notes on methodology

Quantitative analysis of individual bile acids by gas–liquid chromatography: an improved method

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Summary The quantitative analysis of individual bile acids by gas–liquid chromatography has been improved by column oven temperature programming and by a new liquid phase, SP-2401. The method is fast; bile acids are well resolved; retention times are reproducible; detector responses are linear and sensitive to 0.1 μg; and there is little adsorption onto the liquid phase. The method has been successfully used for bile, and it has the potential for use on serum.

Supplementary key words trifluoroacetates · bile · SP-2401 liquid phase · squirrel monkey

The quantitative analysis of bile acids by GLC in the past has had two major deficiencies: *(1)* adsorption of bile acid derivatives on the column packing, which was not the same for each bile acid (1–10); and *(2)* long analysis times of 20 to 70 min (2, 3, 6, 9–12). The adsorption problem was not eliminated, but in 1971 Klaassen (13) did shorten the analysis time to 5 min.

In this paper we report a method which eliminates the adsorption problem while retaining a short analysis time. We have accomplished this by using *(1)* a new liquid phase, SP-2401, a more thermally stable, more efficient, less viscous (14) version of OV-210; *(2)* temperature programming of the column oven; *(3)* a narrow diameter U-shaped glass column; and *(4)* a gas–liquid chromatograph with on-column injection ports and no transfer line from column exit to detector. Our method can accurately quantitate less than 0.1 μg of each bile acid injected onto the column.

Abbreviations: GLC, gas–liquid chromatography; R_t, retention time; RR_t, relative retention time; RWR, relative weight response; ID, inside diameter.

Experimental

Preparation of column packing (1.0% (w/w) SP-2401). Ten g of dry Gas Chrom Q, 100/120 mesh (Applied Science Labs., State College, Pa.), were suspended in acetone (Nanograde, Mallinckrodt, St. Louis, Mo.) in a 500 ml Morton flask. The liquid phase, 100 mg of SP-2401 (Supelco, Inc., Bellefonte, Pa.) dissolved in 50 ml of acetone, was added. The Morton flask was turned by a rotary flash evaporator at room temperature under vacuum for thorough mixing. The acetone was removed slowly as the temperature of the flask was increased from 30°C to 50°C. When the packing appeared to be thoroughly dry, with no acetone odor, it was dried at 110°C overnight, cooled in a desiccator at room temperature, and stored thereafter at room temperature in a linear polyethylene bottle. The packing did not decompose after one year of such storage.

Silanization, packing and conditioning of column. The column was U-shaped glass, 4 ft long with 2 mm ID. It was silanized with 5% (v/v) dimethyldichlorosilane (Applied Science Labs.) as described by Chattoraj (15). After packing with SP-2401/Gas Chrom Q, the column was conditioned at 265°C for 35 hr with a N_2 flow of 20 ml/min. Final conditioning may require a few injections of bile acid derivatives. The standard curves were linear after 16 injections. Column deterioration can be detected by a decrease in the separation of cholate from 7-keto-lithocholate. There were

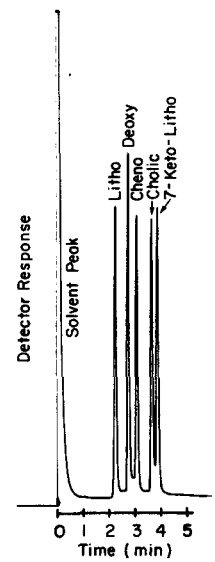

Fig. 1. GLC of a mixture of bile acid reference standards. Approximately equal weights of the trifluoroacetylated methyl esters of four bile acids and an internal standard (7-keto-lithocholate) were dissolved in dichloromethane and injected into a 4 ft (122 cm) × 2 mm ID glass U-column, packed with 1% SP-2401 on 100/120 Gas Chrom Q. The oven temperature was held at 215°C for 1 min after injection, then increased at 20°C/min to 265°C.

at least 350 injections over five months before deterioration was noted.

Preparation of trifluoroacetyl bile acid methyl esters. Diazomethane was prepared from Diazald (Aldrich Chemical Co., Milwaukee, Wisc.) using their Diazald Kit. Cold diazomethane was added to the bile acids in a methanol–ethyl ether solution 3:10 (v/v) at 0°C until the yellow color of excess diazomethane persisted for 15 min. The methylated bile acids were evaporated with N_2 at 37°C, and redissolved in methanol.

The bile acid methyl esters were trifluoroacylated by heating to 37°C for 30 min in undiluted trifluoroacetic anhydride (used without further purification, from Eastman Kodak Co., Rochester, N.Y.). The anhydride was evaporated and the bile acids were dissolved in dichloromethane. The bile acid derivatives were injected into the gas chromatograph on the same or next day. No signs of deterioration of these derivatives were seen when they were kept tightly capped overnight in dichloromethane at 4°C. In fact, standard pools were stable for one week when stored in this manner.

GLC conditions. We used a Bendix model 2500 gas–liquid chromatograph (Bendix Corp., Ronceverte, W. Va.) equipped as described in the Introduction. Bile acid GLC was unsuccessful with another gas chromatograph which had off-column injection ports, a metal column, and a 6-inch stainless steel transfer line. During analysis, the injection port temperature was 280°C, detector temperature 295°C, and oven temperature 215°C initially. Fearing degradation of bile acid derivatives at 280°C, we tried injection port temperatures of 260°C, 240°C, 220°C, and 200°C. We found increased tailing of peaks with decreasing temperature, and no improvement in recovery. One minute after injection the oven temperature was increased at 20°C/min to 265°C and held there until the last derivative (7-keto-lithocholate) eluted. All three temperatures were kept at 215°C during inactive periods. The flow rates were: N_2, 20 ml/min; H_2, 55 ml/min; and air, 450 ml/min. Peak areas were integrated with an Infotronics digital integrator, Model CRS-208, (Columbia Scientific Industries Corp., Austin, Tex.). Peaks were recorded on a Varian Aerograph (Walnut Creek, Calif.) Model A-25 recorder at a chart speed of 0.5 inch/min.

Preparation of standard curves (derived from a method by Gehrke, Kuo, and Zumwalt (16). A solution of four bile acid methyl esters (Steroloids, Inc., Wilton, N. H.) was prepared in methanol (1.6 mg of each bile acid/ml). The bile acid standards were checked for purity of GLC. Those standards selected for use were 98–99% free of other peaks on the chromatogram. This solution was diluted serially with 7-keto-lithocholate methyl ester so that each dilution had a constant concentration of the internal standard. The bile acids were acylated as described above. Approximately 2.6 μl of each solution was injected into the gas chromatograph.

Calculations.

1. Standard curves: The ratio of the area of the bile acid peak divided by the area of the 7-keto-lithocholate peak was graphed as a function of the amount of bile acid injected. Since the concentration of 7-keto-lithocholate was constant for all dilutions, the ratio of areas was corrected for the slight variations in volume of sample injected (16). The lithocholate peak area was corrected for the amount of lithocholate found as a contaminant in the 7-keto-lithocholate.
2. Relative Weight Response (RWR):

$$\text{RWR} = \frac{\text{peak area}_{BA}/\mu g_{BA} \text{ injected}}{\text{peak area}_{IS}/\mu g_{IS} \text{ injected}}$$

where BA is bile acid and IS is internal standard (7-keto-lithocholate).

TABLE 1. Retention times for four bile acids relative to internal standard

Bile Acid	Pool I[a] RR_t[b]			Pool II RR_t		
	$\bar{x}$[c]	SD[d]	SEM[e]	$\bar{x}$	SD	SEM
Lithocholate	0.587	0.003	0.001	0.586	0.002	0.001
Deoxycholate	0.712	0.004	0.001	0.711	0.004	0.001
Chenodeoxycholate	0.794	0.003	0.001	0.795	0.002	0.001
Cholate	0.942	0.003	0.001	0.943	0.003	0.001

[a] The bile acids in pools I and II were weighed into separate volumetric flasks.
[b] RR_t = relative retention time: Retention time of bile acid:retention time of 7-keto-lithocholate, the internal standard. The absolute retention time of 7-keto-lithocholate was 3.89 min, with SD of 0.02 and SEM of 0.006 min.
[c] Mean values (n = 11 for pool I; n = 9 for pool II). No differences in mean values between pools I and II were significant by student's *t* test.
[d] Standard deviation.
[e] Standard error of the mean.

Preparation of bile. Squirrel monkey bile was deconjugated with cholylglycine hydrolase (Schwartz/Mann, Orangeburg, N.Y.) according to the method of Nair (17), except dithiothreitol was substituted for β-mercaptoethanol and the reaction was incubated 30 min before adding 4 N HCl. The deconjugated bile acids were extracted four times with ethyl ether, then methylated and trifluoracetylated as described above, after adding 7-keto-lithocholate as internal standard. Samples analyzed without adding internal standard did not contain peaks with the same R_f as 7-keto-lithocholate.

Results and Discussion

A chromatogram of a standard pool of the four bile acids is shown in **Fig. 1**. The lack of complete

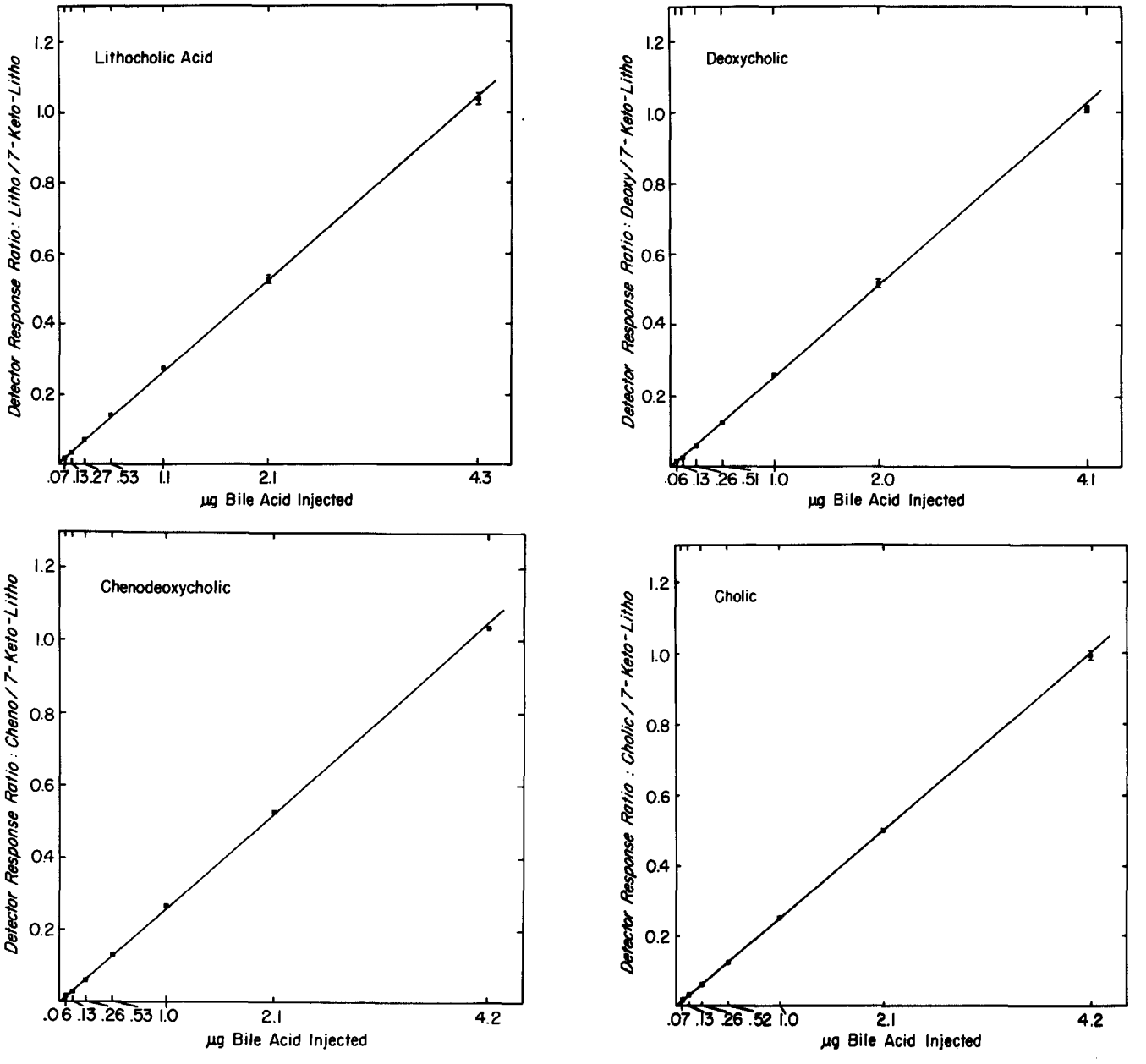

Fig. 2. GLC standard curves of lithocholic, deoxycholic, chenodeoxycholic, and cholic acids. A 1:2 serial dilution of the four bile acids in Fig. 1 was prepared with the internal standard, 7-keto-lithocholate, as diluent. Slight variations in volume of each dilution injected were compensated for by graphing the ratio of bile acid to 7-keto-lithocholate detector response vs. μg bile acid injected, since the concentration of 7-keto-lithocholate was constant in all dilutions. GLC conditions are the same as Fig. 1. Each square (■) represents the mean of six injections. The brackets represent ±3 standard deviations (SD) from the mean ratio. Where brackets appear absent they are inside the squares, indicating that ±3 SD was ≤0.005.

resolution did not affect the linearity or accuracy of the standard curves (described below). The assay time was approximately 4 min. However, the total time per sample is 10–12 min when the time required to cool the oven from 265°C to the initial 215°C is included.

The retention times of these four bile acids relative to the internal standard (RR_t) were reproducible, even with the drastic temperature programming used (**Table 1**). The absolute retention time (R_t) can be calculated from the absolute R_t of the internal standard (see footnote *b*, Table 1). The absolute R_t changed as a given column aged. Therefore, for identification of peaks, a standard pool was injected with each batch of samples for comparison.

Fig. 2 demonstrates the linearity and sensitivity of the hydrogen flame detector response for the four bile acids. All four bile acids can be measured accurately with less than 0.1 μg injected onto the column. This level of sensitivity would make this method applicable to bile acid analyses in serum. The standard curves are quite reproducible, which is illustrated by the very small standard deviations in Fig. 2.

Only slight adsorption of bile acids onto the column packing could be detected, as indicated by the nearly identical slopes of the four standard curves. The adsorption phenomenon can be more easily observed, however, by comparing the RWR's (see Experimental section) of each bile acid to the internal standard. If the RWR of one bile acid is the same as the RWR of another bile acid, then we can assume no adsorption (or equal adsorption) of the two bile acids on the column. The RWR's of bile acid standards are shown in **Table 2**. There is no RWR which is significantly different from any other RWR. This is a marked improvement over previous methods (3–10). Klaassen (13) hypothesized that the greatest sensitivity of the flame ionization detector was for lithocholate, intermediate sensitivity was for deoxycholate and chenodeoxycholate, and the least sensitivity was for cholate. However, he measured peak height only, not area, and did not use temperature programming. Isothermal oven conditions cause lower, broader peaks for bile acids with longer R_t, which explains the apparent lack of equal detector responses. Evrard (18) used temperature programming for his methyl ketone derivatives, but his deoxycholate and chenodeoxycholate derivatives did not separate, and he provided no data on relative detection responses. Crotte, Mule, and Planche, (9) also tried temperature programming, but the detector responses still decreased with increasing R_t.

The applicability of our method to a biological sample (squirrel monkey bile) is demonstrated in **Fig. 3**. The resolution between cholate and 7-keto-lithocholate is not quite as good as that of the standard pool (Fig. 1) because the column was 3.5, not 4 ft long. The resolution in Fig. 3 can also be improved by diluting bile less, or by adding less 7-keto-lithocholate. Using this method one can also use 7-keto-deoxycholate as an internal standard (19), which separates more completely from cholate than does 7-keto-lithocholate.

TABLE 2. Detector responses of bile acids relative to internal standard[a]

Bile Acid	RWR[b]	
	Mean[c]	$\bar{x} \pm 2$ SD[d]
Lithocholate	1.01	1.00–1.02
Deoxycholate	1.05	1.02–1.08
Chenodeoxycholate	1.00	0.97–1.03
Cholate	1.03	1.02–1.04

[a] 7-keto-lithocholate.
[b] Relative weight response (see Experimental section).
[c] Mean of three analyses of one pool of carboxymethylated bile acids.
[d] $\bar{x} \pm 2$ SD = mean ± two standard deviations of the mean.

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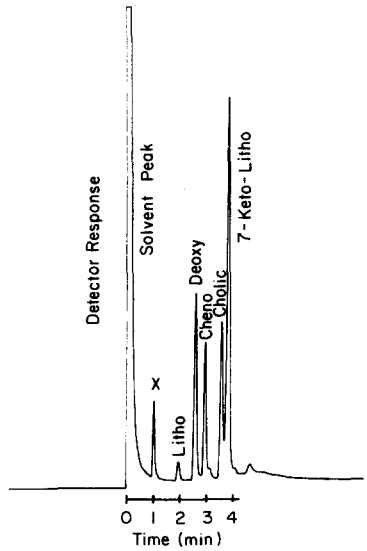

Fig. 3. GLC of squirrel monkey bile. The bile acids were diluted, deconjugated with cholylglycine hydroxylase, extracted, and derivatized to their trifluoroacetyl methyl esters after adding 7-keto-lithocholate as an internal standard. GLC conditions are the same as Fig. 1. All peaks were identified by R_t only. Peak X has the same R_t as cholesterol with these chromatographic conditions.

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