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DEVELOPMENT AND VALIDATION OF A METHOD FOR MEASURING THE GLYCINE AND TAURINE CONJUGATES OF BILE ACIDS IN BILE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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SUMMARY

We developed and validated a simple method for measuring the individual glycine and taurine conjugates of bile acids in bile by high-performance liquid chromatography with a C_{18} reversed-phase column using an isocratic solvent system of acidified methanol—potassium phosphate. Without preliminary derivatization or purification, complete separation of the ten major conjugated bile acids present in bile could be achieved in 65 min. Total bile acid concentrations were identical when measured enzymatically and by summing the individual bile acids determined by high-performance liquid chromatography. Bile acid composition determined by gas—liquid chromatography correlated with results by high-performance liquid chromatography. Finally, measurements of individual glycine and taurine conjugates in human bile and in mixtures of bile acid standards by high-performance liquid chromatography and thin-layer chromatography gave similar results. This high-performance liquid chromatographic system permits simultaneous quantification of total and individual bile acids and their glycine and taurine conjugates in bile.

INTRODUCTION

A thorough description of bile acid metabolism in man requires measurement of total bile acid concentration, bile acid composition, and the glycine and taurine conjugates of bile acids in bile. These analyses generally require extensive preparative techniques and separate methodologies. For example, total bile acid concentration is usually measured by enzymatic assay while bile acid composition is commonly determined by gas—liquid chromatography

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(GLC) after hydrolysis and chemical derivatization. Measurement of the individual glycine and taurine conjugates of bile acids in bile most frequently involves two separate procedures: initial separation by thin-layer chromatography (TLC) and subsequent quantitation by enzymatic assay. These techniques, while well established, require different equipment, utilize separate preparative procedures that can result in artifacts or incomplete recovery, and, taken together, can be quite time-consuming. Therefore, our aim was to develop and validate a single method for separating and quantifying the glycine and taurine conjugates of bile acids in bile using high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Reagents

HPLC-grade organic solvents were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

Bile acid standards

Conjugated bile acid standards were purchased from Calbiochem (La Jolla, CA, U.S.A.) and Steraloids (Wilton, NH, U.S.A.). Additional samples of taurocholic acid (TC), taurochenodeoxycholic acid (TCDC), taurodeoxycholic acid (TDC), and tauroursodeoxycholic acid (TU) were kindly provided by Dr. Martin C. Carey and glycoursodeoxycholic acid (GDC) was kindly provided by Dr. Alan F. Hofmann. All standards were > 97% pure by HPLC. [^{14}C]Chenodeoxycholic acid (50 mCi/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.) and was conjugated to glycine as previously described [2]. [^{14}C]Glycochenodeoxycholic acid (GCDC) was greater than 98% pure by TLC in appropriate solvents.

Chromatographic apparatus

We used a Hewlett-Packard 1084 liquid chromatograph (Hewlett-Packard, St. Paul, MN, U.S.A.) equipped with a Waters μBondapak C_{18} (octadecylsilane) column, 30 cm × 3.9 mm I.D., 10 μm particle size (Waters Assoc., Milford, MA, U.S.A.).

Detection was accomplished with a Schoeffel Spectroflow ultraviolet (UV) detector (Schoeffel Instrument, Westwood, NJ, U.S.A.) at 200 nm. Preliminary experiments by us assessing wavelengths of 195—210 nm had established optimum absorbance of the individual bile acids at 200 nm. Peak area was calculated by slope integration on a programmable Hewlett-Packard 79850B LC terminal.

Preparation of solvent and standards

The mobile phase was methanol—0.1 *M* monobasic potassium phosphate (60:40, v/v), adjusted to pH 4.5 with phosphoric acid. This mixture was then filtered through a 0.45-μm filter (type HA, Millipore, Bedford, MA, U.S.A.) and degassed. The solvent flow-rate was 1.5 ml/min.

To prepare a mixture of the ten conjugated bile acid standards, we dissolved each bile acid individually in methanol at a concentration of 1 mg/ml. A 1-ml

aliquot of each solution was then combined, evaporated under nitrogen, and redissolved in the mobile phase to a volume of 1 ml. A standard elution profile was obtained by injecting 50 μl of the standard mixture representing 50 μg of each individual bile acid.

Sample preparation

Human duodenal bile was obtained from 34 patients undergoing gallstone dissolution therapy [3] and from normal volunteers undergoing bile acid replacement studies [4]. We diluted the bile 1:10 (v/v) in isopropanol, heated it for 10 min at 75° C, centrifuged the sample at 450 *g*, and decanted the supernate. We then filtered the supernate through a Gelman Acrodisc 0.45-μm filter. Of this solution 1 ml was then evaporated under nitrogen and redissolved in the HPLC solvent for injection and subsequent analysis. The injection volume used was generally 100 μl. This volume was sometimes modified to rerun questionable peaks.

Estimation of recovery

[^{14}C]GCDC was used to estimate recovery of the sample [5]. To each ml of sample, we added 10,000 cpm of [^{14}C]GCDC. We collected 2-min fractions (3 ml) of the eluent, added 10 ml of Safety Solve Scintillant (Research Products International, Mount Prospect, IL, U.S.A.), and counted ^{14}C by liquid scintillation spectroscopy with the use of external standardization for quench correction.

Other analytical methods

Total bile acid concentration was measured by an automated modification of the method of Talalay [6]. Bile acid composition was determined by GLC on an AN-600 column (3%), as described elsewhere [7]. Glycine and taurine conjugated bile acids were measured by TLC as previously described [8].

Statistical analysis

Linear regression lines were calculated by the method of least squares and statistical analyses were done by unpaired Student's *t*-test.

RESULTS

Fig. 1A shows the elution profile of the ten major conjugated bile acids prepared as standards representing 50 μg of each individual bile acid. The retention times are given in Table I. The low pH (4.5) contributes to the optimum separation conditions [9], and peak symmetry is maintained by the high salt concentration of the solvent system. Chromatographic runs observed by us as well as other authors [10] show slight asymmetry in the taurolithocholate and glycolithocholate peaks; however, they are integratable by peak area. Free bile acids, cholesterol and phospholipids were not run in this system as their elution patterns were not of specific interest for these analyses.

Fig. 1B shows the separation of the ten major conjugated bile acids in a sample of human duodenal bile with an injection volume of 50 μl.

We established a standard curve for each of the ten conjugated bile acids by

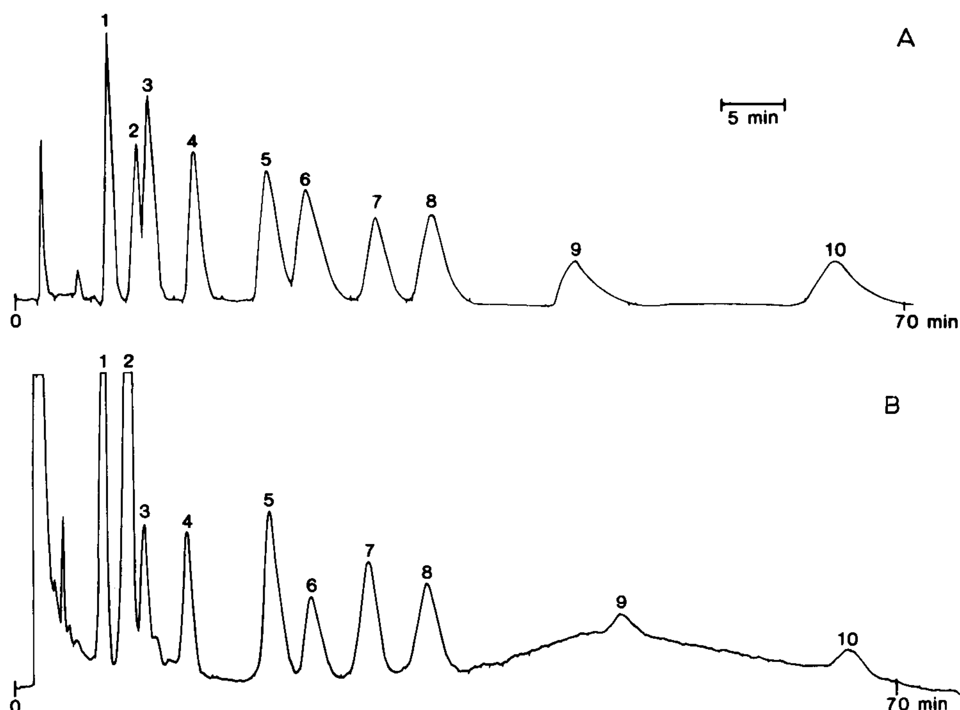

Fig. 1. (A) Elution profile of standard solution of pure bile acids. (B) Elution profile of bile acids of a sample of human duodenal bile. This profile is from bile taken from a patient after twelve months of tauroursodeoxycholic acid treatment resulting in the large percentage of glycoursodeoxycholic and tauroursodeoxycholic acid in the sample. Chromatographic conditions: solvent, methanol—0.1 *M* potassium dihydrogen phosphate, pH 4.5 (60:40); flow-rate, 1.5 ml/min; detector wavelength, 200 nm. For peak identification, see Table I.

TABLE I

RETENTION TIMES AND RELATIVE RETENTION TIMES OF THE TEN MAJOR CONJUGATED BILE ACIDS

Peak number	Bile acid	Retention time (min)	Retention time relative to GCDC
1	Tauroursodeoxycholic	7.4	0.26
2	Glycoursodeoxycholic	9.7	0.34
3	Taurocholic	10.6	0.37
4	Glycocholic	14.3	0.51
5	Taurochenodeoxycholic	19.9	0.71
6	Taurodeoxycholic	22.8	0.81
7	Glycochenodeoxycholic	28.2	1.0
8	Glycodeoxycholic	32.7	1.2
9	Taurolithocholic	44.6	1.6
10	Glycolithocholic	64.6	2.3

plotting concentration injected versus eluted peak area, and obtained linearity from 0.02 to 0.40 μmol. This range compares favorably with the findings of other authors [11]. Correlation coefficients (*r* values) of linear regression for the taurine conjugates ranged from 0.973 to 0.999 ($P < 0.001$) and from 0.997

to 0.999 ($P < 0.001$) for the glycine conjugates. As found by other authors [11], each standard curve was linear, although the slopes differed slightly for each bile acid (Fig. 2).

Total bile acid concentrations in bile (Fig. 3) were virtually identical when measured enzymatically by the 3α-steroid dehydrogenase method [6] and by summing the quantities of individual bile acids determined by eluted peak areas on HPLC ($r = 0.99$; $P < 0.001$). Repeat determination by HPLC of total bile acid concentrations on four separate samples of duodenal bile varied by only 3 ± 1.1% (mean ± S.D.). The four samples included total bile acid concentrations of 44, 70, 106, and 189 μmol/ml showing assay reproducibility within a reasonably broad concentration range.

Bile acid composition of bile determined by GLC (Fig. 4) correlated closely ($r = 0.96$; $P < 0.001$) with results of measurements by HPLC.

A highly significant correlation ($r = 0.96$; $P < 0.001$) existed between measurements by HPLC and TLC for individual glycine and taurine conjugates in human bile (Fig. 5).

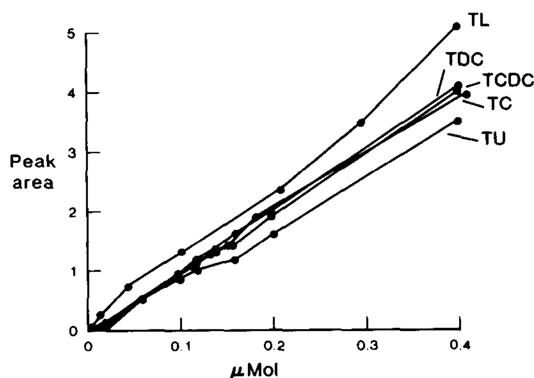

Fig. 2. Relationship of the concentrations of bile acid standards to the peak integrated areas for the taurine bile acid conjugates. Each point represents results for the mean of three determinations; these varied by only 3 ± 1.0% (mean ± S.D.). The same profile and statistics were demonstrated by the glycine bile acid conjugates ($r = 0.973 - 0.999$; $P < 0.001$). For abbreviations of the bile acids, see Table II.

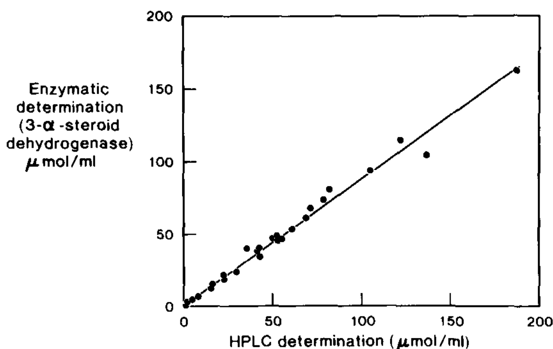

Fig. 3. Concentrations of total bile acids in samples of human duodenal bile obtained from patients with gallstones. The concentration of total bile acid in each of 26 duodenal bile samples was measured enzymatically and by summing the individual bile acid concentrations determined by HPLC ($r = 0.99$; $P < 0.001$).

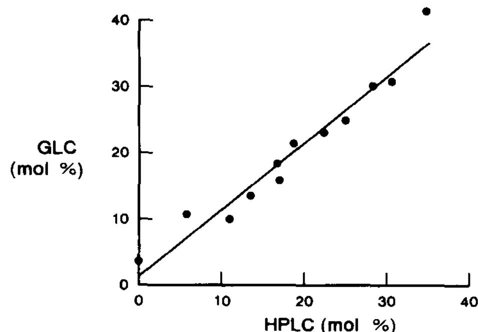

Fig. 4. Bile acid composition of samples of human duodenal bile obtained from normal volunteers undergoing bile acid replacement studies [4]. The bile acid composition in each of ten individual duodenal bile samples was determined separately by HPLC and GLC. This profile shows the correlation between HPLC and GLC for taurodeoxycholic and glycodeoxycholic acid (r = 0.97; $P < 0.001$). The other four bile acid conjugates also showed excellent correlation (r = 0.96; $P < 0.001$).

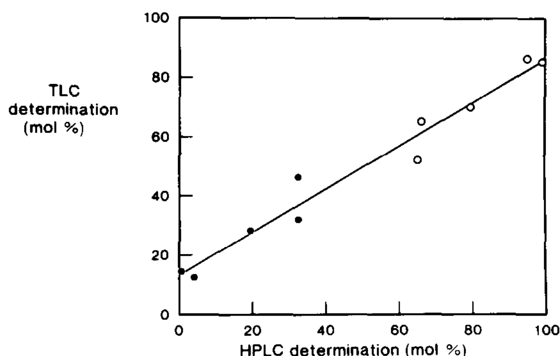

Fig. 5. The amount of individual conjugates of glycine and taurine in samples of human duodenal bile obtained from normal volunteers undergoing bile acid replacement studies [4]. The amount of individual bile acids conjugated to glycine and taurine in each of five samples of duodenal bile was determined by TLC and HPLC. Each point represents the percent of glycine or taurine conjugated bile acids in the bile samples. (○) Glycine conjugates; (●) taurine conjugates (r = 0.96; $P < 0.001$).

TABLE II

MEAN RECOVERY FOR EACH OF THE MAJOR CONJUGATED BILE ACID

Bile acid	Recovery (%)	Bile acid	Recovery (%)
Tauroursodeoxycholic (TU)	86	Glycoursodeoxycholic (GU)	85
Taurocholic (TC)	85	Glycocholic (GC)	88
Taurochenodeoxycholic (TCDC)	91	Glycochenodeoxycholic (GCDC)	89
Taurodeoxycholic (TDC)	85	Glycodeoxycholic (GDC)	90
Taurolithocholic (TL)	85	Glycolithocholic (GL)	94

Mean ± S.D. = 87.2 ± 2.5%

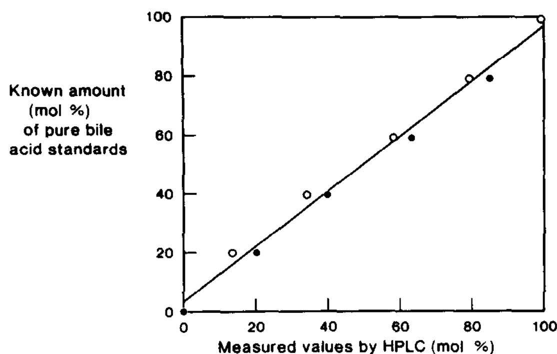

Fig. 6. The amount of individul conjugates of glycine and taurine in each of five mixtures of pure bile acid standards was measured separately and compared to the amount known to be present in each sample. Each mixture was prepared in physiologic proportions of individual bile acids (i.e., 40% cholic, 35% cheno, 15% deoxy, 5% urso, 5% litho). In addition, the mixtures were prepared so that the molar percentages of glycine and taurine, when summed, would equal 100%; for example, 60% glycine plus 40% taurine = 100%. Each point represents the percent of glycine or taurine conjugated bile acids in mixtures of bile and standards. (○) Glycine conjugates; (•) taurine conjugates ($r = 0.99; P < 0.001$).

When mixtures of pure bile acid standards enriched to varying degrees with glycine or taurine conjugates were assessed by HPLC, the measured values correlated significantly ($r = 0.99$; $P < 0.001$) with the known values (Fig. 6).

The mean recovery of [^{14}C]GCDC in all of the validation studies was 91 ± 4.7% (mean ± S.D.). Recovery experiments were also carried out by preparing a mixture of known amounts of each bile acid conjugate added in approximate physiologic proportions and running the mixture three times.

The mean recoveries for each of the bile acids are given in Table II. Mean recovery for all the bile acids was 87.2 ± 2.5% (mean ± S.D.).

DISCUSSION

We have developed a rapid, reproducible, accurate and sensitive technique using HPLC which permits the simultaneous determination of total bile acid concentration, bile acid composition and molar percentages of the individual bile acids conjugated to glycine and taurine in bile. Furthermore, using both samples of human bile and mixtures of bile acid standards, we have systematically compared this technique to the enzymatic assay of total bile acids, the GLC measurement of bile acid composition, and the TLC determination of the glycine and taurine conjugates of biliary bile acids.

Others have described HPLC systems for the separation of biliary bile acids. Armstrong and Carey [9] used HPLC to examine quantitatively the hydrophobic/hydrophilic properties of bile salts in monomeric bile salt and mixed bile salt–cholesterol micellar solutions; they did not, however, study samples of human gallbladder or duodenal bile [9]. Nakayama and Nakagaki [12] reported the successful separation of the glycine and taurine conjugates of the commonly occurring bile acids in bile, as did Bloch and Watkins [11]; neither group, however, provided quantitative data on the actual amount of the individual bile acids present in bile. Rubin and Van Berge-Henegouwen [13]

employed HPLC using a radial compression system for separation of biliary bile acids; they provided a partial validation of the method by comparing the quantification of the primary bile acids, chenodeoxycholic and cholic acids, in bile by HPLC and GLC.

Although others have described HPLC systems for the separation and, to a lesser extent, the quantitation of biliary bile acids, we believe our technique is at least as efficient as these published methods and may have several advantages over these techniques. First, methanol—potassium phosphate is superior to acetonitrile as a solvent system because the solubility of bile acids is greater in methanol [9]; this is an advantage which is particularly important for the quantitation of the poorly soluble lithocholic acid conjugates, which have frequently been difficult to clearly separate. We should add that, in our experience, glycolithocholate has a short shelf life when dissolved in methanol and begins to precipitate within one to three weeks. For this reason, it is important that standard solutions be prepared freshly prior to injection.

Second, the isocratic feature of the system enhances the simplicity and reproducibility of the technique, at least in our experience and with the equipment available to us. Indeed, attempts by us at gradient elution were abandoned because of baseline instability. Third, no derivatization or separation of phospholipid and cholesterol is needed with our technique and, except for deproteinization, little preparation of the specimen is necessary. We have not assessed other methods of sample preparation (i.e., Sep-Pak) which might make sample preparation even simpler or faster [14]. With our method, ten samples can easily be run in 12 h with the use of an automatic injector, once the technique has been established. Fourth, our recovery using [^{14}C] GCDC as an internal standard is better than has been reported for estriol [15, 16], testosterone [10], dexamethasone [13], commonly used internal standards which may actually overlap with the bile acids of interest. Finally and perhaps most importantly, others have not validated the accuracy of HPLC for bile acid analyses by comparing it to the conventional methods used to measure total bile acid concentration, bile acid composition, and the glycine and taurine conjugates of bile acids in bile. This latter determination is of increasing importance since the possibility exists that reversing the normal predominance of glycine conjugates by taurine supplementation may enhance the efficacy of gallstone dissolution.

The only limitation of this technique is that it does not measure unconjugated bile acids. Since these rarely occur in human bile, this disadvantage has little practical significance. Our systematic comparison of this HPLC technique to conventional methodology establishes the validity of using HPLC alone for analyses necessary for a complete description of biliary bile acids.

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