Measurement of human serum bile acids by gas–liquid chromatography

D. H. SANDBERG,* J. SJÖVALL,‡ K. SJÖVALL,‡ and D. A. TURNER§

Biochemistry Research Division, Department of Medicine, Sinai Hospital of Baltimore, Inc., Baltimore, Maryland

SUMMARY Gas–liquid chromatography (GLC) has been used for the separation and quantification of the bile acids of normal and abnormal serum. Recovery of added bile acids from serum samples during the combined processes of extraction with an anion exchanger, hydrolytic cleavage of the conjugates, and purification by alumina chromatography of the bile acid methyl esters, was 60–85%. The main serum bile acids were identified as deoxycholic, chenodeoxycholic, and cholic acids by GLC of their methyl esters, partial trimethylsilyl ethers, and trifluoracetates. Quantification was most satisfactorily achieved with the trifluoracetates of the methyl esters on the stationary phase QF-1.

Values for normal fasting subjects ranged from 0.03 to 0.23 mg/100 ml of serum. In two experiments the level rose from 0.04–0.06 to 0.18–0.32 mg/100 ml after a meal. In a number of patients with liver disease the serum bile acid levels were between 0.39 and 20.1 mg/100 ml. Deoxycholic acid was either absent or made up only a small percentage of the total bile acids in these patients. In two patients examined, 3 and 5% of the bile acids were unconjugated.

KEY WORDS bile acids · human serum · normal · pathological · gas–liquid chromatography · cyanoethyl methyl silicone · fluoroalkyl silicone · trifluoracetates · trimethylsilyl ethers · anion exchanger

BECAUSE OF THE LIMITATIONS inherent in available techniques for the separation and measurement of bile acids, studies of normal serum bile acid levels and patterns have not been satisfactory (1). Further progress has awaited the development of more specific and sensitive analytical methods.

Recent developments in the separation by gas–liquid chromatography (GLC) of bile acid methyl esters (2–8) prompted us to study the application of this method to the analysis of serum bile acids. It is known from paper chromatographic studies that the concentration of bile acids in normal serum is quite low (9). This made it necessary to develop a method for preliminary purification that eliminates interfering substances.

The procedure developed utilizes an initial extraction of the bile acids from serum with a strong anion exchange resin. The conjugates are hydrolyzed and the free bile acids are methylated and purified by passage through an alumina column. The extract is then analyzed by GLC. For identification of the microgram quantities of bile acids in serum, a simple technique was developed which used two different derivatives of the bile acid methyl esters and two columns with different stationary phases. Data on the chromatographic behavior of a variety of bile acids on columns coated with a nitrile silicone rubber gum as the stationary phase are also included. A preliminary report of this investigation has been published (10).

METHODS

Reagents

Fisher "Certified Reagents" were used. All solvents were redistilled twice. Ammonium carbonate was recrystallized from aqueous ethanol, dried by suction on a sintered glass funnel, and stored at −15°. Aqueous solutions were made with twice distilled water.

Amberlyst XN-1006 anion exchange resin in the chloride form (kindly supplied by Mr. R. W. Percival, Rohm and Haas, Philadelphia, Pa.) was washed on a

* Present address: Department of Pediatrics, University of Miami, Miami, Florida.

‡ Present address: Department of Chemistry, Karolinska Institutet, Stockholm, Sweden.

§ Present address: The Research Institute, The Hospital for Sick Children, Toronto, Canada.

sintered glass funnel with 5–10 volumes of the following series of solvents: water, ethanol, hexane, ethanol, and water. To obtain the resin in the hydroxide form, it was recommended by the manufacturer that the ion exchanger first be converted into the bicarbonate form and then into the hydroxide form. The resin is preferably stored in the bicarbonate form in water and the columns are treated with sodium hydroxide and washed to neutrality with water just prior to application of a serum sample.

Neutral aluminum oxide, activity grade I, was heated at 600° to destroy any organic material which might be present, and then stored in a vacuum desiccator over silica gel.

24-C^{14}-Labeled cholic, chenodeoxycholic, and deoxycholic acids were prepared as described by Bergström et al. (11), and had a specific activity of approximately 10, 8, and 3 μc/mg respectively. Labeled "taurocholic acid" was obtained by injecting a bile fistula rat with 5 μc of C^{14}-labeled cholic acid and collecting the bile for 3 hr. An ethanol extract of this bile was evaporated and the residue dissolved in water and used for recovery experiments. Radioactivity was determined in a Packard Tri-Carb Liquid Scintillation Spectrometer. The unlabeled bile acid standards were as used in previous investigations (3, 6).

Preparation of Bile Acid Derivatives

3α-Trimethylsilyl ethers were prepared by dissolving bile acid methyl esters in 0.2 ml of anhydrous acetone to which 0.1 ml of hexamethyldisilazane was added. After 3 hr at 50° (optimal conditions for formation of 3α-, 3β-, 7β-, and 12β-trimethylsilyl ethers with minimal reaction of 7α- and 12α-hydroxyl groups as determined by testing a number of isomeric mono-, di-, and trihydroxycholanic acid methyl esters, Sjövall and Weisz, unpublished data) the solvents were removed by evaporation under a stream of nitrogen and the sample was dissolved in acetonitrile and analyzed by GLC. Acetonitrile was used as solvent for all GLC analyses since it is not ionized in the argon ionization detector.

Trifluoracetates of the bile acids were prepared by addition of 0.2 ml of trifluoracetic anhydride to the sample, heating for 15 min at 35°, and evaporating off the reagent (6). The residue was dissolved in acetonitrile, and an aliquot was used for GLC.

Gas–Liquid Chromatography

An EIR analytical gas chromatograph Model AU8 (Electronic Communications, Inc., Timonium, Md.) with an argon ionization detector (20 mc Sr^{90} foil) was used throughout. The gain was set to give full scale deflection on a 10 mv recorder at 3×10^{-10} amp. Columns were prepared as previously described (3, 6, 8) using Gas Chrom S [treated as described earlier (3) for Gas Chrom P] as support, and as stationary phase either 0.5% (w/w) nitrile silicone (CNSi, 50 moles % β-cyanoethyl-methyl polysiloxane, General Electric 287-108-949) or 0.5–1% (w/w) QF-1 (Dow Corning FS-10065 fluorsilicone fluid). U-shaped glass columns, 6–8 ft × 4–5 mm i.d., were used. Column conditions were similar to those previously described (3, 6, 8). The flash heater temperature should not exceed 240° when the trifluoracetoxy derivatives are chromatographed.

PROCEDURE

A sample of serum (5–10 ml) was diluted 1:1 with distilled water. The anion exchange resin (5 ml) in the bicarbonate form was pipetted into a chromatographic column 1.0 cm in diameter. The resin was then washed with 50 ml of 1 N NaOH and 50 ml of 1 N NaOH in 80% ethanol, followed by distilled water until the effluent was neutral. The serum was adjusted to pH 11 with a few drops of 1 N NaOH, quantitatively transferred into the column and allowed to pass through the resin at a rate of about one drop every 2 sec. The column was then washed to neutrality with distilled water. After successive washing with 20 ml of 95% ethanol, 20 ml of ethanol–ethylene chloride 1:1 (v/v), and 20 ml of 80% ethanol, the bile acids were eluted with 150 ml of 0.2 M $(NH_4)_2CO_3$ in 80% ethanol (12).

The eluate was evaporated to dryness in a flash evaporator and dissolved in small portions of 1 N NaOH to make a total of 20 ml, and the solution was transferred into a 50 ml nickel crucible. Hydrolysis was carried out for 4 hr in an autoclave at 15 psi. A nickel crucible was used since it was found that a variable amount of C^{14}-labeled bile acids was adsorbed by precipitated silica if the hydrolysis was done in Pyrex glass. Since the preparation of this report further studies have shown that somewhat better recoveries are obtained by hydrolysis of the sample for 10 hr with 15% NaOH in 50% aqueous ethanol at 115–120°, as described by Irvin et al. (13). This hydrolysis has been carried out in Teflon containers in Parr bombs.

The sample was subsequently transferred to a separatory funnel and after acidification to pH 1 with 6 N HCl was extracted three times with equal volumes of diethyl ether. The pooled extracts were washed with small portions of distilled water until neutral; the water phases were reextracted with about 20 ml of ether to prevent any loss of bile acids in this step.

The pooled ether extracts were evaporated to dryness and the residue was dissolved in 10 ml of ether plus 1 ml of methanol. Diazomethane was added in excess (as revealed by a persistent yellow color), and the mixture allowed to react for 15 min. This solution was then

TABLE 1 RECOVERY OF 24-C^{14}-LABELED BILE ACIDS ADDED TO SERUM

Stage of Procedure	Deoxycholic Acid		Chenodeoxycholic Acid		Cholic Acid		Taurocholic Acid	
				cpm				
Added to serum	31,400;	31,400	18,670;	18,670	19,600;	19,600	29,700;	29,700
After anion exchange	30,200;	30,000	—	18,100	18,700;	18,300	28,400;	28,200
After ether extraction	27,900;	25,900	16,900;	17,200	17,500;	20,000	—	—
After alumina column	25,400;	25,100	15,100;	14,900	18,400;	17,000	24,300;	25,300
Percentage recovery of added C^{14} for entire procedure	83	82	83	82	96	89	84	87

evaporated to dryness and the residue was dissolved in about 10 ml of anhydrous benzene and applied to an alumina column (prepared in benzene with 2 g of aluminum oxide to which 0.2 ml of water had been added). After the column had been washed with about 40 ml of benzene, the bile acid methyl esters were eluted with 40 ml of methanol–acetone 1:9 (v./v). This eluate was evaporated to dryness and the residue was transferred in acetone to a 2 ml graduated glass-stoppered centrifuge tube.

The acetone was evaporated under a stream of nitrogen, 0.2 ml of trifluoracetic anhydride was added, and the tube was stoppered and incubated for 15 min at 35°. Excess reagent was then removed under a stream of nitrogen and the residue was dissolved in 50–100 μl of acetonitrile. A 5 μl aliquot was injected into the gas chromatograph.

The areas of the peaks corresponding to the trifluoracetates of methyl deoxycholate, chenodeoxycholate, and cholate were measured by triangulation or with a planimeter. In each series of samples analyzed by GLC a standard mixture containing the trifluoracetates of methyl deoxycholate, chenodeoxycholate, and cholate was included. All quantifications were made with loads within the range for which the peak area/microgram ratio had been shown to be constant. A Hamilton syringe with a Chaney adapter was used and the same volume was always injected both from the sample and from the standard solution.

RESULTS

Purity of Reagents

Because of the small quantities of bile acids present in normal serum, the use of highly purified solvents and reagents was essential. Solvents were carefully redistilled to eliminate residues that give rise to interfering peaks in the gas chromatograms. After distillation, all solvents were tested by evaporation of 100 ml aliquots to dryness. The residue was washed into a centrifuge tube with a small volume of the same solvent. The solvent was then evaporated and the sample dissolved in 50 μl of acetonitrile. The absence of artifactual GLC peaks arising from the solvent residue was tested by injecting 5 μl of the acetonitrile solution into the gas chromatograph. Except for a small solvent front, no peaks should be seen at the appropriate sensitivity setting.

The purity of the ammonium carbonate, the aluminum oxide, and the ion exchanger was tested by extraction with the solvents to be used and analysis of the concentrated extracts by GLC. Purification of these reagents as described was found to be necessary to eliminate interfering peaks in the gas chromatograms. Thoroughly cleaned glassware should be kept separate for use only in the analysis of serum bile acids.

Recovery of Bile Acids from Serum

Preliminary recovery experiments employing the addition of C^{14}-labeled bile acids to serum showed that the added compounds could be quantitatively extracted by addition of serum, dropwise, to 20 volumes of ethanol. After filtration, the ethanol extract was diluted with water until it was 70% with respect to ethanol and extracted three times with half the volume of hexane to remove most of the lipids. This procedure resulted in a loss of about 5% of the least polar bile acid tested (deoxycholic acid) and less for the other acids. However, subsequent hydrolysis and purification of such an extract failed to give a sample pure enough to be analyzed by GLC. Other solvents were studied for the extraction until it was found that strong anion exchangers in the hydroxide form were able to extract added bile acids quantitatively from serum. Amberlyst XN-1006 was the best of the ion exchangers tested, since it did not markedly change volume on change of solvent and did not adhere to the walls of the chromatography column.

The recovery of added C^{14}-labeled bile acids was tested at various stages throughout the extraction procedure by counting an aliquot of the sample in a scintillation counter. This was initially studied when the serum was put on the ion exchange column at pH 7.4. The recovery data are listed in Table 1. However, when the procedure was tested with the addition of unlabeled

compounds using glycine conjugates of cholic and deoxycholic acid, the percentage recovery was lower as measured by GLC. We have not been able to explain this difference fully, but it was considered to be partly the result of protein binding of serum bile acids (14), and so the same test with added unlabeled compounds was made after preliminary addition of enough 5 N NaOH to make the pH approximately 11. Recoveries of added taurine and glycine conjugates were improved by this procedure and are listed in Table 2. The differences in the recoveries between Tables 1 and 2 are due largely to destruction of the bile acids in the hydrolytic step. Thus autoradiography of thin-layer chromatograms of hydrolyzed pure C^{14}-bile acids showed faint spots of compounds other than the expected bile acid. Hydrolysis of pure conjugated bile acids using the procedure of Irvin et al. (13) gave recoveries between 70 and 100% as measured by GLC, but 100% recovery of C^{14} was usually found.

Thus, it appears that with the present procedure, recoveries of both conjugated and free acids, whether added to serum or already present, are between 59 and 87% for the entire procedure (Table 2).

Gas–Liquid Chromatography

Different stationary phases were tried for the GLC analyses of the minute amounts of bile acids present in serum. A batch of methyl-cyanoethyl polysiloxane previously used (8), at 0.5% (w/w) on silanized Gas Chrom S, was found to give the least tailing with trihydroxycholanates. The relative retention times of a number of substituted methyl cholanates on columns of this kind are given in Table 3. A chromatogram of a synthetic mixture of the common serum bile acids is shown in Fig. 1, and Fig. 2 illustrates the result of a similar chromatography of a normal serum extract. Although a linear detector response could be obtained in the 0.1–0.9 μg range, the use of the CNSi phase for quantification of serum bile acids as their methyl esters had some drawbacks. As seen in Fig. 2, an interfering compound which could not be removed appears in the tail of methyl chenodeoxycholate, and the long retention time of methyl cholate is inconvenient.

Because of the large separation factors that can be obtained by using trifluoracetoxy (TFA) derivatives of bile acids on columns with QF-1 as stationary phase (6), conditions were tested for the quantification of these derivatives. As seen in Fig. 3, the peak area/microgram response curve for the trifluoracetate of methyl deoxycholate was linear in the range of 0.2–0.45 μg. All quantitative measurements were made after injection of a constant volume (usually 4 or 5 μl) from suitable dilutions of the standard and sample solutions. Reproducibility of the injections was satisfactory with a 10 μl Hamilton syringe equipped with a Chaney adapter, as seen in Fig. 3. The trifluoracetates of methyl deoxycholate and chenodeoxycholate usually gave the same response per microgram, whereas that of the cholic acid derivative often gave a lower response (calculated on the weight of the methyl ester). Responses between 72 and 100% of that of the deoxycholic acid derivative were obtained with different columns. These variations indicate destruction of the trifluoracetates to different extents on different columns. In fact, several columns were made which could not be used in quantitative work because of the appearance of sig-

TABLE 2 Recoveries of Unlabeled Glycine and Taurine Conjugated Bile Acids Added as Sodium Salts to Serum from Normal Subjects and Carried Through the Entire Procedure

Bile Salt Added	Serum	Amount Added	Amount Recovered	Recovery
	ml	*μg*	*μg*	%
Taurocholate	10	26.0	18.4	71
	10	26.0	20.7	79
Glycocholate	10	5.3	3.1	59
	5	0.42	0.31	74
	5	0.48	0.32	67
Taurodeoxycholate	10	32.1	19.4	60
	10	32.1	23.5	73
Glycodeoxycholate	10	47.5	37.2	78
	5	0.70	0.61	87
	5	0.70	0.51	73
	5	0.35	0.22	63
	5	0.43	0.32	74

TABLE 3 Retention Times Relative to Methyl Deoxycholate of Some Bile Acid Methyl Esters on 0.5% CNSi Columns

Substituted Methyl Cholanate*	Relative Retention Time	Substituted Methyl Cholanate*	Relative Retention Time
12α	0.24	3-keto,7α	1.74
12β	0.26	3,12-diketo	1.56
3β	0.34	3,7-diketo	1.61
3α	0.37	3α,7α,12α	3.08
12-keto	0.28	3α,7β,12α	3.20
3-keto	0.49	3α,6α,7α	3.51
3β,12α	0.91	3α,6α,7β	3.12
3α,12α	1.00	3α,6β,7α	3.71
3α,12β	1.06	3α,6β,7β	2.82
3α,7α	1.10	3α,6β,12α	3.41
3α,7β	1.18	3α,12α,7-keto	4.07
3α,6α	1.48	3α,7α,12-keto	4.19
3α,12-keto	1.23	3-keto,7α,12α	4.97
3-keto,12α	1.44	3α,7,12-diketo	3.98
3α,7-keto	1.14	3,7,12-triketo	4.55

Column temperature 225°, argon inlet pressure 24 psi.
* Hydroxyl groups denoted by Greek letters, keto groups by -keto.

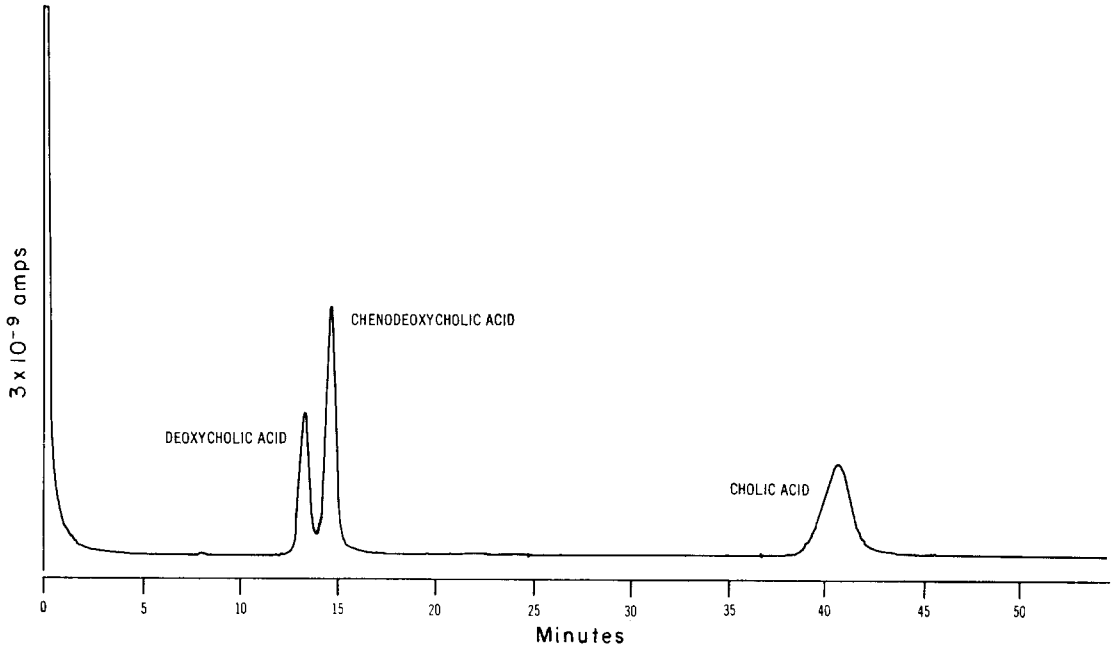

FIG. 1. Separation of a synthetic mixture of the methyl esters of deoxycholic, chenodeoxycholic, and cholic acids on a 6 ft × 4 mm column of 0.5% (w/w) methyl-cyanoethyl polysiloxane (CNSi) on 120–140 mesh Gas Chrom S. Column temperature 225°. Argon inlet pressure 24 psi.

nificant degradation peaks, especially with the chenodeoxycholic and cholic acid derivatives.

The degradation was particularly evident when small amounts (<0.5 μg) were analyzed. The appearance of degradation products as noted by a very slight rise in the baseline can be easily verified by mass spectrometry of the effluent from the gas chromatography (P. Eneroth, B. Gordon, R. Ryhage, and J. Sjövall, to be published). It is then seen that the tendency for decomposition depends on the nature, position, and stereochemistry of the substituents. In the present investigation only deoxycholic, chenodeoxycholic, and cholic acids were measured, and all quantitative estimations were based on comparison with appropriate standards in a linear range. In the course of this investigation, a report was published describing the use of trifluoracetates for quantitative bile acid analysis by GLC (15). A flame ionization detector was used and differences in molar response between bile acids containing a different number of hydroxyl groups was noted, but differences between various columns were not discussed.

Chromatography of trifluoracetoxy derivatives of the serum bile acids on 0.6% QF-1 columns is illustrated in Fig. 4. The separation factors are greater, there are no interfering peaks, and the analysis can be carried out more rapidly than on CNSi.

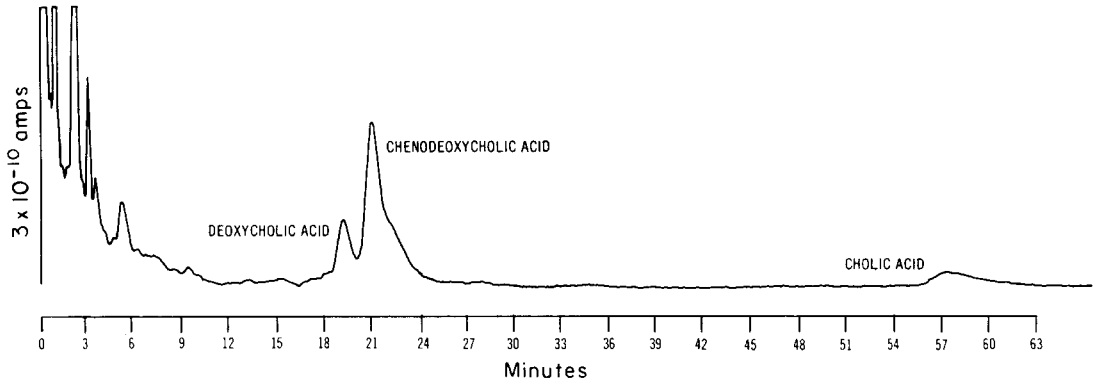

FIG. 2. Gas chromatogram of the methylated bile acid fraction from normal serum. Conditions as for Fig. 1, except for column temperature 220° and argon inlet pressure 20 psi.

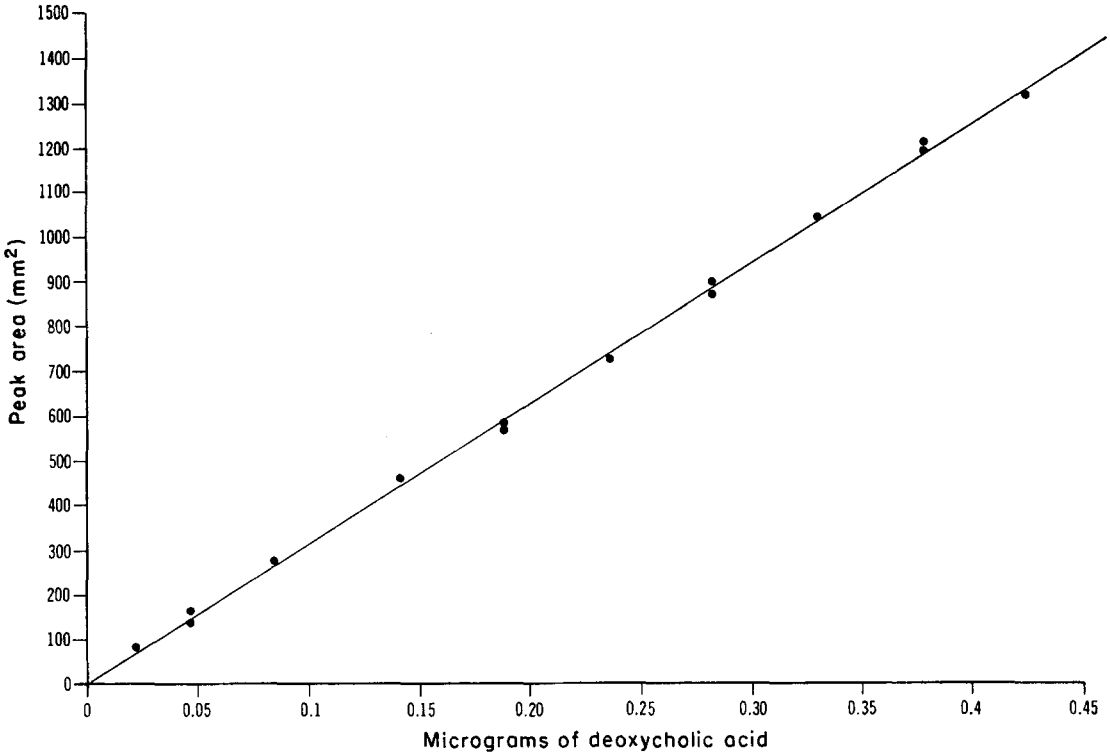

FIG. 3. Standard curve, deoxycholic-TFA derivative. Relationship between peak area and amount of deoxycholic acid analyzed as the trifluoracetylated methyl ester on a 0.6% QF-1 column.

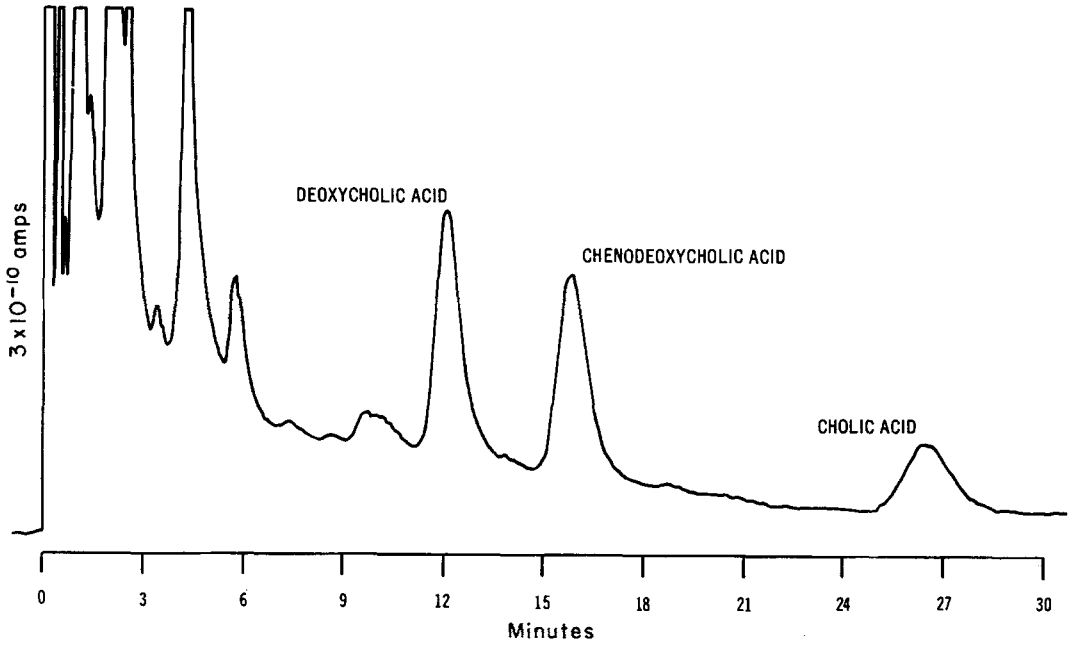

FIG. 4. Analysis of the trifluoracetylated methyl esters of bile acids in normal serum. Column 6 ft × 4 mm, 0.6% QF-1 on 100–120 mesh Gas Chrom S. Column temperature 210°. Argon inlet pressure 25 psi.

In order to find out whether the peaks in the chromatograms of serum extracts were in fact due to the presence of bile acids and that they represented only bile acids, a simple peak shift technique was developed. The 3α-trimethylsilyl ethers were first prepared and after GLC analysis the solvent was removed under nitrogen and trifluoracetates of the same sample were prepared. The trimethylsilyl ethers immediately hydrolyzed when dissolved in trifluoracetic anhydride. Table 4 shows the retention times of the various derivatives of the serum compounds and reference compounds, and Fig. 5 a chromatogram obtained from serum by this procedure. The results strongly indicate that the observed peaks in the serum samples are attributable to deoxycholic, chenodeoxycholic, and cholic acids. Further confirmation of the specificity of the method has been obtained by mass spectrometric analysis of the bile acid derivatives as they emerge from the GLC column (R. Ryhage, J. Sjövall, and K. Sjövall, unpublished results).

Serum Bile Acid Levels

Table 5 lists some values obtained for the bile acid levels in serum from apparently healthy individuals. No correction was made for losses in arriving at the values in the tables. The reproducibility of the method is illustrated by the results of analyses of plasma and serum obtained at the same time from some subjects. In 8 samples containing between 0.16 and 0.45 μg of individual bile acids per milliliter of serum (total bile acids, 0.73–1.11 μg/ml serum), GLC analyses were carried out in duplicate. Aliquots corresponding to an original serum volume of 0.2 and 0.5 ml were injected. The differences between the duplicates were between 0 and 0.15 μg/ml (mean 0.05 μg/ml) for the individual bile acids. For total bile acids the differences were 0.04–0.21 μg/ml (mean 0.13 μg/ml). In another experiment six GLC analyses were made of the same serum extract. The following ranges were found: deoxycholic acid 0.40–0.46 (mean 0.43), chenodeoxycholic acid 1.25–1.32 (mean 1.29), and cholic acid 0.31–0.35 (mean 0.33) μg/ml serum. By omitting the hydrolysis step in the procedure, one can get values for free bile acids. These values (subject 16, Table 5 and subjects 1 and 2, Table 6) show that most of the serum bile acids are conjugated.

Table 6 gives the results of analyses of serum bile acids in some patients; Fig. 6 shows a typical chromatogram. Deoxycholic acid makes up a very small part of the markedly increased amounts of serum bile acids in the cases with liver diseases. In two patients (1 and 2) with portal cirrhosis, the amount of free bile acids was also estimated. The level was high compared with normal bile acid levels, but the concentration of free as a percentage of total bile acids was low (less than 5%). Analyses were also made on different serum samples from a patient (no. 11) with marked hyperlipemia. The bile

TABLE 4 COMPARISON BETWEEN RELATIVE RETENTION TIMES OF SERUM BILE ACID DERIVATIVES AND REFERENCE COMPOUNDS ON COLUMNS WITH CNSi AND QF-1 AS STATIONARY PHASES

	Retention Times Relative to Methyl Deoxycholate on					
	QF-1*			CNSi†		
Compound	OH‡	TMS‡	TFA‡	OH	TMS	TFA
Methyl deoxycholate	1.00	0.57	0.67	1.00	0.42	0.23
Serum peak	1.00	0.59	0.68	1.00	0.43	0.23
Methyl chenodeoxycholate	1.15	0.65	0.86	1.10	0.48	0.31
Serum peak	1.15	0.65	0.86	1.10	0.48	0.31
Methyl cholate	2.33	1.24	1.34	3.08	1.27	0.48
Serum peak	2.33	1.26	1.35	3.08	1.24	0.48

* Column: 6 ft × 4 mm, 0.6% QF-1 on 120–140 mesh Gas Chrom S. Temperature: 221°, argon inlet pressure 21 psi.

† Column: 6 ft × 4 mm, 0.5% CNSi on 120–140 mesh Gas Chrom S. Temperature: 225°, argon inlet pressure 24 psi.

‡ OH, free hydroxyl groups; TMS, 3α-trimethylsilyl ethers; TFA, trifluoracetates.

TABLE 5 ANALYSES OF SERUM SAMPLES FROM HEALTHY INDIVIDUALS

Subject (Sex)	Deoxycholic Acid	Chenodeoxycholic Acid	Cholic Acid	Total
	μg bile acid per ml serum			
1. C. B. (F)	0.43	0.44	0.33	1.20
2. B. P. (F)	0.13	0.13	0.03	0.29
3. L. F. (F)	0.06	0.41	0.40	0.87
4. C. P. (M)	0.18	0.22	0.06	0.46
5. D. V. (F)	0.45	0.05	0.07	0.57
6. A. P. (M)	0.13	0.07	0.09	0.29
7. J. M. (M)	0.17	0.15	0.08	0.40
8. W. C. (M)	0.35	0.07	0.04	0.46
9. J. S. (M)	0.13	0.07	0.09	0.29
10. A. L. (M)	0.22	0.34	0.12	0.68
11. B. F. (M)	0.15	0.14	0.02	0.31
12. D. M. (F)	0.29	0.90	0.65	1.84
13. E. M. (M)	0.36	1.30	0.60	2.26
14. A. K. (M)	0.42	0.71	0.25	1.38
15. E. H. (M)	0.30	0.38	0.21	0.89
16. D. S. (M)				
Serum	0.06	0.14	0.17	0.37
Plasma	0.05	0.18	0.21	0.44
Unhydrolyzed	0.02	0.02	0.09	0.13
17. K. S. (F)				
Plasma*	0.13	0.10	0.15	0.38
Plasma*	0.12	0.11	0.18	0.41
Serum†	0.49	0.56	0.77	1.82
18. K. S. (F)				
Serum*	0.20	0.16	0.22	0.58
Serum*	0.19	0.16	0.21	0.56
Serum†	0.80	1.10	1.83	3.73

* Fasting samples.

† Samples taken 1 hr after lunch.

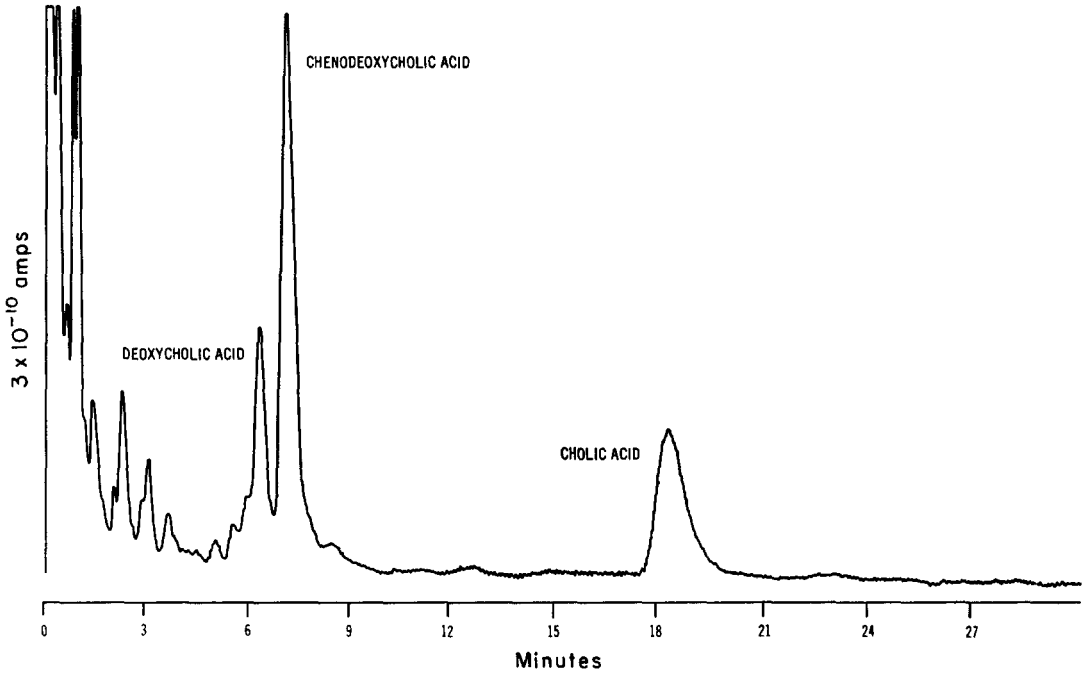

FIG. 5. Gas chromatogram of the 3α-trimethylsilyl ethers of the methylated bile acid fraction from normal serum. Column conditions as for Fig. 1.

acid levels fell within the normal range, but showed considerable differences on different days. No interfering peaks or excessive front peaks were seen in the gas chromatograms of these samples, suggesting that the method of purification is satisfactory even with markedly hyperlipemic sera.

DISCUSSION

Previously Described Methods

The first demonstration of the presence of bile acids in normal human serum other than by nonspecific colorimetry was made by Carey (9). He found spots on paper chromatograms of hydrolyzed serum extracts that corresponded to cholic, chenodeoxycholic, and deoxycholic acids. Isotope techniques have been used to measure quantitatively the bile acids in rat portal blood (16) and in the peripheral blood of rats, rabbits, and monkeys (17–19). Because of the large amount of isotope needed, these methods are not generally applicable to human studies. Many colorimetric methods have been described; a summary of these with the reported results has been published (1). Other recent methods include those by Levin and Johnston (20), Osborn and Wootton (21), Karbach (22), Yamagishi (23), and Ayad (24). Although some of these colorimetric methods can be used to determine elevated serum bile acid levels, lack of specificity and sensitivity precludes their use in the determination of normal and slightly elevated levels (see below).

Colorimetric methods, usually combined with preliminary chromatographic separation to increase the specificity, have been used to study the ratio of trihydroxy to dihydroxycholanic acids in sera with elevated bile acid levels (14, 25–27). Since the metabolic pathways of dihydroxycholanic acids are very different [chenodeoxycholic acid is formed from cholesterol in the liver and deoxycholic acid from cholic acid in the intestinal tract (28)], an interpretation of values for total dihydroxycholanic acids in various pathological conditions is difficult.

Features of Present Method

A method which can separate and measure the three major bile acids with relative ease will therefore be of service in the investigation of bile acid metabolism. The main advantage of the technique here described is the combined extraction and purification achieved in one step by direct application of diluted serum to a column of the strong anion exchanger Amberlyst XN-1006 in the hydroxyl form. The conjugated bile acids are then hydrolyzed. Quantitative hydrolysis of conjugated bile acids has always presented a difficult problem which has been studied by, for example, Irvin et al. (13) and Levin et al. (29). It is probable that some of the loss of bile acids in the present procedure occurred in the hydrolytic step. In the later stages of this work we have used the

TABLE 6 ANALYSES OF SOME PATIENTS WITH DISEASES AFFECTING THE LIVER

Patient (Sex) (Disease)	Deoxycholic Acid	Chenodeoxycholic Acid	Cholic Acid	Total
		μg bile acid per ml serum		
1. L. S. (F) (portal cirrhosis)				
Hydrolyzed sample	0.06	37.30	22.10	59.50
Unhydrolyzed; pH 7.4*	—	0.93	0.81	1.74
2. A. M. (M) (portal cirrhosis)				
Hydrolyzed sample	0.23	11.60	10.10	21.90
Unhydrolyzed	—	0.53	0.62	1.15
3. F. J. (F) (hepatitis)	0.13	21.40	22.60	44.10
4. A. G. (M) (infectious hepatitis)	0.52	31.30	102.30	134.10
5. G. L. (F) (common duct stone)	0.09	1.09	2.76	3.94
6. M. S. (F) (carcinoma of pancreas) pH 11	1.25	6.47	11.10	18.80
7. N. H. (F) (carcinoma of pancreas with metastases)	0.08	1.91	3.03	5.02
8. S. G. (F) (bile duct atresia)	—	13.70	187.00	200.70
9. D. C. (M) (sickle cell disease)	0.75	3.72	0.97	5.44
10. C. L. (F) (sickle cell disease with microinfarcts of liver)	—	4.88	3.43	8.31
11. J. K. (F) (marked hyperlipemia)†	0.20	0.10	0.02	0.32
11. J. K. (F) (marked hyperlipemia)†	0.24	0.35	0.30	0.89
12. A. J. (F) (infectious hepatitis) pH 11	—	22.20	29.50	51.70
13. L. D. (F) (infectious hepatitis) pH 11 (died)	—	7.70	0.67	8.37
14. C. G. (F) (infectious hepatitis) pH 11	—	8.30	16.20	24.50
15. C. H. (F) (bile duct atresia) pH 11	—	40.00	54.00	94.00

* pH values refer to pH of serum when extracted for bile acids.
† Two serum samples obtained on different days.

method of Irvin et al. (13) and in our hands this hydrolysis has given the highest recoveries of several methods tried. Further improvements in this step may have to await the development of an enzymatic method for hydrolysis of conjugated bile acids.

A number of methods were tried for the purification of the extract containing bile acids after hydrolysis. A two-stage countercurrent extraction between 70% ethanol and petroleum ether gave good recovery of bile acids, but the sample contained a large number of components that were eluted with the solvent in the subsequent GLC. The bile acid derivatives often appeared on the slope of a trailing solvent peak, which made quantification difficult. Chromatography of the extract on silicic acid and cellulose columns was tried but was found to give less pure extracts than the alumina chromatography of methyl esters finally used.

TABLE 7 RESULTS OF SERUM BILE ACID ANALYSES OF A POOLED SERUM SAMPLE USING DIFFERENT METHODS FOR THE EXTRACTION OF BILE ACIDS FROM SERUM

Extraction Procedure	Deoxycholic Acid	Chenodeoxycholic Acid	Cholic Acid
		μg bile acid per ml serum	
Ion exchanger, serum at pH 7.4	0.10	0.03	0.02
Ion exchanger, serum at pH 11	0.17	0.13	0.14
Ethanol extraction followed by ion exchanger, serum at pH 7.4	0.14	0.20	0.13

The quantitative GLC analyses have been made with trifluoracetoxy derivatives, and compared with similar analyses of standard mixtures of the same compounds as those present in the serum samples. The advantages of using an internal standard in GLC are well known, but no satisfactory compound has so far been found. However, good results are obtained by the injection of constant volumes of both the sample and the standard solution.

Bile Acid Levels in Normal Serum

The values for total concentration of bile acids obtained with the present method ranged from 0.03 to 0.23 mg/100 ml serum in fasting persons. The lower range of values was obtained with the serum at pH 7.4. It should be noted that in two experiments the bile acid level was increased after a meal. This might indicate that serum bile acids increase after gall bladder contraction, which can occur during fasting. This will widen the range of values obtained. Deoxycholic, chenodeoxycholic, and cholic acids were the major bile acids present, mainly in the conjugated form.

Our values are lower than those obtained by many methods recently published. The specificity of our GLC method was clearly shown with a peak shift technique and has later been confirmed by mass spectrometric analysis of the GLC eluates. The specificity of the colori-

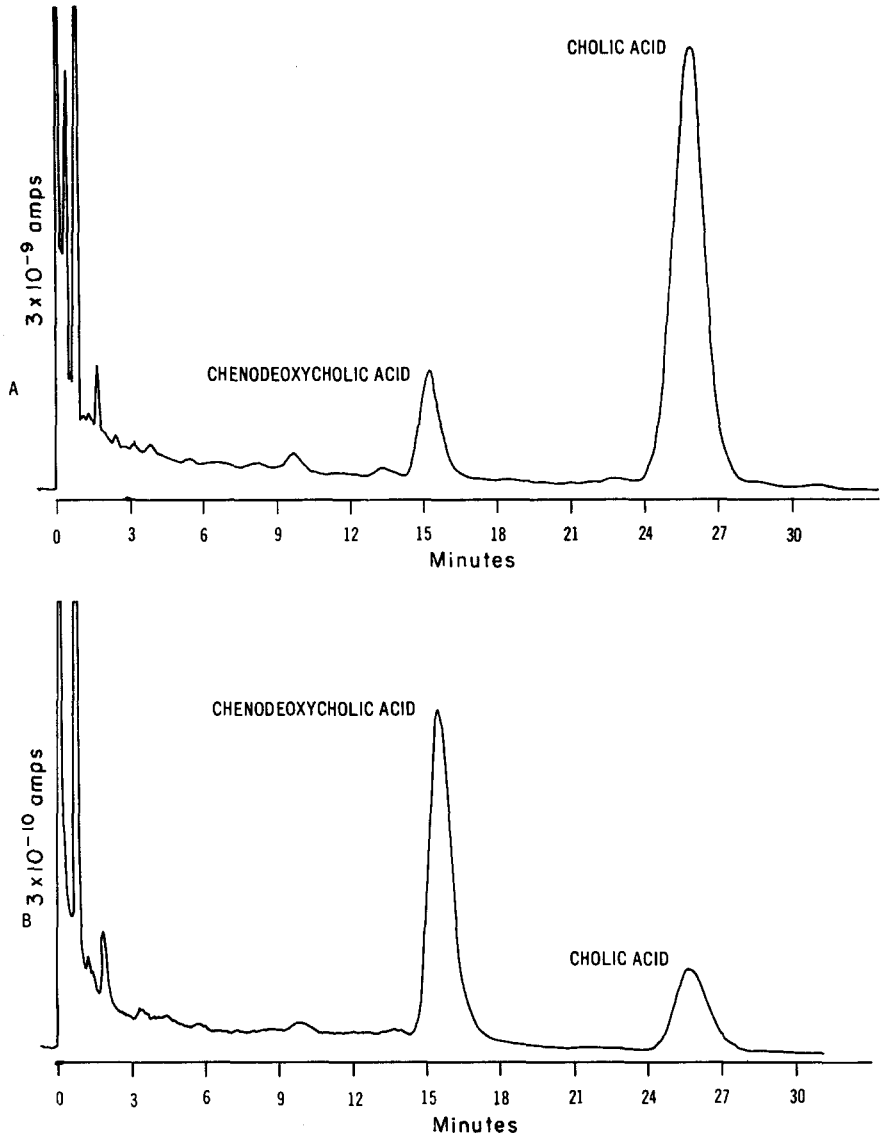

FIG. 6. Analysis of methylated and trifluoracetylated bile acids in patients with liver disease. Upper curve: bile duct atresia (S. G.). Lower curve: portal cirrhosis (L. S.). Column conditions as in Fig. 4.

metric methods is difficult to establish since even extensively purified extracts contain much material besides the bile acids and there may be unknown substances present that give color or fluorescence with the reagents used. In papers describing methods for human serum bile acids giving normal values of, for example, 1 mg/100 ml for total bile acids, positive proof of the absence of nonspecific reactions has not been presented. By contrast, the chromatographic studies of Carey (9) and of Rudman and Kendall (14) indicate that the serum bile acid levels are lower than 1 mg/100 ml.

The recoveries of various amounts of added bile salts were between 59 and 87%. It must be realized that the achievement of a given recovery of added compounds does not necessarily mean that the bile acids originally present in the sample are recovered to the same extent. In favor of the present method are the facts that extraction of endogenous bile salts with the ion exchanger gave results similar to conventional ethanol extraction (Table 7), which has given quantitative recoveries of endogenously C^{14}-labeled bile acids in rat and rabbit serum (16–18), and that the over-all GLC method gives values for rabbit serum deoxycholic acid that are of the same order of magnitude as those obtained with a method using internal C^{14}-labeling (18).

The reproducibility of the method is satisfactory even for low values, as shown in Table 5. It permits measurement of an increased bile acid level in the peripheral

circulation after a meal, as shown by the examples (subjects 17, 18) in Table 5.

Pathological Sera

The results of analyses of sera from some patients with liver disease confirm and extend the results obtained by Carey (25) and by Rudman and Kendall (14). The ratios between the three main bile acids in these cases are similar to the ratios found between biliary bile acids in patients with liver disease (30). The relative amount of deoxycholic acid is very low, both in cases with portal cirrhosis and in cases with obstructive jaundice. The lack of deoxycholic acid in the serum in obstructive jaundice is explained by the absence of an enterohepatic circulation in these patients (28, 30).

This study has been restricted to the determination of the three common bile acids in serum, but it has been noted that a number of serum samples have shown a small peak with a retention time equal to that of the trifluoracetate of methyl ursodeoxycholate. This peak has not been definitely identified as yet. One patient with infectious hepatitis showed a large peak with the retention time of the methyl ester trifluoracetate of lithocholic acid. Attempts to identify this peak are also in progress.

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