Bile acid analysis in biological fluids: a novel approach

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Abstract In contrast to current methods of bile acid analysis that require the separation of bile acids into different groups prior to their analysis, the HPLC method using a reverse phase column and gradient elution that we developed permits the separation and detection of nonconjugated, glycine-conjugated, and esterified bile acids as their fluorescent dimethoxycoumarin esters. The mild conditions for ester formation make possible the identification of allylic bile acids characteristic of metabolic errors in bile acid synthesis. Quantification is obtained using $7\alpha,12\alpha$-dihydroxy-5β-cholanoic acid as an internal standard. In addition to identification based on retention time, peak-shift strategy is used by treatment of aliquots with cholyglycine hydrolase and/or solvolysis. Loss of the parent peak and appearance of the derivative provide further assurance of the identity of each bile acid in biologic fluids that contain other organic acids.—**Budai, K., and N. B. Javitt.** Bile acid analysis in biological fluids: a novel approach, *J. Lipid Res.* 1997. **38:** 1906–1912.

Supplementary key words HPLC • 4-bromomethyl-5-6-dimethoxy-coumarin • fluorescence detection • cholylglycine hydrolase • solvolysis • reverse-phase column • allylic bile acids • metabolic errors

A variety of methods exist for the analysis of bile acids both by gas-liquid chromatography (GLC) and by high-performance liquid chromatography (HPLC). Many of these methods are designed for the analysis of gallbladder bile, hepatic bile, or duodenal aspirates after gallbladder contraction, i.e., fluids in which the concentration of bile acids is much greater than that of other organic acids. Therefore, the preparation of an appropriate carboxylic acid ester derivative and the finding of a characteristic retention time by GLC or HPLC are usually sufficient for identification. Use of a mass selective detector provides significantly greater assurance with regard to the identity of the chromatographic peak but is often not available.

Analysis of biologic fluids for bile acids in which they represent a relatively small fraction of the total organic acids that are present is more difficult and the problems that are encountered have not been fully addressed. Two of the major problems are to identify individual bile acids based on retention time when mass spectrometry is not available and to identify labile compounds that decompose during derivative preparation.

Although HPLC has the potential for directly addressing both these problems (1–4), most of the published methods continue to utilize preparative techniques that were introduced for the analysis of bile acid by GLC (5). Thus, extensive multiple column chromatographic separations are done to group the bile acids into conjugated, nonconjugated, and esterified groups prior to analysis. Often, after group separation, solvolysis and hydrolysis are done prior to analysis, with loss of allylic bile acids. Thus these procedures, which have been useful for the characterization of bile acids in urine and other biologic fluids, are not generally applicable in most laboratories and have discouraged routine analysis of bile acids in the differential diagnosis of cholestatic syndromes.

We have taken a novel approach utilizing methods that were already established for organic acid and bile acids analysis. Using gradient elution HPLC and reverse phase columns we have developed a solvent system that separates glycine-conjugated, nonconjugated, and esterified bile acids as fluorescent esters in a single analysis. After the detection of conjugated and/or esterified bile acids, peak-shift analysis can be applied to appropriately treated aliquots. Loss of the retention time for the parent compound and the appearance of the ex-

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pected derivative provide a powerful analytical tool for specific identification.

MATERIALS AND METHODS

Chemicals

Bile acids, including the internal standard 7α,12α-dihydroxy-5β-cholanic acid, were purchased either from Calbiochem (La Jolla, CA) or Sigma Chemical Co. (St. Louis, MO). The allylic bile acid 3α,7α-dihydroxy-5-cholenoic acid was prepared as described previously (6) and was treated with cholesterol oxidase to yield 7α-hydroxy-3-oxo-chol-4-enoic acid (7). The C_{27} bile acid 3β-hydroxy-cholest-5-enoic acid was prepared from 27-hydroxycholesterol by Jones oxidation of the 27-hydroxy-cholesterol-3-acetate. Authentic tetrahydroxylated bile acids were a gift from Dr. Mizhuo Une at Hiroshima University, Japan and bile acid glucuronides were a gift from Dr. Jan Pyrek. Dicyclohexano-18-crown-6 (DCC), 4-bromomethyl-6,7-dimethoxycoumarin (BMC), cholylglycine hydrolase, and creatinine kit #555-A were also purchased from Sigma Chemical Co. Solvents for HPLC and anhydrous solvents (less than 0.2% H_2O) for esterification were obtained from Aldrich Chemical Co. (St. Louis, MO). Bile acid standards (0.1 mg/ml) were prepared using 90% methanol. DCC (1.5 mg/ml) and BMC (3 mg/ml) were dissolved in anhydrous acetonitrile. These reagents, when kept at room temperature and shielded from light, were stable for at least 1 month.

Equipment

Gradient HPLC was done using dual Shimadzu pumps Model # LC-6A and a systems controller Model # SCL6B with a C_{18} reverse phase ODS-AM column 3 × 100 mm purchased from YMC Inc. (Wilmington, NC). A Chrompack, Inc. (Raritan NJ) fluorescence detector Cat# 505154 was used for detection of the fluorescent ester derivatives. Thin-layer chromatography was done using silica gel G plates (cat# 01511) containing binder purchased from Analtech (Newark, DE).

Sample preparation and derivatization

To a volume of urine containing 0.1 mg of creatinine, usually between 0.5 and 1.5 ml, 500 ng of 7α,12α-dihydroxy-5β-cholanoic acid (Calbiochem) was added and the pH was adjusted to 5.6 with 0.1 M phosphate buffer.

For the analysis of glycine-conjugated and/or non-conjugated bile acids and their esters, the sample was passed directly through a 500-mg bed of octadecylsilane (C-18) bonded silica (Sep-Pak, Waters). Standard techniques were used to prepare the cartridge and to adsorb and elute the bile acids (8).

For total bile acid analysis, the sample was incubated with 10 units of cholylglycine hydrolase (Sigma Chemical Co., St. Louis, MO) and the mixture was allowed to stand for 2 h at room temperature. The sample was then adjusted to pH 5.6 and was passed through the octadecylsilane cartridge. To the methanol eluate containing the bile acids, 0.1 ml of 1.8 mM methanolic KOH was added and the sample was taken to dryness.

To the dried sample, 50 μl of acetonitrile was added, followed by 20 μl of DCCE (1.5 mg/ml acetonitrile) and 20 μl of BMC (3.0 mg/ml). The tubes were sealed with parafilm and placed in a heating block at 60°C for 30 min.

After removal of the parafilm, a 50-μl aliquot was plated onto a silica gel G plate and was developed in a tank containing chloroform for a distance of 20 cm.

After drying, the plate was visualized under long-wave UV light (365 nm) and the fluorescent zone extending from the origin to approximately 2 cm up the plate was scraped with a razor blade onto weighing paper and then transferred to a 6 × 50 mm glass tube. Acetonitrile (200 μl) was added to the tube. After the tube was vortexed and the silica gel was allowed to settle, an 8-μl aliquot equivalent to approximately 5.0 ng of internal standard was injected onto the HPLC column.

For solvolysis (9), a 50-μl aliquot was taken to dryness, 100 μl of freshly prepared solvolysis mixture (dimethoxy-propane: ethyl acetate–methanol–10 N HCl 5:4:4:0.1 was added, and the tube was kept at room temperature for 2 h. The reaction mixture was neutralized with 1 drop of ammonium hydroxide (1 M) and then taken to dryness. It was redissolved in 50 μl of acetonitrile and 2-μl aliquot was injected.

HPLC analysis

Each analysis takes 55 min. During this time the pump pressure falls gradually because of the progressive increase in the proportion of acetonitrile and methanol. After completion of the program, approximately 10 min is required to re-equilibrate the column, which is recognized by return of the pressure to the initial value.

Solvent A is a mixture of water–acetonitrile–methanol 3:2:1. Solvent B is a mixture of acetonitrile–methanol 2:1. One ml of 7.6 M ammonium acetate is added to each 500 ml of solvents A and B, respectively. The flow rate is constant at 0.6 ml/min. The gradient elution program begins with 100% solvent A. The propor-

TABLE 1. HPLC analysis of bile acids by reverse phase column chromatography as the 6,7-dimethoxycoumarin esters

		Minutes
Tetrahydroxy bile acids[a]		
1	1β,3α,7α,12α-TetraOH-5β-cholanoic	14.8
2	3α,6β,7α,12α-TetraOH-5β-cholanoic	15.3
Glycine conjugates		
3	Glycocholic acid	16.7
4	Glycochenodeoxycholic acid	27.8
8	Glycodeoxycholic acid	29.8
14	Glycolithocholic acid	36.2
Bile acid ester sulfates		
3A	3β,7α-Dihydroxy-chol-5-enoic a-3-sulfate[b]	23.7
5	Chenodeoxycholic acid-3-sulfate	28.2
6	Chenodeoxycholic acid-7-sulfate	28.7
9	3β-Hydroxy-5-cholenoic acid sulfate	31.5
13	Lithocholic acid sulfate	34.6
Nonconjugated bile acids		
7	Ursodeoxycholic acid	29.1
10	Cholic acid	32.5
11	3β,7α-Dihydroxy-chol-5-enoic acid[b]	33.3
12	7α-Hydroxy-3-oxo-chol-4-enoic acid[c]	34.1
16	Chenodeoxycholic acid	39.3
17	Deoxycholic acid	39.7
18	7α,12α-Dihydroxy-5β-cholanoic a. (internal std)	41.9
19	3β-Hydroxy-5-cholenoic acid	42.4
20	Lithocholic acid	45.9
21	3β-Hydroxy-5-cholestenoic acid	47.6
Bile acid ether glucuronides		
15	Lithocholic acid	37.6

[a]Normal constituents of premature and neonatal urine.

[b]Major component of urine in 3β-hydroxy-C_{27}-steroid dehydrogenase/isomerase deficiency.

[c]Major component of urine in Δ^4-3-oxosteroid 5β-reductase deficiency.

tion of solvent B is increased from 0 to 95% over a 45-min period and is then kept constant for an additional 10 min prior to recycling to initial conditions.

The detector is set at an excitation wavelength of 340 nm and an emission wave length of 430 nm.

RESULTS

Table 1 indicates the retention times of all the bile acids that we have analyzed thus far. Although each of the standards can be resolved using pure mixtures, co-elution sometimes occurs either because the column may age and lose resolving power or because unknown constituents in biological samples may have slight effects on the retention time of some components. Each bile acid has been assigned a number according to its order of elution; these are used for reference in the figures.

Figure 1 is the HPLC tracing of a mixture of the glycine conjugates of cholic, chenodeoxycholic, deoxycholic, and lithocholic acids, the nonconjugated cholic,
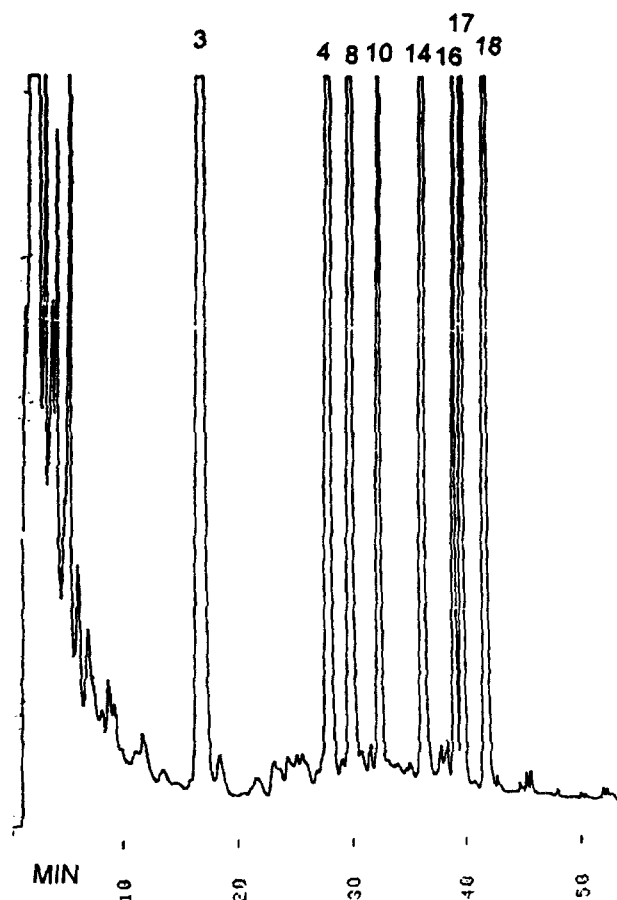

Fig. 1. HPLC analysis of 5,6-dimethoxy esters of glycine-conjugated and nonconjugated bile acids. Using a reverse phase column and gradient elution, the internal standard, 7α,12α-dihydroxy bile acid (#18), has a longer retention time than other nonconjugated and glycine conjugated bile acids. (See Table 1 for identity of other peaks.)

chenodeoxycholic, and deoxycholic acids, and the internal standard, all detected by fluorescence of their 6,7-dimethoxycoumarin esters. Because of the reverse-phase packing, the elution pattern is in the order of decreasing polarity. The internal standard, 7α,12α-5β-cholanoic acid (#18), is apparently less polar than other dihydroxy bile acids.

Figures 2, 3, and 4 represent the same standard mixture of taurocholic and glycodeoxycholic acids and the carboxylic acid-3 sulfates of chenodeoxycholate, 3α-hydroxy-5-cholenoate, and lithocholate, which were added to an aliquot of urine and then adsorbed onto and eluted from a Sep-Pak column.

In **Fig. 2** derivatization was done on an aliquot of the Sep-Pak eluate prior to hydrolysis with cholyglycine hydrolase. Although taurocholic acid was present, only glycodeoxycholic acid and the 3-ester sulfates were detected, together with the internal standard. In this anal-
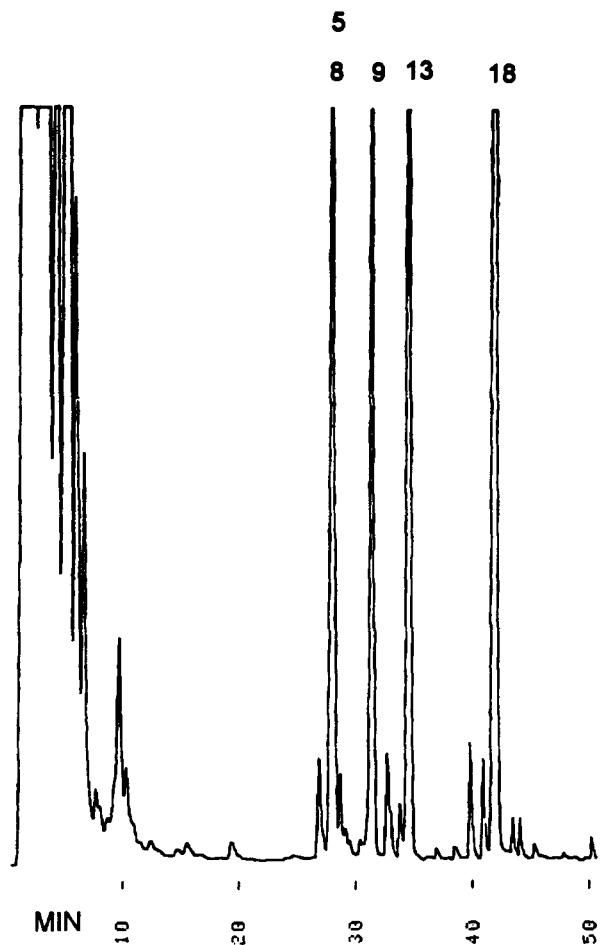
Fig. 2. HPLC analysis of derivatized eluate from Sep-Pak cartridge prior to enzyme hydrolysis and solvolysis. Although taurocholic acid was present in the eluate, none was detected. In this analysis chenodeoxy-3-sulfate (#5) and glycodeoxycholic acid (#8) co-eluted. However, as shown in Figs. 3 and 4 their presence could be established. See Table 1 for identity of other bile acids.

Fig. 3. HPLC analysis of the same bile acid mixture (Fig. 2) after incubation with cholylglycine hydrolase. Comparison with Figs. 1 and 2 indicates the appearance of peaks corresponding to cholic acid (#10) and deoxycholic acid (#17). A peak corresponding to chenodeoxycholic acid-3-sulfate (#5) is unchanged. See Table 1 for the identity of other bile acids.

ysis, chenodeoxycholic acid-3-sulfate was not resolved from glycodeoxycholic acid.

After enzyme hydrolysis (**Fig. 3**), both deoxycholic acid (#17) and cholic acid (#10) were detected together with the ester sulfates of chenodeoxycholic acid (#5), 3β-hydroxy-5-cholenoic acid (#9), and lithocholic acid (#13).

A 50-μl aliquot taken from the enzyme-hydrolyzed sample was solvolyzed for 2 h. HPLC analysis (**Fig. 4**), indicated that the peaks corresponding to the ester sulfates are no longer detectable and new peaks representing chenodeoxycholic acid (#16), 3β-hydroxy-5-cholenoic acid (#19), and lithocholic acid (#20) were detected. Although complete recovery of these bile acids was obtained after solvolysis, when the method was applied to the 3-sulfate of 3β,7α-dihydroxy-chol-5-enoic acid,
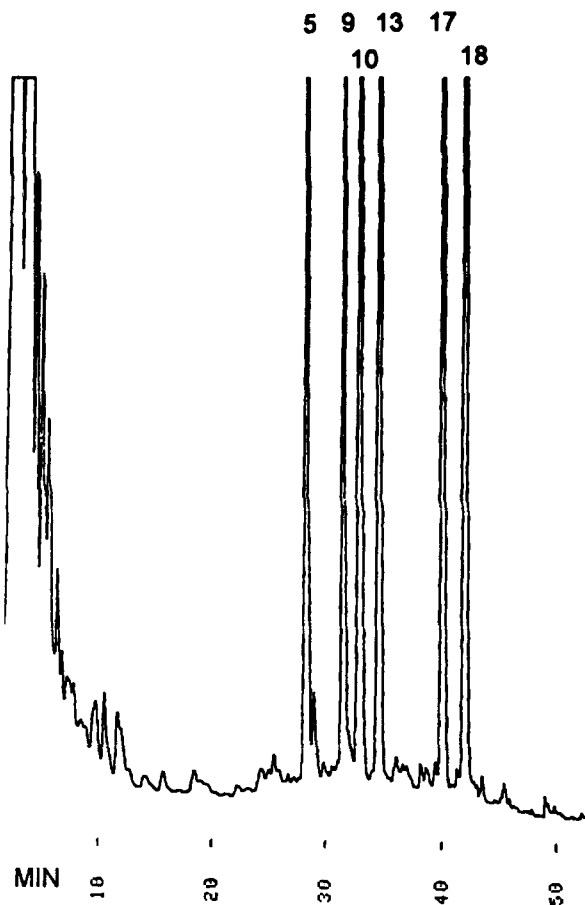
an allylic bile acid found in relatively large amounts in the urine of infants with 3β-hydroxy-C_{27}-steroid dehydrogenase/isomerase enzyme deficiency (10), decomposition occurred and the nonsulfated derivative was not recovered. The use of trifluoroacetic acid for solvolysis as described (11) also did not give recovery of the expected non-sulfated allylic bile acid.

Figure 5 indicates the analysis of a typical urine sample in a normal neonate. An aliquot of urine containing 0.1 mg of creatinine was reduced in volume and enzyme-hydrolyzed prior to Sep-Pak column adsorption and elution and derivative formation. The major peak has a retention time identical to that of cholic acid and other peaks corresponding to those of tetrahydroxy-5β-cholanoic acids, typical of neonatal urine (2, 4), and chenodeoxycholic acid 3-sulfate are seen. No chenodeoxycholic acid was detected but after solvolysis
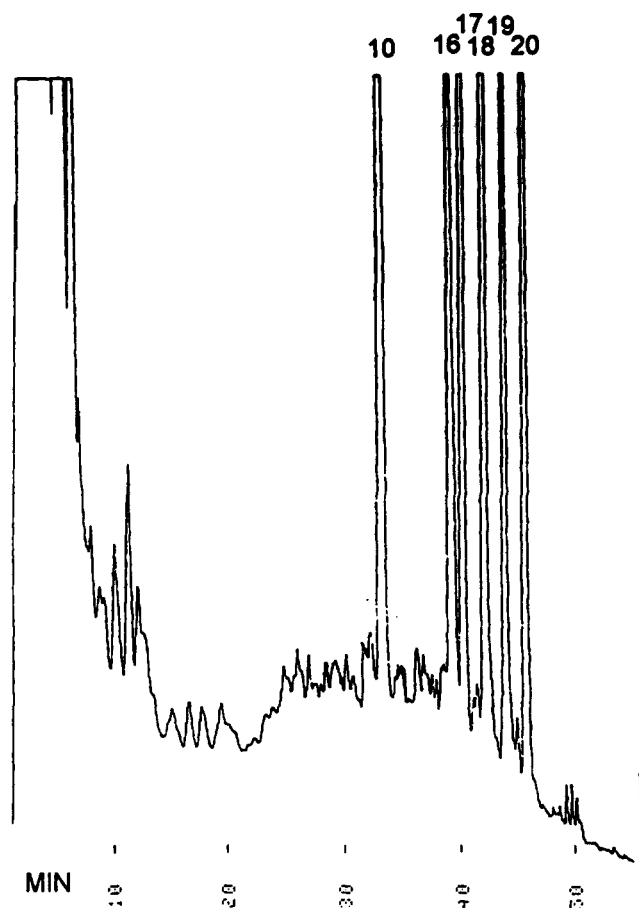
Fig. 4. HPLC analysis of an aliquot taken from the enzyme-hydrolyzed sample after solvolysis for 2 h. After the incubation with cholylglycine hydrolase and 2-h solvolysis, the peaks attributable to ester sulfates (#5, 9, and 13) are no longer present and new peaks corresponding to chenodeoxycholic acid (#16), 3β-hydroxy-5-cholenoic acid (#19), and lithocholic acid are found (#20). See Table 1 for identity of other bile acids.

Fig. 5. HPLC analysis of urine from a normal neonate. An aliquot containing 0.1 mg of creatinine was incubated with cholylglycine hydrolase, adsorbed onto and eluted from a Sep-Pak cartridge, derivatized, and, after elution from a thin-layer plate, injected onto a reverse phase column. Cholic acid (#10) is the major peak followed by a peak with the retention time corresponding to 1β,3α,7α,12α-tetrahydroxy-5β-cholanoic acid and (#1) chenodeoxycholic acid 3-sulfate (#5).

peak #5 disappeared and was replaced by a peak corresponding to chenodeoxycholic acid (data not shown). Other peaks remained unchanged after solvolysis.

DISCUSSION

Our initial experience with HPLC analysis of bile acids using fluorescent detection was with 1-bromoacetylpyrene (12, 13) and brought us the realization that the reagent had major limitations when utilized to detect bile acids in biologic fluids in which the bile acids are relatively minor components of the organic acids that may be present. These limitations are of two types: the generation of a variety of fluorescent de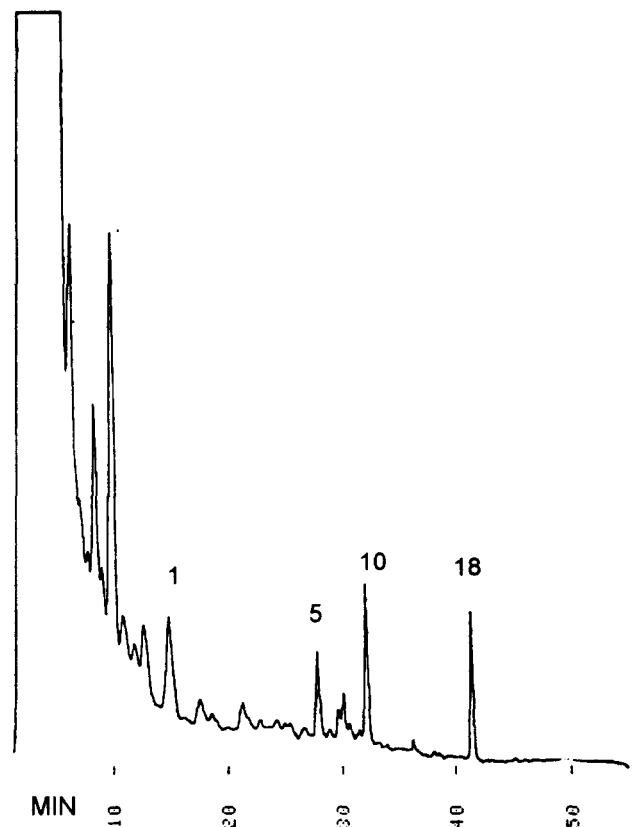composition peaks and the formation of non-bile acid fluorescent esters. Because of the potential advantages of an HPLC method for bile acids that is applicable to urine and other biologic fluids, we set about mostly by trial and error to resolve these problems.

As a guide to possible reagents for the preparation of fluorescent derivatives, we initially evaluated a number of fluorescent reagents listed in review articles (10, 14). As most fluorescent reagents permit detection in the picomole or femtomole range, adequate sensitivity is usually not a concern. Using a low sensitivity range for the detector, we find that a 5-ng injection of a 6,7-dimethoxycoumarin nonconjugated bile acid ester gives an excellent signal/noise ratio as illustrated in the figures depicting bile acid standards.

Our major goals were to maintain accurate quantification by avoiding multiple column separations prior to HPLC analysis as losses of up to 40% of the bile acid ester sulfates in urine are known to occur (2) and to prepare stable carboxy acid esters under mild conditions so that both allylic bile acids and bile acids esteri-

fied at the 3-hydroxy position as either glucuronides or sulfates can be analyzed directly. As indicated in Table 1, bile acids with allylic structures can now be detected and quantitated directly. We are not aware of previous HPLC methods that permit direct analysis of esterified allylic bile acids.

When we found thin-layer chromatographic conditions that kept the 6,7-dimethoxycoumarin derivatives of the glycine-conjugated, nonconjugated, and ester sulfate bile acid derivatives together as a single group at the origin, this criterion made it the reagent of choice, particularly as fatty acid ester derivatives and other unidentified fluorescent peaks, perhaps decomposition products, all migrated up the TLC plate and could be eliminated prior to HPLC without losses.

It was noted that taurine conjugates of bile acids also remain at the origin but do not form fluorescent esters with 4-bromo-6,7-dimethoxycoumarin and are beyond the scope of these studies. However, fluorescent reagents that form stable adducts via the 3-hydroxy group common to most bile acids have been described (15) and can be used for the detection of taurine conjugates. Thus potentially, the TLC purification step method can be adapted for the analysis of both taurine and glycine conjugates. However, for the present we maintain quantitation by utilizing enzyme hydrolysis to yield the nonconjugated bile acids and their ester sulfates and glucuronides.

The careful studies that were done when 4-bromomethyl-6,7-dimethoxycoumarin was introduced as a reagent (16) also encouraged our further evaluation. Thus it was shown that the quantum yield was greater than for 4-bromomethyl-7-methoxycoumarin (17) and more importantly, it remained constant during gradient elution when the mixture of solvents changes. These useful features, established for fatty acids, were also found to be true for the nonconjugated bile acids that we analyzed. Thus the quantum yield per mole is constant and equal to the internal standard, all of which co-elute without loss from the thin-layer plate after development with chloroform.

Additional useful features of the reagent also noted previously (16) are that a relatively low molar excess is required, the reaction rate that occurs goes to completion rapidly under mild conditions, and the esters have been found to be stable for at least several months when stored at room temperature in the dark.

However, although the method is satisfactory for standard mixtures, a large number of unidentifiable peaks appeared on the HPLC tracing when urine was analyzed without preliminary TLC, attributable presumably to other organic acids that form 6,7-dimethoxycoumarin esters.

Analysis by thin-layer chromatography using a long-wave ultraviolet light of these derivatized mixtures as compared with those containing the various standards revealed many fluorescent areas that were less polar than the bile acids. By varying the solvents used for TLC, we found that most of these non-bile acid peaks could be eliminated by applying the derivatized mixture to a thin-layer plate and using chloroform by itself for development. Under these conditions, with the exception of lithocholic acid and 3β-hydroxy-5-cholenoic acid, all the nonconjugated bile acids including the internal standard and the more polar ester sulfates and the glycine and taurine conjugates remained at the origin. Authentic standards of lithocholic acid and 3β-hydroxy-5-cholenoic acid were clearly visualized on the thin-layer plate above the origin and no fluorescence corresponding to these zones was noted in the samples of urine that were analyzed. The observations agree with previous reports indicating that these bile acids in urine are esterified and/or conjugated and therefore will remain at the origin and will not be lost during the TLC purification.

Most of the non-bile acid peaks migrated above the origin and could be eliminated by removing only the group of compounds at the origin. No systematic attempt was made to determine the identity of the less polar non-bile acid constituents. It was reasoned that they probably represent both short- and long-chain organic acids that may also contain hydroxyl groups. Amino acids for the most part are probably either lost during the adsorption step using the Sep-Pak cartridge (8) or, if derivatized, remain together with the bile acids at the origin. Because amino acid fluorescent derivatives are likely to be more polar than the bile acids, they will appear mostly as a group at or near the solvent front before elution of the bile acids.

Thus, by choosing a reagent that generates relatively few extraneous peaks and finding a thin-layer purification step that eliminates many non-bile acid components, we were able to develop a gradient HPLC method that can identify virtually all the glycine-conjugated and nonconjugated bile acids and their esters. Nevertheless, non-bile acid peaks are not entirely eliminated and it is useful to utilize peak-shift analysis that can further establish the identity of a compound.

One of the most useful techniques is a 2-h solvolysis on an aliquot that has been derivatized after elution from the Sep-Pak cartridge either before or after enzyme hydrolysis. Disappearance of the presumed ester sulfate peak and appearance of a characteristic non-sulfate bile acid peak is unlikely to be mimicked by other constituents. With this peak-shift strategy, used previously for GLC analysis, it is our experience thus far that most of the chenodeoxycholic acid in urine is present as an ester sulfate, as we detect little or no

chenodeoxycholic acid until after solvolysis. Although previous analyses of normal urine always identified chenodeoxycholic acid-3-sulfate, losses attributable to extensive column separation were not corrected (2).

Solvolysis of allylic bile acids without decomposition remains an unachieved goal. Although we did not find solvolysis using trifluoroacetic acid helpful for preventing decomposition, it is possible that the larger amounts that were processed using this procedure (11) yielded some non-decomposed product. However, because the method that has been developed provides direct analysis of these compounds, the peak-shift technique is still applicable as non-allylic bile acids will not be decomposed by the procedure.

Because the method outlined in this report utilizes equipment and techniques that can be easily applied at most major medical centers, it should encourage bile acid analysis early in the course of evaluating cholestatic syndromes of unknown cause (18).

Although most of our efforts in this study were directed to the development of a practical method for the analysis of bile acids in urine, we have applied the method to the analysis of bile acids in serum and in breast cyst fluid. No difficulties have been encountered thus far in the analysis of stored serum samples obtained from normal adults and children with cholestatic liver disease. However, breast cyst fluid appears to contain many more fluorescent unidentified peaks than urine. Ideally, coupling a mass selective detector to HPLC will further resolve many of these analytic problems.▮

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