC retention times for the *n*-butyl and methyl ester-TMS ether derivatives of several common bile acids are given in Table 1 and a representative GC chromatogram is shown in Fig. 1. The retention times of all bile acid derivatives were highly reproducible and for amounts of bile acids ranging from 0.02–0.2 μg injected onto the column, the detector response, as shown by the integrator, was linear. Both the methyl ester and the *n*-butyl ester-TMS ether derivatives of all bile acids showed similar resolution from each other (Fig. 1). Although the *n*-butyl ester-TMS ethers of chenodeoxycholic acid and cholic acid did not show baseline resolution while the corresponding methyl ester-TMS ethers were better resolved (Fig. 1), when known amounts of these bile acid derivatives were injected together into the column, the detector response, as shown by the integrator, corresponded to the amounts when injected in a range of 0.02–0.2 μg of the bile acid. Cholesterol and sitosterol were resolved from all bile acids and from nor-cholic acid and eluted earlier than the acids (Table 1). The retention times of the *n*-butyl ester-TMS ethers of the bile acids were larger than those of the corresponding methyl ester-TMS ethers. However, the detection limits for both esters of the bile acids were similar (0.01 μg for both

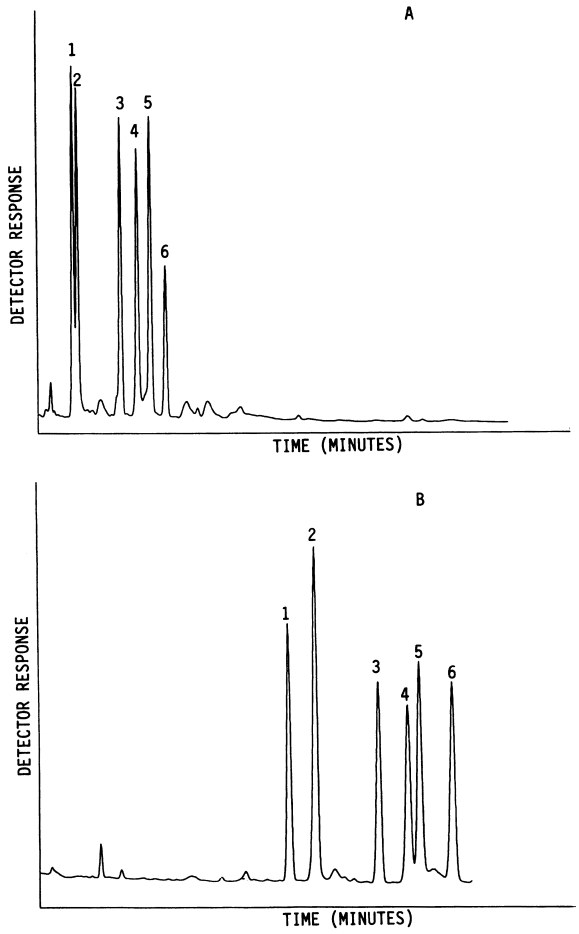

Fig. 1. GC chromatogram of bile acids: 1–2 μl of hexane solution containing 0.05–0.1 μg bile acid ester-TMS ether was injected onto the GC column. Chromatographic conditions as described in Section 2.2. (A) Methyl ester-TMS ethers; (B) *n*-butyl ester-TMS ethers. Peak identification, derivatives of: 1=nor-cholic acid; 2=lithocholic acid; 3=deoxycholic acid; 4=chenodeoxycholic acid; 5=cholic acid; 6=ursodeoxycholic acid.

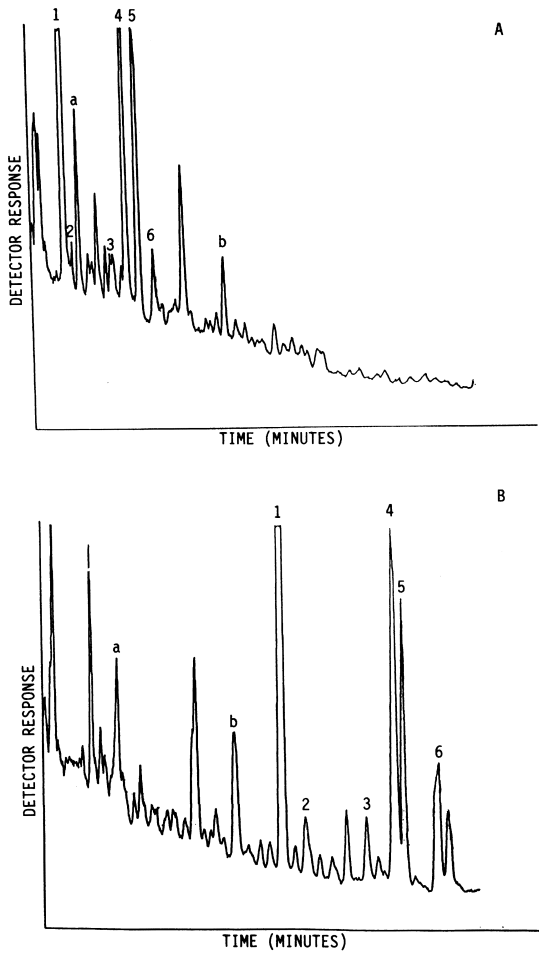

Fig. 2. GC chromatogram of plasma bile acids from a patient with sitosterolemia: 1 ml plasma containing 10 μg nor-cholic acid was passed through Sep-Pak and bile acids were isolated and derivatized as described in Section 2.4. After dissolving in 100 μl hexane, 5 μl was injected onto the GC column. Chromatographic conditions as described in Section 2.2. (A) methyl ester-TMS ethers of the bile acids; (B) *n*-butyl ester-TMS ethers of the bile acids. Peak identification: 1–6, same as in Fig. 1; a=cholesterol; b=sitosterol.

the *n*-butyl esters and the methyl esters injected into the column).

This derivatization method was employed for plasma bile acid analysis, where bile acids in the plasma were isolated by passing through a pre-washed Sep-Pak cartridge and eluting with acetone. Fig. 2A shows the GC chromatogram of the methyl ester-TMS ethers of plasma bile acids in a patient with sitosterolemia obtained by the Sep-Pak method as described above and Fig. 2B shows the GC chromatogram of the corresponding *n*-butyl ester-TMS ether derivatives. As can be seen from the figures, substantial amounts of sterols were still present after passing the plasma through Sep-Pak. However, the peaks due to the derivatized bile acids were distinctly separated from those of sterols when injected as the *n*-butyl ester-TMS ethers (Fig. 2B) while peaks due to the methyl ester-TMS ethers of the bile acids were clustered together with those of

the TMS ether derivatives of the sterols (Fig. 2A). The identification of *n*-butyl ester-TMS ethers of bile acids in presence of sterols allows for a simplified method for concentration of plasma bile acids where the bile acids are enzymatically deconjugated and the incubation mixture is directly passed through Sep-Pak cartridge and bile acids are extracted out with acetone, converted into their *n*-butyl ester-TMS ether derivatives and applied to the GC column.

In summary, we have shown that *n*-butyl ester-TMS ether derivatives of several common bile acids can be quantitated by GC and are well resolved from each other and from cholesterol and plant sterols. Their relatively high GC retention times have led us to develop a simplified method for quantification of plasma bile acids where multiple solvent extraction steps are avoided and interference due to plasma sterols is eliminated. The method can be adapted to routine plasma bile acid analysis.

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References

[1] T. Iida, T. Momose, T. Tamura, T. Matsumoto, J. Goto, T. Nambara, F.C. Chang, J. Chromatogr. 389 (1987) 155.
[2] A.K. Batta, S.K. Aggarwal, G.S. Tint, M. Batta, G. Salen, J. Chromatogr. 704 (1995) 228.
[3] P. Child, M. Aloe, D. Mee, J. Chromatogr. 415 (1987) 13.
[4] P.A. Szczepanik, D.L. Hachey, P.D. Klein, J. Lipid Res. 17 (1976) 314.
[5] A.K. Batta, G. Salen, M. Batta, D. Earnest, D. Alberts, J. Chromatogr. A 766 (1997) 286.
[6] T. Iida, I. Komatsubara, F.C. Chang, J. Goto, T. Nambara, Lipids 25 (1990) 753.
[7] S. Barnes, D.G. Pritchard, R.L. Settine, N. Geckle, J. Chromatogr. 183 (1980) 269.
[8] C. Tsaconas, P. Padieu, G. Maume, M. Chessebeuf, N. Hussein, N. Pitoizet, Anal. Biochem. 157 (1986) 300.
[9] H. Miyazaki, M. Ishibashi, K. Yamashita, Biomed. Mass Spectrom. 5 (1978) 469.
[10] G. Salen, V. Shore, G.S. Tint, T. Forte, S. Shefer, I. Horak, E. Horak, B. Dayal, L. Nguyen, A.K. Batta, F.T. Lindgren, P. Kwiterovich Jr., J. Lipid Res. 30 (1989) 1319.
[11] C.D. Schteingart, A.F. Hofmann, J. Lipid Res. 29 (1988) 1387.
[12] A.K. Batta, R. Arora, G. Salen, G.S. Tint, D. Eskreis, S. Katz, J. Lipid Res. 30 (1989) 1953.
[13] A.K. Batta, S. Shefer, M. Batta, G. Salen, J. Lipid Res. 26 (1985) 690.