Determination of individual conjugated bile acids in human bile

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ABSTRACT A method has been developed and validated for the determination of the six major conjugated bile acids, cholesterol, and total phospholipids in bile of human subjects previously injected with 4-^{14}C-cholesterol.

The procedure is designed for use with 5–10 ml of duodenal or T-tube bile and eliminates difficulties associated with existing methods for bile acid determination, in particular the requirement for preliminary saponification under pressure or the use of paper chromatography. Saponification under pressure is employed only in steps where partial destruction of the steroid moiety of conjugated bile acids is not a crucial matter.

A preliminary Folch extraction and washing step separated free cholesterol and phospholipids (bottom layer) from the six major conjugated bile acids (top layer). The conjugated bile acids were then fractionated cleanly by thin-layer chromatography to give four groups, the ^{14}C content of each of which was determined. A second aliquot of the top layer was used to determine (after deconjugation) the radioactivity ratio of deoxycholic acid to chenodeoxycholic acid for the two unresolved groups (dihydroxycholanoic acid conjugates with glycine and taurine, respectively). A third aliquot was used for determination of specific activities of the methyl esters of cholic, chenodeoxycholic, and deoxycholic acids derived from the total bile salts. Appropriate calculations yielded the concentration in bile of all six major bile acid conjugates.

SUPPLEMENTARY KEY WORDS thin-layer chromatography · glycine conjugates · taurine conjugates · cholic acid · deoxycholic acid · chenodeoxycholic acid · cholesterol · phospholipids · ^{14}C-steroids

When human bile samples have been obtained during lengthy metabolic studies of cholesterol metabolism, it is often desirable that reliable and complete analyses of steroidal components are made. A survey of methods used in recent studies indicated certain deficiencies. Most procedures require preliminary alkaline saponification of conjugated BA under pressure; examples are gas–liquid chromatographic analysis of BA derivatives (1) and spectrophotometric determination of unconjugated BA in 65% sulfuric acid solution (2). Unfortunately, all methods in general use for converting conjugated to unconjugated BA result in variable and often large losses of components (3–6).

The enzyme cholylglycine hydrolase may prove useful for obtaining good yields of unconjugated BA without alteration of their steroid structure (7). However, complete analysis of bile requires knowledge of the concentrations of individual conjugated BA as they exist in bile; such information is lost during hydrolysis by any method. Attempts to separate the six major conjugated BA in human bile cleanly have not been very successful.

TLC has not so far been shown to separate adequately the isomeric dihydroxycholanoic acid conjugates of either glycine or taurine. Paper chromatography is the basis of a method for separation of conjugated BA (8); in our hands this procedure has not been successful, because of the spreading of bands on paper and overlap of components, and other workers have reported difficulties with

Abbreviations: BA, bile acids; C, cholic acid; CD, chenodeoxycholic acid; D, deoxycholic acid; FC, free cholesterol; FFA, free fatty acids; GC, glycocholic acid; GCD, glycochenodeoxycholic acid; GD, glycodeoxycholic acid; G-Di, glycodihydroxycholanoic acids; Me-C, methyl cholate; Me-CD, methyl chenodeoxycholate; Me-D, methyl deoxycholate; PC, phosphatidyl choline; PL, phospholipids; POPOP, 1,4-bis[2,5-phenyloxazolyl)]benzene; PPO, 2,5-diphenyloxazole; SA, specific activity; TC, taurocholic acid; TCD, taurochenodeoxycholic acid; TD, taurodeoxycholic acid; T-Di, taurodihydroxycholanoic acids; TG, triglycerides; TLC, thin-layer chromatography.

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this method, particularly during determination of the taurine conjugates (9).

A recent paper (10) has claimed good resolution of all six conjugates in human bile by column adsorption chromatography on silicic acid. The order of elution found is rather unexpected for this adsorbent and quantitation was by direct spectrophotometric determination of conjugated bile acids.

In view of these considerations, the following method has been devised and tested and has been applied in the analysis of some hundreds of human bile samples obtained from the duodenum or directly from the liver via a T-tube

GENERAL METHODS, MATERIALS, AND APPARATUS

Solvents. Solvents were reagent grade. They were redistilled before use, with the exception of *n*-butanol and acetic acid. Solvents were removed under a nitrogen stream in a water bath at about 80°C.

Thin-Layer Chromatography. TLC was carried out with 0.5 mm layers of Silica Gel G (E. Merck AG, Darmstadt, Germany) on 20 × 26 cm plates. The plates were washed (to remove adsorbent contaminants from the working area) by allowing a mixture of chloroform–methanol–water 85:35:5 (v/v) to rise to the top of the plates; they were then dried and activated at 120°C for 1 hr. In use the solvent was allowed to travel the full 26 cm length of the plates. Separated components were detected by brief exposure to iodine vapor, or by light spraying with 0.01% (w/v) Rhodamine 6G in ethanol solution. After the position of separated components had been marked, about 10 min was allowed for evaporation of iodine or ethanol.

Recovery of Separated Components. The area of silica gel containing a required component was scraped off quantitatively with a razor blade, transferred to a small elution column, and eluted with 50 ml of the appropriate solvent under slightly increased pressure.

Reference Standards. The glycine and taurine conjugates of cholic, deoxycholic, and chenodeoxycholic acids were obtained from Cal-Biochem (Los Angeles, Calif.) and were suitable for use as markers on TLC. Cholic, chenodeoxycholic, and deoxycholic acids were obtained in the unconjugated form from Cal-Biochem and each was purified by TLC before use. 4-^{14}C-cholesterol was obtained from Volk Radiochemical Company (Burbank, Calif.) and was purified by TLC on Silica Gel G before use. Absence of radioactive saturated sterols was verified by TLC on Silica Gel G impregnated with silver nitrate.

Methylation of Unconjugated Bile Acids. Excess ethereal diazomethane was added to a counting vial containing a solution of the acid(s) in a few drops of methanol. After 15 min, the volatile components were removed under nitrogen. The procedure was repeated to ensure complete conversion to methyl esters.

Spectrophotometric Determination of Methyl Cholate, Chenodeoxycholate, and Deoxycholate. The procedure used was based on that of Sjövall (8). Appropriate aliquots of solutions containing unknown amounts (less than 80 μg) of the methyl ester of cholic, chenodeoxycholic, or deoxycholic acid were placed in 10-ml tubes and the solvent was evaporated. 4 ml of freshly prepared 65% sulfuric acid was added and mixed thoroughly, and the tubes were heated at 60 ± 1°C for 1 hr. The tubes were then transferred to a cold water bath for a few minutes to stop further production of UV-absorbing substances. Measurement of UV absorption on a Beckman DU spectrophotometer was started 15 min after the tubes had been removed from the hot water bath. Cholic acid methyl ester was measured at 320 nm, chenodeoxycholic acid at 380 nm, and deoxycholic acid at 385 nm against a blank of 4 ml of 65% sulfuric acid, which was treated in the same way as the unknown samples. Known amounts of purified methyl esters of each BA were treated in the same way at the same time. All determinations were made in duplicate.

Determination of ^{14}C. Determination was made in a Packard Tri-Carb liquid scintillation spectrometer. The scintillation fluid was composed of 4 g of PPO and 0.1 g of dimethyl POPOP dissolved in 1 liter of toluene. *Conjugated* BA were usually dissolved in methanol before the scintillation fluid was added; for these samples quenching was corrected for by the internal standardization method.

Duodenal Bile. Bile samples were obtained by aspiration following intubation of subjects and intravenous injection of cholecystokinin (Cecekin; Vitrum AB, Stockholm, Sweden). *T-tube bile* samples were obtained from post-cholecystectomy patients with either a conventional or a Baldwin T-tube inserted in the common bile duct. The cholesterol and bile acids of subjects studied were labeled with ^{14}C about 20 days prior to bile sampling. The method does not require that all three unconjugated bile acids closely approach isotopic equilibrium since individual SA values are determined. 30–100 μc of purified 4-^{14}C-cholesterol dissolved in about 2 ml of sterile propylene glycol was administered intravenously by slow injection.

QUANTITATIVE DETERMINATION OF BILIARY BILE ACIDS AND CHOLESTEROL

General Description

The method developed is designed for use with duodenal or T-tube bile from human subjects injected intravenously

with 30–100 μc of 4-^{14}C-cholesterol about 20 days prior to sampling. Fig. 1 shows in outline the procedure used. Bile containing bile acids and cholesterol labeled with ^{14}C was extracted and washed by the Folch method (11) to give a top layer (containing the conjugated bile acids) and a bottom layer (containing the cholesterol and phospholipids).

A sample of free cholesterol was obtained from the bottom layer and purified via the digitonide for determination of its specific activity. This value, together with the amount of radioactivity (in dpm/ml of bile) present in the bottom layer, allowed the concentration of total cholesterol per milliliter of bile to be calculated. An aliquot of the bottom layer was also used for determination of total organic phosphorus (P × 25 = total PL).

The top layer contained the six conjugated BA: GC, GCD, GD, TC, TCD, and TD, together with traces of others which have been neglected. These conjugates were cleanly separated into four groups by TLC, giving the amount of radioactivity (dpm/ml of bile) present in GC, (GCD + GD), TC, and (TCD + TD). After alkaline hydrolysis of another aliquot of the top layer, methylation, and TLC, the specific activity of the methyl esters of each of the three major deconjugated BA (Me-C, Me-CD, and Me-D) was determined and the concentration in bile of GC and TC was obtained directly by division.

To know the concentrations of the individual glycine and taurine dihydroxy conjugates, we used a further aliquot of the top layer to isolate the two mixed conjugate bands (G-Di and T-Di). Each mixture was hydrolyzed, then methylated; Me-D and Me-CD were separated by TLC and their radioactivity was determined. From this and the SA of Me-D and Me-CD, the ratio *k*:

$$\frac{\text{mass D/ml of bile}}{\text{mass CD/ml of bile}}$$

was calculated for each conjugated group. From the total radioactivity in each mixed conjugate band and the SA of Me-D and Me-CD, the concentration of GCD, GD, TCD, and TD in bile could be calculated (Table 1).

In this way the concentration in bile of all six major conjugated bile acids has been determined.

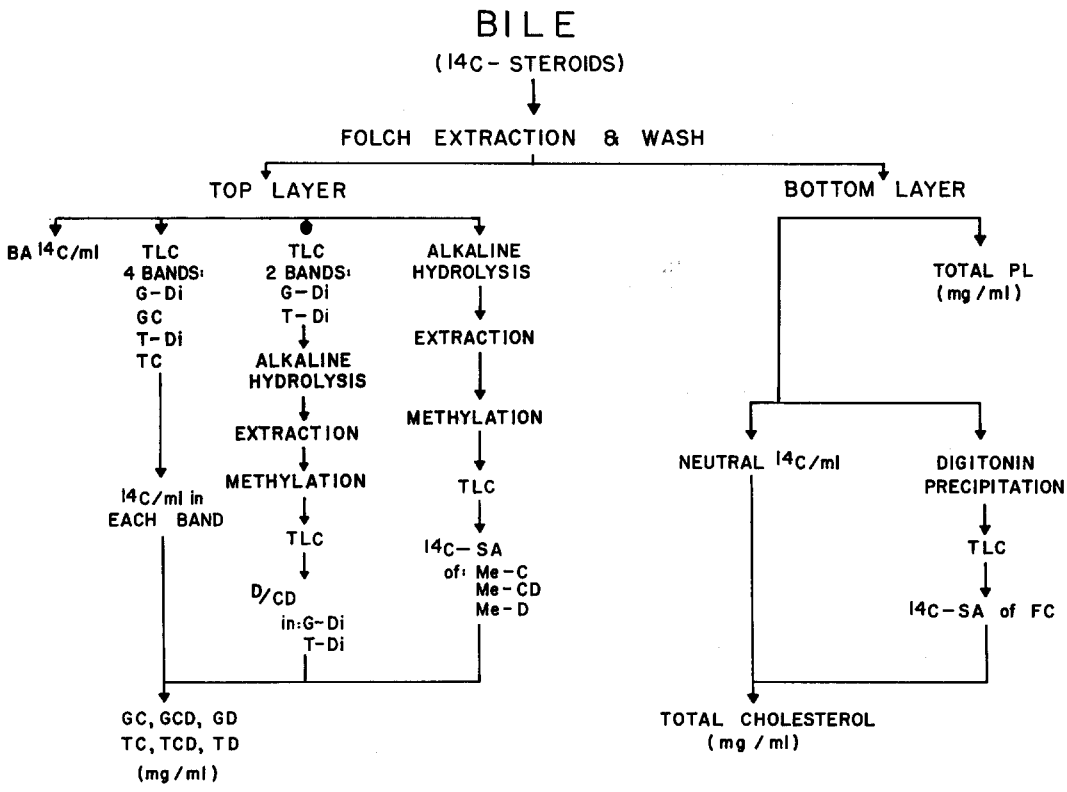

FIG. 1. Outline of procedure for determination of total phospholipids, total cholesterol, and individual conjugated bile acids in bile. G and T = glycine- and taurine-conjugated. Di = the two dihydroxycholanoates D and CD. "TLC—4 Bands" means that all four bands of bile acids are removed from the TLC plate and the total radioactivity in each is determined. "TLC—2 Bands" means that only two (G-Di and T-Di) of the four bands are removed and further processed (hydrolysis, methylation, separation by TLC into Me-D and Me-CD, and determination of the radioactivity in each of these). From this information, and a knowledge of the SA of the deconjugated bile acid methyl esters (Me-C, Me-CD, and Me-D), the mass of all six conjugates per milliliter of bile can be calculated.

TABLE 1 ILLUSTRATION OF CALCULATIONS FOR BILE ACID ANALYSIS*

	dpm	Corrected dpm
^{14}C in: top layer	66300	
G-Di	15500	16200
GC	15600	16300
T-Di	15600	16300
TC	16900	17600
Total recovered	63600	66400
Recovery, %	95.9	
Specific activity of: Me-C	1540 dpm/mg	
Me-CD	1670 dpm/mg	
Me-D	1740 dpm/mg	
"k" for: G-Di	0.728	
T-Di	0.629	

		BA as Methyl Esters	Conversion Factor	BA as Conjugates
		mg		*mg*
Glycine conjugates				
GC $\frac{16300}{1540}$	=	10.6	1.102	11.7
GCD $\frac{16200}{(0.728 \times 1740) + 1670}$	=	5.52	1.106	6.11
GD 0.728×5.52	=	4.02	1.106	4.45
Total (glycine conjugates)		20.1		22.3
Taurine conjugates				
TC $\frac{17600}{1540}$	=	11.4	1.220	13.9
TCD $\frac{16300}{(0.629 \times 1740) + 1670}$	=	5.90	1.229	7.25
TD 0.629×5.90	=	3.71	1.229	4.56
Total (taurine conjugates)		21.0		25.7
Total (all conjugates)		41.1		48.0
Glycine/taurine	=	$\frac{22.3}{25.7}$ = 0.868		

* ^{14}C and BA concentrations are per milliliter of bile.

Folch Extraction and Washing

A measured volume of bile (5–10 ml) was added, with swirling, to 20 times the volume of chloroform–methanol 2:1 and the solution was filtered through glass wool, with washing of the small protein residue on the filter, into a graduated, stoppered cylinder. Distilled water equal to 0.2 times the volume of the solution was added and the mixture was shaken and allowed to separate into two clear layers. The top layer was removed as completely as possible by siphoning, additional distilled water equal to 0.2 times the volume of the lower layer was added to the lower layer, and the mixture was shaken again. After separation, the second top layer was removed and combined with the first top layer.

The bottom layer was evaporated under nitrogen, made up with chloroform–methanol 9:1 to the volume of bile (5–10 ml) from which it was derived, and stored at 4°C. This was designated *bottom layer, stock solution.*

The combined top layers were evaporated to dryness under reduced pressure in a rotary evaporator at 75°C, and the residue was dissolved in methanol–water 85:15 and made up to 50 ml in a volumetric flask. This was designated *top layer, stock solution.*

Bottom Layer

Duplicate 0.5 ml aliquots of the bottom layer stock solution were placed in 20-ml counting vials and evaporated, a few drops of 30% hydrogen peroxide were added, and the vials were capped, allowed to stand overnight, and then heated under a stream of nitrogen to remove the peroxide solution (no decrease in detectable radioactivity resulted from this bleaching treatment—unpublished data). 10 ml of scintillation fluid was added, and the mean radioactivity was determined, corrected for any residual quenching, and expressed as dpm/ml of bile.

3–8 ml of bottom layer stock solution was placed in a 50 ml tube and evaporated and 15 ml of acetone–ethanol 1:1 was added to dissolve the residue. 15 ml of a 1% (w/v) solution of digitonin in ethanol–water 1:1 was added and the mixture was shaken and allowed to stand overnight. The precipitate of cholesterol digitonide was centrifuged down and washed first with acetone–diethyl ether 1:2 and then with diethyl ether, each time with centrifugation. Cholesterol was released from the digitonide by treatment with pyridine at 100°C for 1 hr followed by cooling, addition of excess diethyl ether, filtration, and evaporation of the filtrate to dryness. The recovered cholesterol was further purified by TLC in petroleum ether–diethyl ether–acetic acid 60:40:1. The free cholesterol band, made visible with Rhodamine 6G, was scraped off and the cholesterol was eluted with 50 ml of diethyl ether into a weighed counting vial. The resulting weight was corrected by subtraction of a small weighing blank obtained by eluting a similar portion of silica gel with 50 ml of diethyl ether. Scintillation fluid (toluene-based) was added and radioactivity was determined.

Duplicate aliquots of 0.05 ml of the bottom layer stock solution were taken for determination of total organic phosphorus (12).

Top Layer

Total Radioactivity in Top Layer. Duplicate aliquots of top layer stock solution (corresponding to 0.1 ml of original bile) were placed in 20-ml counting vials, evaporated to

dryness, and dissolved in 1 ml of methanol, and 10 ml of scintillation fluid was added.

Separation of Four Conjugate Groups. Top layer stock solution (corresponding to 0.4 ml of original bile) was evaporated and applied as a streak (in methanol–water 20:1) to a TLC plate. A solution of standards containing the six conjugated BA (about 35 μg of each acid) was also applied as a 1 cm streak on each side of the sample. The plate was dried in air and developed in *n*-butanol–acetic acid–water 10:1:1. The plate was dried in an air current under a fume hood for 30 min and briefly placed in an iodine vapor tank. After the bands had been delineated and the iodine had evaporated, the four main bands were scraped from the plate with a razor blade into a column for elution with 50 ml of methanol–acetic acid 100:1. The eluate was concentrated, transferred to a 20 ml counting vial, and evaporated to dryness. 1 ml of methanol was added to dissolve the conjugates, followed by 10 ml of scintillation fluid for radioactivity determination with quenching. Over-all recovery of ^{14}C was calculated by comparison with the total radioactivity found in the top layer before TLC. The radioactivity present in each of the four bands was then corrected for losses (averaging 4%) and these data were used in the calculations.

SA of C, CD, and D. Top layer stock solution (equivalent to 2 ml of original bile) was placed in a 200 ml Teflon bottle with Teflon screw cap (Nalge Co., Rochester, N.Y.), the methanol was evaporated, and 10 ml of 1 N aqueous NaOH was added. The bottle was tightly capped and heated in an autoclave at 15 psi for 3 hr. The mixture was acidified to pH 1 with 3 N HCl, transferred to a glass separatory funnel, and extracted six times with 30 ml of diethyl ether. The combined extracts were washed twice with 20 ml of distilled water and the solution was evaporated to dryness.

The mixture of deconjugated BA so obtained was converted to methyl esters and applied as a streak to a TLC plate in chloroform–methanol 2:1. A mixture of Me-C, Me-CD, and Me-D was used as a reference standard. The plate was developed in petroleum ether–isopropyl ether–acetic acid 2:1:1, dried for 1 hr in an air current, and lightly sprayed with Rhodamine 6G. The bands (R_f values: Me-C, 0.35; Me-CD, 0.60; Me-D, 0.66) were delineated under UV radiation, removed, and eluted with 50 ml of diethyl ether. This procedure gives adequate (but not quantitative) yields of each methyl ester, but does not elute the rhodamine. The methyl esters were weighed and dissolved in 10 ml of methanol.

Duplicate aliquots of the methanol solution, containing 10–80 μg of methyl ester, were taken for determination of Me-C, Me-CD, and Me-D, using spectrophotometric measurement of the sulfuric acid chromophores (see General Methods).

5 ml of methanol solution was evaporated to dryness in a counting vial and the radioactivity was determined; quench correction was unnecessary. SA was calculated.

Determination of Mass Ratio: Me-D/Me-CD in the Glycodihydroxy Conjugates and in the Taurodihydroxy Conjugates. Top layer stock solution (equivalent to 0.8 ml of original bile) was applied to a TLC plate and the four conjugate groups were separated as described earlier. Of the four bands, two were scraped off (GCD + GD and TCD + TD) and their conjugated bile acids were separately eluted with 50 ml of methanol–acetic acid 100:1 and saponified under pressure as described earlier, except that 2 N NaOH was used. The deconjugated acids were recovered, methylated, and separated into Me-CD and Me-D by TLC in petroleum ether–isopropyl ether–acetic acid 2:1:1. The recovered methyl esters were counted in 10 ml of scintillation fluid, without quench correction. Using the previously determined SA of Me-D and Me-CD, the ratio (k) of mass of Me-D to mass of Me-CD in both the glycine and the taurine conjugates was calculated.

Calculations

Phospholipids. The amount of P per milliliter of original bile was converted to amount of phospholipid (as lecithin) by multiplying by 25.

Cholesterol. Bile total cholesterol (virtually all of it free cholesterol) was calculated by dividing the total radioactivity in the bottom layer by the specific activity of free cholesterol in this layer, and expressing it per milliliter of bile.

Glycocholic and Taurocholic Acid. The mass of GC per milliliter of bile (as methyl cholate) was calculated by dividing the total radioactivity in the GC band (per milliliter of bile) by the specific activity of methyl cholate. A similar calculation yielded mass of TC per milliliter of bile (as methyl cholate).

To convert from mass as the methyl esters to mass as the conjugated cholic acids (carboxyl form), the factors used were 1.102 for the glycine conjugate and 1.220 for the taurine conjugate.

Glycine and Taurine Conjugates of D and CD. For *glycine* dihydroxy conjugates, the ratio k was calculated from:

$$\frac{\text{dpm in D per ml of bile}}{\text{SA of Me-D}} \div \frac{\text{dpm in CD per ml of bile}}{\text{SA of Me-CD}}$$

The mass of GCD was then calculated (as Me-CD) by dividing the total radioactivity in the (GD + GCD) band (per milliliter of bile) by $\{(k \cdot \text{SA of Me-D}) + (\text{SA of Me-CD})\}$.

The mass of GD per milliliter of bile (as Me-D) = k (mass of GCD per milliliter of bile).

To convert from mass as the methyl esters to mass as

glycine-conjugated dihydroxy acids (carboxyl form), we used the factor 1.106 for both GD and GCD.

For *taurine* dihydroxy conjugates (TCD and TD), similar calculations gave mass per milliliter of bile (as methyl esters), converted to mass as taurine-conjugated dihydroxy acids (carboxyl form) by means of the factor 1.229 for both TD and TCD.

Total Unconjugated Bile Acids per milliliter of bile (as methyl esters) were obtained by addition of the calculated masses of Me-C, Me-CD, and Me-D derived from both glycine and taurine conjugates.

Total Conjugated Bile Acids per Milliliter of Bile (Carboxyl Form) were obtained by addition of the calculated masses of GC, GCD, GD, TC, TCD, and TD.

The Ratio of Glycine to Taurine Conjugates was calculated.

Calculations for bile acid components are illustrated in Table 1.

RESULTS

The major steps in the procedure have been validated as follows.

Extraction and Washing

The efficacy of the extraction and washing procedure with respect to separation of cholesterol and phospholipids (bottom layer) from the conjugated BA (top layer) has been frequently checked and is illustrated in Fig. 2 for a sample of duodenal bile. In particular, the virtual absence of ^{14}C-labeled BA from the bottom layer was shown by the recovery of 100 ± 2.1% (mean ± SD for 18 samples) of total ^{14}C after passing this fraction through a basic ion-exchange resin column known to remove BA completely.

Bottom Layer

Table 2 shows the good reproducibility obtained during replicate determinations of the following: total PL; SA of FC; and radioactivity per milliliter of bile in the bottom layer. For this sample the mean concentration of total cholesterol was therefore 1630/1530 = 1.07 mg/ml of bile. The mean SA of biliary FC (1530 dpm/mg) may be compared with those of 1530 and 1750 dpm/mg for FC and esterified cholesterol, respectively, of plasma taken from the patient on the same day.

TABLE 2 REPRODUCIBILITY OF ANALYTICAL PROCEDURES ON BILE EXTRACT BOTTOM LAYER

Determination	n	Mean ± SD
Total phospholipid* (mg/ml bile)	4	5.94 ± 0.05
SA of FC (dpm/mg)	5	1530 ± 12
^{14}C concentration (dpm/ml bile)	6	1630 ± 20

* As phosphatidyl choline.

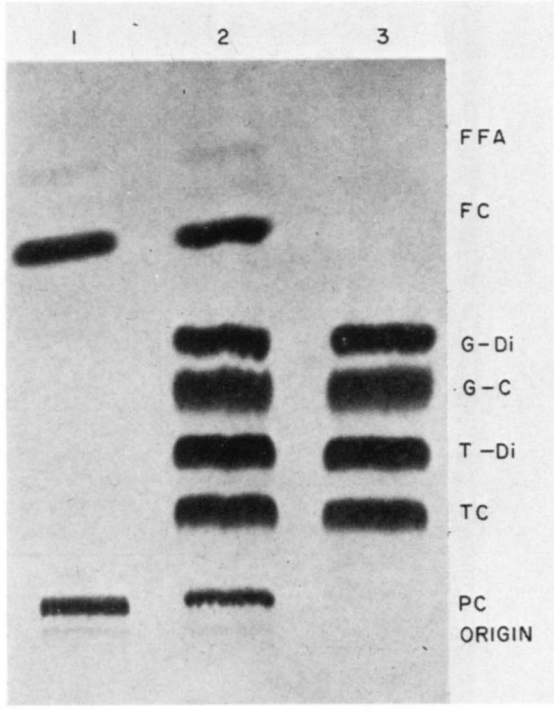

FIG. 2. TLC of a duodenal bile extract, showing clean separation of free cholesterol and phosphatidyl choline in Folch bottom layer, *1*, and of conjugated bile acids in Folch top layer, *3*. *2*, total Folch extract before washing step. TLC: 0.5 mm Silica Gel G plate; solvent, *n*-butanol–acetic acid–water 10:1:1; visualization, phosphomolybdic acid spray and heating for 20 min at 110°C.

Top Layer

^{14}C in Four Conjugate Groups. Table 3 shows the satisfactory reproducibility obtained during determinations of ^{14}C present in the four conjugate groups separated initially (Fig. 2). Exhaustive further elution of the silica gel with methanol–acetic acid mixtures has frequently been carried out and has indicated that elution with 50 ml of methanol–acetic acid 100:1 (as described in the method) removes at least 98% of ^{14}C from the adsorbent for each of the four conjugate groups. The mean recovery of total applied ^{14}C for the sample of duodenal bile shown in Table 3 was 95.9%; in analyses of more than 75 samples of bile in which bile acids were labeled, recoveries in the range of 92–102% have been found.

SA of Unconjugated BA Methyl Esters. The excellent reproducibility obtained is illustrated in Table 4. When expressed on a micromolar basis, the rank order of specific activities of the bile acids and of FC (obtained simultaneously from bile and plasma) was as would be predicted from their respective precursor–product relationships (samples in this experiment were taken 69 days after intravenous 4-^{14}C-cholesterol): secondary (i.e., bac-

TABLE 3 REPRODUCIBILITY OF ^{14}C DETERMINATIONS IN BILE EXTRACT TOP LAYER AND IN FOUR CONJUGATE BANDS

	Mean ± SD (n = 4)
	dpm/ml bile
Top layer	66300 ± 921
Glycodihydroxycholanoic acids	15500 ± 134
Glycocholic acid	15600 ± 323
Taurodihydroxycholanoic acids	15600 ± 481
Taurocholic acid	16900 ± 234
Total conjugated acids	63600 ± 256
	%
^{14}C recovered (mean)	95.9

TABLE 4 ^{14}C-SPECIFIC ACTIVITIES OF STEROIDS IN DUODENAL BILE AND PLASMA SAMPLES TAKEN SIMULTANEOUSLY FROM THE SAME SUBJECT

	Specific Activity	
	dpm/mg	*dpm/μmole*
	Mean ± SD (n = 4)	
Bile		
Methyl cholate	1540 ± 14	651 ± 6
Methyl chenodeoxycholate	1670 ± 11	679 ± 4
Methyl deoxycholate	1740 ± 21	707 ± 9
	Mean (n = 2)	
Free cholesterol	1580	611
Plasma		
Free cholesterol	1660	642

terially produced) Me-D was higher than its precursor, primary (i.e., hepatic-synthesized) Me-C; and all bile acids showed specific activities higher than precursor cholesterol, as measured in two parts of its miscible pool.

The method presented rests on the assumption that more than 20 days after the injection of tracer the SA of each unconjugated BA will be the same for conjugates of the particular acid with either glycine or taurine. This point was tested in five bile samples obtained from four subjects at various time intervals. Results (shown in Table 5) indicate the close agreement of SA for a given BA in the two conjugates, in samples taken 24–92 days after the administration of labeled cholesterol.

^{14}C in Conjugated Dihydroxy Acids. Good reproducibility (Table 6) was obtained during replicate determinations of ^{14}C present in the deoxycholic and chenodeoxycholic acid components of both glycine- and taurine-conjugated bile acids. The mean recovery of ^{14}C after the entire procedure was not the same for the two conjugate groups; however, *complete* recovery is not essential to the validity of the procedure provided that the loss of ^{14}C in deoxycholic acid is essentially the same as the loss in chenodeoxycholic acid.

TABLE 5 SIMILARITY OF SPECIFIC ACTIVITIES OF METHYL ESTERS FROM GLYCINE- AND TAURINE-CONJUGATED BILE ACIDS IN DUODENAL BILE OF FOUR SUBJECTS TAKEN AT VARIOUS TIMES AFTER IV INJECTION OF 4-^{14}C-CHOLESTEROL

Subject	Days After Injection	Bile Acid Methyl Ester	Mean Specific Activity (n = 2)	
			Glycine Conjugate	Taurine Conjugate
			dpm/mg	
1	24	Me-D	702	754
		Me-CD	581	594
		Me-C	644	640
2	33	Me-D	1200	1210
		Me-CD	1070	1080
		Me-C	929	953
3	44	Me-D	111	115
		Me-CD	126	121
		Me-C	103	101
4	62	Me-D	127	124
		Me-CD	119	122
		Me-C	127	125
2	92	Me-D	325	339
		Me-CD	320	323
		Me-C	289	285

TABLE 6 REPRODUCIBILITY OF MEASUREMENT OF ^{14}C IN GLYCINE- AND TAURINE-CONJUGATED DIHYDROXYCHOLANOIC ACIDS

	Mean ± SD (n = 4)
^{14}C content (dpm/ml bile)	
Glycodihydroxycholanoic acids	15500 ± 134
Methyl deoxycholate from G-Di	6300 ± 184
Methyl chenodeoxycholate from G-Di	8270 ± 198
^{14}C recovery (%)	94.0 ± 3.5
^{14}C content (dpm/ml bile)	
Taurodihydroxycholanoic acids	15600 ± 481
Methyl deoxycholate from T-Di	5600 ± 191
Methyl chenodeoxycholate from T-Di	8500 ± 184
^{14}C recovery (%)	90.4 ± 3.9

A study was made to test this point. Highly purified samples of 4-^{14}C-labeled Me-D and Me-CD were prepared by TLC from duodenal bile of a subject previously injected intravenously with 4-^{14}C-cholesterol. Known amounts of each methyl ester-^{14}C were separately subjected to alkaline saponification, extraction of unconjugated BA, methylation, and quantitative purification by TLC. The recovered ^{14}C is shown in Table 7 for each methyl ester. Excellent reproducibility was found for both components and the ratio of ^{14}C recovered in Me-D as compared with recovery in Me-CD was 0.990, which indicated no significant difference in percentage loss of ^{14}C between the two dihydroxy isomers.

Reproducibility of the determined mass of Me-D and Me-CD and of the ratio k was excellent (Table 8).

TABLE 7 Recovery of ^{14}C in Methyl Deoxycholate Compared to Methyl Chenodeoxycholate After Alkaline Hydrolysis, Methylation, TLC, and Elution from Silicic Acid

	Mean Initial ^{14}C (n = 2)	Recovered ^{14}C Mean ± SD (n = 4)	Recovery Mean ± SD (n = 4)
	dpm	*dpm*	%
Methyl deoxycholate	2930	2650 ± 25	90.4 ± 0.8
Methyl chenodeoxycholate	11500	10500 ± 106	91.3 ± 0.9

Ratio of recoveries in deoxy- and in chenodeoxycholates = 0.990.

band was scraped off and quantitatively eluted with methanol. Radioactivity determination indicated an over-all recovery of 81.5% for Me-C during these procedures.

Concentration of Individual BA. The reproducibility obtained during determination of concentrations of individual BA and of total conjugated BA (the calculations for which are illustrated in Table 1) was excellent and is illustrated in Table 10. The glycine/taurine ratios for both of these samples (which are presented only for the purpose of illustration) are rather less than unity. They were both taken from subjects on diets that in our experience lead to low glycine/taurine ratios in bile. Much higher ratios (up to 11:1) have been found in other circumstances, for instance during ingestion of cholestyramine (13).

TABLE 8 Reproducibility of Mass Ratios for Methyl Deoxycholate and Methyl Chenodeoxycholate in Glycine and Taurine Conjugates of a Duodenal Bile Sample

	Methyl Deoxycholate	Methyl Chenodeoxycholate	*k**
	mg/ml bile		
Glycine conjugates	3.61 ± 0.031	4.97 ± 0.149	0.728 ± 0.010
Taurine conjugates	3.21 ± 0.109	5.10 ± 0.111	0.629 ± 0.026

All values are mean ± SD (n = 4).
* k = (mass of Me-D):(mass of Me-CD) in a given conjugate.

TABLE 9 Recovery of ^{14}C in Methyl Cholate After Alkaline Hydrolysis

		Mean (n = 2)	Recovery
		dpm	%
^{14}C initially in methyl cholate		4700	
^{14}C recovered after saponification, acidification, successive diethyl ether extractions:	I	3620	76.9
	II	601	12.8
	III	104	2.2
	IV	18	0.4
	V	4	0.1
^{14}C in total extract		4350	92.6
^{14}C in purified methyl cholate		3830	81.5

TABLE 10 Reproducibility of Values for Individual Conjugated Bile Acids in a Duodenal Bile Sample

	Mean ± SD (n = 4)
	mg/ml bile
Glycocholic acid	11.2 ± 0.26
Glycochenodeoxycholic acid	5.86 ± 0.095
Glycodeoxycholic acid	4.26 ± 0.027
Taurocholic acid	11.4 ± 0.28
Taurochenodeoxycholic acid	6.92 ± 0.38
Taurodeoxycholic acid	4.37 ± 0.10
Total conjugated bile acids	44.0 ± 0.30

Losses of Cholic Acid During Saponification and Recovery. When pure samples of Me-C, Me-D, or Me-CD were subjected to alkaline saponification (1 N NaOH, 15 psi, 3 hr) and then recovered, there was a considerable loss in each case, Me-C consistently showing the greatest loss (Table 9). After saponification under pressure and acidification of a sample of highly purified Me-C-^{14}C, five extractions of free acids with diethyl ether resulted in over-all recovery of only 92.6% of the initial activity (Table 9). After methylation of the extracted acid, TLC showed the presence of several bands both above and below the major band of Me-C, presumably attributable to reaction products formed during hydrolysis. The Me-C

DISCUSSION

Much significant work on cholesterol metabolism has been done with human subjects. Often in such studies the body steroids are labeled by means of intravenous cholesterol-^{14}C as a preliminary to studying the turnover of cholesterol in plasma (14) or quantitating fecal steroid excretion rate (15–17). It is usually neither difficult nor undesirable to collect bile samples of about 10 ml volume, so that micromethods of analysis are not mandatory.

In the method presented, an initial solvent partition (Fig. 1) cleanly separates free cholesterol and total phospholipids on the one hand from total conjugated BA on the other (Fig. 2).

Care has been taken in the initial treatment of the bile top layer to avoid the inevitable losses of conjugated BA attendant upon alkaline hydrolysis under pressure (3–6). Preliminary separation of conjugate groups is achieved by TLC, which in our hands has proved preferable to paper chromatography for this purpose by virtue of its simplicity, speed, and superior resolution. The ^{14}C content of the BA has enabled the average overall recovery during TLC to be estimated at about 96%; the unrecovered part is largely the result of handling losses but includes very minor amounts of BA other than the six major conjugates. Since the extent of the loss is known for each sample, correction to 100% may be made on the assumption that handling losses are equally distributed.

The SA of the three unconjugated BA methyl esters (Me-C, Me-CD, and Me-D) have been determined using a spectrophotometric method for mass determination. Direct weighing of the methyl esters followed by counting has also been used and gives closely similar results; however, in certain metabolic conditions it is difficult to obtain sufficient methyl ester (3–5 mg) for accurate weighing, especially in the case of deoxycholic acid. As with bile cholesterol, accurate SA values for the individual unconjugated BA are often required in other aspects of studies on cholesterol metabolism, so that their determination is usually not solely for analytical purposes.

Two assumptions inherent in the procedure described have been shown to hold within the limits of experimental error: (*a*) that the SA of a particular unconjugated BA is identical for a given bile sample whether the acid is conjugated with glycine or taurine (Table 5); and (*b*) that when Me-D and Me-CD are isolated from the conjugates, the hydrolytic and other losses are identical for the two bile acids (Table 7).

The considerable losses of BA resulting from alkaline hydrolysis under pressure are illustrated for Me-C in Table 9; TLC of the material recovered by extraction into diethyl ether from the hydrolysate showed the presence of significant amounts of compounds not identical with cholic acid, as has been observed previously (6). The method presented employs alkaline saponification under pressure, but only in steps where partial destruction of the steroid moiety of conjugated BA is not a crucial matter.

The use of certain procedures for checking the reliability of a new analytical method was considered unlikely to be helpful in this case. Thus, comparison with an established method was not possible since there is no established procedure for detailed analysis of individual conjugated BA. The recovery of known amounts of added pure components was considered but rejected since considerable uncertainty existed regarding the purity of even the best available samples of taurine-conjugated BA. The reliability of the procedure described rests upon the careful validation of individual steps; the use of the ^{14}C content of the component BA to correct for small losses during the critical initial fractionation of conjugated BA; and the good reproducibility consistently found during replicate analyses (Table 10).

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