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A Rapid Flexible Method for Determining Bile Lipids

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A rapid flexible method has been developed for the quantitative determination of bile lipids in gallbladder and hepatic bile and duodenal aspirates. Quantification of bile salts involves separation of bile salt conjugates from one another and other bile lipids by thin layer chromatography. The separated salts are determined using 3-hydroxysteroid dehydrogenase and gas liquid chromatography. Cholesterol is determined in petroleum ether extracts of saponified bile by application of the Lieberman-Burchard reaction. Phospholipid phosphorus is determined in purified bile lipid extracts by oxidation followed by application of Bartlett's modification of the Fiske-SubbaRow method.

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Recent findings suggest that gallstone formation is initiated by precipitation of cholesterol from bile which is supersaturated with this sterol. Studies have shown that the soluble cholesterol of bile is a component of colloidal particles called micelles which are composed of bile salts, lecithin and cholesterol. The stability of these micelles and consequently the solubility of cholesterol in bile depends on the ratio of the lipids in these particles.[1-8] Using these relationships, several investigators have shown that in the majority of cases it is possible to predict the probability of gallstone development.[9-14] To facilitate studies such as these, we have developed a rapid flexible procedure which enables the analyst to determine: (a) bile salts, (b) cholesterol, and (c) lecithin in relatively small samples of gallbladder and hepatic bile or duodenal aspirates. The analyst has the option of determining, independently, either the quantities of individual bile salts or the total bile salt concentration. Bile salt determinations involve separation of the salts from other bile components by thin layer chromatography. This separation is followed by quantification according to a modification of the method of Iwata and Yamasaki.[15] Cholesterol is determined by a modification of the method of Beher and Anthony[16] and phospholipid phosphorus by Bartlett's method.[17]

Materials

A. *General*

1. All reagents used in the determinations were chemically pure. All solvents were distilled prior to use.

B. *Reagents and Materials Used in Bile Salt Determinations*

1. *Conjugated Bile Salts*
These reagents were purchased from Calbiochem, San Diego, CA. They were checked for purity by thin layer and gas liquid chromatography. If impurities were found, the salts were purified by preparative thin layer chromatography.

2. *Pyrene Spray*
This reagent, which is a 0.05% solution of pyrene in hexane, is used for detecting the positions of bile salts on chromatographic plates. It must be highly pure. Although pyrene has never been shown to possess carcinogenic properties, the impure material may contain carcinogens. In addition, certain oxidation products of pyrene are difficult to remove from the chromatographic plates prior to enzymatic assay of bile salts. It is advisable to store this reagent under nitrogen.

3. *Bile Salt Standard*
18.8 mg of chromatographically pure sodium taurocholate is dissolved in 90 ml of 0.1 M pH 10 pyrophosphate buffer and diluted to 100 ml with buffer. This solution contains 0.348 mM of taurocholate per ml.

4. *β-Nicotinamide Adenin Dinucleotide (β-NAD) Solution*
45 mg of β-NAD (Sigma Chemical Company, St. Louis, MO) is dissolved in 100 ml of water. This solution is stable for several weeks if stored at 4°C.

5. *Pyrophosphate Buffer*
The 0.1 M pH 10 buffer is prepared by dissolving 44.61 g of sodium pyrophosphate decahydrate in 900 ml of distilled water. The pH of the solution is adjusted to 10 by careful addition of 0.1 M hydrochloric acid. The buffer is then diluted to one liter with water. This solution is stable for six months when stored at 5°C.

6. *Hydrazine Hydrate Solution*
12.5 g of 99-100% hydrazine hydrate (Matheson, Coleman and Bell) is diluted to 90 ml with 0.1 molar pH 10 pyrophosphate buffer. The pH of the solution is adjusted to 10 with 6 M hydrochloric acid and it is then diluted to 100 ml with pyrophosphate buffer. The solution contains 3μM of hydrazine hydrate per 1.2 ml and is stable for several weeks.

7. *3-Hydroxysteroid Dehydrogenase Solution*
100 mg of dried *pseudomonas testosteroni* (Sigma Chemical Company, St. Louis, MO) is homogenized for five minutes together with 10 ml of 0.1 M pH 10 pyrophosphate buffer in an ice bath. The contents of the homogenizer are transferred to a high speed centrifuge tube and spun at 50,000 g for twenty minutes. The supernatant solution, containing 3-hydroxysteroid dehydrogenase, is decanted into a test tube and stored in an ice bath until used. The solution can be frozen and kept for 24 hours. After that, it rapidly loses activity even when frozen. Dried extracts of *pseudomonas testosteroni* are also available and, depending on activity, are simply dissolved in pyrophosphate buffer prior to use.

8. *Triflouroacetic Anhydride*
This reagent was purchased from Aldrich Chemical Company, Inc, Milwaukee, WI, and assayed 99+%.

9. *Thin Layer Chromatography Tanks*
23L x 24H x 12W (cm) glass tanks designed for ascending chro-

matography are used. The inside surfaces of the tanks are lined with Whatman No. 1 filter paper. The paper serves two purposes: (a) It insulates the tanks, and (b) It helps to saturate their atmosphere uniformly with the solvent being used. All solvent systems are prepared on a v/v basis and are introduced into the tanks at least one hour prior to use.

10. *Silica Gel Coated Thin Layer Chromatography Plates*
    30 g of silica gel G (E. Merck, Darmstadt, Germany) is mixed with 70 ml of distilled water acidified with 5 drops of glacial acetic acid. The mixture is stirred until it just begins to thicken (about 8 minutes) and is then rapidly spread on 20 x 20 cm glass plates using a spreading device to produce 250μ silica gel layers. After spreading, the plates are allowed to air dry and are then stored in dessiccators over silica gel. Prior to use, the plates are channeled so that each plate has 12 to 14 separate channels. Prior to applying bile salt samples, the channeled plates are activated in a drying oven at 100°C for one hour.

11. *Preparation of Gas-Liquid Chromatography Columns*
    Four-foot long, 1/8-inch internal diameter, glass U-shaped columns are silanized by filling with 3% dichlorodimethylsilane (Analabs, Inc, North Haven, CT) dissolved in toluene and allowing them to stand overnight. The silanized columns are rinsed with acetone, dried, and then packed with 1% QF-1 supported on 100-120 mesh gaschrome P (Applied Science Laboratories, Inc, State College, PA). Glass wool silanized with 3% dichlorodimethylsilane is inserted at the exit and injection areas of the column. The packed column is placed in the gas chromatograph oven and the column and injector temperatures raised to 150°C. A minimum flow of gas is started. 100 to 200 μl of hexamethyldisilazane (Analabs, Inc, North Haven, CT) is injected in 10 μl quantities. Gas flow is then discontinued and the column is allowed to stand overnight at 150°C. The column temperature is then increased to 240°C, the injector to 260°C, and gas flow is started and maintained at 60 ml/min. The column is conditioned for eight hours.

C. *Reagents Used in Lecithin Determinations*

1. *Fiske-SubbaRow Reagent*
   0.5 g of 1-amino-2-naphthol-4-sulfonic acid is dissolved in 250 ml of a solution containing 30 g of sodium bisulfite and 6 g of sodium sulfite. This reagent should be prepared fresh just before use.

D. *Reagents Used in Cholesterol Determinations*

1. *Digitonin Solution*
   500 mg of digitonin is dissolved in 100 ml of 50% ethanol. Gentle heating is necessary. The reagent is stable for several months.

2. *Alcoholic Potassium Hydroxide*
   Alcoholic potassium hydroxide is prepared fresh daily by diluting 10 ml of 50% aqueous potassium hydroxide with 90 ml of 95-100% ethanol.

3. *Phenolphthalein Indicator*
   This is a 1% solution of phenolphthalein in 50% ethanol.

4. *Cholesterol Standard*
   10 mg of chromatographically pure cholesterol is dissolved in 100 ml of glacial acetic acid. The solution contains 0.1 mg of cholesterol per ml and is stable indefinitely.

5. *Lieberman-Burchard Reagent*
   100 ml of acetic anhydride is cooled in an ice bath. 5 ml of concentrated sulfuric acid is added slowly, with constant mixing. The reagent is stored in an ice bath and must be used within one hour.

Procedures

Flow diagrams of the procedures appear in Figures 1 and 2 and will be useful in following the detailed instructions.

A. *Preparation of the Bile Lipid Extract*

From 5 to 20 ml of hepatic bile, gallbladder bile, or duodenal aspirate are placed in 250 ml round bottomed boiling flasks. The contents of the flasks are frozen and lyophylized. After drying, 30 ml of chloroform:methanol 2:1 (v/v) is added to each flask. The contents are refluxed for 30 minutes. After cooling, the solid and liquid phases are separated by centrifugation at 2500 RPM. The supernatant fluids are decanted, partially evaporated (caution!! not to dryness), transferred to volumetric flasks and made up to volume with chloroform: methanol. Experience has shown that extracts resulting from 20 ml samples of hepatic and gallbladder bile are best made up to 5 and 10 ml respectively. Occasional samples require more or less dilution. This extract is called the bile lipid extract. If it is necessary to determine the percentage of solids in the samples, weights of the empty flasks and the flasks plus wet and dry contents must be determined.

B. *Bile Salt Determination*

1. *Separation of conjugated bile salts by thin layer chromatography.*

   Duplicate nine to fifteen μl aliquots of each bile lipid extract are applied to individual channels of 20 x 20 cm thin layer chromatography plates. Twelve μl of a solution containing 4μg/μl each of tauro and glyco cholic and deoxycholic acids is applied to one or two channels to aid in identification of the bile salts. If total bile salt concentrations *only* are being determined, the plates are developed using chloroform:methanol: water 80:25:3 (v/v). If it is desired to determine individual bile salts, the plates are developed in tanks containing chloroform:methanol: water:acetic acid 65:25:4:2 (v/v). After developing, the plates are air-dried and then lightly sprayed with 0.05% pyrene dissolved in hexane. After the plates are dry, the separated bile salts are visualized using short wave ultra violet radiation. The positions of the salts are marked by outlining the spots with a needle. The plates are now placed (within one hour) in tanks containing petroleum ether:ethyl ether 3:2 (v/v) and allowed to develop. The plates are then viewed with ultra violet radiation to make sure that all of the pyrene has been removed from the bile salt spots. If not, development is repeated. As a rule, two developments are necessary; however, they are very rapid and little time is consumed. It is important that pyrene is promptly removed since this hydrocarbon interferes with subsequent colorimetry. After pyrene has been removed, the marked areas containing the bile salts are quantitatively transferred to 15 ml centrifuge tubes by scraping the silica gel from the plates with the squared end of a stainless steel spatula.

2. *Bile salt determination using 3-hydroxysteroid dehydrogenase.*

   Three types of samples are used in the determination: (a) a blank, (b) standards containing a known amount of bile salt, and (c) unknowns containing the separated bile salts absorbed on silica gel G. One ml of NAD solution and 1.2 ml of hydrazine hydrate solution is added to each tube. 0.5 ml of 0.1 M pH 10 pyrophosphate buffer is added to the blank and unknown tubes. 0.5 ml of

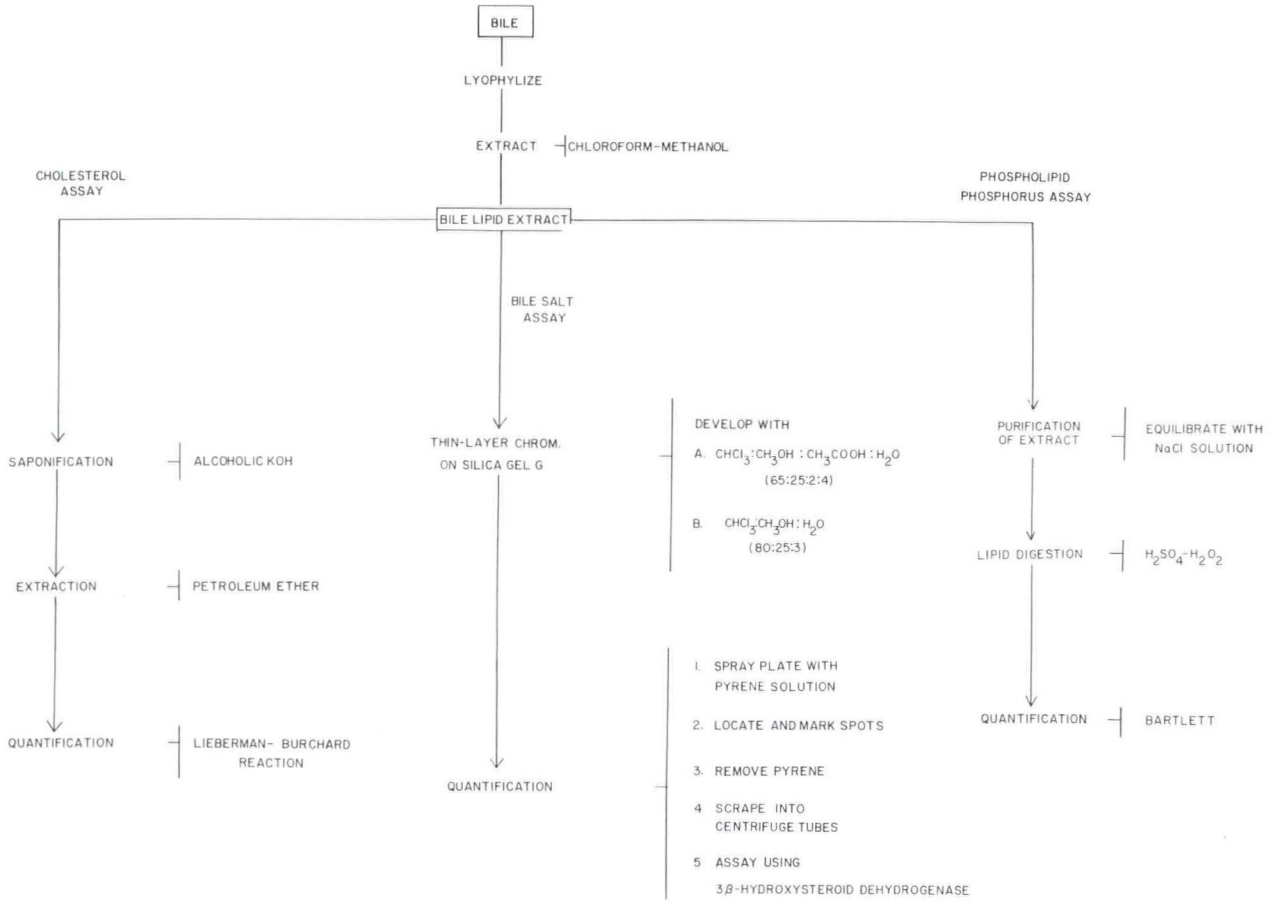

Figure 1
The Separation and Quantification of Bile Lipids.

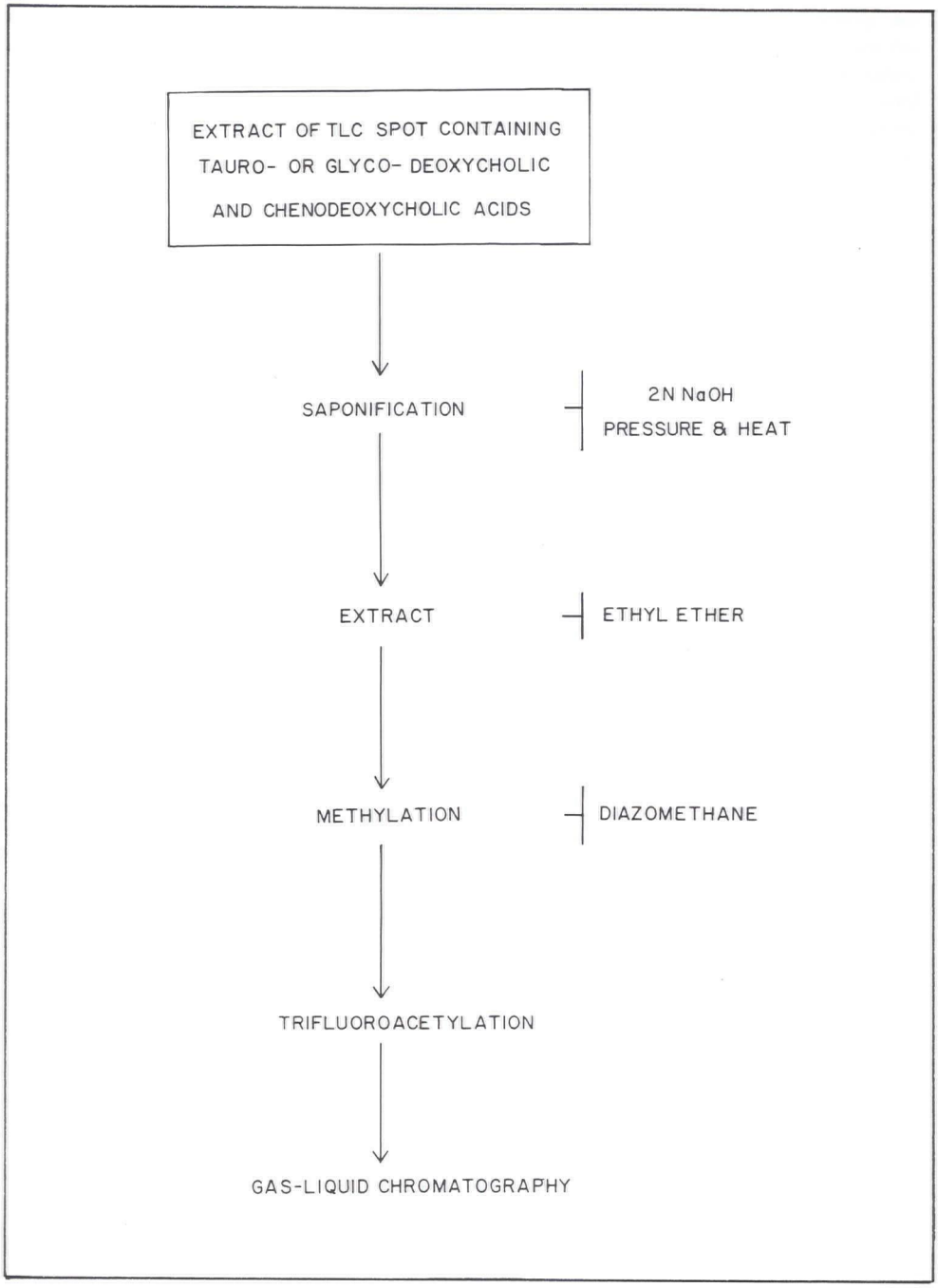

Figure 2
The Separation and Determination of Percentages of Chenodeoxycholic and Deoxycholic Acids in Thin Layer Chromatography (TLC) Spots.

standard bile salt solution is added to the standard tubes. The contents of each tube is thoroughly mixed and 0.3 ml of fresh 3-hydroxysteroid dehydrogenase solution is added.

After thorough mixing, the tubes are placed in a water bath (37°C) for 30 minutes. Agitation is unnecessary. Following incubation, the tubes containing the unknown samples are centrifuged at 2500 RPM for five minutes to remove silica gel. The contents of all of the tubes are transferred to cuvettes and their optical density read at 340 nm. The quantity of bile salts per liter of bile is calculated according to Equation I, Table I (page 100).

3. *Quantitative determination of conjugated deoxycholate and chenodeoxycholate contained in thin layer chromatographic spots.*

Since no solvent system has been discovered which will separate the glycine and/or taurine conjugates of deoxycholic and chenodeoxycholic acids from one another on thin layer plates, some alternate method must be used to determine these bile acids. We have approached this problem by determining the percentage of deoxycholic and chenodeoxycholic acids in thin layer chromatographic spots containing their taurine and glycine conjugates by gas liquid chromatography. The quantities of individual bile salts in the spots can then be calculated by multiplying the total bile salt content of the spots by the percentage of deoxycholic and chenodeoxycholic acids. The method is highly accurate and quantitative procedures are unnecessary since only percentages are determined. The procedure follows.

Twelve to 18 μl aliquots of bile lipid extracts are applied to thin layer chromatographic plates. The conjugated bile salts in the extracts are separated and the spots containing the glycine and taurine conjugates of deoxycholic and chenodeoxycholic acids located and outlined as described in section (B) of Procedures. Each outlined spot is transferred to a tube and the tubes are placed momentarily in a water bath at 80°C. Each tube is thoroughly mixed to insure elution of bile salts. The tubes are then centrifuged at 2500 RPM for five minutes to separate silica gel. Separation of the gel is necessary since it interferes with subsequent steps in the procedure. The supernatant fluid in the centrifuge tubes, which contains the conjugated bile salts, is transferred to nickel crucibles or teflon tubes and evaporated to dryness. Two ml of 2M sodium hydroxide is added to each sample. The crucibles or tubes are placed in an autoclave and heated at 250°F for three hours to effect bile salt deconjugation. The hydrolysed samples are transferred to 15 ml glass stoppered tubes and acidified with 1 ml of 12 M hydrochloric acid. The bile acids (now free) are extracted from the acidified solution with 3 ml of peroxide-free ethyl ether. The extract is washed twice with 1 ml of water. The ether extract is then evaporated to dryness in a stream of nitrogen and the last traces of moisture removed *in vacuo*. Before the free bile acids in the tubes can be separated by gas liquid chromatography, volatile derivatives must be prepared by methylating their carboxyl group and trifluoroacetylating their hydroxyls. To effect methylation of the carboxyl, the dried samples are dissolved in 2 ml of ether containing 10% methanol and are treated with diazomethane according to Schlenk and Gellerman.[18] Following methylation, the solution is evaporated to dryness. It is very important that all traces of moisture are removed at this point since it interferes with trifluoroacetylation in the next step. Trifluoroacetylation is

accomplished by adding 0.2 ml of trifluoroacetic anhydride (Hood!!) to each sample. The tubes are stoppered (glass) and placed in a water bath at 30-35°C for 15 minutes. At the end of this time the excess trifluoroacetic anhydride is removed in a stream of nitrogen (Hood!!). The bile acid methyl ester trifluoroacetates, in each tube, are dissolved in 0.1 ml of acetone. A Barber-Coleman Series 5000 gas liquid chromatograph, equipped with an argon ionization detector, was used for the separation and determination of percentages of deoxycholic and chenodeoxycholic acids in the samples. The conditions used were the following: The column was packed with 1% QF-1 supported on gas-chrom P. The temperatures used were: injector 250°C; column 235°C; and detector 280°C. Argon flow was maintained at 60 ml per minute. Peak areas were measured by planimetry. Suitable corrections were made for the difference in response of the detector to deoxycholic and chenodeoxycholic acids when calculating percentages of acids in the samples.

C. *The Determination of Bile Lecithin*

Bile lecithin is determined by assaying bile for its lipid phosphorus content using an adaption of Bartlett's method.[17] Before lipid phosphorus can be determined in bile lipid extracts, nonlipid phosphorus must be removed. To effect this, one ml aliquots of bile lipid extracts [see A of Procedures] are diluted to 25 ml with chloroform:methanol 2:1 (v/v). These solutions are equilibrated with 5 ml of 0.73% aqueous sodium chloride in separatory funnels. The organic phase (bottom) resulting from the equilibration, in each case, is separated and diluted to 25 ml with chloroform:methanol 2:1 (v/v). Tubes marked at 5 ml are used for digestion and color development and are set up as follows: The blanks contain 1 ml of chloroform:methanol 2:1 (v/v); the standards 1 ml of chloroform:methanol and 0.2 ml of 0.5 mM potassium dihydrogen phosphate. The unknowns contain 1 ml aliquots of the purified diluted bile lipid extracts. The contents of each tube is evaporated to dryness and 0.5 ml of 5 M sulfuric acid added. The tubes are placed in an oven at 160°C for three hours, they are then removed and allowed to cool. Two drops of 30% hydrogen peroxide is added to each tube and they are returned to the oven for an additional 1.5 hours. Each tube should be examined 30 minutes after adding hydrogen peroxide. If it is colored, two more drops of hydrogen peroxide should be added. At the end of the digestion, the contents of each tube *must* be colorless. If not, the addition of hydrogen peroxide and heating is repeated until the contents are colorless. Following digestion, 0.2 ml of 5% ammonium molybdate is added to each tube. The contents of the tubes are thoroughly mixed. 0.2 ml of Fiske-SubbaRow reagent is then added and the tubes are mixed again. The tubes are then placed in a water bath and maintained at 100°C for ten minutes. The colored solutions are diluted to 5 ml and transferred to cuvettes. Their optical density is read at 830 nm. The millimoles of bile lecithin per liter of bile are calculated according to Equation II, Table I.

D. *Bile Cholesterol Assay*

One ml aliquots of the bile lipid extracts are placed in screw capped vials marked at 20 ml and evaporated to dryness. Ten ml of alcoholic potassium hydroxide is added to each vial. The vials are capped and incubated in a water bath (100°C) for thirty minutes. The samples are agitated occasionally during incubation. This procedure effects hydrolysis of any cholesterol esters in the lipid extracts. At the end of incubation, the vials are cooled and the contents made up to 20 ml with ethanol. Five ml aliquots of the hydrolysed extracts are transferred to screw capped vials, five ml of water and 10 ml of petroleum ether are added, and the vials vigorously shaken to effect quantitative transfer of cholesterol to the petroleum ether phase. It is helpful to

add two or three drops of phenolphthalein indicator solution to each vial at this point to clearly delineate the boundary between the petroleum ether and water phases. Duplicate four ml aliquots of the petroleum ether phase are transferred to colorimeter tubes and evaporated to dryness. For colorimetry the following solutions are set up in colorimeter tubes: a) the blank: 2 ml of glacial acetic acid; b) the standards: 2 ml of glacial acetic acid solution containing 0.1 mg of cholesterol per ml; c) the unknowns: prepared by dissolving the contents of the tubes containing the residue from the evaporation of petroleum ether in 2 ml of warm glacial acetic acid. At one-minute intervals, 5 ml of Lieberman-Burchard reagent is rapidly added to each tube, the contents are thoroughly mixed, and the tubes are placed in a water bath maintained at 25°C. Light should be excluded from samples during color development. At the end of thirty minute intervals, the tubes are removed from the water bath and their optical density determined at 600 nm. The mM of cholesterol per liter of bile are calculated according to Equation III, Table I.

Experimental

A. *Solvent Systems for Thin Layer Chromatography of Conjugated Bile Salts*

Conjugated bile salts are highly polar substances with solubility characteristics somewhat similar to the phosphatidyl cholines. A number of solvent systems were investigated in an effort to formulate two. One was designed to effect separations of

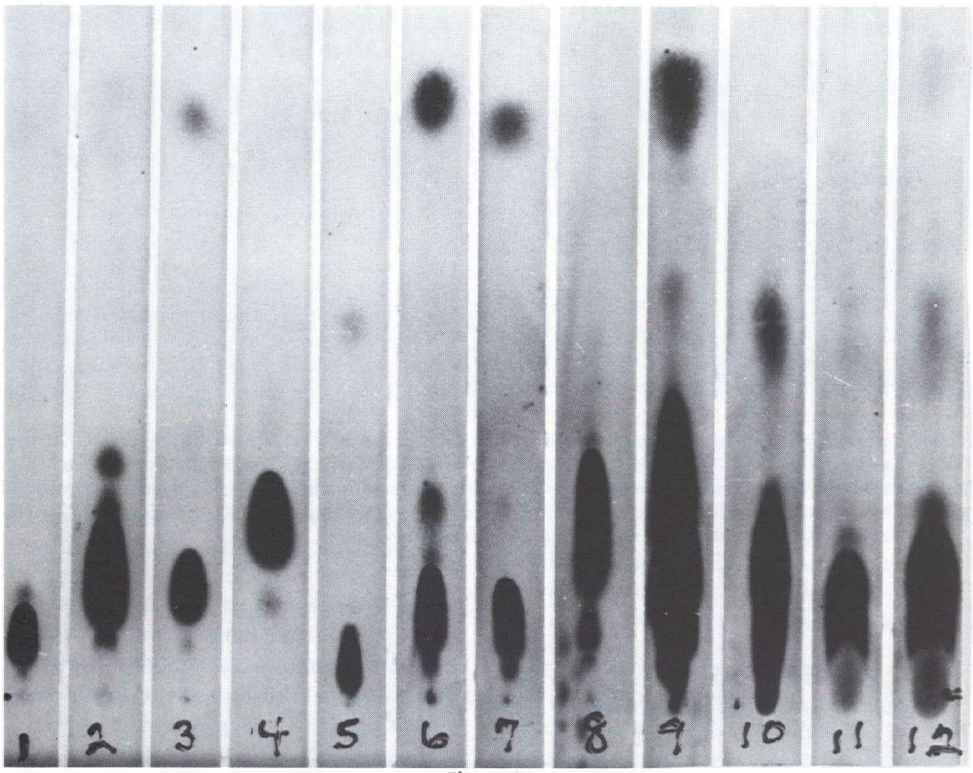

Figure 3

The thin-layer chromatographic separation of conjugated bile acids on Silica Gel G, using $CHCl_3$:MeOH:H_2O (160:50:6, v/v) as the developing solvent. Channel 1, taurocholic acid; 2, taurochenodeoxycholic acid; 3, taurodeoxycholic acid; 4, taurolithocholic acid; 5, glycocholic acid; 6, glycochenodeoxycholic acid; 7, glycodeoxycholic acid; 8, glycolithocholic acid; 9, mixture of 1-8; 10, 11 and 12, human bile samples.

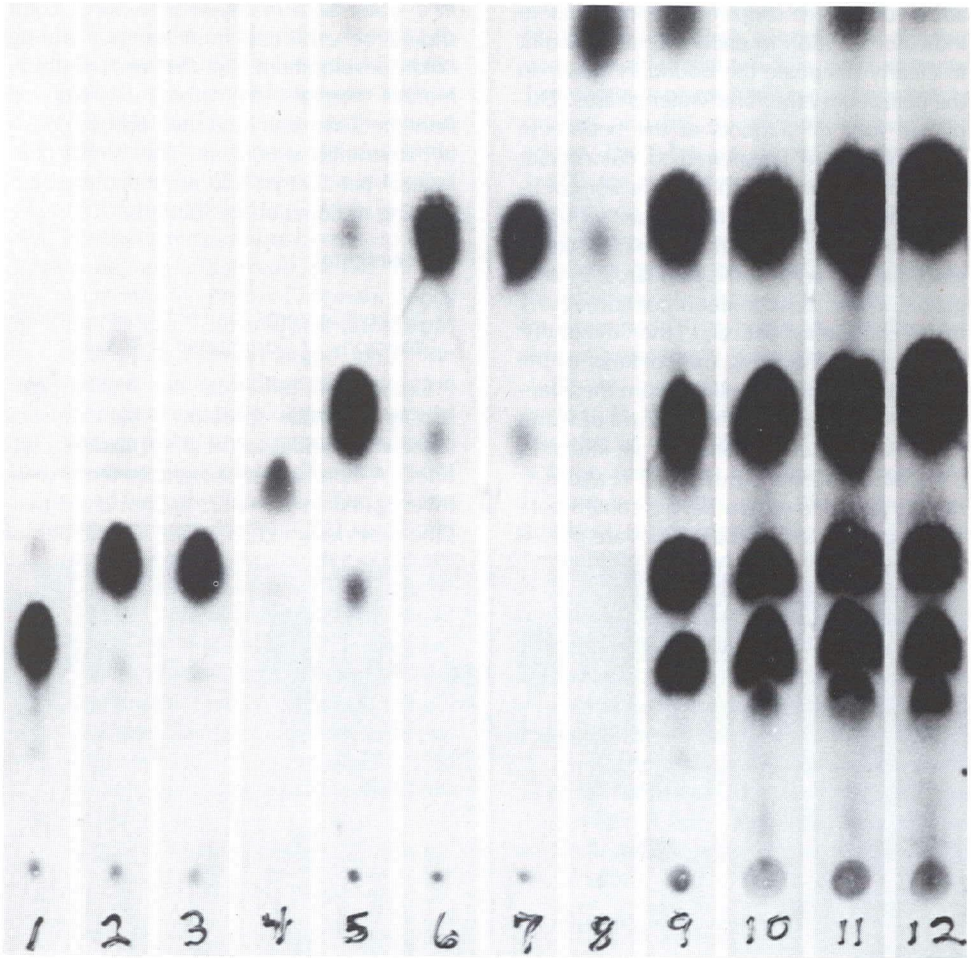

Figure 4

The thin-layer chromatographic separation of conjugated bile acids on Silica Gel-G, using $CHCl_3$:MeOH:HOAc:H_2O (130:50:4:8, v/v) as the developing solvent. Channel 1, taurocholic acid; 2, taurochenodeoxycholic acid; 3, taurodeoxycholic acid; 4, taurolithocholic acid; 5, glycocholic acid; 6, glycochenodeoxycholic acid; 7, glycodeoxycholic acid; 8, glycholithocholic acid, 9, mixture of 1-8; 10, 11 and 12, human bile samples.

glycine and taurine conjugated mono-, di-, and trihydroxy bile salts from one another and from other lipids; the other to effect separation of conjugated bile salts as a group from other lipids. After a number of trials two satisfactory solvent systems were formulated. Chloroform:methanol: water:acetic acid 65:25:4:2 v/v which separates the individual bile salts and chloroform:methanol:water 80:25:3 v/v which separates the bile salts as a group from most other lipids. Photographs of chromatograms, illustrating the results obtained using these solvent systems, are shown in Figures 3 and 4. These solvent systems effect complete separation of conjugated bile salts from cholesterol, cholesterol esters and triglycerides. They do not separate the salts

from lecithin. This poses no problem since lecithin *does not* interfere with bile salt determinations involving the oxidation of their 3α-hydroxyl groups catalyzed by 3-hydroxysteroid dehydrogenase.

B. *The Bile Salt Determination*

Since many of the procedures used in the bile salt determination are original, it was necessary to investigate the precision and accuracy of several of the steps.

1. *The validity of bile salt assays utilizing crude extracts of Pseudomonas testosteroni (ATCC 11996) containing 3-hydroxysteroid dehydrogenase.*

The method used for bile salt determination is dependent on the oxidation of bile salt 3α-hydroxyl groups catalyzed by 3-hydroxysteroid dehydrogenase according to the reactions outlined in Equation IV, Table I.

Hydrazine is used as a trapping agent to drive the reaction to completion. The quantity of bile salt oxidized is proportional to the quantity of NADH formed. NADH is determined by measuring its absorbance at 340 nm.

The validity of the method depends on the exclusive oxidation of bile salt 3α-hydroxyl groups. Most investigators who have developed methods of bile salt assay, based on the use of crude extracts of *Pseudomonas testosteroni* (ATCC 11996) that contain 3-hydroxysteroid dehydrogenase (3α-hydroxysteroid:NAD(P) oxidoreductase 1.1.1.50), have reported that the extracts exclusively oxidize 3α-hydroxyl groups of bile salts.[15,19,20] Skålhegg[21] has recently reported that crude extracts of *Pseudomonas testosteroni* (ATCC 11996) may, in fact, contain enzymes which catalyze the oxidation of the 7α and 12α hydroxyl groups of bile acids. The use of such preparations would, of course, yield erroneous results if used in assays of mixtures of bile salts which do and do not contain 7α and 12α hydroxyl groups. Since such combinations are the rule in bile and serum samples, use of such extracts would result in inaccurate determinations. We have, therefore, made careful studies of the specificity of oxidation of bile salt hydroxyl groups using crude extracts of *Pseudomonas testosteroni* (ATCC 11996) derived from three different sources. They were Worthington hydroxysteroid dehydrogenase lot-L3J055 (Worthington Biochemical Corp, Freehold, NJ), Sigma hydroxysteroid dehydrogenase Type I, lot-102C-6800-1 (Sigma Chemical Company, St. Louis, MO), and Sigma hydroxysteroid dehydrogenase grade II, lot-94C-6920. To test these extracts, 0.5 ml aliquots of solutions containing 0.05, 0.10, 0.15 and 0.20 μM of sodium taurocholate, sodium glycodeoxycholate, and sodium taurochenodeoxycholate were placed in small test tubes. 1 ml of NAD solution, 1.2 ml of hydrazine hydrate solution, and 0.3 ml of one of the enzyme-containing extracts was added to each tube. The tubes were incubated for 30 minutes at 37°C. The absorbance of the contents of each tube was read at 340 nm. This procedure was repeated using each of the enzyme-containing extracts.

Figure 5 shows the results. Since the curves nearly coincide in each case, it is obvious that none of these preparations contained significant amounts of a 12α-hydroxysteroid dehydrogenase. If such a dehydrogenase had been present the slopes of the curves, resulting from the oxidation of taurocholate and glycodeoxycholate which have 12α-hydroxyls, would have been greater than the slope of the curve resulting from the oxidation of taurochenodeoxycholate which lacks a 12α-hydroxyl. The results also eliminate the possibility that the crude enzyme-containing extracts contained significant amounts of a 7α-hydroxysteroid dehydrogenase since the curves of taurochenodeoxycholate and glycodeoxycholate (bile salts with and without 7α-hydroxyl groups respectively) have similar slopes in each case.

The results show that currently available crude extracts of *Pseudomonas testosteroni* (ATCC 11996) are suitable for bile salt

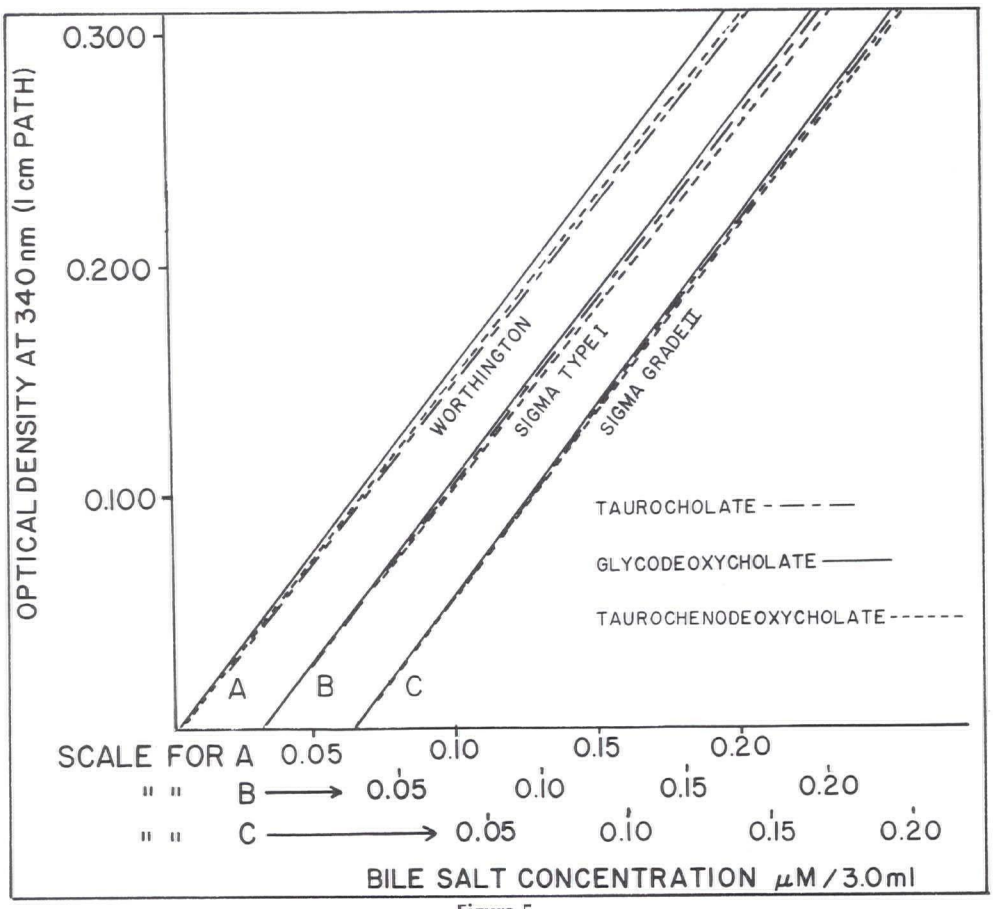

Figure 5

The oxidation of taurocholate, glycodeoxycholate and taurochenodeoxycholate by three crude extracts of Pseudomonas testosteroni containing hydroxysteroid dehydrogenases.

assays. It is conceivable that, some time in the future, mutations might occur which would result in the presence of 7α and/or 12α-hydroxysteroid dehydrogenases. Although this is a remote possibility, since 3α-hydroxysteroid dehydrogenase is an induced enzyme, it might be prudent to check for this possibility when using extracts derived from new batches of bacteria. This could be done by simply comparing the absorbance of NADH solutions resulting from the oxidation of equimolar quantities of cholate, deoxycholate and chenodeoxycholate.

C. *The Precision and Accuracy of Bile Salt Quantification*

The operations included in the precision and accuracy studies were (a) application of bile extracts to the plates, (b) development of the plates, (c) location and marking of areas containing bile salts, (d) pyrene removal, (e) transfer of marked areas on plates to sample tubes, and (f) bile salt determination using 3-hydroxysteroid dehydrogenase.

Two separate 12-channel thin layer chromatography plates were used. 12 μl of a solution containing 47 μg each of sodium

taurocholate, sodium taurochenodeoxycholate, sodium glycocholate and sodium glycodeoxycholate was applied to each of the twelve channels on each plate. The plates were developed, the bile acids located and determined, as outlined under procedures. The results of the study are presented in Table II. Three things should be noted: 1) The results are precise, 2) There is excellent agreement between the results obtained using different plates, and 3) Recoveries of each bile salt were excellent. *In toto* the method is precise and accurate.

D. *The Precision and Accuracy of the Gas Liquid Chromatographic Determination of the Percentage of Deoxycholate and Chenodeoxycholate Present in Thin Layer Chromatographic Spots*

Solutions containing 4 μg of sodium glycochenodeoxycholate and 4 μg of sodium glycodeoxycholate per μl (I); 4 μg of sodium glycochenodeoxycholate and 2 μg of sodium deoxycholate per μl (II); and 2 μg of sodium glycochenodeoxycholate and 4 μg of sodium deoxycholate per μl (III) were prepared. 18 μl of I was applied to each of channels 1-4 of a thin layer chromatography plate. 18 μl of II was applied to each of channels 5-8 and 18 μl of III was applied to each of channels 9-12. The plates were developed and the spots containing the mixtures of bile salts located and transferred to tubes. The bile salts absorbed on the silica gel were extracted and prepared for gas liquid chromatography. All of these steps were according to the methods outlined under procedures.

The results of the gas liquid chromatography determinations are presented in Table III. Examination of the table shows that the determined percentages of bile salts in the spots agreed well with the theoretical; the deviations were small. It can be concluded that use of this method yields both precise and accurate determinations of the percentages of deoxycholate and chenodeoxycholate in thin-layer chromatographic spots. These percentages, when multiplied by the total number of micrograms of bile salts in the spots, yield accurate quantitative determinations of chenodeoxycholate and deoxycholate.

E. *Cholesterol Assay*

The cholesterol assay omits the usual purification steps which are necessary for precise serum cholesterol analyses. This was done to reduce the amount of time involved, and the number of steps required in the procedure.

Although the conditions for cholesterol extraction from bile were satisfactory and no troublesome emulsions were encountered, there was concern that certain bile pigments might be extracted and interfere with the Lieberman-Burchard color reaction. To test for this possibility, we chose a number of random bile samples and determined their cholesterol concentration using the method outlined under procedures and a method involving cholesterol purification through digitonin precipitation and subsequent washing. The procedure for purification was the following: After extracting cholesterol from bile with petroleum ether (See procedures), duplicate 4 ml aliquots were transferred to centrifuge tubes. After evaporation, 6 ml of ethanol:acetone (1:1 v/v) 1 drop of 10% acetic acid and 3 ml of digitonin solution were added, and the contents of the tubes mixed. The samples were allowed to stand for 24 hours to insure complete precipitation of cholesterol digitonide. The digitonide was washed once with ether:acetone (2:1 v/v) and twice with peroxide-free dry ether. After washing, the samples were heated to 100°C in a sand bath then dissolved in 2 ml of glacial acetic acid. These solutions were treated with Lieberman-Burchard reagent and their absorbance read at 600 nm as outlined under procedures.

Seven different bile samples were chosen for comparison of the methods. These differed widely in cholesterol concentration

and apparent pigment content. The cholesterol content of six aliquots of each bile extract was determined by the method outlined under procedures and by the method involving cholesterol purification through digitonide precipitation and washing. A comparison of the results can be made from the data in Table IV. Two things should be noted: 1) Standard deviations are small, showing that either method is precise; and 2) Similar results are obtained by either method, suggesting that both methods are accurate. While this data suggest that purification of extracts of bile containing cholesterol is unnecessary in most cases, it is possible that an occasional sample may require purification if the highest degree of accuracy is necessary. Highly-colored extracts should be suspect.

Discussion

Figure 6 illustrates the type of information that can be obtained by application of the methods outlined above. The flexibility of the procedure can be illustrated by a few examples. If the goal of a study is simply to determine the degree of cholesterol saturation of different samples of bile, the data needed are (a) the total bile salt concentration, (b) the lecithin concentration and (c) the cholesterol concentration of the bile samples. To obtain this data, an abbreviated bile salt assay is used since no separation of glycine and taurine bile salt conjugates is necessary. The conjugates are separated from other bile lipids as a group, (Figure 3), and the total molar concentration of the salts in the mixture determined, using 3-hydroxysteroid dehydrogenase. Limiting the procedure in this way saves time since it eliminates three quarters of the bile salt determinations and the gas chromatography step involved in the comprehensive determination. The method becomes slightly more involved if the study calls for determinations of the degree of cholesterol saturation of bile samples and, in addition, determinations of the ratio of glycine to

TABLE I

Equation I:

$$\text{mM Bile Salt/liter Bile} = \frac{\text{unknown reading}}{\text{Std. reading}} \times \frac{\mu\text{M of Bile Salt Std.}}{\text{ml Bile Ext. Applied to plate}} \times \frac{\text{Vol. (ml) Bile lipid extract}}{\text{ml of Bile Lyophilysed}}$$

Equation II:

$$\text{mM Lecithin/lt} = 2.5 \times \frac{\text{Vol. Bile Lipid Ext. (ml)}}{\text{Vol. of Bile Lyophilysed (ml)}} \times \frac{\text{Unknown reading}}{\text{Standard reading}}$$

Equation III:

$$\text{mM Bile Cholesterol/lt} = 5.172 - \frac{\text{Unknown reading}}{\text{Standard reading}} \times \frac{\text{Vol. of Bile Lipid Ext. (ml)}}{\text{Vol. of Bile Extracted (ml)}}$$

Equation IV:

$$3\alpha\text{-hydroxy bile salt} \xrightarrow[\text{NAD (pH 10)}]{3\alpha\text{-hydroxysteroid dehydrogenase}} \text{3-oxo bile salt} + \text{NADH} + \text{H}^+ \xrightarrow{\text{NH}_2\cdot\text{NH}_2\cdot\text{H}_2\text{O}} \text{bile salt oxime}$$

TABLE II

THE PRECISION AND ACCURACY OF THE BILE SALT DETERMINATION

Bile Salts*	Taurocholate		Taurochenodeoxycholate		Glycocholate		Glycodeoxycholate	
Plate Number	I	II	I	II	I	II	I	II
Recovery (μg)	48.5 ± 0.52** (12)†	47.2 ±0.28 (12)	47.6 ±0.32 (12)	47.1 ±0.21 (12)	46.3 ±0.56 (12)	46.1 ±0.72 (12)	46.8 ±0.76 (12)	46.3 ±0.82 (12)

*47 μg of the sodium salt of each bile acid applied to each channel of two thin layer chromatography plates

†Number of determinations

**Standard Deviation

TABLE III

PRECISION AND ACCURACY OF THE GAS LIQUID CHROMATOGRAPHIC DETERMINATION OF THE PERCENTAGE OF CHENODEOXYCHOLATE AND DEOXYCHOLATE IN THIN LAYER CHROMATOGRAPHIC SPOTS

	I		II		III	
Mixture Applied	**Deoxycholate** 50%	**Chenodeoxycholate** 50%	**Deoxycholate** 33.3%	**Chenodeoxycholate** 66.7%	**Deoxycholate** 66.7%	**Chenodeoxycholate** 33.3%
Determined Percentage	49.7±1.50*(10)†	50.3±1.36(10)	33.4±1.31(10)	66.6±1.31(10)	67.6±1.84(9)	32.4±1.84(9)

*Standard Deviation

†Number of Determinations

taurine bile salt conjugates in the samples. In this case, bile salt analysis would involve the separation of glycine conjugates from taurine conjugates (Figure 4), followed by determinations of the molar concentrations of the total bile salt conjugates of the separated groups. A procedure such as this would be shorter than a complete bile salt analysis since fewer bile salt determinations would be involved and gas liquid chromatography would be unnecessary. If it were necessary to determine the ratio of di- to trihydroxy bile salts, in addition to the total bile salt concentration and the ratio of glycine to taurine conjugates in the bile samples, then it would be necessary to determine the bile salt concentration in the individual spots of each channel on the thin layer chromatography plates. However, gas liquid chromatography would still be unnecessary. If a study demanded all of the information in Figure 5, eight bile salt determinations, gas liquid chromatography, phospholipid analysis and cholesterol determination are necessary for each bile sample. Even so, many samples can be simultaneously handled in a relatively short period of time.

TABLE IV

COMPARISON OF THE RESULTS OF BILE CHOLESTEROL DETERMINATIONS OBTAINED BY METHODS INCLUDING AND OMITTING PURIFICATION OF CHOLESTEROL EXTRACTS BY DIGITONIDE PRECIPITATION AND WASHING

Bile Sample	I		II		III		IV	
Method	P*	NP	P	NP	P	NP	P	NP
Bile Cholesterol mM/1t	6.59 * ±0.23 ** (6)†	6.60 ±0.36 (6)	1.22 ±0.07 (6)	1.33 ±0.06 (6)	2.35 ±0.08 (6)	2.39 ±0.04 (6)	0.60 ±0.07 (6)	0.61 ±0.02 (6)

Bile Sample	V		VI		VII	
Method	P	NP	P	NP	P	NP
Bile Cholesterol mM/1t	3.25 ±0.14 (6)	3.23 ±0.10 (6)	0.60 ±0.05 (6)	0.63 ±0.07 (6)	1.63 ±0.05 (6)	1.65 ±0.12 (6)

*P = Extract Purified NP = Extract not Purified ** Standard Deviation † Number of Determinations

TAURINE CONJUGATES: (mM/Lt)
- TAUROLITHOCHOLATE
- TAURODEOXYCHOLATE
- TAUROCHENODEOXYCHOLATE
- TAUROCHOLATE

TOTAL TAURINE CONJUGATES

GLYCINE CONJUGATES: (mM/Lt)
- GLYCOLITHOCHOLATE
- GLYCODEOXYCHOLATE
- GLYCOCHENODEOXYCHOLATE
- GLYCOCHOLATE

TOTAL GLYCINE CONJUGATES

GLYCINE CONJ. / TAURINE CONJ.
DIHYDROXY BILE SALTS / TRIHYDROXY BILE SALTS
CHOLESTEROL (mM/Lt)
LECITHIN (mM/Lt)
CHOLESTEROL SATURATION INDEX

Figure 6
The data gathering capability of the outlined method. The triangular coordinate diagram relates the millimolar concentrations of cholesterol bile salts and lecithin which determine the solubility of cholesterol in bile samples.

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