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QUANTITATIVE DETERMINATION OF BILE ACIDS IN BILE WITH REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Separation and quantitation of glycine and taurine conjugates of commonly occurring bile acids in bile, i.e. lithocholic, deoxycholic, chenodeoxycholic, ursodeoxycholic and cholic acids in their naturally occurring states have been successfully accomplished using high-performance liquid chromatography. No preliminary purification of bile acids is required except ethanol extraction of bile. A μBondapak C_{18} column and acetonitrile—methanol—phosphate buffer and ultraviolet detector at 200 nm were used. Detection limit was 0.05 μg and linearity was observed in the range up to 16 μg. Bile acid composition of ten randomly chosen normal human gallbladder bile samples is given. A large difference in bile acid composition between glycine and taurine conjugates was found to be present.

INTRODUCTION

Rapid expansion of our knowledge on bile acid metabolism in recent years is only made possible by the refinement of analytical techniques for bile acids, such as the introduction of column chromatography, gas—liquid chromatography, thin-layer chromatography (TLC), gas chromatography—mass spectrometry (GC—MS), radioimmunoassay and high-performance liquid chromatography (HPLC). The last has several advantages over the preceding methods, i.e. minimal sample preparation, requiring only ethanol extraction, no hydrolysis, good separation and sensitivity of detection. In the present communication, successful separation of glycine and taurine conjugates of commonly occurring bile acids in bile such as lithocholic, deoxycholic, chenodeoxycholic, ursodeoxycholic and cholic acids has been accomplished in a single run using HPLC. Quantitative application to human bile is presented.

MATERIALS AND METHODS

Samples

Human bile was obtained by puncture aspiration of gallbladder content during laparotomy from patients suffering from diseases other than those of the hepatobiliary tree. The bile samples obtained were kept frozen at −20°C until analyzed.

Reagents

The solvents were reagent grade and redistilled prior to use. Those for HPLC were analytical grade reagents prepared specifically for the purpose. Glycine and taurine conjugates of lithocholic, deoxycholic, chenodeoxycholic and cholic acids were obtained from Calbiochem (San Diego, CA, U.S.A.) or from Steraloid (Wilton, NH, U.S.A.). Glyco- and tauroursodeoxycholic acids were kindly supplied by Tokyo Tanabe (Tokyo, Japan).

Chromatography

The HPLC equipment used was a Waters ALC/GP C 202 equipped with a Model 6000 solvent delivery system and a U6K universal injector. The detector used was a Shimadzu variable ultraviolet spectrometer Model SPD-1 (Shimadzu Seisakusho, Kyoto, Japan) which had variable wavelengths between 190 and 750 nm. The μBondapak C_{18} column (30 cm × 3.9 mm I.D.) (CN 27324) and the guard column containing 400 mg of Bondapak C_{18}/Corasil (CN 27248) were supplied by Waters Assoc. (Milford, MA, U.S.A.). Peak area was computed with the use of Shimadzu Chromatopac E1A (Shimadzu Seisakusho). Bile samples were extracted with 20 volumes of ethanol, brought to boiling in a hot water bath, cooled to room temperature, left overnight and filtered through Toyo Roshi 5A filter paper, Toyo Roshi (Tokyo, Japan) (comparable to Whatman No. 43). The filtrate was passed through a Millipore filter, pore size 0.45 μm. To an aliquot of the final filtrate the internal standard of testosterone acetate was added in a form of ethanol solution 200 μg/ml and used for HPLC. An aliquot of 5—10 μl of the ethanol extract of bile containing 5—20 μg of solid and 1.0 μg of internal standard was injected. The solvent system used was a mixture of acetonitrile—methanol—0.03 *M* phosphate buffer, pH 3.40 (10:60:30, v/v/v), prepared just before use and the pH was adjusted exactly to three digits. The flow-rate was 0.5 ml/min with 600 p.s.i. isobaric flow. Detection was made at 200 nm.

RESULTS

Separation of conjugates of commonly occurring bile acids in bile

Baseline separation was obtained between the glycine and taurine conjugates of lithocholic, deoxycholic, chenodeoxycholic, ursodeoxycholic and cholic acids as shown in Fig. 1. Ursodeoxycholic acid conjugates emerge before cholic acid conjugates. Taurine conjugates always precede glycine conjugates. However, the conjugates of each bile acid were grouped together except deoxycholic and chenodeoxycholic acid conjugates, i.e. in the order of taurochenodeoxycholic, taurodeoxycholic, glycochenodeoxycholic and glycodeoxycholic acids.

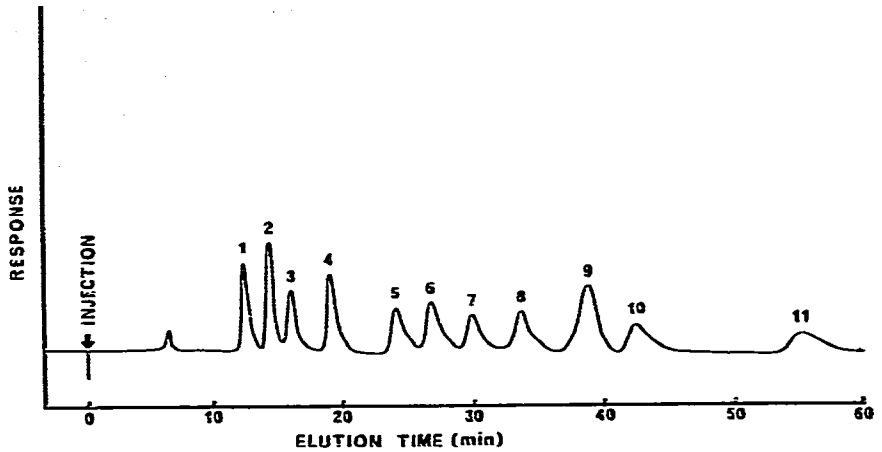

Fig. 1. Separation of standard mixture of conjugated bile acids. Peaks: 1, tauroursodeoxycholic; 2, glycoursodeoxycholic; 3, taurocholic; 4, glycocholic; 5, taurochenodeoxycholic; 6, taurodeoxycholic; 7, glycochenodeoxycholic; 8, glycodeoxycholic; 9, testosterone acetate (internal standard); 10, taurolithocholic; 11, glycolithocholic.

TABLE I

RELATIVE ELUTION VOLUME OF BILE ACID CONJUGATES

Bile acid	Relative elution volume*
Tauroursodeoxycholic acid	0.46
Glycoursodeoxycholic acid	0.53
Taurocholic acid	0.60
Glycocholic acid	0.71
Taurochenodeoxycholic acid	0.90
Taurodeoxycholic acid	1.00
Glycochenodeoxycholic acid	1.12
Glycodeoxycholic acid	1.26
Taurolithocholic acid	1.59
Glycolithocholic acid	2.07
Testosterone acetate (internal standard)	1.45

*Relative to taurodeoxycholic acid = 1.00.

The elution volume for each conjugate is given in Table I. In order to effect good separation it is important to adjust the pH exactly to three digits.

Quantitation of bile acid in bile

The calibration curve for each bile acid conjugate is shown in Fig. 2. Linearity was observed up to 16 μg. Good reproducibility was obtained (Table II). Recovery experiments carried out by adding a known amount of each bile acid conjugate to the bile of known bile acid composition gave good results (Table III). The detection limit was found to be 0.05 μg.

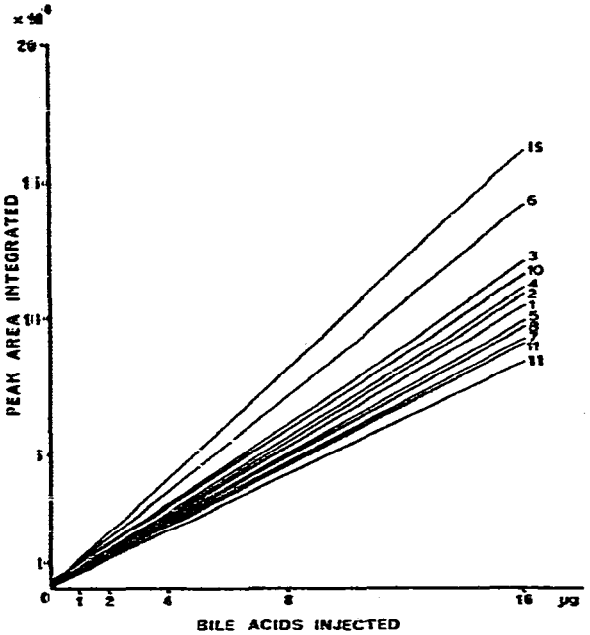

Fig. 2. Calibration curve for each conjugated bile acid and testosterone acetate, used as internal standard. Correlation coefficients of linear regression ranged from 0.996 to 0.999. Designations as in Fig. 1.

TABLE II

REPRODUCIBILITY OF DETERMINATION OF BILE ACID IN HUMAN GALLBLADDER BILE BY HPLC

n = 5. Coefficient of variation expressed in per cent of total bile acid.

Bile acid	Mean ± S.D. (mg/ml)	Coefficient of variation (%)
Glycolithocholic	—	—
Taurolithocholic	—	—
Glycodeoxycholic	45.0 ± 2.2	4.8
Taurodeoxycholic	7.1 ± 0.4	5.9
Glycochenodeoxycholic	40.6 ± 2.0	4.8
Taurochenodeoxycholic	13.4 ± 0.5	3.9
Glycoursodeoxycholic	2.9 ± 0.1	4.5
Tauroursodeoxycholic	1.0 ± 0.04	3.9
Glycocholic	29.2 ± 1.9	6.4
Taurocholic	7.5 ± 0.3	4.3

Bile acid composition of normal human gallbladder bile

Ten human gallbladder bile samples were subjected to HPLC. Representative separation of bile acid from human gallbladder bile is shown in Fig. 3. Their bile acid composition is presented in Table IV. The major bile acids in bile were chenodeoxycholic acid and cholic acid conjugates in concordance to the earlier reports. Minor unidentified peaks appeared occasionally between glycoursodeoxycholic and taurocholic and between taurocholic and glycocholic acid. The bile acid composition of glycine and taurine conjugates showed considerable variation in individual cases.

TABLE III

RECOVERY OF BILE ACID ADDED TO HUMAN GALLBLADDER BILE

$n = 3$.

Compound added	Originally present (mg/ml)	Added (mg/ml)	Calculated (mg/ml)	Found (mg/ml)	Recovery of added compound (%)
Glycolithocholic	0.0	10.2	10.2	9.2	90.4
		51.0	51.0	52.0	102.0
Taurolithocholic	0.0	10.2	10.2	9.0	89.1
		50.8	50.8	47.3	93.2
Glycodeoxycholic	45.0	9.7	54.7	57.8	86.4
		48.5	93.5	105.0	112.3
Taurodeoxycholic	7.1	9.8	16.9	14.4	85.4
		49.0	56.1	47.8	85.2
Glycochenodeoxycholic	40.6	9.7	50.3	41.8	83.1
		48.5	89.1	96.3	108.1
Taurochenodeoxycholic	13.4	9.4	22.8	18.7	81.9
		47.0	60.4	54.3	89.9
Glycoursodeoxycholic	2.9	9.9	12.8	11.9	92.7
		49.5	52.4	55.8	106.4
Tauroursodeoxycholic	1.0	9.9	10.9	11.6	106.2
		49.5	50.5	53.6	106.1
Glycocholic	29.2	9.6	38.8	37.3	95.9
		48.0	77.2	65.5	84.8
Taurocholic	7.5	10.0	17.5	17.1	97.5
		50.0	57.5	54.2	94.1

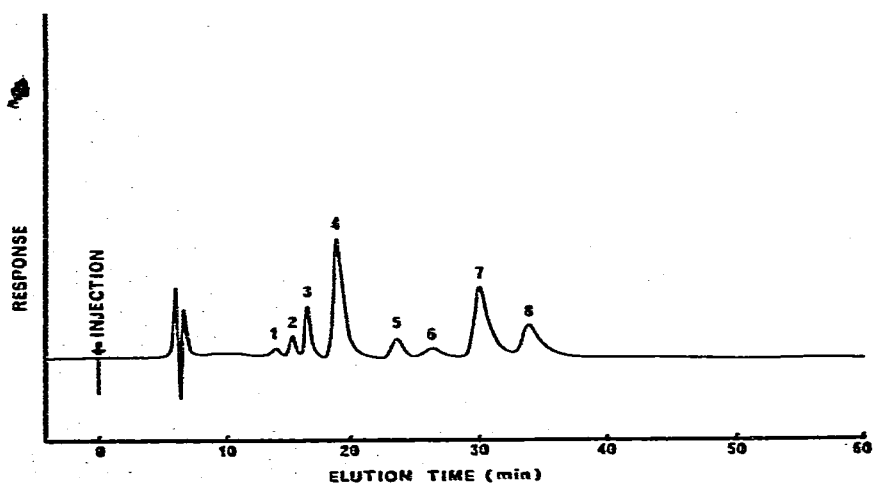

Fig. 3. Separation of bile acids from human gallbladder bile. Identification of peaks as in Fig. 1.

TABLE IV

BILE ACID COMPOSITION OF HUMAN GALLBLADDER BILE

Expressed in per cent of total bile acid. Unidentified peaks were calculated as having the same peak area response as glycodeoxycholic.

Bile acid	Case No.									
	1	2	3	4	5	6	7	8	9	10
Tauroursodeoxycholic	0	0	0.8	0.6	0	0.2	0	0.7	0	0
Glycoursodeoxycholic	0.9	0	1.1	3.6	3.6	0.1	0.2	0.1	0	0
Taurocholic	9.2	18.3	13.8	2.3	2.4	12.1	6.4	17.5	10.2	10.3
Glycocholic	30.8	27.4	21.3	31.3	23.7	41.0	32.7	36.6	43.5	39.6
Taurochenodeoxycholic	6.1	12.0	12.8	1.9	1.6	5.5	3.7	13.4	6.7	6.4
Glycochenodeoxycholic	25.2	42.2	31.8	46.3	32.9	22.9	32.9	31.5	34.5	32.8
Taurodeoxycholic	3.1	0	3.4	0.3	0.9	2.6	1.7	0.1	0	1.2
Glycodeoxycholic	18.9	0.1	15.0	12.2	32.9	14.6	20.3	0	5.1	10.2
Taurolithocholic	0	0	0	0	0	0	0	0	0	0
Glycolithocholic	2.2	0	0	0	0	0	0	0	0	0
Unidentified peak 1*	0	0	0	0.1	0.1	0	0.2	0.1	0	0
Unidentified peak 2**	3.6	0	0	1.4	2.2	1.0	1.9	0	0	0

*Appearing between glycoursodeoxycholic and taurocholic.

**Appearing between taurocholic and glycocholic.

DISCUSSION

The GC methods used for the analysis of bile acids commonly occurring as conjugates of glycine and taurine, usually require prior hydrolysis to enhance their volatility and to simplify the analytical procedure. The inclusion of hydrolysis steps has inherent disadvantages, i.e. it may remain incomplete and may produce artefacts. Furthermore, our knowledge on conjugate forms in which bile acids occur may be important in view of the differences in micelle formation [1] and during the intestinal passage [2]. Recent introduction of HPLC made it possible to analyse bile acids conjugates without prior hydrolysis [3—16]. However, the methods thus far proposed do not permit clear cut separation of glycoursodeoxycholic acid either from taurodeoxycholic and glycocholic acid [11] or from taurodeoxycholic acid [16], or require two runs for late emerging lithocholic acid conjugates [7]. The present method gives a baseline separation of glycine and taurine conjugates of all commonly occurring bile acids in bile, i.e. lithocholic, deoxycholic, chenodeoxycholic, ursodeoxycholic and cholic acids. Since unconjugated bile acids occur in bile only in rare cases and in only minute proportions [17], prior separation of unconjugated from conjugated bile acids is usually not necessary when analyzing bile acid in bile.

Determination of the glycine and taurine conjugation ratio depends on either determination of bile acid after the separation by TLC [18, 19], or lipophilic gel column chromatography [7, 20] or determination of amino acid moieties after hydrolysis of conjugated bile acid separated [21]. These methods suffer from disadvantages, i.e. from the possibility of incomplete recovery during the chromatographic procedure or incomplete hydrolysis of conjugates. The

present HPLC method permits fingerprinting of bile acid conjugates in their naturally occurring state. Large differences were found in bile acid composition of glycine and taurine conjugates (Table IV).

The advantages offered by HPLC of bile acids are the ease of preparation of the samples, i.e. circumventing hydrolysis of bile acid conjugates as well as preliminary separation of phospholipids and cholesterol which usually emerge with the solvent front and does not interfere with the determination of the later emerging bile acids, and the sensitivity of detection of bile acid conjugates comparable to GC with flame ionisation detection.

The bile acid concentration in icteric sera comes well within the range of detection. However, at the present state of development of the method, the determination of bile acid sulfates requires their preliminary separation [22]. Determination of unconjugated bile acid poses another difficulty, i.e. ultraviolet absorption of bile acid in the unconjugated form is much less than in the conjugated form, approximately one tenth. To enhance detectability, pre- [3, 9, 14] and post-labelling of bile acids have been reported. So far prelabelling of taurine-conjugated bile acid has been unsuccessful. Post-labelling of bile acids with 3α-hydroxysteroid dehydrogenase coupled with resazurin has been successful but the expense involved limits its routine use [23].

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