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13. ABSTRACT *(Maximum 200)*

Although it is known that bile acids including lithocholic acid are present in breast cyst fluid, analysis by gas-liquid chromatography-mass spectrometry requires the preparation of volatile derivatives. To identify the natural state of the bile acids in breast cyst fluid, HPLC was utilized because of the potential for identifying both conjugated and esterifed bile acids. For detection, preparation of the 5,6-dimethoxycoumarin carboxy ester derivatives was found more useful than either the 1-bromoacetylpyrene or pyrene 1-carbonyl azide derivatives. Using a binary gradient elution system conditions were found for the separation and identification of glycine conjugated, nonconjugated and esterified bile acids. A peak-shift technique using cholylglycine hydrolase and/or solvolysis was found useful for identifiying esterified and taurine conjugated bile acids. Application of the technique to the identification of conjugated and esterified bile acids in breast cyst fluid was found limited by the finding of other overlapping organic acids. For definitive identification coupling of HPLC with fluorescence detection to a mass spectrum detector can distinguish the bile acids from these other components.

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PI - Signature Date

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5. INTRODUCTION: The discovery, using conventional methods of GLC-MS analysis, that high concentrations of bile acids, including lithocholic acid, are present in breast cyst fluid (1) led to support of a project for developing a method of analysis that would permit their detection as they occur in the fluid rather than as the volatile derivatives required for gas-liquid chromatographic analysis.

6. BODY:

A. Experimental Methods: Because the naturally occurring bile acids are nonvolatile compounds, HPLC provides an analytical technique that can permit their separation without the need to prepare volatile derivatives. Use of fluorescent reagents that react with the hydroxy group at C3, common to all bile acids, or the carboxyl group common to glycine and nonconjugated bile acids can provide derivatives that can be detected at the nanogram level. Therefore, this approach was used in an attempt to develop newer methods of analysis.

1. Preliminary studies: Based on our previous experience in developing methods of bile acid analysis (2), we knew that a preliminary purification procedure would be necessary prior to injection of a fluorescent derivative onto an HPLC column. Thus, we screened fluorescent derivatizing reagents (3) that (a) would react either with the hydroxyl group or with the carboxyl group and (b) would yield bile acid derivatives that could be removed as a single group after a thin-layer chromatographic separation that also functions as a purification procedure by eliminating the excess reagent and decomposition products with Rf,s different from the fluorescent bile acid group.

Some of the reagents that were studied were 1- bromoacetylpyrene, pyrene 1-carbonyl azide, and 4-bromomethyl-6,7-dimethoxycoumarin (4). Although the reaction conditions for forming 1-bromoacetylpyrene derivatives were satisfactory, many decomposition products occurred and it was not possible to achieve a satisfactory purification by TLC on silica gel G using a variety of different solvent systems. Although azide derivatives via the C3-OH group were obtained, reactions did not go to completion and the reagent was unstable when stored in the cold in light-protected tubes. Many decomposition products were also detected by TLC and satisfactory purifcation could not be achieved. Preliminary studies of 4-bromomethyl-6,7-dicoumarin appeared very promising. Thus, the reaction went to completion under mild conditions and the bile acid derivatives could be obtained as a group, free of reagent and other products. Studies using this reagent were therefore carried forward.

2.Bile acid analysis using 4-bromomethly-6,7-dimethoxycoumarin:

Equipment: Shimadzu LC 6A System

Column: ODS-AM (3 x 150 mm, 3 μm, YMC)

Mobile Phase:

A: water/MeCN/MeOH= 3/2/1 (v/v/v/) containing $AcONH_4$ (7.6M, 1 ml/500 ml solvent A)

B: MeCN/MeOH = 2/1(v/v) containing $ACONH_4$ (7.6M, 1 ml/500 ml of Solvent B)

Derivatization Conditions:

1. A 500 ng sample of each bile acid dissolved in 95% methanol is added to 100 µl of 1. 8mM KOH and evaporated in vacuo.
2. The dried residue is redissolved in 50 µl acetonitrile followed by the addition of (a) 20 µl DCC (1.5 mg/ml acetonitrile of dicylcohexano-18-crown-6)
   (b) 20 µl BMC (3.0 mg/ml acetonitrile 4-bromomethyl-6,7,dimethoxycoumarin
3. Heat at 60°C, 30 min

Purification by Thin-layer chromatography

4. evaporate in vacuo, redissolve in methanol and transfer to Silica gel G plate 20 x 5 cm, develop in chloroform
5. Visualize under UV light and scrape fluorescent area at origin onto glassine paper and transfer to 6x50 mm glass test tube
6. add 200 µl of 1/1 (v/v) acetonitrile/methanol, suspend silica by vortex and centrifuge
7. Inject 2 to10 µl aliquots onto HPLC column

HPLC analysis:
Gradient program:
0 min A/B 100/0 Linear gradient to A/B 5/95 45 min; isocratic 15 min
Flow rate: 0.6 ml/min Column temp: ambient
Detection: Ex 340 nm, Em 430 nm

Peak-shift technique:
As indicated in the Results section, because of the large number of acidic compounds found in breast cyst fluid, an attempt was made to improve identification by subjecting aliquots to further procedures that would shift the retention time to that of known standards.

B. Results:

Summary:

1. Although overlap occurs with some species of bile acids, the method is applicable to glycine conjugates of bile acids, nonconjugated bile acids, and esterified bile acids.
2. The fluorescence yield of bile acids as glycine conjugates on a molar basis is less than that of the nonconjugated bile acids.
3. The fluorescence of nonconjugated bile acids and their esters is equivalent on a molar basis.
4. As an internal standard, commercially available 7α,12α-dihydroxy-5β-cholan-24-oic acid (#18) was chosen because it is not naturally occurring and has a retention time (41.9 min) that falls in between nonconjugated di- and trihydroxy bile acids and monohydroxy bile acids.

5. Taurine conjugated bile acids cannot be analyzed directly. Use of the enzyme cholylglycine hydrolase specifically converts the taurine and glycine conjugated bile acids to the nonconjugated species followed by derivatization.
6. Esterified bile acids, particularly the naturally-occurring ester sulfates (#28-31) have distinct retention times.

7. Acid-sensitive allylic bile acids with the C_5=C_6, C_7OH configuration can be detected without destruction (# 15,17,18,19)

Peak-shift technique:
1. Cholylglycine hydrolase enzyme hydrolysis:
a. Glycine conjugates and esters could be further identified by a shift in retention time to the analogous nonconjugated species.
b. The presence of ester sulfates could be specifically recognized by solvolysis of an aliquot to yield the glycine conjugated or nonconjugated derivative. The technique was not successful for allylic sulfated bile acids which decompose under the acidic conditions needed for solvolysis. Attempts to utilize sulfatase enzymes were not successful.
c. Acetate derivatives were prepared but satisfactory peaks were not obtained with the column and solvents that were used.

Figure 1 indicates the retention times of the 5,6-dimethoxy esters of glycine conjugated and nonconjugated bile acid standards compared to the internal standard, 7α,12α-dihydroxy-5β-cholanoic acid (#18) that is not known to be natuarally-occurring.

Some of the bile acid standards representative of those that might be found in breast cyst fluid were analyzed following elution from a Sep-Pak cartidge as illustrated in figure 2. In this analysis it was found that chenodeoxycholic acid-3-sulfate (#5) co-eluted with glycodeoxycholic acid (#8). However, as shown in figure 3, following incubation of an aliquot with cholylglycine hydrolase these two bile acids were resolved since a peak corresponding to deoxycholic acid (#17) was obtained.

Further confirmation of bile acid ester sulfates was obtained by a solvolysis procedure. Figure 4, is an HPLC tracing obtained after solvolysis of an aliquot of the enyzyme-hydrolyzed sample. The peaks corresponding to the ester sulfates of chenodeoxycholic, lithocholic and 3β-hydroxy-5-cholenoic acids (#5,9 and 13) are no longer present and peaks corresponding to the 5,6-dimethoxy esters of the respective bile acids (#16,19, and 20) are found.

B. Bile acid analysis of breast cyst fluids: Most of the effort of the project went into the development of a method that would allow the identification of naturally occurring bile acids without the need to prepare volatile derivatives. Additional time without additional funds was requested to complete development of the method.

After completion of the method aliquots of samples of breast cyst fluid (0.1 to 0.5 ml) were evaporated in vacuo together with 1 μg of internal standard (7α,12α-dihydroxy-5β-cholanoic acid) and derivatized and analyzed as described above.

A typical HPLC tracing (breast cyst fluid D-024) is shown in figure 6.
It is evident that many more fluorescent peaks, presumably unidentified organic acids, are present than can be accounted for from the known bile acid composition of human breast fluid.

Although peaks corresponding to both glycine conjugated and esterified bile acids including glycolithocholate-3-sulfate and glycolithocholate were detected in some breast cyst fluid specimens, it is not possible to exclude that the peaks represent unknown organic acids.

Thus definitive identification must depend on more specific methods.
Going beyond the present workscope, we elected to collect the peaks obtained by HPLC analysis and further analyze them by GLC-MS to confirm the presence of lithocholic acid. The data are shown in Table 2.

Table 1. HPLC analysis of bile acids by reverse phase column chromatography as the 5,6-dimethoxycoumarin esters

Bile acid	minutes
1. 1β,3α,7α,12α-tetrahydroxy-5β-cholanoic acid	14.8
2. 3α,6β,7α,12α-tetrahydroxy-5β-cholanoic acid	15.3
3. glycocholic acid	16.7
4. glycochenodeoxycholic acid	27.8
5. chenodeoxycholic acid-3-sulfate	28.2
6. chenodeoxycholic acid-7-sulfate	28.7
7. ursodeoxycholic acid	29.1
8. glycodeoxycholic acid	29.8
9. 3β-hydroxy-5-cholenoic acid sulfate	31.5
10. cholic acid	32.5
11. 3α,7α-dihydroxy-chol-5-enoic acid	33.3
12. 7α-hydroxy-3-oxo-chol-4-enoic acid	34.1
13. lithocholic acid sulfate	34.6
14. glycolithocholic acid	36.2
15. lithocholic acid glucuronide	37.6
16. chenodeoxycholic acid	39.3
17. deoxycholic acid	39.7
18. 7α,12α-dihydroxy-5β-cholanoic acid (internal std.)	41.9
19. 3β-hydroxy-5-cholenic acid	42.4
20. lithocholic acid	45.9
21. 3β-hydroxy-5-cholestenoic acid	47.6

FIg 1

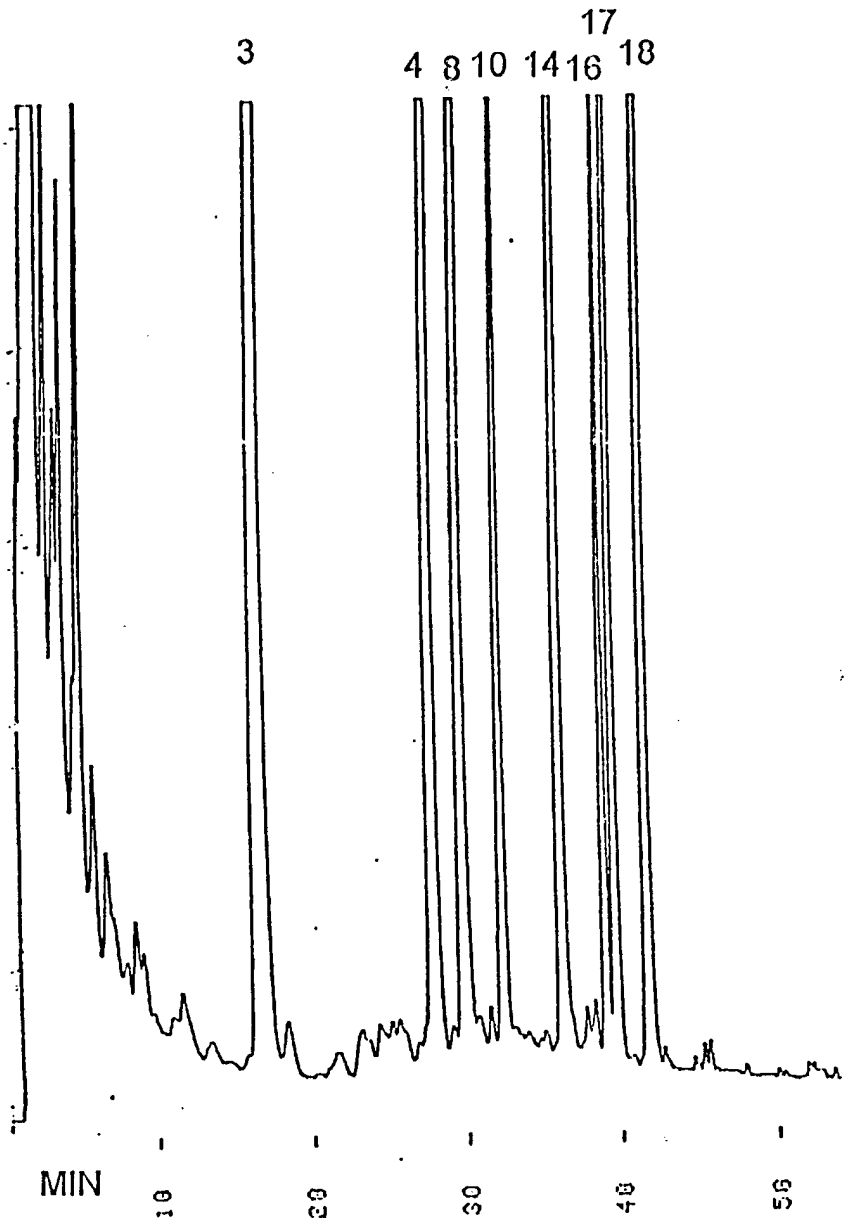


Figure 1. HPLC analysis of 5,6-dimethoxy esters of glycine conjugated and nonconjugated bile acids. Using a reverse phase column and gradient elution the internal standard, 7α,12α-dihydroxy bile acid (#18) has a longer retention time than other nonconjugated and glycine conjugated bile acids. See table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

Fig 2

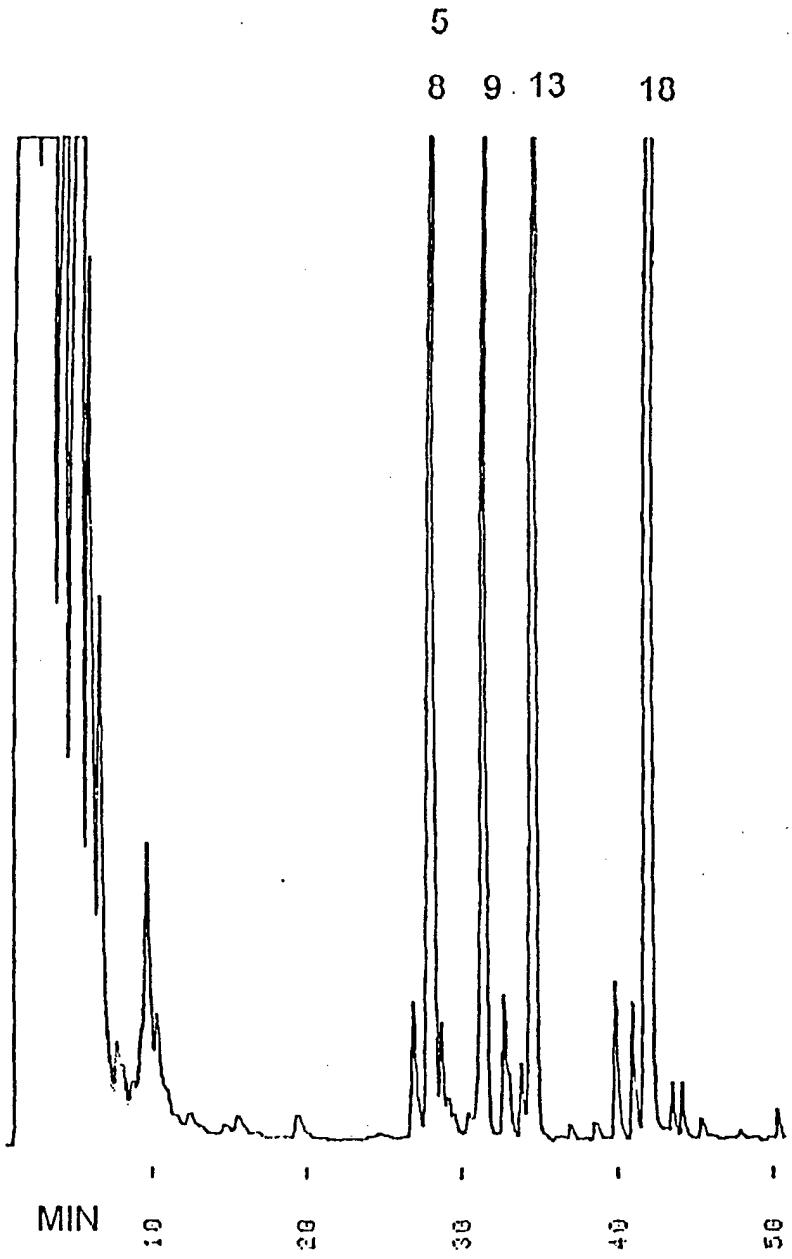


Figure 2. HPLC analysis of derivatized eluate from Sep-Pak cartridge prior to enzyme hydrolysis and solvolysis. Although taurocholic acid was present in the eluate none was detected. In this analysis chenodeoxy-3-sulfate (#5) and glycodeoxycholic acid (#8) co-eluted. However, as shown in figure 3 and 4 their presence could be established. See Table 1 for identity of other bile acids.

Fig 3

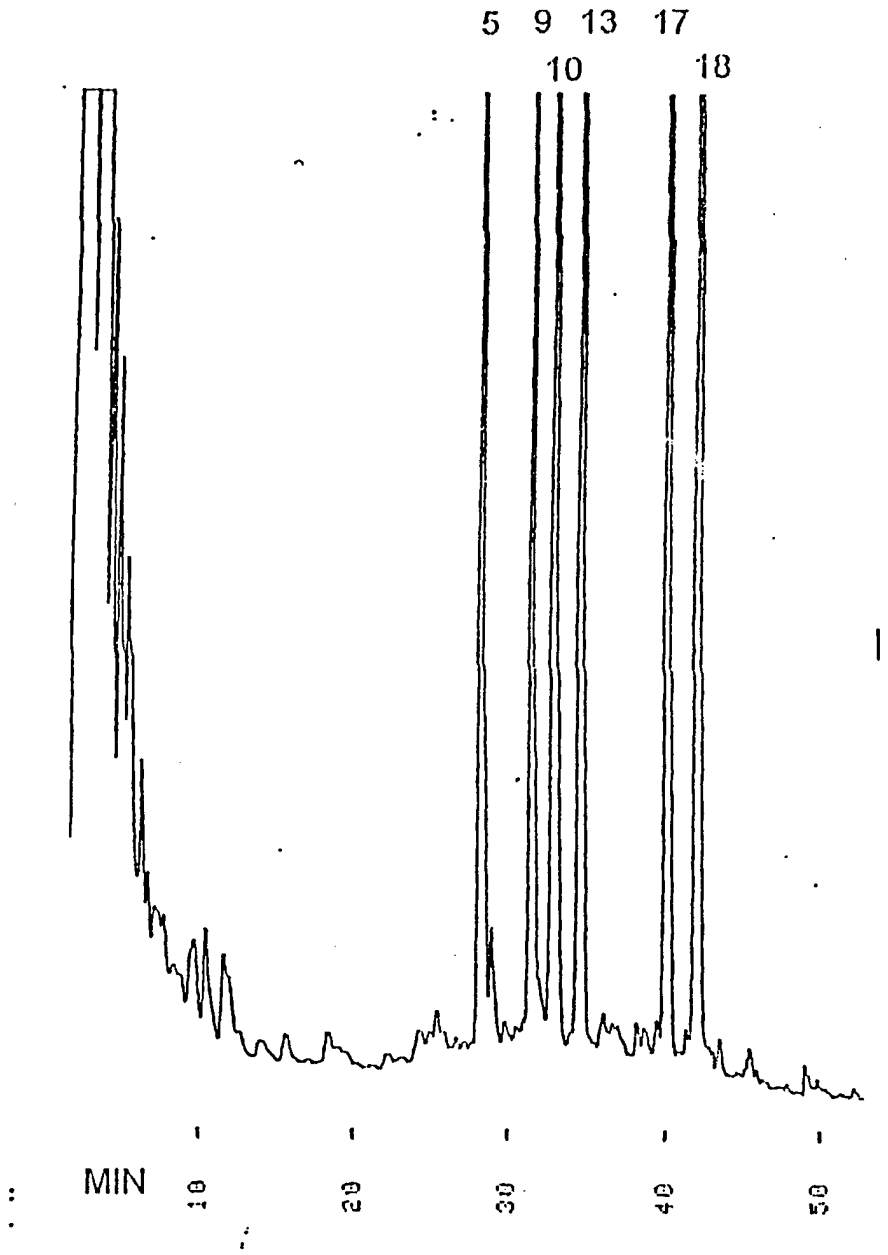


Figure 3. HPLC analysis of the same bile acid mixture (figure 2) after incubation with cholylglycine hydrolase. Comparison to figure 1 and 2 indicates the appearance of peaks corresponding to cholic acid (#10) and deoxycholic acid (#17). A peak corresponding to chenodeoxycholic acid-3-sulfate (#5) is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

Fig 4

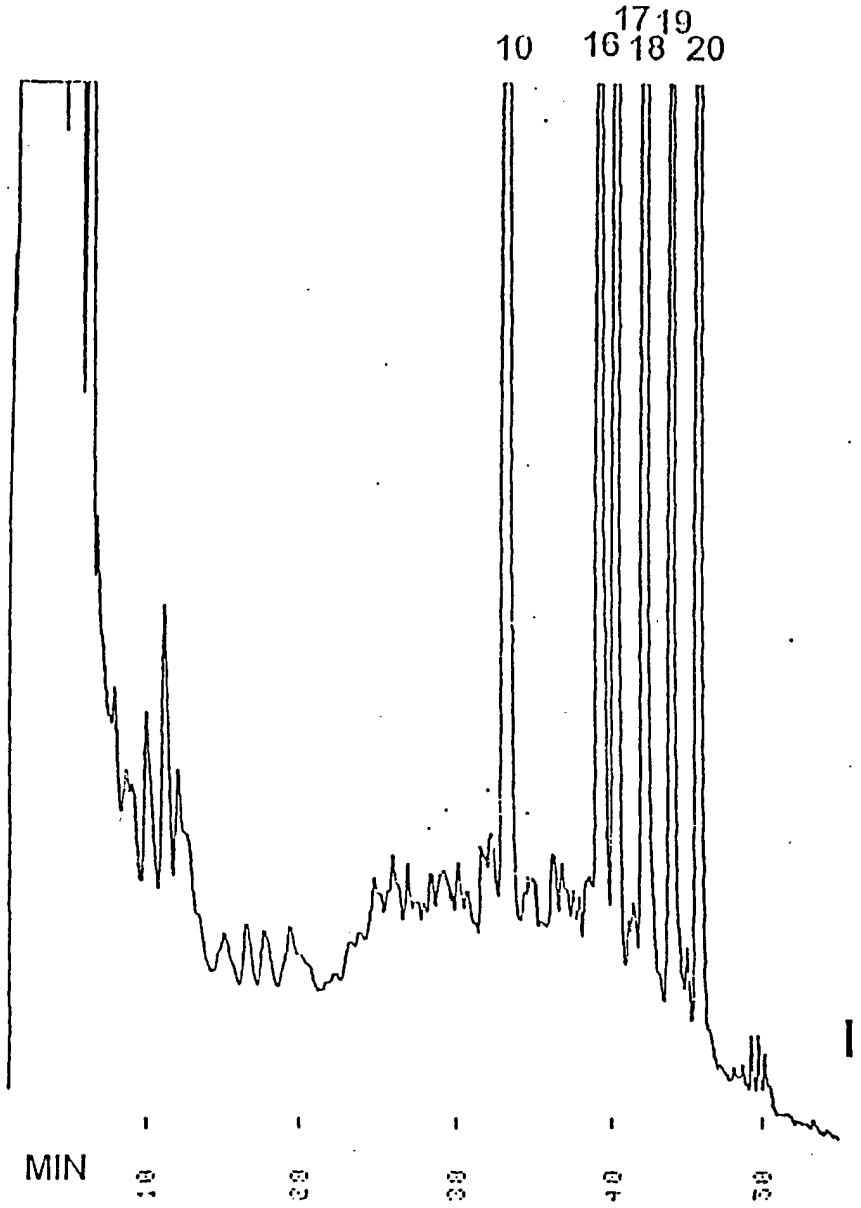
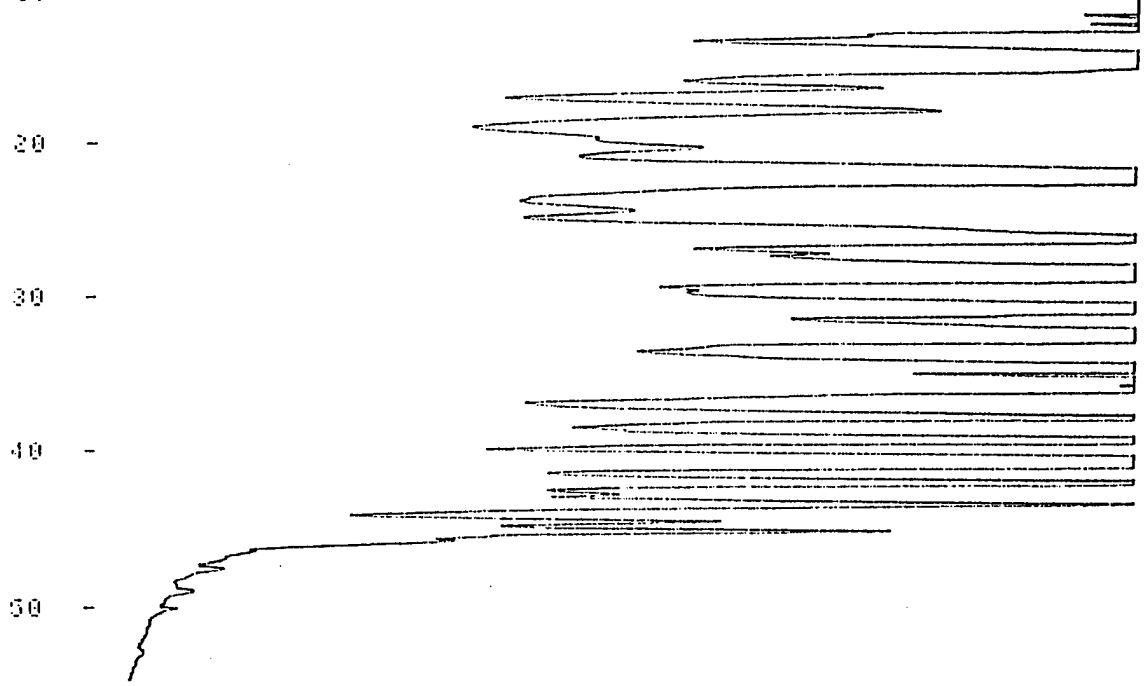


Figure 4. HPLC analysis of an aliquot taken from the enzyme hydrolyzed sample after solvolysis for 2 hours. Following the incubation with cholylglycine hydrolase and 2-hr solvolysis the peaks attributable to ester sulfates (#5,9, & 13) are no longer present and new peaks corresponding to chenodeoxycholic acid (#16), 3β-hydroxy-5-cholenoic acid (#19) and lithocholic acid are found (#20). See Table 1 for the identity of other bile acids.

97/01/08 10:33:54 D-024

Fig. 5

Dimethoxycoumarin esters of organic acids found in breast cyst fluid. Some of the peaks correspond to authentic standards of glycine conjugated, nonconjugated and esterified bile acids.



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97/01/08 11:34:03
CHROMATOPAC C-R3
SAMPLE NO 0
REPORT NO 212

FILE 9
METHOD 0811

D-024

P/NO	TIME	AREA	MK	IDNO	CONC	NAME
1	13.917	10305785			9.1286	
2	16.242	1028051			0.9106	
3	17.767	2937261			2.6017	
4	20.358	422416			0.3742	
5	21.767	11346350			10.0503	
6	26.192	6351560			5.626	
7	27.267	911104	V		0.807	
8	28.017	5511318	V		4.8818	
9	28.658	5666424	V		5.0192	
10	30.608	9032614	V		8.0008	
11	32.2	15142128	V		13.4125	
12	34.45	6721804	V		5.954	
13	35.217	3957243	V		3.5052	
14	35.867	4164098	V		3.6884	
15	37.742	4040550	V		3.579	
16	39.158	6194444	V		5.4869	
17	40.733	7842869			6.947	
18	42.083	3825117	V		3.3882	
19	43.542	3700046	V		3.2774	
20	44.717	1159501			1.0271	
21	45.317	2463149	V		2.1818	
22	47.725	83867			0.0743	
23	49.15	88381			0.0783	
	TOTAL	112896008			100	

Table 2

HPLC ANALYSIS OF BILE ACIDS IN BREAST CYST FLUID									
DIMETHOXYCOUMARIN ESTERS									
BREAST CYST FLUID	B-013	B-014	B-022	B-024	B-028	C-023*	D-024*	A-029*	C-013*
Bile acid** μg/ml									
glycocholic a.	2				1.7		0.5	1.0	
glycolithosulfate	3.5								
chenodeoxycholic 3-sulfate	6.6	1.6	2	0.7	1.3			6.4	2.2
chenodeoxycholic-7-sulfate									
glycochendeoxycholic a.	6.4	2.4	2.4	3.0	3.2	5.8	2.8	5.0	
glycodeoxycholic a.	7.4	2	7.6	3.4	3.4	3.8		5.8	
3β–OH-5-cholenoic-3-sulfate	18.6	2.4	11.4	8.8	10.4			7.0	
glycolithocholic a.		13.8					7.9	8.0	3.0
lithocholic-3-sulfate	10	11.2		2.0	2.0	5.2	3.6	3.8	3.8
chenodeoxycholic a.	6.8	12.8	7.4	3.6	5.8	10.4	3.2	2.4	
deoxycholic a.	7.4	14.8	7.6		5.8			3.8	2.4
lithocholic a.		5.6	3.2	1.6	2.8	3.0	1.9	1.8	3.2

* bile acid confirmed by GLC-MS analysis after hydrolysis and solvolysis and preparation of methyl ester acetate

** identification based only on retention time requires further confirmation by HPLC-MS or other procedure

(7) CONCLUSIONS:

A. An HPLC method was developed that permits the separation and detection of many of the naturally occurring bile acids after preparation of the fluorescent 5,6-dimethoxycoumarin derivative.

B. Because of the occurrence of many other organic acids in breast cyst fluid that overlap with those of the standard bile acids, definitive identification must depend on a more precise method of identification than fluorescence.

C. If further interest exists in more precise identification of the naturally occurring species of bile acids in breast cyst fluid then extension of the methods that have been developed in this project to the use of mass spectrometry in conjunction with HPLC and fluorescent derivative formation would be a practical approach that can provide definitive identification.

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(9) APPENDICES: none

(11) Final report

a. Bibliography

1. Budai, K. and N. B. Javitt. Bile acid analysis in biologic fluids: a novel approach. J. Lipid Res. 38: 1906-1912, 1997.

b. Personnel:

1. Norman B. Javitt
2. Kornelia Budai
3. Tadeusz Warchol
4. Dario Gargiulo

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 7α,12α-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-dimethoxycoumarin • 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-

Abbreviations: HPLC, high performance liquid chromatography; GLC, gas–liquid chromatography; DCC, dicyclohexano-18-crown-6; BMC, 4-bromomethyl-6,7-dimethoxycoumarin.

[1]To whom correspondence should be addressed.

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 (dimethoxypropane: 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.

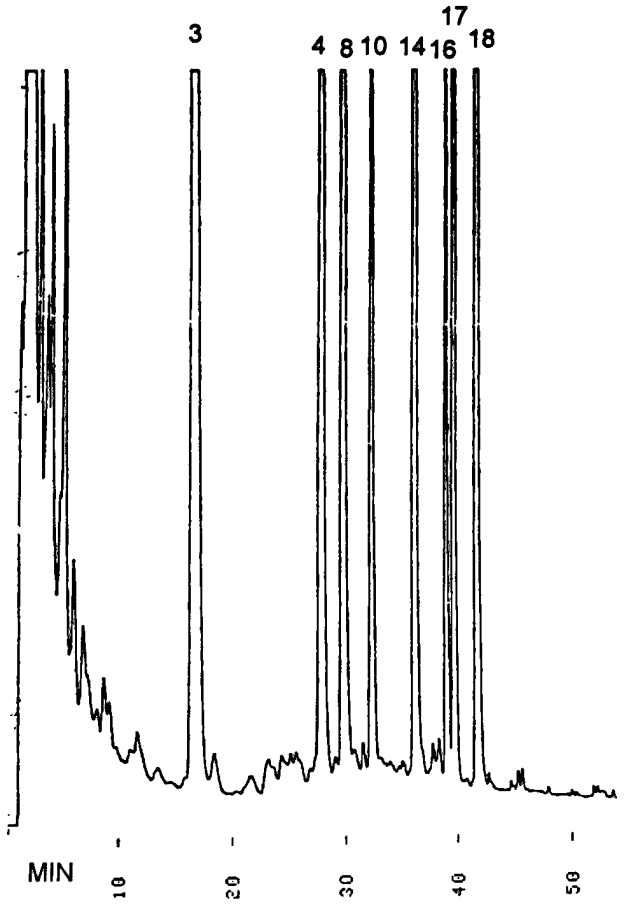

Fig. 1. HPLC analysis of 5,6-dimethoxy esters of glycine-conjugated and nonconjugated bile acids. Using a reverse phase column and gradient elution, the internal standard, 7α,12α-dihydroxy bile acid (#18), has a longer retention time than other nonconjugated and glycine conjugated bile acids. (See Table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

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, coelution 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, 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-

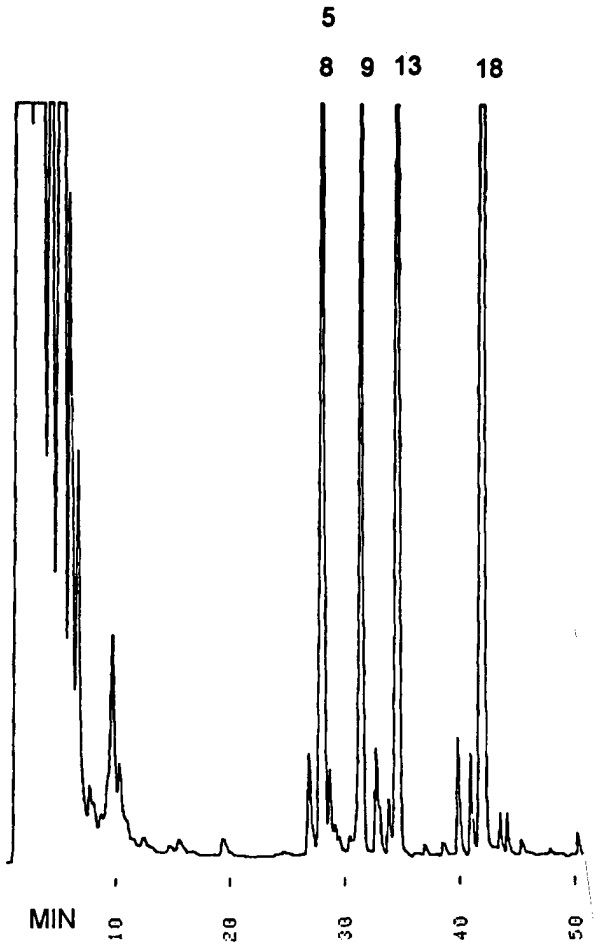

Fig. 2. HPLC analysis of derivatized eluate from Sep-Pak cartridge prior to enzyme hydrolysis and solvolysis. Although taurocholic acid was present in the eluate, none was detected. In this analysis chenodeoxy-3-sulfate (#5) and glycodeoxycholic acid (#8) co-eluted. However, as shown in Figs. 3 and 4 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

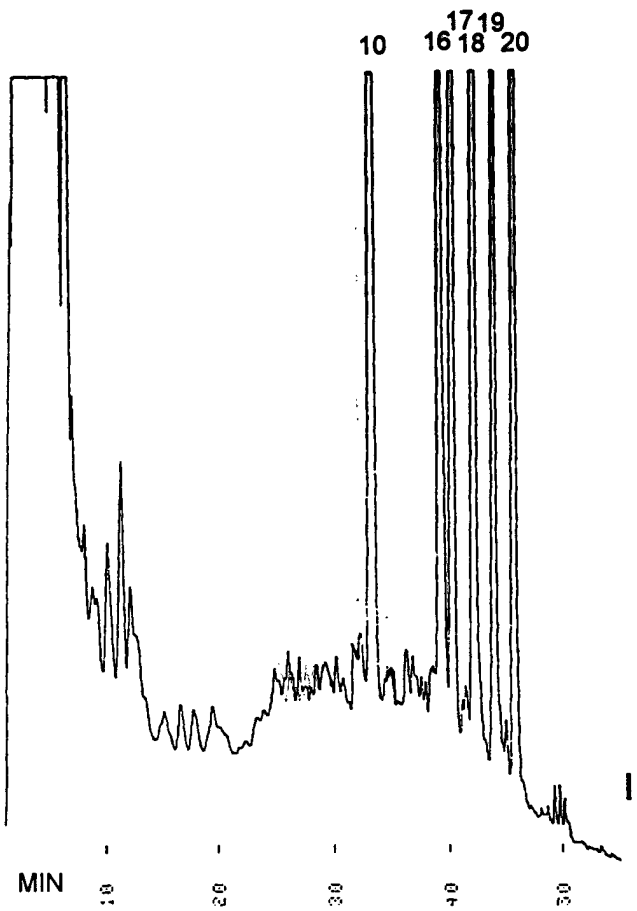

Fig. 4. HPLC analysis of an aliquot taken from the enzyme-hydrolyzed sample after solvolysis for 2 h. After the incubation with cholylglycine hydrolase and 2-h solvolysis, the peaks attributable to ester sulfates (#5, 9, and 13) are no longer present and new peaks corresponding to chenodeoxycholic acid (#16), 3β-hydroxy-5-cholenoic acid (#19), and lithocholic acid are found (#20). See Table 1 for identity of other bile acids.

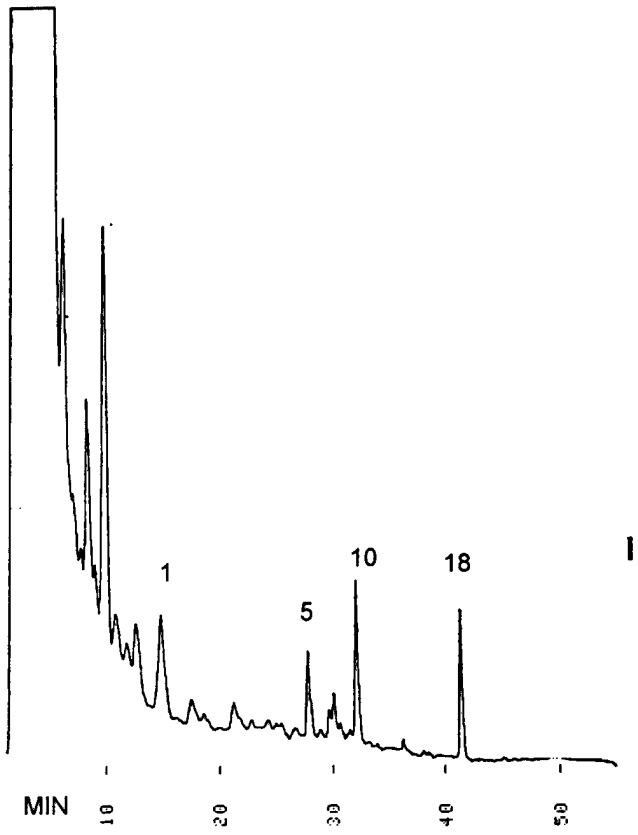

Fig. 5. HPLC analysis of urine from a normal neonate. An aliquot containing 0.1 mg of creatinine was incubated with cholylglycine hydrolase, adsorbed onto and eluted from a Sep-Pak cartridge, derivatized, and, after elution from a thin-layer plate, injected onto a reverse phase column. Cholic acid (#10) 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 decomposition 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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