Gas-Liquid Chromatography of Bile Acids

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Abstract

Methods are reviewed for the preparation, gas-liquid chromatographic (GLC) separation, identification, and quantitative estimation of the trifluoroacetyl derivatives of bile acid methyl esters. Of the stationary phases tried (SE-30, QF-1 and XE-60) methylfluoroalkyl silicone (QF-1) was best suited for analysis of the trifluoroacetates. This phase (1–2% QF-1 on 100–120 mesh Gas Chrom P) allowed an orderly resolution of conformational isomers and consistently gave GLC columns (stainless steel tubes, ⅛ in. O.D. × 3 ft) from which the bile acid derivatives could be recovered in high yield. Applications to biological materials are illustrated with bile acid samples from animal biles and from human duodenal drainage and feces. Identifications of the major bile acids made by the GLC of the trifluoroacetates were confirmed by results obtained with bile acid methyl esters and bile acid methyl ester acetates on QF-1 and the other liquid phases investigated. For most mixtures of bile acids, however, it appears that GLC of methyl esters and methyl ester trifluoroacetates on QF-1 is sufficient for a reliable recognition of common bile acids. Overall accuracy of the estimates was of the order of ± 5%, but it varied with the nature and concn of the component.

Introduction

POTENTIAL USEFULNESS of gas-liquid chromatography (GLC) for separation of bile acids has been well established (1) and much new knowledge has been expected to result from application of this technique in research. Qualitative separations of bile acids have been extensively explored (2–5) and advantages of using selective liquid phases fully recognized (6–8) but only a few quantitative studies have been reported (5,9,10). Quantitative determination of these compounds has been difficult because of varying response observed with different steroids in the detector and increasing losses on the column with increasing degree of hydroxyl-substitution.

The present report summarizes recent studies which show that losses on columns can be greatly reduced by employing more volatile bile acid derivatives and short GLC columns prepared with thin films of highly selective liquid phases. Quantitative interpretation of the results is greatly facilitated by use of a hydrogen flame ionization detector which gives a correct weight response for combustible carbon content of bile acid derivatives.

Analytical Conditions

A quantitative analysis of natural bile acids by GLC depends upon availability of suitable procedures for their isolation, conversion to appropriate derivatives, satisfactory separation by GLC and a meaningful translation of the resulting information into analytical data. The following is a short account of methods that have permitted apparently quantitative determinations of bile acids as the trifluoroacetates in certain biological materials.

Isolation of Bile Acids

For the isolation of conjugated bile acids, 10 ml samples of gallbladder bile were mixed with 10 volumes of absolute ethanol and heated to boiling on a steam bath. After cooling the precipitate was filtered off, the solution made up to 100 ml with 0.05 N NaOH and continuously extracted with ethyl ether (24 hr) to remove neutral lipid. The alkaline phase was neutralized with dilute acetic acid and reduced in vacuo to small volume. After a further purification and desalting on a Dowex 50 cation exchange column, the glycine and taurine conjugates were recovered separately from a Dowex-1 anion exchange column using a gradient of HCl in aqueous alcohol or acetone (11). The column effluents were monitored by thin-layer chromatography (TLC) (12). For the isolation of free bile acids the individual conjugates (1–5 mg) were heated with 5 ml of 1.25 N NaOH in sealed tubes in an autoclave at 120C and 15 psi. The free acids were recovered by ether or chloroform extraction of the acidified (dil. HCl) hydrolysate. The extracts were washed with distilled water to remove chlorides, evaporated to small volume and dried with benzene.

The fecal bile acids were extracted from freeze-dried fecal samples with a mixture of methanol and ethylene chloride (1:3) either at room temp or in a Soxhlet apparatus (48 hr). The crude lipid extract was reduced to a small volume in a rotatory evaporator, diluted with ethyl ether and the acidic fraction isolated by extraction with 5% K_2CO_3. The mixed acids were recovered by ether extraction following acidification of the carbonate solution. The fatty acid and bile acid portions were separated by silicic acid chromatography of their methyl esters (obtained by diazomethylation as described below). The fatty acid esters were eluted with 1% ethyl ether in petroleum and the bile acid esters with 50% acetone in ethyl ether. To insure a quantitative transfer of the difficultly soluble bile acid methyl esters to the column, all containers and the top of the column were carefully rinsed with the 50% acetone-ether mixture prior to the elution of the bile acid methyl esters. Any more polar residual material was removed with a final methanol wash. Smaller samples of mixed methyl esters may be effectively separated by scaling-up the micro-TLC system of Hofmann (13) where the fatty acid esters are carried near the solvent front. All or any one of the bile acid ester fractions may be recovered from the plate by methanol elution. After evaporation of the solvents the ester fractions can be gas-chromatographed as such or after a conversion into the trifluoroacetates.

Preparation of Derivatives

The methyl esters of the bile acids were prepared by dissolving the sample (1 to 50 mg) in a small volume of dry methanol and adding freshly distilled diazomethane in ether (14) until a persistent excess of the reagent was attained. Although TLC examinations of such reaction mixtures indicated that the methylation was practically instantaneous, the solutions were allowed to stand for about an hour before the excess reagent and the solvents were evaporated.

The acetates and trifluoroacetates were prepared from the bile acid methyl esters by dissolving the sample (a few milligrams) in a small volume of dry benzene and adding an excess of the anhydride along with a trace of dry pyridine. Complete trifluoroacetylations were obtained in 10 to 15 min on a steam bath (45C) or after an overnight standing at room temp. For complete acetylation 30 min at reflux temp were sufficient. The inclusion of pyridine in the reaction mixture ensures complete acylation of hindered hydroxyl groups. For gas chromatography with hydrogen flame ionization detectors it was convenient to inject the entire reaction mixture into the gas chromatograph. The reagents appear at the solvent front. In order to avoid excessive solvent fronts in this detector it is advisable to remove the excess reagents and solvents by evaporation under nitrogen and injecting the sample into the gas chromatograph in carbon disulphide solution.

Gas-Liquid Chromatography

The instrumentation and the general conditions of bile acid gas chromatography as employed in the subsequently described studies have been reported previously (10,15). The specific elution conditions are given in the legends accompanying the figures. The column packings were either obtained from the Applied Science Laboratories (1% QF-1, 2% QF-1) or were prepared in the laboratory (2% XE-60, 2.25% SE-30) by the solvent evaporation technique. In the latter case the coatings were applied to Anachrom ABS (110–120 mesh) from toluene solutions in 2 to 3 increments of 0.5 to 0.75% (w/w) at a time. The QF-1 phase was the Dow Corning FS 1265 fluorosilicone fluid of 10,000 cts, and the XE-60 a General Electric cyanoethylmethyl and dimethyl silicone, both sold by the Applied Science Laboratories. The SE-30 methyl silicone rubber gum was purchased from Wilkens Instrument and Research, Inc. The siliconized supports had been prepared by techniques similar to those described by Horning et al. (1). Although it was important to have the support well siliconized before applying the liquid phase, heavily siliconized supports gave inefficient columns. In order to avoid decomposition of the bile acid derivatives all columns were operated at the lowest practicable temps. When working with the trifluoroacetates the flash evaporator temp was not allowed to exceed that of the column (maximum of 235C), because the ester groups may be eliminated if very high column or injector heater temps are used (1).

Separation of Standards

The separation of the methyl esters of the common bile acids can be carried out by extensions of the usual gas chromatographic methods. The nonpolar silicone phase SE-30 used in the original separations (16) has been later abandoned in favor of the more polar polymers SE-52 (3,4), F-60 (1) and more recently QF-1 (6,8,10) and the silicone nitrile polymers (8,17). The structures and sources of these liquid phases have been described (1).

Figure 1 compares the separations of five common bile acids as methyl esters on the SE-30 and the QF-1 columns. The same ester mixture representing about equal weights of all components was analyzed on both columns. The more efficient separation of the chenodeoxycholic (*3*) and hyodeoxycholic (*4*) acid esters on the QF-1 column may be attributed to the higher selectivity of the fluoroalkyl silicone liquid phase.

It has been demonstrated (18) that with polycyclic systems such as those found in steroids, the effect found for a hydroxyl group on QF-1 depends upon its stereochemical configuration and location in the molecule. Axial hydroxyl groups usually lead to shorter retention times than equatorial hydroxyl groups in the same position. It is therefore possible to use QF-1 for the separation of epimers not resolved with a nonselective phase. Furthermore, with this phase ketones show unusually high retention times and are readily separated from the corresponding alcohols. The relative retention behaviour of different functional groups on QF-1 has been considered from a quantitative standpoint and it has been shown (19) that the logarithm of the relative retention time of a compound is made up of the additive contributions of the individual groups together with the contribution of the nucleus to which these groups are attached. Theoretical retention times can therefore be computed for the various bile acids and their derivatives and can help in the identification of unknowns (17).

Despite a rather low polarity of all of these liquid phases (SE-30, XE-60 and QF-1) the methyl ester peaks tail even when siliconized supports are used and the tailing becomes more apparent at low temps. There is an incomplete resolution of the dihydroxy cholanates and with increased degree of hydroxyl substitution an increased loss of the methyl esters on the column. Methyl cholate, for example, may suffer extensive loss by adsorption or decomposition under gas chromatographic conditions. In order to reduce the polarity of these steroids, shorten the retention times, and decrease the losses on the column, Vanden Heuvel et al. (20) prepared the trifluoroacetyl derivatives of bile acid methyl esters. Although trifluoracetyl esters may undergo thermal elimination of the ester group, the bile acid derivatives investigated are stable under the conditions employed. Though some of the desirable changes in the chromatographic behaviour of the bile acid methyl esters were realized, the separations of the trifluoroacetates of the common bile acids on the nonselective liquid phases were of limited value since the usual order of elution of several of these was reversed and there was undesirable overlapping. The increased volatility of these compounds, however, was sufficient to provide effective separations at lower temps on the more polar QF-1 columns (6,10). Figure 2 compares the behaviour of the trifluoroacetates on SE-30 and QF-1 columns. Even though the resolution shown in the first part of the figure for the SE-30 phase may be improved by lowering the temp, the crowding of the peaks resulting from the increased volatility of tri- and dihydroxy derivatives remains. The unidentified peak eluting at about 15 min in the upper print has the retention time of cholanic acid. Because the stereochemical selectivity shown by the QF-1 phase for the methyl esters has been retained for the methyl ester trifluoroacetates, the separations obtained with it were superior to any realized previously. Similar observations have been made by Sjövall (8), who investigated a much larger collection of standards.

Figure 3 illustrates the separation of the methyl ester trifluoroacetates of lithocholic, 3-beta-12-alpha dihydroxy cholanic, deoxycholic, chenodeoxycholic, hyodeoxycholic, cholic, and 7-keto-lithocholic acids on QF-1. The resolving power of the selective liquid phase is best demonstrated by the complete resolution of the four isomeric dihydroxy bile acid derivatives. The utilization of short (3 ft) $\frac{1}{8}$ in. diameter columns permitted the completion of the runs at significantly lower temps and with shorter retention times than

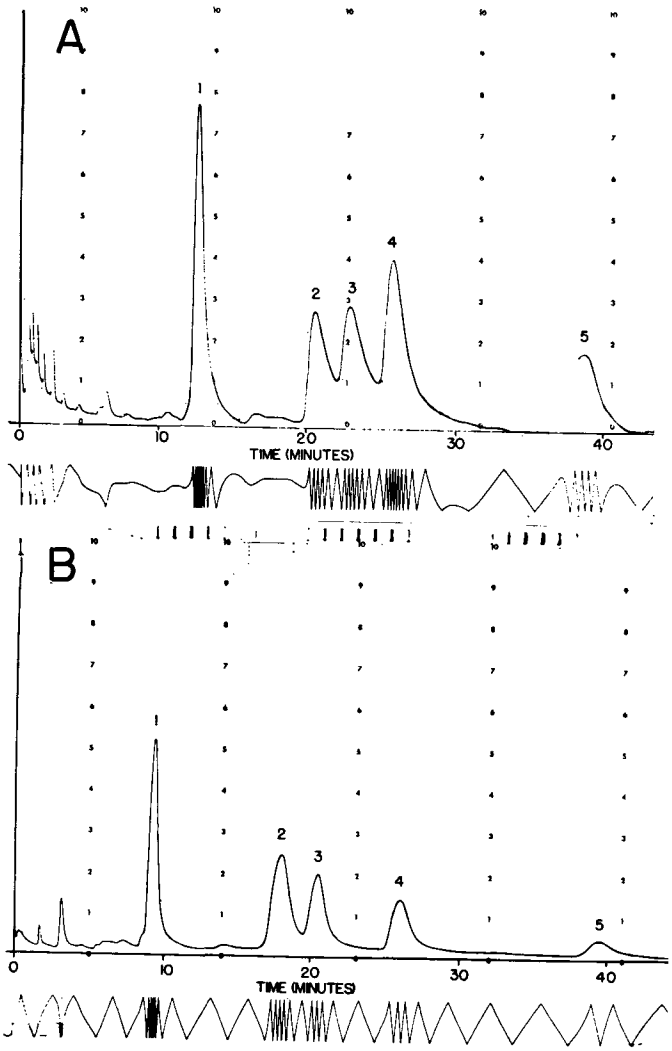

FIG. 1. Gas chromatographic separation of bile acids as methyl esters on SE-30 (A) and QF-1 (B) columns: (*1*) lithocholic, (*2*) deoxycholic, (*3*) chenodeoxycholic, (*4*) hyodeoxycholic, (*5*) cholic. Conditions: (A) 2.25% SE-30 on Chromosorb W (60–80 mesh); 5 ft × ⅛ in. O.D. column; 195C isothermal. Instrument: Aerograph Model A-100 with hydrogen flame ionization kit, Brown 1 mv recorder. (B) 1% QF-1 on Gas Chrom P (100–200 mesh); 3 ft × ⅛ in. O.D. column; 200C isothermal. Instrument as in (A).

comparable columns of 5–6 ft lengths and ¼ in. diameter. Many of the features of the fluoroalkyl silicone are also exhibited by the silicone nitrile polymer XE-60 which make them both exceptionally well suited for the separation of complex bile acid mixtures. The elution sequence parallels that of the mol wts, with the monohydroxy cholanic acid derivative being eluted first and the trihydroxycholanic acid derivative last. The affinity of the QF-1 phase for the keto group was so great that the 7-ketolithocholate was retained longer than either of its corresponding di-trifluoroacetyl derivatives (cheno- and urso-deoxycholates) and the tri-trifluoroacetyl cholate. The order of elution of the dihydroxy bile acid esters followed that noted for other steroid derivatives in which the equatorial derivatives have been found to be retained longer. The diaxial (3-beta-12-alpha) cholanate was eluted first, followed by the mixed axial-equatorial derivatives of deoxy-(3-alpha-12-alpha) and chenodeoxy-(3-alpha-7-alpha) cholates, which were eluted ahead of the di-equatorial derivatives of hyodeoxycholate (3-alpha-6-alpha-).

The more rapid elution of the deoxycholic acid ester in comparison to the ester of the chenodeoxycholic acid

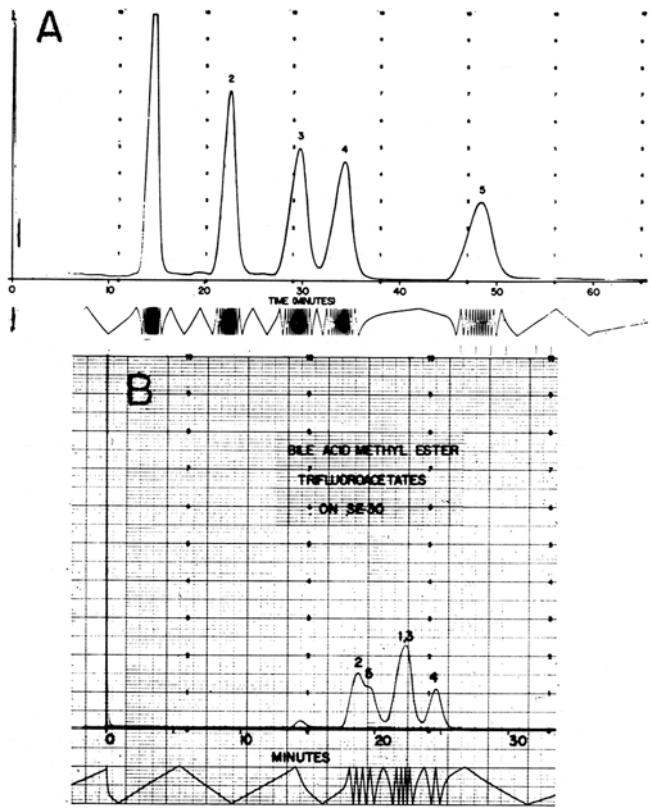

FIG. 2. Gas chromatographic separation of bile acid methyl ester trifluoroacetates on SE-30 (A) and QF-1 (B) columns. Chromatography conditions and peak identification as in Fig. 1.

was probably due to the shielding of the 12-alpha-position by the side chain. Inspection of molecular models has shown (21) that the bile acid side chain in some of the many positions which it may occupy can provide considerable shielding of position 12. That such a shielding reduces the vulnerability of a 12-alpha-hydroxyl group to acetylation, as compared to a 7-alpha-hydroxyl group, has been indicated by the greatly increased rate of acetylation realized at the 12 position when the side chain is eliminated.

The acetates of the methyl esters of bile acids were prepared as a substitute for the trifluoroacetates (10). Although these derivatives are stable under the chromatographic conditions used and recoveries were higher than for the methyl esters, their utilization was limited due to inversions in the elution patterns and undesirable overlapping. Figure 4 compares the behaviour of the bile acid acetates on the SE-30 and the QF-1 columns. While the separation of the methyl esters of acetylated dihydroxy derivatives on the SE-30 column was greatly improved over that noted for the methyl esters, the emergence of the cholate triacetate between the diacetates of cheno- and hyodeoxycholates resulted in undesirable crowding. Surprisingly, the improved recoveries of the bile acid methyl esters following acetylation could also not be exploited on the QF-1 columns. The overlap of cheno-, hyo- and urso-deoxycholic acid derivatives was complete. Evidently some of the selectivity of the QF-1 for the stereochemical configuration of the bile acids was lost on acetylation. Though impractical for the separation of complex mixtures, the changes noted in the elution sequence of the bile acid methyl esters following acetylation may be useful as an aid in characterization of unknown bile acids by gas chromatographic means. Qualitative separations of the bile acid methyl ester acetates have also been made on the SE-52 silicone polymer (3,22).

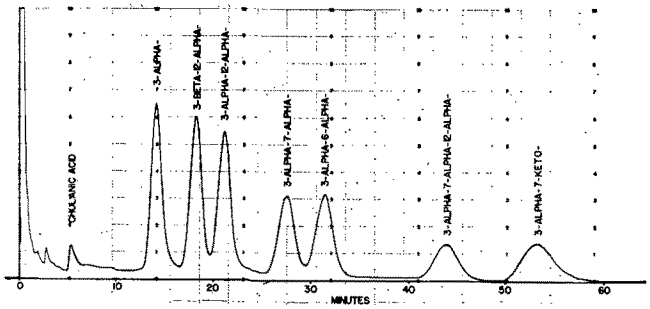

FIG. 3. Gas chromatographic separation of some isomeric bile acid methyl ester trifluoroacetates and ketones on QF-1. Peak identity as indicated. Chromatography conditions as in Fig. 1.

Besides acylation, the polarity of hydroxyl substituted bile acids may be reduced by ether formation. Ethers have gas chromatographic properties very similar to those of hydrocarbons (1). They are thermally stable and are rarely adsorbed on column packings. While the conditions for the preparation of the methyl ethers are generally unsatisfactory for steroids with ester and ketone functional groups, the silyl ethers may be prepared (9) under conditions which do not affect other functional groups. Although the latter ethers are hydrolyzed with great ease, satisfactory gas chromatograms have been obtained when injected immediately after preparation. Of the stationary phases tried, the polar cyclohexanedimethanol polysuccinate (Hi Eff-8B) yielded the best separations with standards and unknowns from rat feces (9). For most of the silyl ethers an inverse relationship between the retention time and the number of substituent hydroxyl groups was observed. The equatorial hydroxyl groups (7-beta and 6-alpha) increased the retention time. As noted for the trifluoroacetates on QF-1, the mixed axial-equatorial derivative (disilyl ether of chenodeoxycholate) was eluted ahead of the diequatorial derivative (disilyl ether of ursodeoxycholate). Keto groups brought about a significant increase in the retention time of the methyl cholanate derivatives. Although the selected liquid phase allowed considerable crowding of the peaks, it was possible to tentatively identify and quantitate the principal bile acids found in rat feces.

The trimethylsilyl derivatives of methyl glycolithocholate and methyl glycocholate have been separated (23) on a number of silicone polymer phases (SE-30, SE-52, QF-1 and F-60; 0.5–1.25%). A mixture including the derivative of methyl glycodeoxycholate in most cases gave only two peaks for the three conjugates. Under the same conditions a mixture of the corresponding acetates gave three well-defined peaks. Of the methylated conjugates only glycolithocholate gave a good peak.

Identification of Unknowns

Although the bile acid methyl esters have permitted the identification of the common bile acids in human (2,24) and animal (25,26) biles, the more complex fecal bile acid mixtures have been effectively separated by gas chromatography only after trifluoroacetylation (28–30) or silylation (9). The elution patterns obtained with the methyl esters have been used, however, for qualitative comparisons of fecal bile acid excretion during different dietary periods (27,31). Despite the rigid order of elution noted with the more selective liquid phases, no completely adequate system for the detection and identification of these derivatives has yet been devised, and reliance upon comparative retention times in any single system is unwise, particularly with the simpler derivatives. Compounds having the gas chromatographic properties of bile acids have proven not to possess even the steroidal ring structure (32). The possible presence of bacterial hydroxy fatty acids in fecal bile acid preparations is an ever present danger. If gas chromatography is to be used as the sole means of bile acid identification, the use of more than one type of derivative and several liquid phases is mandatory.

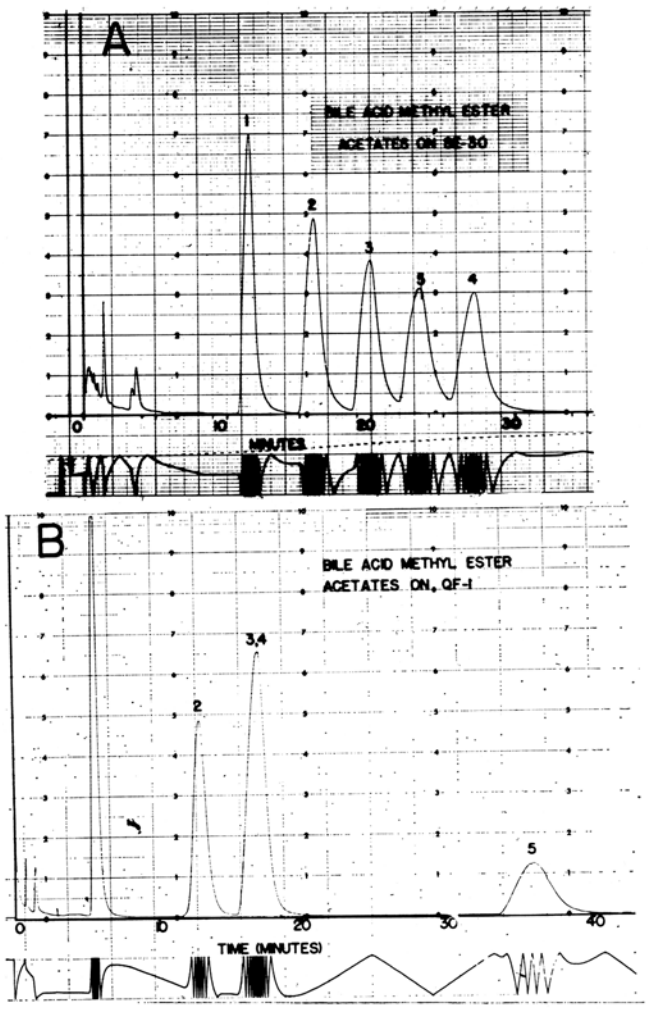

FIG. 4. Gas chromatographic separation of five bile acid methyl ester acetates on SE-30 (A) and QF-1 (B) columns. Chromatography conditions as in Fig. 1.

Using the silicone polymers SE-30, QF-1 and XE-60 for the separation of the bile acid methyl esters, their acetates and trifluoroacetates, we have examined the bile acid composition of the gallbladder bile of man, rabbit, dog, ox, sheep and pig (33), and the fecal bile acids of man on a variety of experimental diets (28,30, 31). In all cases the most complete separations were obtained with the trifluoroacetates on the QF-1 columns. The separations obtained with some of the more complicated mixtures illustrate the consistency of the gas chromatographic findings. Some of the more extensively investigated bile samples from man were those aspirated from the duodenum during a dietary study (11). Figure 5 shows the gas chromatographic elution patterns recorded for the glycine and taurine conjugates on a free choice diet. In both conjugate groups peaks are seen for deoxycholic (*9*), chenodeoxycholic (*11*), ursodeoxycholic (*13*) and cholic (*16*) acid derivatives. It is interesting to note that the trifluoroacetate of the diequatorial ursodeoxycholic (3-*a*-

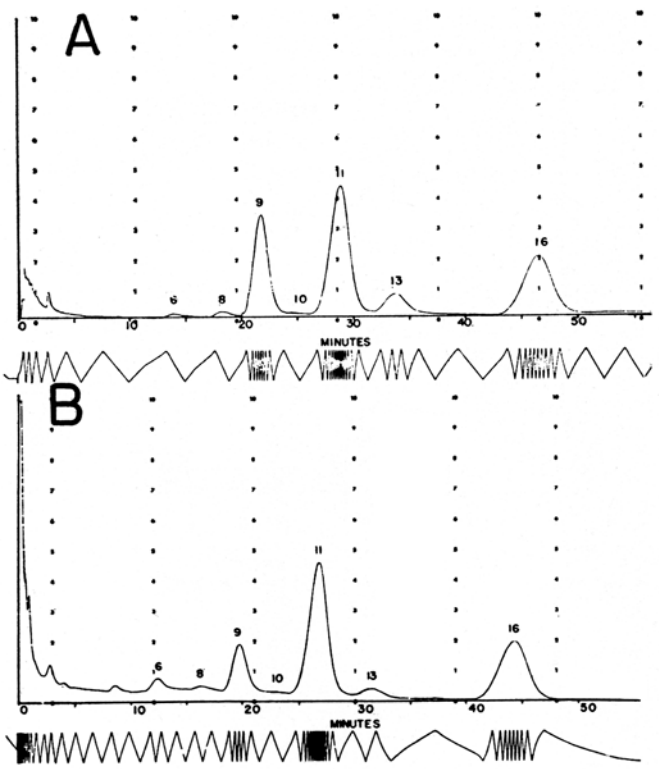

FIG. 5. Gas chromatographic separation of human bile acids as the methyl ester trifluoroacetate on QF-1. (A) glycine conjugates, (B) taurine conjugates. Lithocholic (*6*), 3-β-12-α-dihydroxycholanic (*8*), deoxycholic (*9*), chenodeoxycholic (*11*), ursodeoxycholic (*13*), cholic (*16*). Chromatography conditions as in Fig. 1.

7-β-) acid methyl ester is eluted at almost exactly the same position where the diequatorial hyodeoxycholic (3-α-6-α-) acid derivatives would have been anticipated. The identity of the peak with the ursodeoxycholate derivative was determined from runs at lower temps in which small amts of added hyodeoxycholate produced a definite advanced shoulder on the ursodeoxycholate peak.

Figure 6 represents the gas chromatographic elution patterns obtained for the trifluoroacetyl derivatives

TABLE I

Tentative Identifications of Fecal Bile Acids of Man as Established by Gas Chromatographic Means (35,37)

Peak Number	Chemical structure	Retention times		
		Fecal bile acid esters	Standards[a]	
1.	Unknown	0.11	.....	
2.	Unknown	0.12	.....	
3.	Cholanic acid	0.22	0.22	[0.22]
4.	Unknown	0.28	.....	
5.	12α (axial, shielded) cholanic	0.34		[0.33]
6.	7-β (equatorial) cholanic	0.45		[0.48]
7.	3-α (equatorial) cholanic	0.63	0.69	[0.63]
8.	3-β (axial), 12-α (axial, shielded)	0.81	0.83	[0.86]
9.	3-α (equatorial), 12-α (axial, shielded)	1.00	1.00	[1.00]
10.	Unknown	1.11		
11.	3-α (equatorial), 12-β (equatorial, shielded)	1.15		(1.18)
12.	3-α (equatorial), 7-α (axial)	1.24	1.28	(1.27)
13.	3-α (equatorial), 7-β (equatorial)	1.47	1.48	[1.50]
14.	Unknown	1.65		
15.	3-α (equatorial), 7-β (equatorial), 12-α (axial, shielded)	1.93		[1.98]
16.	3-α (equatorial), 7-α (axial), 12-α (axial, shielded)	2.01	2.01	(2.07)
17.	3-α (equatorial), 12-keto (shielded)	2.28	2.28	(2.30)
18.	3-α (equatoria'), 7-keto	2.42	2.45	(2.51)

[a] Values in brackets represent retention times obtained by Sjövall (27) for a larger selection of bile acid trifluoroacetates under similar GLC conditions.

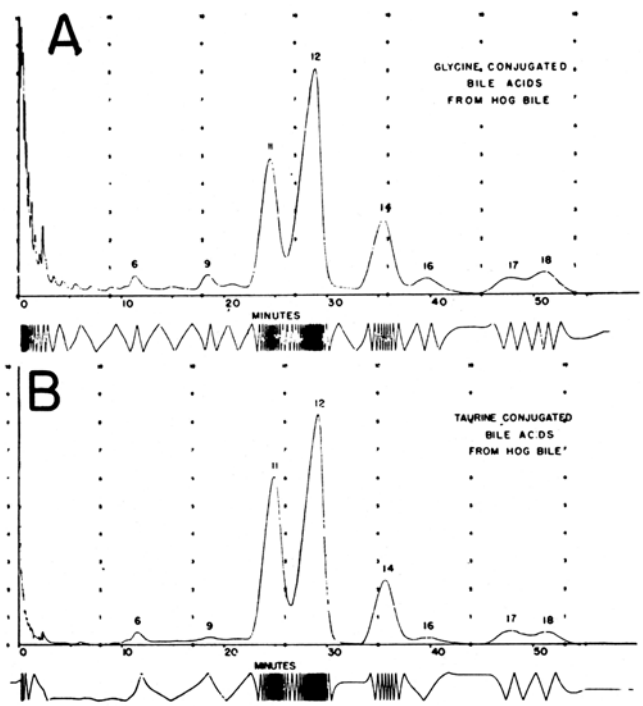

FIG. 6. Gas chromatographic separation of hog bile acid methyl ester trifluoroacetates on QF-1. (A) glycine conjugates, (B) taurine conjugates. Peaks (*6, 9, 11* and *16*) as in Figure 5; hyodeoxycholic (*12*), hyocholic (*14*), 3-α-12-keto hydroxycholanic (*17*), 3-α-7-keto hydroxycholanic (*18*).

of the methyl esters of pig bile acids. This was the most complicated mixture found for any of the animal biles thus far examined. Peaks in order of decreasing prominence were found for hyodeoxycholic (*12*), chenodeoxycholic (*11*), hyocholic (*14*), and cholic (*16*) acid derivatives. In addition, two others peaks, with retention times similar to those recorded for a pair of peaks from human feces were also found (*17* and *18*). There appears to be an inconsistency in the usual elution order of the mixed axial and equatorial derivatives. The diequatorial (3-α-6-α) monoaxial (7-α) hyocholate is eluted ahead of the monoequatorial (3-α) diaxial (7-α-12-α) cholate. It has been suggested (17) that this apparent abnormality might arise from a vicinal group interaction (positions 6 and 7) resulting in a relative reduction in the affinity between the solute and the liquid phase and a lower retention time. Such a complete reversal in the elution sequence however, is not observed with the free hydroxy compounds which emerge in the anticipated order. The behaviour of all the major pig bile acid esters on all three liquid phases (SE-30, QF-1, XE-60) was consistent with that indicated above for the standards.

The above gas chromatographic systems were equally well suited for the investigation of the composition of the fecal bile acids of man. Figure 7 shows a gas chromatogram of the fecal bile acids of a middle-aged subject on a fat-free diet (28). When compared to the fecal bile acid samples from other diets and other subjects on the fat free diet, there was considerable variation from individual to individual, although all samples contained essentially the same bile acids. In all cases identification of peaks was accomplished by co-chromatography with standards in the form of two or three derivatives on both the SE-30 and the QF-1 columns. Where standards were not available, comparisons were made between retention times recorded for the unknowns by us and those for standards by Sjövall (8). (Table I). It must have been due to

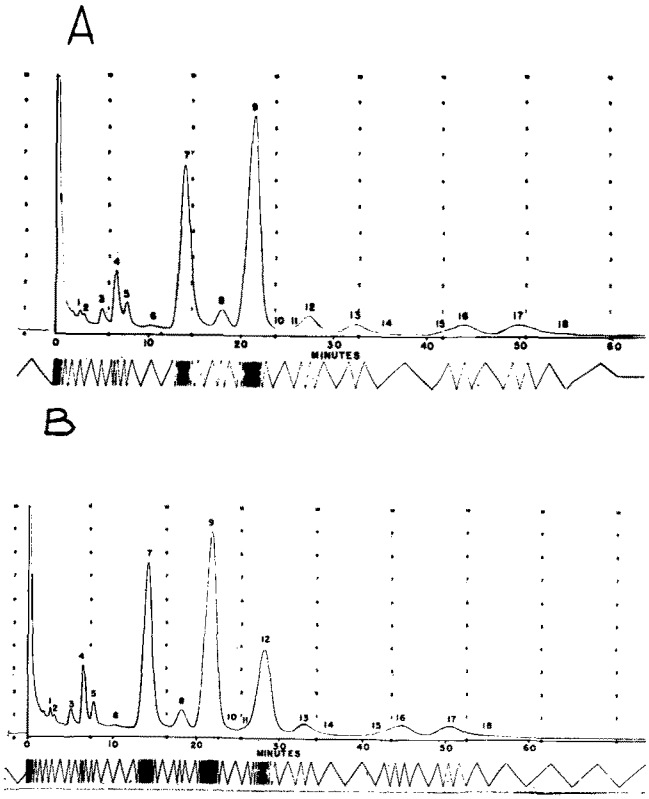

FIG. 7. Gas chromatographic separation of fecal bile acids of man as the methyl ester trifluoroacetates on QF-1. Chromatography conditions as in Figure 1. (A) original sample, (B) original sample plus chenodeoxycholate standard. Tentative peak identifications: (*1*) and (*2*) unknown, (*3*) cholanic acid, (*4*) unknown, (*5*) 12-α-cholanic, (*6*) 7-β-cholanic, (*7*) lithocholic, (*8*) 3-β-12-α-dihydroxycholanic, (*9*) deoxycholic, (*10*) unknown, (*11*) 3-α-12-β-dihydroxycholanic, (*12*) chenodeoxycholic, (*13*) ursodeoxycholic, (*14*) unknown, (*15*) 3-α-7-β-12-α-trihydroxycholanic, (*16*) cholic, (*17*) 3-α-12-keto-hydroxycholanic, (*18*) 3-α-7-keto-hydroxycholanic.

the extreme selectivity and reproducibility of the QF-1 liquid phase that the retention times recorded in the two laboratories for the standards differed only in the second decimal place. Additional support for the nature of the fecal bile acids was derived from thin-layer chromatographic separations of the free acids and a gas chromatographic examination of the recovered materials (31). Figure 8 shows the TLC separations obtained for the free bile acids during a solvent fractionation of the fecal lipid extract. The predominance of the lithocholic and deoxycholic acids in relation to the cholic acid is obvious and there is a striking resemblance between the TLC and GLC patterns of the total acid mixture. On the basis of these data 13 out of a total of 18 peaks were identified. The remaining unknown peaks, accounting for less than 10% of the total peak area appeared not to be hydroxy acids as their retention times did not change following acetylation or trifluoroacetylation. Tentative identification of the bile acids is given in Table I. In every case, except peaks *15* and *16*, where the diequatorial (3-alpha-7-beta-) monoaxial (12-alpha) derivative was eluted ahead of the monoequatorial (3-alpha) diaxial (7-alpha-12-alpha) derivative, the order of elution was fully consistent with the behaviour anticipated from stereochemical considerations. Since the possibility of incomplete trifluoroacetylation under the working conditions of any one of the trihydroxy derivatives appears to be ruled out by previous work with individual standards (10), the reversal in the elution order of the two trihydroxy derivatives requires an explanation based on the behaviour of fully trifluoroacetylated de-

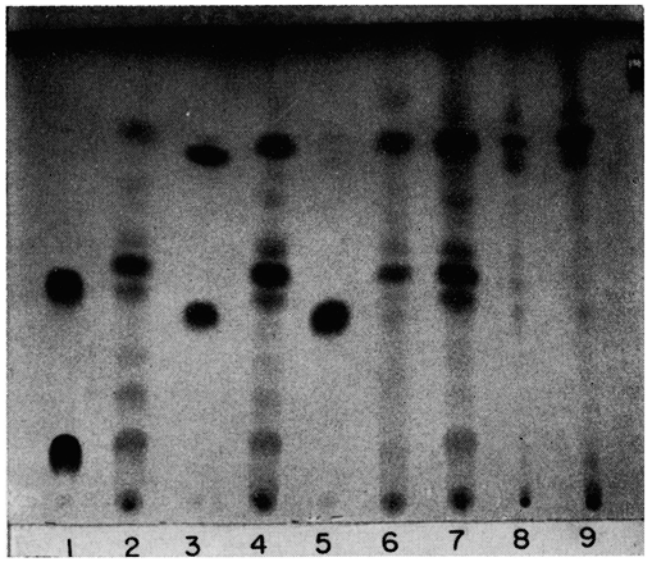

FIG. 8. Thin-layer chromatographic separation of fecal bile acids of man. TLC conditions as given by Hofmann (13) for free bile acids. (1) cholic and deoxycholic acids in ascending order; (3) chenodeoxycholic and lithocholic acids in ascending order; (5) ursodeoxycholic acid; (6), (4) and (2) first and second petroleum extracts and a final ethyl ether extract, respectively, from subject A; (9), (8) and (7) first and second petroleum extracts and a final ethyl ether extract, respectively, from subject B.

rivatives. A plausible hypothesis subject to further experimental verification has been advanced on the basis of a possible interaction between the vicinal substituents of carbons 6 and 7 of the trihydroxycholanic acid molecule (17). The more rapid elution of the 12-substituted derivatives (ketones included) can be attributed in all cases to the shielding of this position by the side chain.

Intramolecular interactions of bulky functional (substituent) groups among themselves or with the side chain must be taken into account when calculating theoretical retention times of steroids by the addition of the retention effects of individual nuclear substituents. These values are of interest as potential means of identifying unknowns on the basis of their gas chromatographic behaviour. The calculation of the related steroid number (34) seems to hold the greatest promise as it is based on retention times relative to two reference steroids rather than one and is independent on temp changes over a rather wide range. A more effective way of determining the structure of steroids in gas chromatograph effluents is provided by the newly developed (35) GLC-mass-spectrometry instrument. One microgram of a single sterol or bile acid injected into the combined instrument was sufficient to give a good mass-spectrum of good quality. The usefulness of this instrument in the analysis of bile acids and steroids from biological materials has been demonstrated.

Quantitation

Despite rapid progress in gas chromatographic separation and identification of steroids and bile acids, quantitation has been relatively unsatisfactory. It was concluded (36) that to apply gas chromatography to the quantitative determination of steroids in a mixture, the detector must be calibrated with each of the steroids present. When working with complex biological mixtures such an approach is impossible. Unknowns are usually present and reference standards may be unavailable or of doubtful purity. For flame ionization detectors the ion yield varies with the structure

and the combustibility of the material, the latter effect becoming more important for the trifluoroacetates. The high reproducibility of the characteristic detector response recorded for the trifluoroacetates, however, has suggested that simple correction factors might be found that would permit the calculation of either the weight or mole ratios from the observed area response. Since the resolutions of the trifluoroacetates on the QF-1 columns are not complicated by distortion, asymmetry or overlapping, attempts have been made to relate the variability of the detector response with chemical structure to the peak area recoveries, which in the absence of decomposition or adsorption should reflect the combustibility of the material.

Since the sensitivity of the flame ionization detector is roughly proportional to the carbon content of the solute, Ongkiehong (37) had introduced the C-factor (= M.W./(No. C atoms X 12) as a means of correcting for the differences in the hetero-atom content. Multiplication of the recorded peak area by the corresponding C-factor and normalization of the corrected areas has given a fairly good relation between detector response and weight per cent composition for hydrocarbons and chlorohydrocarbons. For oxygen containing substances a better correction has been obtained (38) by eliminating all oxygen-bonded carbon atoms in computing the C-factor. Attempts to apply these correction factors to the methyl ester trifluoroacetates of the bile acids failed to give the original weight percentages. Approximately correct molar compositions, however, could be obtained when the average area response was corrected for differences in the molar hydrogen content of the trifluoroacetates (10). It appears that equating the hydrogens provides a means for eliminating the oxygen- and halogen-bonded or noncombustible carbon atoms. The good agreement between the mole proportions in the samples and the calculated area recoveries suggests that the common bile acid trifluoroacetates are essentially completely recovered from the QF-1 columns. Although the results have been reproduced on other QF-1 columns, it is not known what contribution, if any, the design of the instrument has made to these studies.

Similar computations based on the molar hydrogen content for other bile acid derivatives showed significant differences in the mole or weight proportions between the recovered and tested materials. These discrepancies were particularly high with the simple methyl esters and could probably be attributed to degradation and adsorption on the packing. For the bile acid acetates, approximately correct molar proportions could be obtained after elution from SE-30 columns by correcting for differences in oxygen content. Since better agreement was obtained when the area response was corrected for differences in both the oxygen and hydrogen contents, it appears that with the acetates, peak areas must be corrected for on-column losses and differences in detector response.

For bile acids in natural mixtures the desired per cent ratios of the trifluoroacetates have been derived (15,31) from the peak ratios using correction factors based on differences in molar hydrogen content. With samples containing only readily identifiable acids this is easily done. Unknown peaks, on the other hand, may have to be ignored or tentative structures assigned to them. Since the structure assignments necessary for effective application of correction factors do not need to be any more complete than the segregation of unknowns into ketonic and nonketonic mono-, di- and trihydroxy derivatives (which present no special problem), relatively reliable quantitative estimates may be obtained for most bile acid peaks even for complex fecal bile acid mixtures (28,30,31). Where the actual weights of the sample cannot be obtained, the addition of an internal standard (chenodeoxycholic acid for fecal extracts, for example) provides the necessary information for total as well as fractional quantitation. The overall accuracy of such estimates has been of the order of $\pm$ 5%, but it varies with the nature and concn of the component. The recoveries of the trifluoroacetates would appear to compare favourably with those noted for the silyl ethers (9), 92 to 98% of which could be eluted from the cyclohexanedimethanol polysuccinate columns. The somewhat less satisfactory methyl ester separations on the SE-30 columns were accurate to only $\pm$ 10% using cholestane as internal standard (5). The analytical errors of 1 to 2% observed for fatty acids under optimum conditions of gas chromatography have not yet been achieved with the bile acids.

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REFERENCES

1. Horning, E. C., W. J. A. VandenHeuvel and B. G. Creech, in "Methods of Biochemical Analysis," edited by D. Glick, Interscience Publishers, New York, 1963, Vol. XI, pp. 69–147.
2. Blomstrand, R., Proc. Soc. Exp. Biol. Med. *107*, 126–128 (1961).
3. Sjövall, J., C. R. Meloni and D. A. Turner, J. Lipid Res. *2*, 317–320 (1961).
4. Holmes, W. L., and E. Stack, Biochim. Biophys. Acta *56*, 163–165 (1962).
5. Bloomfield, D. K., Anal. Chem. *34*, 737–741 (1962).
6. Gordon, B. A., and A. Kuksis, Proc. Can. Fed. Biol. Soc. *5*, 31 (1962).
7. Ellin, R. I., A. I. Mendeloff and D. A. Turner, Anal Biochem. *4*, 198 (1962).
8. Sjövall, J., Acta Chem. Scand. *16*, 1761–1764 (1962).
9. Makita, M., and W. W. Wells, Anal. Biochem. *5*, 523–530 (1963).
10. Kuksis, A., and B. A. Gordon, Can. J. Biochem. Physiol. *41*, 1355–1366 (1963).
11. Gordon, B. A., A. Kuksis and J. M. R. Beveridge, Can. J. Biochem. Physiol. *41*, 77 (1963).
12. Hofmann, A. F., J. Lipid Res. *3*, 127–128 (1962).
13. Hofmann, A. F., Anal. Biochem. *3*, 145–149 (1962).
14. de Boer, T. J., and H. S. Backer, Rec. trav. chim. *73*, 229–234 (1954).
15. Gordon, B. A., A. Kuksis and J. M. R. Beveridge, Can. J. Biochem. *42*, 897-905 (1964).
16. VandenHeuvel, W. J. A., C. C. Sweeley and E. C. Horning, Biochim. Biophys. Res. Commun. *3*, 33–36 (1960).
17. Sjöval, J., in "New Biochemical Separations," Ed. by A. T. James and L. J. Morris, D. Van Nostrand Company Ltd., 1964, pp. 66–79.
18. VandenHeuvel, W. J. A., E. O. A. Haahti and E. C. Horning, J. Am. Chem. Soc. *83*, 1513–1514 (1961).
19. Knights, B. A., and G. H. Thomas, J. Chem. Soc. 3477–3480 (1963).
20. VandenHeuvel, W. J. A., J. Sjövall and E. C. Horning, Biochim. Biophys. Acta *48*, 596–599 (1961).
21. Fieser, L. F., and M. Fieser, "Steroids," Reinhold Publishing Corp., New York, 1959. p. 222.
22. Tsuda, K., Y. Sato, N. Ikekawa, S. Tanaka, H. Higashikuze, S. Suzuki, H. Ohkubo, and Y. Anazawa, Chem. Pharm. Cull. *12*, No. 6, 710–713 (1964).
23. Hanaineh, L., and C. J. W. Brooks, Biochem. J. *92*, No. 1, 9P–10P (1964).
24. Lin, T. H., R. Rubinstein and W. L. Holmes, J. Lipid Res. *4*, 63–67 (1963).
25. Hellstrom, K., and J. Sjövall, J. Lipid Res. *3*, 397–404 (1962).
26. Gordon, B. A., Ph.D. Thesis (1963) Queen's University, Kingston, Canada.
27. Hellstrom, K., J. Sjövall and G. Wigand, J. Lipid Res. *3*, 405–412 (1962)
28. Ali, S. S., A. Kuksis, and J. M. R. Beveridge, Proc. Can. Fed. Biol. Soc. *6*, 6 (1963).
29. Danie'sson, H., P. Eneroth, K. Hellstrom, S. Lindstedt and J. Sjövall, J. Biol. Chem. *238*, 2299–2304 (1963).
30. Beveridge, J. M. R., S. S. Ali and A. Kuksis, Proc. Can. Fed. Biol. Soc. 7, 26 (1964).
31. Ali, S. S., A. Kuksis and J. M. R. Beveridge, in preparation.
32. Danielsson, A., A. Kallner and J. Sjövall, J. Biol. Chem. *238*, 3846–3852 (1963).
33. Gordon, B. A., and A. Kuksis, in preparation.
34. Hamilton, R. J., W. J. A. VandenHeuvel and E. C. Horning, Biochim. Biophys. Acta *70*, 679–687 (1963).
35. Ryhage, R., and J. Sjövall, Biochem. J. *92*, No. 1, 2P–3P (1964).
36. Sweeley, C. C., and T. Chang, Anal. Chem. *33*, 1860–1863 (1961).
37. Ongkiehong, L., Ph.D. Thesis (1960). The Technical University Eindhoven, Netherlands, cited in reference 38, p. 220.
38. Dal Nogare, S., and R. S. Juvet, Jr., "Gas Chromatography," Interscience Publishers, New York, 1962, p. 220.