$$\boxed{\textit{review}}$$

# The quantitative estimation of bile acids and their conjugates in human biological fluids

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**Abstract** This review attempts to provide a concise and critical summary of modern methods for the analysis of bile acids and their conjugates in human biological fluids. Most emphasis is given to more up-to-date procedures that have been applied to the study of human disease and attention is drawn to previous reviews in areas that have not been covered here. An increasing awareness of the possibility that bile acids may be involved in the etiology of a number of disorders, or that such disorders may give rise to changes in bile acid concentration, has stimulated the study of bile acid methodology. Although many procedures have been described using, for example, high-pressure liquid chromatography (HPLC), gas–liquid chromatography (GLC), gas–liquid chromatography–mass spectrometry (GLC-MS), and radioimmunoassay (RIA), no simple but comprehensive procedure for the estimation of bile acids and their conjugates has yet been published. Further study in this area is still required in order to establish the role of bile acid estimations in the routine diagnosis and treatment of disease.—**Street, J. M., D. J. H. Trafford, and H. L. J. Makin.** The quantitative estimation of bile acids and their conjugates in human biological fluids. *J. Lipid Res.* 1983. **24:** 491–511.

**Supplementary key words** immunoassay • estimation • gas–liquid chromatography • mass spectrometry • bile acids • high-pressure liquid chromatography

## INTRODUCTION

Bile acids are major metabolites of cholesterol and facilitate its elimination in the feces by the formation of micelles that solubilize the cholesterol in the bile (1). Conjugation of the bile acids with the amino acids glycine and taurine occurs in the liver (2), before storage in the gallbladder and subsequent secretion into the duodenum via the bile duct. Within the intestinal lumen, bile acids interact with lipases (3) and assist the lipolysis and absorption of fats, including fat-soluble vitamins, by the formation of mixed micelles (4). During enterohepatic circulation (5), the primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA)

Abbreviations: CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; GCA, glycocholic acid; GCDC, glycochenodeoxycholic acid; GDC, glycodeoxycholic acid; TCA, taurocholic acid; TCDC, taurochenodeoxycholic acid; TDC, taurodeoxycholic acid; LCA, lithocholic acid; UDCA, ursodeoxycholic acid; HSD, hydroxysteroid dehydrogenase; TFA, trifluoroacetate; PC, paper chromatography; GLC, gas–liquid chromatography; MS, mass spectrometry; HPLC, high-pressure liquid chromatography; TLC, thin-layer chromatography; TMS, trimethylsilyl; RIA, radioimmunoassay; NMR, nuclear magnetic resonance.

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which are synthesized in the liver (6), may be modified by intestinal bacteria to form secondary bile acids, principally deoxycholic acid (DCA), lithocholic acid (LCA), and ursodeoxycholic acid (UDCA) (7–9). In health, only small quantities of bile acids are found in peripheral circulation and urine. However, in hepatobiliary and intestinal disease, disturbances of synthesis and clearance by the liver and absorption by the intestine will affect the level and the pattern of serum bile acids.

Therefore it is possible that bile acid analysis may be useful in the evaluation of disturbed liver or intestinal function and in the diagnosis of related diseases. At present the conclusions that can be drawn from some of the results obtained from studies carried out on such patients are equivocal (10–13). However it has been shown that serum bile acid levels are more sensitive indices of liver disease than conventional liver function tests (14–17) and that the primary bile acid ratio, CA/CDCA, may be diagnostically useful (18–22), or offer prognostic guidance (23, 24) in a number of liver diseases. Bile acid analysis may also prove useful in monitoring the efficacy of surgically created portosystemic shunts in cirrhotic patients (25), the diagnosis of congenital chloride diarrhea (26), cerebro-hepato-renal syndrome (27), and benign recurrent intrahepatic cholestasis (28), and as an aid to oral cholecystogram interpretation (29). During the last 10 years there has been a great increase in interest in bile acid analysis because of the development of a possible treatment for gallstones by the administration of CDCA and UDCA. Bile acid measurements have been useful in studying the efficacy of this technique (30–33). Furthermore it has been suggested that bile acids may be involved in the etiology of myotonic muscular dystrophy (34, 35). It has also been postulated that bile reflux into the stomach may promote gastritis and gastric cancer (36, 37) and that increased concentrations of fecal bile acids, particularly the secondary bile acids, may initiate colon carcinogenesis (38–40), although some reports have failed to substantiate this (41, 42). In a study using controls matched for age, sex, and gut transit time, colonic absorption of DCA and the proportion of secondary bile acids in duodenal bile were shown to be significantly higher in patients with adenomatous polyps than in the controls (43). Studies on the correlation of gallstones with several common human cancers have led to the suggestion that they may share common risk factors (44). Further research on the role of bile acids in the etiology of some cancers is indicated, particularly in the light of the report of increased levels of some bile acids in the serum of two breast cancer patients (45). However the evaluation of the use of bile acid analysis as a diagnostic tool is hampered by the lack of a simple, comprehensive assay.

This review is concerned primarily with analytical methods used for the measurement of the common bile acids in human biological fluids that may be of use in the diagnosis, treatment, and study of the etiology of disease. We do not discuss in detail the rigorous identification of bile acids and therefore neglect a number of relatively specialized but valuable techniques such as infra-red analysis and many aspects of nuclear magnetic resonance (NMR) and mass spectrometry (MS). A possible criticism of many analytical methods is that rigorous identification of the analyte has not always been carried out. However, mass spectrometry is increasingly being used both for identification and quantitation (12) and as a reference method against which other procedures can be evaluated (46). Other physicochemical techniques such as NMR, which are not at present sufficiently sensitive, may increasingly come into use in the future.

## EXTRACTION

The estimation of bile acids in biological fluids by gas–liquid chromatography (GLC), gas–liquid chromatography–mass spectrometry (GLC–MS), high-pressure (or high performance) liquid chromatography (HPLC), colorimetry, fluorimetry, or enzymatic means generally requires an initial isolation step. There are a number of methods available for the extraction of bile acids from biological samples. These include solvent extraction, binding to uncharged resins, extraction with solid ion exchangers, and 'Sep-pak' extraction. Several comprehensive reviews of extraction methods exist (47–49) including a review of methodology for bile acid determination in tissues (50). The method of extraction chosen will depend on the biological fluid being assayed since concentration, extent and type of conjugation, and degree of protein binding varies widely. Solid materials present particular problems because of the need to disrupt the matrix before extraction by homogenization, saponification, enzymatic digestion, or lyophilization.

### Solvent extraction

Bile acids, being steroids, are basically hydrophobic and hence capable of extraction from aqueous media by organic solvents. The ease of extraction of bile acids from biological samples is dependent upon four factors: the extent and type of conjugation, the number of hydroxyls, the state of ionization of the carboxyl group, and the degree of binding to proteins. An increase in the number of hydroxyls or the presence of sulfates, glucuronides, and to a lesser extent glycine- and taurine-conjugates, will greatly increase the relative polarity (water solubility) of the bile acid. The state of ionization primarily depends upon the $pK_a$ of the terminal group

of the side chain, which varies from around 5.8 for the carboxyl group in the free bile acids to 4.3 for the glycine-conjugates and 1.9 for the sulfonic acid group of the taurine-conjugates (1). Although it would be expected that a decreased pH would favor extraction of bile acids because of the suppression of ionization, in fact bile acid extraction is, in many instances, favored at alkaline pH, presumably because of the reduced protein binding that occurs.

The protonated glycine- and taurine-conjugated bile acids are sufficiently nonpolar to be extracted from an aqueous solution by n-butanol (47). Other factors such as binding to protein, inhibited above pH 11 (51), may necessitate more rigorous procedures including the use of alcoholic alkali (51–53), acidified ethanol (54), glacial acetic acid–toluene (55), methanol–acetone (56), and methanol–chloroform (57, 58). Soxhlet extraction or refluxing may be necessary with solid material (52, 58, 59). Using these procedures, quantitative recoveries of a comprehensive selection of added unlabeled bile acids have been demonstrated from serum or plasma (60), duodenal aspirates (61), bile (62, 63), rat feces containing cholestyramine (54), and rat liver homogenates (52). Quantitative extraction of various radiolabeled bile acids has been demonstrated from meconium (64), fasting gastric aspirates (37), serum (65), feces (66), and rat muscle and liver homogenates (67). However, using radiolabeled bile acids, extractions of taurocholic acid from tissue homogenates and taurocholic acid trisulfate from serum were unsatisfactory using alcoholic alkali (65, 67). Furthermore, alcoholic alkali saponification and acidic ethanol extraction may lead to deconjugation or solvolysis and hence to loss of information. Acidic ethanol and acetic acid–toluene will cause varying degrees of esterification of the carboxyl and acetate formation on the hydroxyls of the bile acids necessitating subsequent hydrolysis.

In feces, certain bile acids are firmly bound to bacteria (68) and it has been suggested (48) that the extraction of these acids cannot be evaluated by conventional recovery experiments but only by the use of feces from subjects fed labeled bile acids (58). The possibility has been raised that the degree of binding varies between species and it has been shown that it is more difficult to extract from human and rabbit feces (58, 69) than from rat feces (70). Recovery experiments on rat tissues, which have commonly been used to evaluate extraction techniques, may therefore not be applicable to human tissues. The recovery of bile acids from human tissues using solvent extraction has not to our knowledge been examined.

It should be remembered that although there is a wide selection of methods available for the extraction of bile acids, there is no general method that covers all eventualities and each analytical procedure must establish that the system used quantitatively extracts those bile acids of interest.

Hydrolysis of the bile acids is sometimes carried out prior to extraction, in which case subsequent extraction of the hydrolyzate mixture, after acidifying to pH 1, can be carried out with diethyl ether (71) or ethyl acetate (47). Quantitative recoveries of added unlabeled bile acids have been obtained using these procedures (47, 71, 72). Sulfated bile acids are not extracted, a factor that has been used for their differential analysis (72).

## Extraction with neutral resins

Many of the more recent methods for the analysis of bile acids have used the uncharged resins, Amberlite XAD-2 or XAD-7, for the initial isolation step (73, 74). Quantitative recoveries of radiolabeled bile acids from urine (12), plasma (10, 74), and liver tissue (75) have been achieved and both batch and column methods have been evaluated (74). Highest recoveries (85–90%) were obtained with the batch procedure using XAD-7 (74). Recoveries of some bile acids are pH-, flow-, and solvent-dependent (12) and low recoveries of some sulfated bile acid conjugates have been reported (65, 76, 77). Satisfactory elution of the most polar sulfates necessitates the use of a highly acidified ethanolic solution, a procedure that causes partial solvolysis of the bile acid sulfates. At high flow rates on XAD-2 columns, unconjugated nonpolar bile acids are not recovered quantitatively (12). Several investigators (74, 78) have found considerable batch variation in the binding capacity of these resins, necessitating careful evaluation of each batch before use. The extraction of bile acids from tissue using Amberlite XAD-2 rather than solvents has been recommended because it is quantitative and presents a relatively pure extract (50). However, the method used in this study did not permit a comprehensive analysis of the various conjugated forms of the bile acids and only the recovery of free CA was studied (50).

## Anion exchange extraction

Despite the fact that bile acids can be ionized, very few analytical procedures have used anion exchange resins for their extraction direct from biological samples. However quantitative extraction of added ^{14}C-labeled unconjugated bile acids from dilute serum using Amberlyst A26 (XN1006) has been reported (79) and utilized in the measurement of serum bile acids in pregnancy (80). In general, ion exchange chromatography has been used for the purification and separation of the bile acids following initial extraction with solvents and will be discussed in a later section.

### Sep-pak

It was suggested that recoveries of free and conjugated bile acids using Sep-pak $C_{18}$ cartridges are pH-dependent, since quantitative recoveries were obtained at pH 7 but not at lower pH (81). Quantitative recoveries of labeled TCA and CDCA have however been obtained at pH 4 from enzyme hydrolyzates and acetylation mixtures (82). Quantitative recovery at pH 4 of taurine-conjugated bile acids from various biological fluids using Sep-pak $C_{18}$ cartridges has also been demonstrated using various methods including recovery experiments using radiolabeled TCA (83). In this study flow rates of up to 20 ml/min were found to be effective and a single cartridge was found to be capable of concentrating up to 50 $\mu$g of TCA or taurolithocholate sulfate (83). Only small volumes (ca. 5 ml) of organic solvents are necessary for complete elution of all the common bile acids and their conjugates and sulfates from these cartridges (83, 84). They have been found to be particularly useful in sample preparation for isotope ratio determinations where XAD-2 and XAD-7 resins were unsatisfactory (84). In general, the use of Sep-pak obviates the need for lengthy column chromatography and offers a simple and rapid method of extraction.

## HYDROLYSIS OF BILE ACID ESTERS AND CONJUGATES

Bile acids may be conjugated with glycine or taurine as N-acyl conjugates on the C-24 carboxyl group and may form sulfuric esters or conjugates of glucuronic acid on any of the hydroxyls, although esterification is predominantly at the 3-OH position (85, 86).

The percentage of glycine- and taurine-conjugated bile acids in serum is not well documented but a recent study on two healthy persons found that 30% and 53% were unconjugated (10). This is a larger proportion than previously reported (60, 79, 87). A similar study in urine (five subjects) found only 3–10% of the total bile acids were unconjugated (12). All the sulfated bile acids were found to be conjugated with glycine or taurine (12). The percentage of unconjugated bile acids in bile is negligible (88).

The formation of sulfate esters was first demonstrated as a possible metabolic pathway for C-24 bile acids by Palmer and Bolt (89). Sulfate esters have been reported as constituting up to 81% of the bile salts present in the urine of healthy subjects and up to 100% in the urine of patients with hepatobiliary disease (76). In serum, although the percentage of sulfated bile acids is quite small in healthy subjects (2.7–13.3%, ref 76; 9%, ref. 77), this percentage may rise to 83% in he-

patobiliary disease (76). Levels of sulfated bile acids have been shown by some investigators to be very low in bile (76, 90) although Stiehl et al. using similar extraction techniques reported levels of 12% in patients with cirrhosis (91) and using solvent extraction reported levels of 10% in patients with intrahepatic cholestasis (92). Although it has been suggested that they may form a substantial proportion of the fecal bile acids (66), methodology used in this report has been questioned (93). Bile acid glucuronides have been shown to occur in the urine of healthy subjects (94) and of cholestatic patients (95).

Hydrolysis of the conjugates may be carried out directly on the biological fluid under examination prior to extraction (71, 96), although most procedures carry out hydrolysis after extraction. However hydrolysis of the bile acids without separation of the conjugate groups may obscure information about the state of conjugation or esterification of the bile acid in the original material. Comprehensive analytical systems should aim to analyze the bile acids in the state in which they naturally occur or appropriate fractionation should be undertaken prior to deconjugation. Different methods are required for the hydrolysis of the peptide bonds in the glycine- and taurine-conjugates than those used for hydrolysis of the ester sulfate and glucuronide bonds.

### Taurine- and glycine-conjugates

Alkaline hydrolysis is a vigorous procedure and some loss of bile acids occurs, although studies on pure bile acid mixtures have shown that these losses are mainly confined to the free and conjugated keto bile acids, which are only present in appreciable amounts in feces (97, 98). Although the losses of mono- and dihydroxy bile acids were shown to be small, losses of trihydroxy bile acids of up to 13% were reported and it has been suggested that losses may be larger when biological extracts rather than pure bile acids are subjected to these conditions (47). Comparative studies of recoveries of free bile acids from material augmented with labeled and unlabeled bile acid conjugates have shown that, in general, lower recoveries are obtained with alkaline hydrolysis in alcoholic solutions than with aqueous (98) or aqueous dioxane (58) media. The use of 20% KOH–ethylene glycol at reflux temperatures has also been recommended because quantitative recoveries were obtained in 15–20 min (55) rather than the several hours required when autoclaving with aqueous or aqueous alcoholic alkali was used (65, 97).

In an attempt to compensate for losses due to alkaline hydrolysis, internal standards such as 23-nor-deoxycholic acid (99) or deuterated CA or DCA (96, 100) have been used although differential losses probably occur. Using alkaline hydrolysis, some authors have

found that solvolysis of the sulfates and formation of bile acid artefacts mainly from sulfated bile acids can occur (65, 89).

Glycine- and taurine-conjugates may also be hydrolyzed quantitatively by the enzyme choloylglycine hydrolase (EC 3.5.1.24) prepared from *Clostridium welchii* (71, 72, 77), a technique pioneered by Nair and Garcia (101). Quantitative enzyme hydrolysis of the glycine-conjugates occurs five times faster than for the taurine-conjugates although, with the exception of certain tri-keto bile acid conjugates, rapid quantitative hydrolysis may be achieved where sufficient enzyme is used (97). No sulfatase activity or formation of degradation products is observed when sulfated bile acids are submitted to enzymatic hydrolysis, although hydrolysis times are prolonged (65, 72). Therefore enzymatic hydrolysis is recommended as the best method of deconjugation because it minimizes destruction and artefact formation.

### Sulfate esters and glucuronide conjugates

A number of methods have been used to hydrolyze sulfates of bile acids (12, 60, 77, 102, 103). Few recovery experiments have been carried out but quantitative solvolysis has been demonstrated by Cantafora et al. (77) and Parmentier and Eyssen (103).

Prolonged extraction with diethyl ether from acidified media hydrolyzes the C-3 sulfate group (104) but does not remove sulfate groups from C-7 or C-12 (65). The position of the sulfate moiety in monosulfated bile acids may be established by the oxidation and derivatization of the unsulfated hydroxyls with subsequent solvolysis and GLC–MS analysis (12).

Bile acid glucuronides, like other steroid glucuronides, may be hydrolyzed by β-glucuronidase from a number of sources, including the digestive juice of *Helix pomatia* and bovine liver, with subsequent determination by GLC (92, 95). The liver enzyme tends to be contaminated with traces of bile acids whereas the *Helix pomatia* enzyme, although uncontaminated, contains sulfatase (92). The sulfatase activity may be inhibited by including phosphate in the incubation buffer. Total bile acid glucuronides can be analyzed semi-quantitatively by GLC–MS after formation of methyl ester, trimethylsilyl ether (TMS) derivatives (94).

Transformation of the bile acid glucuronide into a formate ester by periodate oxidation, followed by oxidation with chromic acid and conversion of keto groups to O-methyl oximes, permits GLC–MS determination of the site of glucuronide conjugation (94).

## SEPARATION AND QUANTITATION

Following extraction and preliminary purification if required, bile acids need to be separated to permit their identification and quantitation. The choice of method of separation will depend on the concentration and the nature of the bile acids to be measured. Absorption chromatography (105), liquid chromatography (106), paper chromatography (PC) (48), and counter-current distribution (107) have all been used for bile acid separation although they have been largely superseded by thin-layer chromatography (TLC) (87), ion-exchange chromatography (12), GLC (12), and HPLC (108). Solvent systems suitable for the separation of conjugated and free bile acids by counter-current distribution have been described (107). Although counter-current distribution was not widely adopted, the solvent systems developed served as an important basis for many partition chromatographic procedures. The use of PC for the quantitative separation of free, conjugated, and derivatized bile acids has been extensively reviewed (47, 49).

Initially bile acids, like steroid hormone metabolites, were measured colorimetrically as a group (109, 110). These methods, reviewed by Bartos and Pesez (111), suffer from a lack of specificity although the sensitivity may be increased by the use of fluorimetric measurement (87). However, as new methods of analysis have developed, these early, simple, and nonspecific colorimetric and fluorimetric techniques for the determination of bile acids have been progressively superseded by direct estimation using GLC, HPLC, and radioimmunoassay (RIA) procedures, although enzymatic methods using fluorimetry are still in general use. **Table 1** summarizes the minimum detectable limits of the methods presently available for the measurement of bile acids.

### Column and thin-layer chromatography

Many of the early techniques for column chromatography, which have been extensively reviewed elsewhere (47–49), are now seldom used for the analysis of bile acids, so that only studies carried out over the last 10 years will be discussed in detail here. The most useful column materials at present are alumina or the neutral polymers, Amberlite XAD-2 and XAD-7, for purification, and the gels Sephadex LH20, diethylaminohydroxypropyl (DEAP) Sephadex LH20, and piperidinohydroxypropyl (PHP) Sephadex LH20 for separation of various bile acid groups.

Silica gel columns have been used for the separation of the bile acids into mono-, di-, and trihydroxy fractions (105), group separation of the glycine- and taurine-conjugates, and for the separation of bile acid methyl esters (132); quantitative recovery from augmented bile of the common di- and trihydroxylated glycine- and taurine-conjugated bile acids has also been demonstrated (133). However, reproducibility problems experienced with many chromatographic methods (47, 105) and the long chromatographic time required (105) have led to its

abandonment as a separation method. Alumina chromatography has been used for the separation of bile acid methyl (47) or ethyl esters (134) but proved less suitable for conjugated or free bile acids (134). Alumina has greater resolving power than silicic acid but has the disadvantage that destruction may occur during chromatography (48). Recently it has been used principally as a purification step prior to GLC (79, 135).

Separation of nonpolar and polar bile acids has been achieved with reverse-phase column chromatography (47, 49) on siliconized diatomaceous earth (Hyflosupercel) (106), polyethylene powder (Hostalene) (136), and methylated Sephadex (137). Straight phase partition chromatography has been used for the separation of free bile acids (138, 139) and for group separation of glycine- and taurine-conjugates (140). Partition chro-

TABLE 1.   Minimum limits of detection reported for various methods in the measurement of bile acids

| Method | Bile Acids Measured | Minimum Limits of Detection | Reference |
|---|---|---|---|
| 1. Densitometry | all | 0.5–0.1 nmol | 112 |
| 2. Colorimetry | free deoxycholic acid | 5 nmol | 56 |
| | glycine-conjugated bile acids | 12.5 nmol | 113 |
| 3. Fluorimetry | all | 1.2–3.1 nmol | 87 |
| 4. Enzymatic | | | |
| i) With spectrophotometric detection | 3-hydroxy bile acids | 10 nmol | 114 |
| ii) With fluorimetric detection | 3-hydroxy bile acids | 0.5 nmol | 115 |
| | 3-hydroxy bile acids | 0.1 nmol | 116 |
| | 3-hydroxy bile acids | 0.3 pmol | 117 |
| 5. Radioimmunoassay | conjugated cholic acid | 10 pmol | 118 |
| | conjugated cholic acid | 9.5 pmol | 119 |
| | conjugated cholic acid | 3.7 pmol | 120 |
| 6. High performance liquid chromatography | | | |
| i) Differential refractometer | conjugated bile acids | 3–4 nmol | 121 |
| | conjugated bile acids | 20 nmol | 88 |
| ii) Electrochemical detector | free bile acids | 1.25 nmol | 122 |
| iii) UV absorbance | free bile acids | 1 nmol | 81 |
| | phenacyl esters of free bile acids | 10–265 pmol | 123 |
| | p-bromophenacyl esters of free bile acids | 2 pmol | 124 |
| | conjugated bile acids | 0.1 nmol | 125 |
| | conjugated bile acids | 0.1–0.2 nmol | 121 |
| | pure conjugated bile acids | 20 pmol | 124 |
| | 3-sulfated free and conjugated bile acids | 10 pmol | 126 |
| iv) UV absorbance with ion pair reagents | free bile acids | 0.5 nmol | 127 |
| v) Fluorescence detector | free and glycine bile acid derivatives | 20–30 pmol | 128 |
| vi) Immobilized enzyme | free and conjugated bile acids | 20–30 pmol | 129 |
| vii) Immobilized enzyme and electrochemical detector | free and conjugated bile acids | 20 pmol | 45 |
| 7. Gas–liquid chromatography | | | |
| i) Packed column FID[a] | trifluoroacetates methyl esters | 0.2 nmol | 73 |
| ii) WCOT[b] column FID | DMES ether methyl esters | 1–20 pmol | 130 |
| iii) Electron capture | trifluoroacetates methyl esters | 0.2 pmol | 131 |
| iv) WCOT column mass fragmentography | DMES ether ethyl esters | 0.04–1 pmol | 75 |
| v) Packed column mass chromatography | TMS ether methyl esters | 10–50 pmol | 12 |

[a] FID, flame ionization detector.
[b] WCOT, wall-coated open tubular.

matography was an important early technique for the fractionation of bile acids but is tedious and time-consuming and has been replaced by simpler procedures.

Sulfated and nonsulfated bile acids can be separated into two fractions using Sephadex LH20, eluting the nonsulfated bile acids with chloroform–methanol and the more polar sulfates with methanol (76, 77). Using this system, Pageaux et al. (65) found that free bile acids were completely separated from bile acid sulfates but conjugated–nonsulfated and unconjugated–sulfated bile acids were not resolved. Lepage, Roy, and Weber (72) reported that nearly 100% of free sulfated LCA and more than 40% of glycolithocholic sulfate were eluted with the nonsulfated fraction. Problems occur with the use of Sephadex LH20 with crude extracts from urine because of sensitivity to overloading, memory effects, and the need to use the sodium salts of the bile acids (12). Lipidex 5000 (alkoxypropyl Sephadex LH20) has also been used for the separation of methyl esters of free bile acids (141) and bile acid glucuronides (94).

The many techniques of ion-exchange chromatography used for the purification and separation of bile acids have been discussed by Kuksis (142). Although quantitative recoveries, high capacity, and large separation factors have been reported (142), the methods have not been generally adopted, probably because of problems with strong adsorption to the resins so that large volumes of solvent were required for elution (48) and the need for the use of strongly acidic solutions for quantitative elution in many cases (142). Recently the lipophilic strong anion exchanger, DEAP Sephadex LH20, has been used in the analysis of bile acids in urine and plasma, separating the bile acids from the neutral lipids and allowing fractionation into unconjugated, glycine-conjugated, taurine-conjugated, monosulfated, and disulfated fractions (10–12); bile acid glucuronides eluted with the glycine- and taurine-conjugated fractions. Stepwise elution of the bile acid fractions was achieved by increasing the pH of the eluting buffer. Recoveries of added labeled bile acids were quantitative and overlap between the fractions was minimal (10, 12), although some loss of glycocholic acid (GCA) occurred with plasma extracts (10). With the conditions used, no separation of individual bile acids within each group was observed and further chromatography on Sephadex LH20 would be required for the fractionation of the various sulfated bile acids on the basis of glycine and taurine conjugation. DEAP Sephadex LH20 offers many advantages including high capacity, negligible adsorption effects, chemical inertness, and rapidity of separation was also claimed (12). Ion-exchange chromatography of the bile acids on PHP Sephadex LH20 has also been used to separate the free, glycine-conjugated, and taurine-conjugated fractions (62, 88). Both meth-

ods require prior removal of cations, which interfere with separation, by purification on a column of Amberlyst A-15. Elution of a comprehensive selection of labeled bile acids from Amberlyst A-15 has been shown to be quantitative (12). Desalting has also been achieved using Sep-pak $C_{18}$ cartridges (45) and XAD-2 (103).

TLC has largely replaced PC because of its greater simplicity and speed (47). TLC methods for bile acids have been reviewed elsewhere (47, 143). Layers of polyamide (144) and Anasil B (143) have been used for TLC of bile acids but most methods use silica gel G (56, 75, 87, 145). A number of TLC methods serve simply to separate the bile acids from the neutral lipids (87, 146, 147). In general, several solvent systems are required in order to separate all the bile acids (56, 87). Unconjugated bile acids may be separated in the free form (148, 149) or as the methyl (143) or methyl ester acetate derivatives (150). Conjugated bile acids may be separated into mono-, di-, and trihydroxy groups by a number of solvent systems (148, 151, 152). However, these methods do not separate the isomeric conjugated dihydroxy bile acids glycochenodeoxycholic acid (GCDC) and glycodeoxycholic acid (GDC) or the corresponding taurine-conjugates (TCDC and TDC). Goswami and Frey (145) described a solvent system run on silica gel 60 plates that separated these conjugated dihydroxy isomers along with other conjugated bile acids on the same plate. A similar separation has been obtained using $KC_{18}F$ reverse-phase TLC plates (153) and a recent report details the separation of the glycine- and taurine-conjugates of three dihydroxy bile acids: UDCA, CDCA, and DCA (154). Solvent systems for the separation of bile acid glucuronide methyl derivatives (155), bile acid sulfates (103, 156, 157), the $3\alpha$- and $3\beta$-cholenoic acid isomers (158), and the $5\alpha$-bile acids from their $5\beta$-analogues (143), have also been described. As a means of preliminary purification, TLC offers many advantages: the technique is inexpensive, rapid, and susceptible to batch analysis.

After TLC, bile acids may be detected by a number of destructive and nondestructive techniques. The reagent mixtures anisaldehyde–glacial acetic–sulfuric acid (159) or sulfuric acid–water (143) sprayed onto the chromatograms and heated will give (in visible and UV light) a variety of colors that may be useful for identification. A number of other reagents containing sulfuric acid may be used (160–162). Bile acids have been determined by direct densitometry after spraying and heating thin-layer chromatograms with a variety of reagents including concentrated sulfuric acid (163), 20% phosphomolybdic acid in ethanol (164), 10% sulfuric acid (153), and by spraying with anisaldehyde reagent described above (112). Fluorimetric determination has been used with direct scanning of TLC plates after

spraying and heating with phosphoric acid (165) and after treatment of the silica with concentrated sulfuric acid following removal from the plates (87).

Nondestructive techniques for the detection of bile acids include spraying with water (152), although sensitivity of this method is reported as being low (143), pilot chromatograms (12), exposure to iodine vapor (56, 87), and spraying with a dilute solution of iodine in acetone (166). The absorbent areas containing the bile acids may then be removed and extracted with various eluants (146, 151, 158, 166, 167). GLC (167), enzymatic techniques (151, 166), or colorimetry (56) may then be used for quantitation.

A novel procedure for the thin-layer chromatography of bile acids involves the use of chromarods, thin rods of quartz-like material with a boundary sintered layer of silicic acid as stationary phase. However, satisfactory separation of the dihydroxy bile acids, particularly the conjugates, was not achieved (168).

### Enzymatic estimation

$3\alpha$-Hydroxy (OH) bile acids (114), like $3\alpha$-OH neutral steroids (169), may be estimated by using NAD-linked $3\alpha$-hydroxysteroid dehydrogenase ($3\alpha$-HSD) (EC.1.1.1.50) from *Pseudomonas testosteroni*, with spectrophotometric determination of the NADH formed, but this method is not sufficiently sensitive to measure bile acids in normal sera. Sensitivity is increased 20-fold by the use of fluorimetric determination of NADH (115).

Crude preparations of the $3\alpha$-HSD enzyme may contain $3\beta$- and $17\beta$-hydroxysteroid dehydrogenase (166, 169), malate dehydrogenase (170), $12\alpha$-hydroxysteroid dehydrogenase ($12\alpha$-HSD) (171), and possibly $7\alpha$-hydroxysteroid dehydrogenase ($7\alpha$-HSD) (171) and alcohol dehydrogenase (172). As a possible consequence of contaminating enzymes, it has been reported that values found with crude enzyme preparations are usually higher than with purified enzyme, particularly with low bile acid concentrations, and that there are great variations in activity from batch to batch and type of crude enzyme (173). This has been disputed by Beher and Lin (174) who, using three crude extracts of *Pseudomonas testosteroni*, found no significant amount of $12\alpha$-HSD or $7\alpha$-HSD activity. Furthermore, this $3\alpha$-HSD assay is affected neither by the nature of the oxygen function at the 7 or 12 position nor by its orientation at the 7 position, thereby supporting the specificity of this preparation (175).

Possible recycling of NADH to $NAD^+$ may be prevented by the use of trapping agents such as hydrazine (56), although quantitative recoveries have been obtained in its absence (117, 176). The need for initial extraction of the bile acids from sera may be avoided by the inactivation of the serum enzymes by heating (116, 177). Solubility problems may be overcome by the use of a sufficient proportion of methanol in the incubation medium (178). Individual bile acids may be estimated using TLC (56, 166) or HPLC (179) prior to enzymatic determination, although until recently the common dihydroxy bile acids (DCA and CDCA) could not be separated. This problem was overcome by measuring the total dihydroxy bile acids enzymatically, while DCA conjugates were measured colorimetrically (56) or by using $3\alpha$-, $7\alpha$-, and $12\alpha$-HSDS with subsequent calculation of the CA:CDCA:DCA ratio by solving three simultaneous equations (151, 176).

3-Keto bile acids may also be determined using $3\alpha$-HSD (169). Using sodium borohydride, they are first reduced to the $3\alpha$-OH bile acids (97) or directly assayed by measuring the decrease in NADH spectrophotometrically (180). Interference from hydroxylated bile acids in the latter case is minimal because of the low affinity of the $3\alpha$-OH bile acid for the enzyme at the pH used (180).

In order to increase the sensitivity of the enzymatic method, a number of amplification systems have been used (116, 117, 177). These utilize the transfer of the hydrogen of the NADH produced in the reaction to acceptor compounds such as resazurin (116, 177) or dehydroepiandrosterone (117). In the latter method the $NAD^+$ is constantly recycled such that there is net stoichiometric production of the chromophore testosterone which may be determined spectrophotometrically. Using the enzyme diaphorase (EC. 1.6.4.3), resazurin is converted to resorfin which may be measured fluorimetrically at 585 nm, at which wavelength endogenous sample fluorescence is minimized (115).

None of the methods described will measure bile acids with sulfates or glucuronides at the 3 position. This may be important in hepatobiliary disease where up to 82% of the bile acids in serum has been found to be sulfated (76). Estimations by enzymatic means of total bile acids present in normal fasting serum are shown in **Table 2.**

### Radioimmunoassay

The first application of RIA to the measurement of serum bile acids was made by Simmonds et al. in 1973 (185). This assay, along with other early methods for the RIA of bile acids, used tritium-labeled bile acids as the radioligands (186–188), which in general require high concentrations of antisera. More recent methods employ $^{125}I$-labeled cholylglycylhistamine with which, probably because of greater affinity of antibody for ligand, antisera may be used at a greater dilution (119, 120, 189).

The bile acid–bovine serum albumin (BSA) immu-

TABLE 2. Bile acid values using enzymatic determination in normal fasting plasma

| Total Bile Acid | Number of Normal Subjects Studied | Reference |
|---|---|---|
| ng/ml | | |
| 0–3219 | 24 | 115 |
| 301–2077[a] | 20 | 117 |
| 589–3611 | 23 | 181 |
| 118–3650 | 34 | 182 |
| 510–2826 | 46 | 173 |
| 471–3454 | 14 | 183 |
| 353–2473 | 12 | 184 |
| 471–3415 | 22 | 112 |
| 1413–4945 | 13 | 116 |

[a] Range calculated using mean ± two standard deviations (2 SD).

nogen utilized in most of the methods may be prepared by the mixed anhydride (186) or the carbodiimide (185) techniques. In most instances, GCA–BSA complex has been used as the immunogen for the production of antibodies to CA conjugates (185, 186, 188, 189), although increased antibody titres have been obtained with CA–BSA (120, 190) and CA–thyroglobulin (119). This may indicate that the amide linkage between the carrier protein and the bile acid carboxyl group acts as an immunogenic determinant. The use of a spacer (hexanoic acid) between the CA hapten and the carrier protein produced an immunogen that was substantially less effective in producing antibodies to conjugated CA (120).

Considerable differences in the specificity of the antisera produced by these methods are indicated by their reported cross-reactivities (see **Table 3**). Specificity of antisera may vary between individual animals (120) or depend on the antiserum dilution (191). However, after establishing the necessary conditions under which each antiserum can be used specifically, RIA offers a simple

method for the measurement of individual bile acids. RIA kits for the determination of GCA and GCDC are available and have been critically evaluated (192). Specific antisera for GDC and sulfolithocholylglycine have also been prepared (118).

RIA has been used for the determination of primary bile acids in plasma (189) and urine (188). Although most methods employ initial extraction of the bile acids, some authors suggest this may be unnecessary (120). RIA is designed for the measurement of single bile acids, so for a comprehensive profile a number of different radioimmunoassays must be carried out. Alternatively, the bile acids must be separated and then assayed by an antiserum with a wide cross-reactivity. Because of these limits RIA can only be of use where the bile acid of interest has already been identified by a comprehensive assay. Normal fasting levels of glycine-conjugated bile acids in serum as measured by RIA are shown in **Table 4.**

### Fluoroimmunoassay

A fluoroimmunoassay for conjugated CDCA has been described (194). The method employs a GCDC–fluoresceinthiocarbamyl ethylenediamine conjugate as the label. The use of an antiserum coupled to magnetizable particles provides a simple method for the separation of the bound and free fractions.

### Double isotope derivative assay

A double isotope derivative assay of bile acids has been described using $^{14}$C-labeled bile acids added to serum with subsequent acetylation of both endogenous and labeled bile acids with [$^3$H]acetic anhydride. After fractionation of the labeled derivatives, the radioactivity ratio $^3$H/$^{14}$C measured was used to determine the amount of each bile acid in the serum sample (195).

TABLE 3. Percent cross-reactivities of published RIAs of cholyl conjugates

| Bile Acid | Reference Number | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 185 | 186 | 119[a] | 120 | 120[a] | 189[a] | 190 | 118 |
| Glycocholic | 100 | 100 | 75–112 | 100 | 100 | 33 | 100 | 100 |
| Taurocholic | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 18 |
| Cholic | 4 | 10 | 7.8–18.8 | 20 | 10 | 1.7 | 56 | 8 |
| Glycochenodeoxycholic | 10 | 10 | 0.5–1.4 | 5 | 10 | 26 | 13 | 4 |
| Taurochenodeoxycholic | 10 | 10 | 0.6–2.8 | 8 | 16 | 3.5 | | 0.2 |
| Chenodeoxycholic | 1 | 2.5 | | 0.5 | 0.2 | 1.3 | 5 | 4 |
| Glycodeoxycholic | 1 | 5 | 0.7–2.1 | 8 | 2 | 0.5 | 3 | 1 |
| Taurodeoxycholic | 1 | 5 | | 8 | 9 | | 3 | 0.1 |
| Deoxycholic | 0.2 | 2.5 | | 1 | 0.6 | 0.5 | 1 | 0.1 |
| Glycolithocholic | | 0 | 0.1–0.3 | 0.1 | 0.1 | | 1 | 3 |
| Taurolithocholic | | 0 | | | | | 1 | 0.1 |
| Lithocholic | | 0 | | 0.1 | 0.1 | 0.5 | 1 | 0.1 |
| Hyocholic | | | | 0.1 | 0.1 | | | |

[a] $^{125}$I-Labeled ligand.

TABLE 4. Values for glycine-conjugated bile acids using radioimmunoassay in normal fasting serum

| CA | CDCA | Number of Normal Subjects Studied | Reference |
|---|---|---|---|
| ng/ml | | | |
| 0–400[a] | | 25 | 193 |
| 163–776 | | | 119 |
| 82–450 | 157–785 | 10 | 188 |
| 102–158[a] | 65–121[a] | 25 | 118 |

[a] Normal range calculated using mean ± 2 SD.

Double isotope derivative assays such as these are usually tedious and cumbersome and are not recommended for routine clinical use.

## High-pressure liquid chromatography

The first report of the application of HPLC to the separation of bile acids appeared as recently as 1976 (196). Using Corasil II and a recycling step, it was possible to separate all the common conjugates. However, in the analysis of human bile, the method was found to be unspecific (196). Later methods, using a straight-phase Perisorb A with gradient elution (179) or a reverse-phase $\mu$Bondapak C18 column (197), also encountered difficulty in resolving CDCA and DCA conjugates. However, several methods using $\mu$Bondapak C18 columns (63, 121, 125, 198, 199) or a 'Fatty Acid Analysis' column (Waters Associates) (121, 200) have successfully separated the dihydroxy conjugates in a mixture of conjugated bile acids. The $5\alpha/5\beta$ epimers may be resolved in straight-phase systems such as Corasil II, on recycling in acetonitrile–acetic acid 40:1 (v/v) (121). An anion exchange packing TSK Gel 1 EX 540 DEAE has also been used in the group separation of glycine- and taurine-conjugates, thereby providing a rapid method for the measurement of the glycine/taurine ratio in bile (108).

One method of simplifying the analysis of the complex mixture of bile acids obtained from biological materials is preliminary fractionation into free bile acids, glycoconjugates, and tauroconjugates by TLC (201) or ion-exchange chromatography (88). Each group may then be resolved by HPLC.

Conjugated bile acids may be detected after HPLC by a number of methods including differential refractometry (1, 121) or UV spectrophotometry at 193–210 nm (121, 125, 202). UV detection is more sensitive than differential refractometry (see Table 1) (88, 121) but is still too insensitive for the measurement of bile acids in normal sera.

Free bile acids do not show a strong UV absorbance and therefore generally require the preparation of derivatives such as phenacyl esters (123), $p$-nitrophenyl

esters (197, 203), and $p$-chlorobenzoyl esters (203). These derivatives absorb at 254 nm and very low minimum detection limits have been reported (123, 203) (see Table 1); although, under the conditions described, the use of phenacyl ester derivatives does not permit the separation of LCA from other components of the biological sample (123). A fluorescence derivatizing reagent 4-bromoethyl-7-methoxy coumarin has also been used (128). Some methods describe derivatization and separation of all the carboxylated bile acids (free bile acids and glycine-conjugates) (124, 128, 197). The preparation of $p$-bromophenacyl derivatives for the separation and quantitation of the free acids and their glycine-conjugates has been described recently (124). High sensitivity is reported (see Table 1) but interference by excess derivatizing reagent with the peak of GCA–$p$-bromophenacyl ester does occur and further HPLC is required for separation of the GCA ester. Although sufficiently low limits of detection have been reported, none of the procedures using derivatization of the bile acids has been applied to biological fluids where low concentrations of bile acids are present, such as normal plasma and urine.

The free bile acids may also be analyzed by HPLC using ion pair reagents, such as Hyamine 1622, with UV monitoring of the ion pair at 254 nm (127), although the dihydroxy bile acids are not satisfactorily separated by this method. A recent report describes the HPLC separation of free bile acid standards with direct measurement by electrochemical detection (122). The limit of detection reported suggests the method is less sensitive than other methods described for free bile acids (see Table 1), although it may offer an alternative method for the detection of conjugated bile acids. Before the potential value of either of these techniques may be assessed, their application to the analysis of biological fluids is required.

Both free and conjugated bile acids may be quantitated by collection of HPLC eluent fractions, followed by enzymatic analysis (179, 204). However this method consumes considerable amounts of expensive enzyme, a problem which has been overcome by development of a post-column immobilized $3\alpha$-HSD enzyme in a continuous flow system using HPLC (129). The initial system described did not allow for the separation of GDC and TCDC; furthermore it has been suggested that the linear gradient elution in the HPLC system used may cause problems in the reproducibility and the reliability of the enzymatic reaction. Group separation of the bile acids (i.e., free, glycine- and taurine-conjugated fractions) on PHP Sephadex LH20 prior to HPLC has been recommended (205). The use of electrochemical detection of the reduced $NAD^+$ (45) rather than fluorimetric detection does not appear to enhance sensitivity al-

though specificity may be increased. None of the methods using immobilized enzyme detection measure bile acids where the 3-hydroxyl group is masked.

The resolution of 3-sulfates of the common free and conjugated bile acids by HPLC has been reported (126, 206) on a reverse-phase ODS SC-02 column with UV detection at 205 or 210 nm (see Table 1).

Although HPLC offers a highly selective means of separating bile acids, many of the HPLC detection methods described suffer from a variety of drawbacks, including interference from other constituents of the biological sample (123) or components of the derivatizing reagent (124), and have either not been applied to biological samples or only to samples such as bile or feces with high concentrations of bile acids. Post-column immobilized enzyme detection systems are sufficiently sensitive to permit the detection of bile acids in normal serum (207). However, the detection of bile acids following HPLC separation is an area of bile acid analysis that merits further investigation.

## Gas–liquid chromatography

The separation of bile acids by GLC is determined by the choice of derivative and stationary phase. In general, the bile acids are analyzed by GLC as derivatives of the free bile acids after deconjugation and solvolysis, although the glycine-conjugates have been run directly on a number of siloxane polymer phases as methylated acetate and TMS derivatives (208). As yet, taurine-conjugates have not been successfully run on GLC.

The carboxyl group of the bile acid molecule may be methylated although other alkyl derivatives have been made. Methods of esterification have been discussed elsewhere (209). Although many workers use diazomethane reagent for methylation of the carboxyl group (210), this can lead to byproducts (211) and HCl–2,2'-dimethoxypropane–methanol (212) and acetyl chloride–methanol (209) are probably more suitable. Other alkyl derivatives may be made by substituting the appropriate alcohol in the latter two reagents (209). Methylation of the carboxyl and all available hydroxyl groups, also called permethylation, may be achieved using the Hakomori reagent (213, 214).

The hydroxyl groups on the steroid nucleus may be derivatized as formates (94), acetates (62, 99), trifluoroacetates (TFA) (146), and as silyl derivatives. The methods for the partial and full derivatization of the hydroxyls as TMS ethers have been reviewed (215) and the rate of TMS ether formation for the different hydroxyl groups in the bile acids has also been studied (216). In the partial TMS derivatives, the 6α-, 7α-, and 12α-OH groups are not esterified and hence the partial derivatives of the bile acids containing these groups will be more polar (215). Although TMS and TFA deriv-

atives are more volatile than acetates and more commonly used in bile acid analysis, there are reports that these derivatives may be unstable (12, 48) and TFA derivatives in particular are subject to thermal decomposition (48). Using electron capture detection, TFA derivatives of bile acids have been analyzed in micro quantities of serum with a minimum detection limit of 0.2 pmol (131). However, as with other methods using electron capture detection, the linear range of quantitation is narrow. Dimethylsilyl, dimethylpropylsilyl, and dimethylisopropylsilyl ethers of bile acids have also been prepared (130) as have chloromethyldimethylsilyl ethers of some methylcholanates (217). Retention times of these derivatives were increased (in comparison with the corresponding TMS ethers) but the separation of the individual bile acids on an SE 30 capillary column as dimethylsilyl derivatives was greatly improved (130). Unfortunately the appropriate derivatizing reagents are not commercially available and have to be synthesized.

One-step derivatization and deconjugation has been reported with formation of heptafluorobutyrates (HFB) on the carboxyl and hydroxyl groups (218). However, thermal decomposition of the HFB derivatives may occur and bile acids containing ketones gave two peaks (219), possibly due to formation of enol-HFB derivatives.

A number of internal standards have been used including 5α-cholestane (215), 5β-cholanic acid (60), stigmasterol (63), hyodeoxycholic acid (220), 7-ketolithocholic acid (221), 7-ketodeoxycholic acid (135), ursodeoxycholic acid (64), and 23-nor-deoxycholic acid (99). Quantities of bile acids in biological samples are determined by comparison of peak areas with those of known amounts of reference compounds in a range where a linear detector response is obtained. Detector response has been shown to be linear for acetate and TMS derivatives over the range 0.1–7 μg (222), for heptafluorobutyrates over the range 0.1–10 μg (218), and for trifluoroacetates over the range 50–500 ng (221).

Prior to GLC analysis, the keto groups at C-3 and C-6 on the steroid nucleus may be derivatized as O-methyloximes (94) or at C-3 as dimethyl hydrazones (223). Similarly, hydroxyl groups may be oxidized in chromate–sulfuric acid (223) and keto groups may be reduced with sodium borohydride (224), although rates of reduction differ with the position of the keto group.

The derivatized bile acids may be analyzed on packed columns with a variety of polar and nonpolar stationary phases. These have been reviewed previously (47, 152, 225). Recently the use of PPE 20 (222), AN600 (62, 74), and a nematic liquid crystal phase (226) have been described. In many cases, in order to analyze the highly complex mixtures of bile acids in biological samples, it may be necessary to use two types of derivatives (90,

220) or two types of columns (12, 227). In particular the combined use of HiEff 8BP and SE 30 has been shown to be useful in the analysis of bile acid methyl ester TMS derivatives (10, 12).

Greater sensitivity and improved resolution of the individual bile acids may be achieved with the use of wall-coated open tubular (WCOT) capillary columns (130, 135, 220).

## Mass spectrometry

Sensitivity and specificity may also be improved by the combination of gas–liquid chromatography and mass spectrometry (GLC–MS). The electron impact (EI) mass spectra of bile acids have been well documented (211, 228, 229), with recent reports of the mass spectra of conjugated bile acids (212), bile acid methyl ester acetates (230, 231), cholic acid nitrate esters (232), per-methylated derivatives (213), derivatized bile acid glucuronides (94), dimethylsilyl ether bile acid methyl esters (130), and trifluoroacetyl-hexafluoroisopropyl derivatives (219).

Chemical ionization (CI) mass spectrometry, which provides a much simpler fragmentation pattern with only two or three ions predominating, has been used less commonly. However spectral characteristics of bile acid methyl esters using methane gas (233) and bile acid methyl ester acetates using isobutane (231) or ammonia (234) gas have been documented. In general, the use of ammonia gas seems to confer many advantages (84, 234).

Both methods have been used in the identification of unusual bile acids in biological materials (62, 235–237). The use of a multiple ion detector (MID) unit for specific ion monitoring (SIM), also called mass frag-mentography, provides a highly specific and sensitive method for the estimation of bile acids in serum (96, 100) and human liver tissue (75). Deuterium-labeled analogues of the free bile acids, when used as internal standards, offer a means of compensating for losses of the free bile acids in the deconjugation and derivatization procedures (75, 96, 100, 211) and also, if added in excess, act as carriers greatly diminishing losses brought about by adsorption and degradation on the GLC column (75). At present stable isotope analogues of the various bile acid conjugates have not been used in this way, so that procedural losses prior to deconjugation are not covered. This is one example of the use of the technique of stable isotope dilution. In general the stable isotopes used in these studies are $^{13}C$ and $^{2}H$. Stable isotope-labeled bile acids also find particular application in in vivo metabolic studies on very young children (238) and in pregnant women (239) where the use of radioactive bile acids is not permitted. Furthermore, it has been suggested that their use in studies on bile acid kinetics may permit direct measurement in periph-eral plasma where low bile acid concentrations would necessitate the administration of unacceptably large quantities of radioactivity (84), although very few such studies have been carried out (84, 240).

The in vivo use of deuterium-labeled bile acids is complicated by primary and secondary isotope effects. The large difference between the mass of deuterium compared to that of protium changes the bond stability and may effect rates of cleavage not only of the deuterium bond but also of other bonds in the molecule. These effects also occur with $^{13}C$-labeled bile acids but are negligible when compared with the larger effects seen with deuterium (241). However it is difficult to synthesize $^{13}C$-labeled bile acids, particularly if multipoint labeling is required. Furthermore, the natural abundance of $^{13}C$ in C-24 bile acids means that in vivo kinetic studies using mono-substituted $^{13}C$ analogues are complicated by the significant contribution to the $^{13}C$ from endogenous material, necessitating the use of sensitive assays to distinguish changes in $^{13}C$-labeled bile acids. It is possible to measure the $^{13}C/^{12}C$ ratio in bile acids isolated from 5 ml of serum using GLC–MS in the chemical ionization mode and it has been suggested that sample volume requirement could be reduced by replacing conventional packed columns with capillary columns (84). When stable isotope-labeled bile acids are used as internal standards in mass fragmentographic assays, isotope effects are irrelevant but, since natural isotope abundance problems still remain, deuterated standards are preferred.

A comprehensive analysis, which may be useful for the complex bile acid mixtures found in urine and feces, has been achieved by the combination of mass spectrometry with a computer (mass chromatography) (10, 11, 12, 94, 211, 242). In this method external standards are used, with peak areas in reconstructed specific fragment ion current (FIC) chromatograms converted to total ion current (TIC) equivalent and compared with the peak area given by the known amount of external standard. This is less sensitive than specific ion monitoring with a reported minimum detection limit of 0.01–0.05 nmol (see Table 1) (12).

Most reference spectra are obtained and reported at 70 eV (213, 228) since, at this level, perturbations in electron energy have negligible effect on ion production. With electron ionization mass fragmentography and mass chromatography, however, most workers have used an ionization energy of 20–30 eV (12, 62, 75, 100). Low ionization energies decrease the ionization of the carrier gas, helium (228), and maximize the relative abundance of the diagnostically important ions (12). Miyazaki et al. (211) have reported that, at 20 eV, simultaneous ionization of cholesterol TMS ether resulted in erroneous quantitation of CDCA and suggested that ionization energies of greater than 50 eV

TABLE 5. Bile acid values[a] in normal subjects using gas–liquid chromatography

| Biological Fluid | Number of Normal Subjects | Column | Detector | Total Bile Acids | CA | CDCA | DCA | LCA | Reference | Solvolysis |
|---|---|---|---|---|---|---|---|---|---|---|
| Serum | 24 | packed | MS | 1117 ± 114 | 184 ± 24 | 526 ± 62 | 407 ± 44 | | 100 | No |
| (fasting, ng/ml) | 10 | packed | MS | 1366 ± 385 | 200 ± 65 | 608 ± 126 | 565 ± 224 | | 96 | No |
| | 10 | packed | FID | 431–3454 | 123–2940 | 118–1533 | 118–1769 | 0–678 | 60 | Yes |
| | 13 | packed | FID | 2041 ± 510 | | | | | 243 | Yes |
| | 15 | capillary[b] | FID | 840 ± 82 | 237 ± 53 | 385 ± 67 | 224 ± 35 | 0 | 135 | No |
| | 8 | packed | FID | 687 ± 322 | 72 ± 76 | 389 ± 329 | 234 ± 151 | 0 | 76 | Yes |
| | 3 | packed | FID | 1160–1720 | 310–430 | 600–1030 | 250–720 | | 99 | No |
| | 12 | packed | FID | 377–1366 | 139–409 | 184–775 | 0–353 | 0–75 | 221 | No |
| | 18 | packed | FID | 290–2260 | 20–650 | 50–1300 | 60–450 | | 79 | No |
| | 15 | packed | FID | <2473 | 204–735 | 275–1374 | | | 244 | Yes |
| | 30 | packed | FID | 300–2500 | $t^c$–1000 | $t^c$–1000 | $t^c$–1000 | $t^c$ | 245 | No |
| Urine | 3 | packed | FID | 180–380 | 0–28.6 | 19.6–141.3 | 161–310 | | 76 | Yes |
| (μg/24 hr) | 6 | packed | MS | 2512–4310 | 49–298 | 71–538 | 232–710 | 395–667 | 12 | Yes |
| | 3 | packed | MS | 2759–17788 | | | | | 94 | |
| Whole blood | 3 | packed | FID | 1530–2260 | 500–810 | 510–810 | 240–540 | | 99 | No |
| (ng/ml) | | | | | | | | | | |
| Bile | 17 | packed | FID | 16.0 ± 2.3 | 11.58 ± 3.88 | 12.52 ± 3.52 | 2.59 ± 0.93 | 0.56 | 243 | Yes |
| (mg/ml) | 17 | packed | FID | 27.74 ± 6.84 | 8.41 | 6.34 | 5.06 | | 245 | No |
| | 1 | packed | FID | 19.48 | 15.28 | 12.6 | 12.29 | | 76 | Yes |
| | 1 | capillary[b] | FID | 39.56 | | | | | 63 | Yes |
| Feces | 24 | packed | FID | 8.9 ± 1.3 | 1.1 ± 0.5 | 0.6 ± 0.8 | 3.8 ± 0.8 | 3.5 ± 0.5 | 41 | No |
| (dried stool, mg/g) | | | | | | | | | | |

[a] Values presented as mean ± SD; otherwise as normal range.
[b] Capillary wall-coated open tubular column.
[c] Trace detected.

(above which the phenomenon did not occur) should be used. However a later study did not reveal any interference between the two compounds (12). Total ion current peak area response (computer reconstruction) has been shown to be linear over the range 0–1 nmol methyl cholate TMS ether at 22.5 eV and to decrease with increasing energy of the bombarding electrons up to 70 eV, resulting in decreased sensitivity (12).

The levels of total bile acids and the levels of the individual common bile acids measured in various biological fluids using gas–liquid chromatography are shown in **Table 5.**

## Nuclear magnetic resonance

High resolution NMR has been used for the identification of the common bile acids (246, 247). The $^{13}$C resonances of the common bile acids (CA, DCA, CDCA, LCA) (248) and the proton resonances of CA (249) have been recorded and assigned. NMR has also been employed for the elucidation of the structure of unique bile acids (236, 250, 251), differentiation of stereoisomers (252), and the C-5 epimers (253), and the characterization of permethylated bile acids (213). It has also proved useful in the study of the formation and structure of simple and mixed micelles (246, 254).

The development of high field strength superconducting magnets and improvements in spectrometer design, together with computerized Fourier transform techniques, have dramatically improved sensitivity over the last 20 years (255), such that quantitative analysis of bile acids using NMR becomes a possibility in the future. Proton NMR using 60 mg of individual bile acids has been used for the quantitative analysis of CDCA and UDCA for the purpose of purity determination (256). Fifty-microgram quantities (approx 1 mM) of some pure standard bile acids in organic solvents have been analyzed with a 100 MHz NMR spectrometer and although it has been suggested that as little as 2.5 $\mu$g could be detected, this has not as yet been demonstrated (247). Solutions of CA (4 mM) in aqueous media have also been studied with a 400 MHz NMR spectrometer but only for the purpose of individual proton assignments (249). Both methods required long scanning periods (247, 249). At present, NMR is too insensitive for the quantitative measurement of bile acids in biological fluids but is an invaluable method for identifying bile acids and for use in the investigation of the structure of bile salt micelles.

## CONCLUSION

A resurgence of interest in bile acids over the last 10 years as a result of their use in the treatment of gall-

stones and the implication of their involvement in the etiology of a number of diseases has led to some improvements in the analytical techniques available. In particular, there has been the widespread adoption of solid extraction procedures and enzymatic hydrolysis of conjugated bile acids and progress toward more comprehensive assays which include the determination of glucuronide conjugates, sulfate esters, and of minor constituent bile acids in biological fluids. Furthermore, the specificity and sensitivity of enzymatic measurements of total bile acid concentrations have been improved and sensitive RIA techniques for the measurement of specific plasma bile acids have been introduced, although both these techniques have only a limited application. The use of HPLC, although offering an excellent method of separation, is hampered by detector systems that have not yet been fully evaluated for use when the bile acids are present in low amounts. At present the most powerful tools available for the specific and comprehensive analysis of biological fluids are GLC–MS techniques, particularly mass chromatography, but these techniques are time-consuming, expensive, and unsuitable for routine clinical use.

Despite much effort by investigators in the bile acid field, many methodological problems remain. Simple comprehensive techniques are needed before the role of bile acids in health and disease can be assessed confidently and the value of bile acid measurements in biological fluids as a routine diagnostic and research tool can be determined.█

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