Gas–liquid chromatography of bile acids: a new liquid phase for both acetate and trimethylsilyl derivatives

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Summary Polymetaphenoxylene (PPE-20) has been found to be more useful than cyclohexane dimethanol succinate (HI-EFF-8-BP) for trimethylsilyl derivatives of bile acids and to be preferable to trifluoropropyl substituted silicone (OV-210, QF-1) for analysis of their acetate derivatives.

The methyl ester acetate and trimethylsilyl derivatives of bile acid methyl esters have been well characterized and are frequently used for quantitative analysis (1). The acetates can be easily prepared without heating, using perchloric acid as an accelerator (2), and have the advantage of stability. Because they are stable in water, it is possible to backwash the derivative and remove reactants and other compounds present in biologic mixtures that interfere with GLC analysis of the relatively small amount of bile acids present in serum (3). A disadvantage of the acetate derivatives when analyzed using OV-210 or QF-1 is the closeness of lithocholic acid to the solvent front, in a region where many unidentified peaks occur. For this reason, the trimethylsilyl derivatives, analyzed on HI-EFF-8-BP provide a reverse order of elution from the column, so lithocholic acid follows the trihydroxy and dihydroxy bile acids and is more easily quantitated. Other advantages of the trimethylsilyl derivatives are their ease of preparation and good proportionality in mass to peak area ratios for each bile acid. However, HI-EFF-8-BP is unstable at 250°C and the internal standard frequently used for analysis of acetates (3α,7α-dihydroxy-12-keto-5β-cholanoate) has an unsatisfactorily long retention time. Thus, although dual column GLC ovens are available, a packing with HI-EFF-8-BP cannot be kept in the oven when acetates are analyzed (265°C), and the type of derivatives to be prepared must be chosen prior to addition of the internal standard. We have found that the use of PPE-20, with stability to 375°C, resolves these commonly encountered problems.

Polymetaphenoxylene was obtained as both a 3% and a 0.5% coating on acid-washed, silanized Chromosorb W with mesh size 80–100 (Supelco Inc., Bellafonte, Pa.). Preliminary studies with both mixtures led to a final blending of equal amounts of each packing which, after thorough shaking, was packed into a 6 ft glass column (ID = 2 mm). A single column was used for analyses of both acetate (265°C) and TMSi (240°C) derivatives.

In comparison to OV-210, PPE-20 had certain advantages for analysis of methyl ester acetates (**Table 1**). Lithocholic acid is displaced away from the solvent front and toward deoxycholate (**Fig. 1**). Another major advantage is the distinction between hydroxy and keto bile acids. Thus on OV-210, 3α,7-keto-5β-cholanoate does not have a baseline separation from chenodeoxycholate. However on PPE-20, cholate is displaced toward chenodeoxycholate and is then followed by 3α,7-keto-5β-cholanoate. Further evidence of the longer retention of keto groups by PPE-20 relative to OV-210 is the longer relative retention time of the internal standard, 3α,7α-dihydroxy-12-keto-5β cholanoate (RRT 2.760 vs 2.579) compared to deoxycholate.

In comparison to HI-EFF-8-BP, PPE-20 also has

Abbreviations: GLC, gas–liquid chromatography; RRT, relative retention time; TMSi, trimethylsilyl derivatives.

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TABLE 1. Comparison of retention time of substituted 5β-cholanoates relative to deoxycholate on PPE-20, HI-EFF-8BP and OV-210[a]

Functional Groups	PPE-20 (1.75%)				HI-EFF 8BP (1%)	OV-210 (0.5%)
	Acetates (265°C)		TMS (240°C)		TMS (240°C)	Acetates (265°C)
	RRT[b]	RF[c]	RRT	RF	RRT	RRT
Cholesterol	0.439		0.780			0.304
3α,	0.829	0.968	1.583	1.157	1.762	0.589
3β, 5-Cholenoate	0.983		1.502			
3α, 12α	1.000	1.000	1.000	1.000	1.000	1.000
3α, 7α	1.291	1.169	1.100	1.225	1.082	1.186
3α, 7α, 12α	1.431	1.188	0.711	0.901	0.706	1.986
(5α), 3α, 7α, 12α	1.650		0.670			
3α, 6α	1.748		1.218			1.403
3α, 7β	1.903		1.635			
3α, 6α, 7α	2.002		0.900			
3α, 7-Keto	2.083		3.928			1.321
3α, 7α, 12-Keto	2.760	1.484	2.914	1.317	3.470	2.579

[a] Analysis done using Perkin-Elmer 900 with PEP-1 data processing unit. Column length 6 ft with 2 mm ID. Carrier flow: helium 35 ml/min.
[b] RRT, relative retention time.
[c] RF, response factor.

certain advantages. Because the operating temperature of 265°C is much below the thermal stability of the coating (375°C), the column can be very well conditioned and will maintain efficiency for a longer period of use. Both the response factors and relative retention times of the major bile acids are very similar. However, 3α,7α-dihydroxy-12-keto-5β-cholanoate has a longer relative retention time using HI-EFF-8-BP (3.470 vs 2.760) permitting completion of a single analysis in 30 min rather than 45 min. However, for calculation by triangulation or when electronic integration equipment does not have a tangent skimming mode, the closeness of cholic acid to the solvent front can make quantitation difficult.

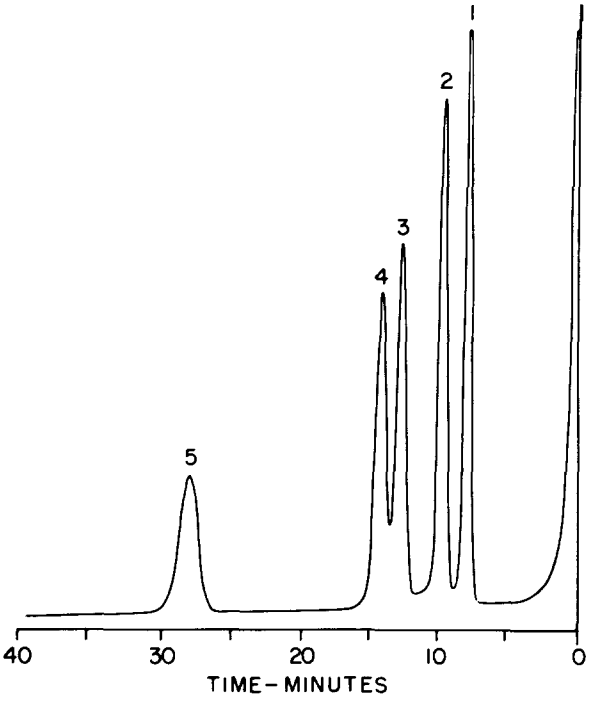

Fig. 1. GLC tracing of bile acid acetates using polymetaphenoxylene (PPE-20). Compared to OV-210, lithocholic acid (1) is displaced away from the solvent front. Other bile acids: deoxycholic (2), chenodeoxycholic (3), cholic (4) and 3α,7α,dihydroxy-12-keto-5β-cholanoate (5).

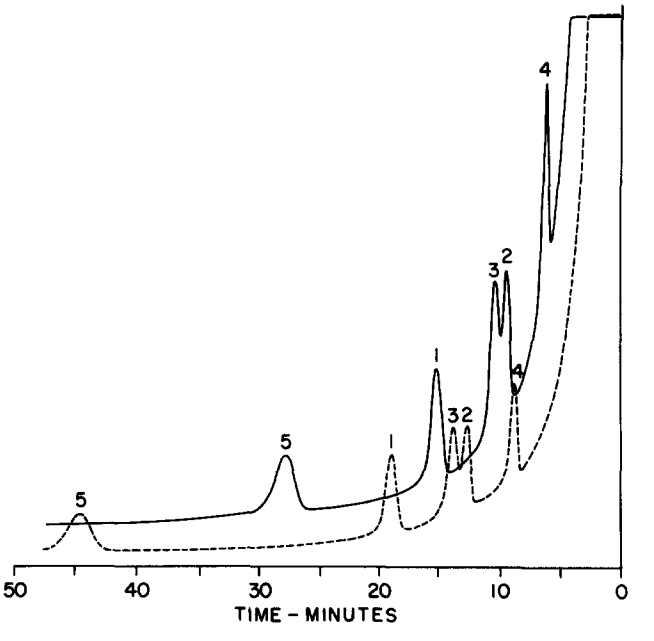

Fig. 2. GLC tracing of bile acid trimethylsilyl ethers using polymetaphenoxylene (PPE-20). Compared to HI-EFF-8-BP (dotted tracing) PPE-20 is more thermostable and provides equal resolution of the bile acids (see Fig. 1). In this illustration the retention time of the internal standard on PPE 20 is 27 min compared to 45 min for H-EFF-8BP. Reduction in carrier flow or temperature will move cholic acid further from the solvent front.

Under these circumstances, reduction of column temperature or carrier flow to increase the retention time of the internal standard to 45 min permits better quantitation.

Linearity of response for the bile acids shown in **Figs. 1** and **2** was tested over the range of 0.1 to 7 μg injected for both the acetate and TMS derivatives. The peak area to mass ratio for the internal standard, 3α,7α-dihydroxy-12-keto-5β-cholanoate remained constant over the entire range. The proportionality of the other bile acids to the internal standard also remained constant, provided adjustments for the integration program were made to compensate for the changing slopes and peak width to insure inclusion of the entire peak area.

Regardless of which bile acid derivative one chooses for routine GLC analysis, the capability of taking a single sample after it has been brought to the methyl ester stage and analyzing it as two different derivatives, using a single column and a single internal standard, has not previously been available. The ease with which this can be accomplished should encourage two derivative-GLC and provide much greater analytical security in regard to compound identification.

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