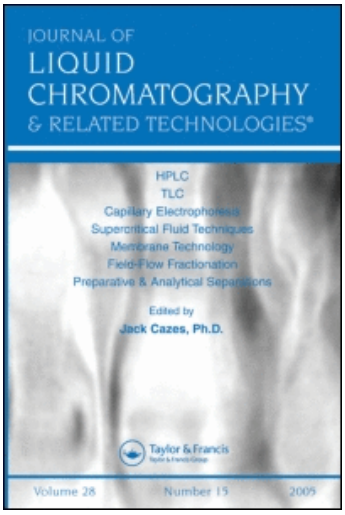


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# Separation and Determination of Bile Acids in Human Bile by High-Performance Liquid Chromatography

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# SEPARATION AND DETERMINATION OF BILE ACIDS IN HUMAN BILE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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## ABSTRACT

A method for simultaneous determination of major bile acids in human bile is described. The unconjugated, glycine- and taurine-conjugated bile acids are extracted with Sep-pak $C_{18}$ and separated into groups by ion-exchange chromatography on a lipophilic gel. Subsequently, resolution of each group into ursodeoxycholate, cholate, chenodeoxycholate, deoxycholate and lithocholate is attained into two stages by high-performance liquid chromatography on a Radial-PAK A column. First, 0.3% ammonium phosphate (pH 7.7)/acetonitrile (19:8, v/v) is used for separation of the latter three as a mobile phase. Ursodeoxycholate and cholate are efficiently separated in 0.3% ammonium phosphate (pH 7.7)/acetonitrile (23:8, v/v). The present method is applicable to quantitation of bile acids in human bile with satisfactory accuracy and precision.

## INTRODUCTION

Considerable attention has been recently directed to the metabolism of bile acids in patients with hepatobiliary diseases (1-3). In a preceding paper of this series we reported a method

for simultaneous determination of unconjugated and conjugated bile acids in human bile which involves the group separation followed by resolution of bile acids of each group by high-performance liquid chromatography (HPLC) on a μBondapak $C_{18}$ column using 0.3% ammonium carbonate (pH 7.8)/acetonitrile as a mobile phase (4,5). However, difficulties were encountered with the resolution of ursodeoxycholate and cholate, and adjustment of a mobile phase to an appropriate pH. In order to overcome these problems, development of a more suitable procedure was undertaken.

The present paper describes a new method for determination of bile acids in human bile involving clean-up with a Sep-pak $C_{18}$ cartridge, group separation by ion-exchange chromatography on a lipophilic gel and subsequent resolution of each group into five bile acids by HPLC on a Radial-PAK A column using 0.3% ammonium phosphate/acetonitrile as a mobile phase.



## MATERIALS AND METHODS

### Instrument

The apparatus used was a Model 6000A solvent delivery system (Waters Assoc., Milford, Mass.) equipped with a Model Uvidec-100 II ultraviolet (UV) detector (Japan Spectroscopic Co., Tokyo) monitoring absorbance at 205 nm, a sample loop injector with an effective volume of 2 ml, and a Model RCM-100 cartridge holder and comparison chamber (Waters Assoc.). A Radial-PAK A column (10 cm x 8 mm i.d.) was used under ambient conditions.

### Materials

The unconjugated bile acids were purchased from Sigma Chemical Co. (St. Louis, Mo.) and purified prior to use. The glyco- and tauro-conjugates were synthesized by the p-nitrophenyl ester method in these laboratories. Estriol and 3α-hydroxysteroid oxi-

doreductase (EC 1.1.1.50) (3α-HSD) were kindly donated by Teikoku Hormone Mfg. Co. (Tokyo) and Daiichi Pure Chemicals Co. (Tokyo), respectively. All the reagents employed were of analytical grade. Solvents were purified by distillation prior to use. Sephadex LH-20 was supplied by Pharmacia Fine Chemicals (Uppsala). Piperidino-hydroxypropyl Sephadex LH-20 (PHP-LH-20) and eluents used for ion-exchange gel chromatography were prepared in the manner previously reported (5). A Sep-pak $C_{18}$ cartridge (Waters Assoc.) was washed successively with methanol (5 ml), acetonitrile (5 ml), and water (10 ml) prior to use.



## Clean-up of bile acids with Sep-pak $C_{18}$ cartridge

The test samples were prepared by dissolving 5 μg of chenodeoxycholate, glycochenodeoxycholate or taurochenodeoxycholate in bile acid-free human bile (100 μl) which was obtained by treatment with charcoal. A bile specimen was diluted with McIlvaine buffer (pH 3.0-5.0) or 0.5 M phosphate buffer (pH 6.0-7.8) (1 ml) and passed through a Sep-pak $C_{18}$ cartridge. After washing with water (2 ml) and 1.5% ethanol (2 ml), the bile acid fraction was eluted with 90% ethanol (4 ml). An aliquot of the effluent was evaporated in vacuo below 40°C and the residue was subjected to the enzymatic assay of bile acids using 3α-HSD (6,7).

## Procedure for determination of bile acids in human bile

A bile sample (10 μl for gallbladder bile and 100 μl for hepatic bile) was diluted with 0.5 M phosphate buffer (pH 7.0) and passed through a Sep-pak $C_{18}$ cartridge. After washing with water (2 ml) and 1.5% ethanol (2 ml), the bile acid fraction was eluted with 90% ethanol (4 ml). The eluate was applied to a column (36 mm x 6 mm i.d.) of PHP-LH-20 acetate form (200 mg). After removal of neutral compounds with 90% ethanol (6 ml), unconjugated, glycine-

and taurine-conjugated bile acids were stepwisely eluted with 0.1 M acetic acid in 90% ethanol (8 ml), 0.2 M formic acid in 90% ethanol (8 ml), and 0.3 M acetic acid-potassium acetate in 90% ethanol (pH 6.5, 7 ml). To the unconjugated and glycine-conjugated fractions were added 200 ng and 2 µg of estriol as an internal standard, respectively and the solution was evaporated in vacuo below 40°C. The residue was redissolved in ethanol (100-200 µl), a 5-30 µl aliquot of which was used for HPLC. In the case of tauro-conjugates, the eluate was redissolved in water (1 ml), passed through a Sep-pak $C_{18}$ cartridge, and washed with water (2 ml) for removal of inorganic salts. The tauro-conjugate fraction was eluted with 90% ethanol (4 ml), added with estriol (600 ng) and then subjected to HPLC.



## Recovery test for bile acids added to human bile

The test samples were prepared by dissolving 4, 20, or 100 µg each of unconjugated and conjugated cholate, chenodeoxycholate and deoxycholate in human gallbladder bile (10 µl). A bile specimen was diluted with 0.5 M phosphate buffer (1 ml) and subjected to clean-up with a Sep-pak $C_{18}$ cartridge and then to the group separation on PHP-LH-20 followed by determination by HPLC in the manner described above.

## RESULTS AND DISCUSSION

An initial effort was directed to the separation of bile acids by means of HPLC. In the previous paper we reported HPLC of bile acids on a µBondapak $C_{18}$ column using 0.3% ammonium carbonate (pH 7.8)/acetonitrile as a mobile phase (4,5). Several attempts have also been made to separate bile acids on ODS columns with acidic mobile phases (8-10). Under these conditions, however, the complete separation was tedious due to prolonged retention

times of unconjugated bile acids. On the other hand, when a neutral mobile phase was used, there was observed little difference in the k' value among the unconjugated, glycine- and taurine-conjugated bile acids (4). Accordingly, the mobile phase adjusted to pH 7.0-7.8 was employed in the present study.

Various combinations of buffer solution and organic solvent were tested to choose the suitable mobile phase on Radial-PAK A, that is a cartridge-type ODS column. The use of an aqueous ammonium phosphate/acetonitrile system appeared to be promising without exhibiting any significant leading and tailing. The chromatographic behaviors of bile acids were initially investigated under these conditions. The k' values of unconjugated and conjugated cholate, chenodeoxycholate and deoxycholate were plotted against the pH value using 0.3% ammonium phosphate/acetonitrile (19:8) as a mobile phase (Fig. 1a). The close similarity in chromatographic behaviors was observed between the glyco- and tauro-conjugate. In contrast, the k' values of unconjugated bile acids decreased along with raising pH and were smaller than those of the corresponding tauro-conjugates at pH 7.8. Elution order of bile acids having a different number of the hydroxyl group on the steroid nucleus was found to be identical with that hitherto reported (4,8-10). The effect of salt concentration on the retention value was then examined with ammonium phosphate (pH 7.0)/acetonitrile (19:8) as a mobile phase (Fig. 1b). The k' value raised with increasing salt concentration of mobile phase up to 0.3%, and this phenomenon was observed more distinctly with tauro-conjugates.

On the basis of these data 0.1% ammonium phosphate (pH 7.0)/acetonitrile (19:8) or 0.3% ammonium phosphate (pH 7.7)/acetonitrile (19:8) was chosen as a suitable mobile phase. The ursodeoxycholate group was efficiently separated from the cholate ($R > 1.4$) when 0.3% ammonium phosphate (pH 7.7)/acetonitrile (23:8) was employed. The k' values of bile acids observed with three solvent systems are listed in Table 1. The detection limits of glycine-, taurine-conjugated and unconjugated bile acids by the

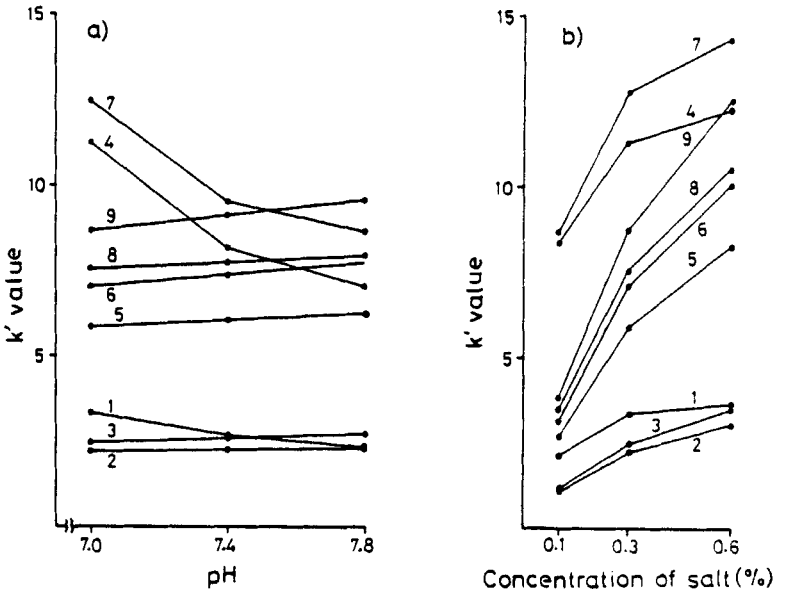


FIGURE 1 Effects of pH and Salt Concentration on k' Values and Separation of Cholates, Chenodeoxycholates and Deoxycholates
1: cholate, 2: glycocholate, 3: taurocholate, 4: chenodeoxycholate, 5: glycochenodeoxycholate, 6: taurochenodeoxycholate, 7: deoxycholate, 8: glycodeoxycholate, 9: taurodeoxycholate. Conditions: column, Radial-PAK A; mobile phase, aq. ammonium phosphate/acetonitrile (19:8, v/v), a) salt concentration 0.3%, b) pH 7.0, 2.0 ml/min; detection, 205 nm.

UV monitoring at 205 nm were 50, 150 and 500 ng, respectively. The calibration curve was constructed by plotting the ratio of peak area of each bile acid to that of estriol against the weight of bile acid.

Examination was then made on the utility of a Sep-pak $C_{18}$ cartridge for clean-up of bile acids in biological fluids. For this purpose a porous polystyrene resin, Amberlite XAD-2, has been widely used. This method, however, is not always favorable

TABLE 1

Capacity Ratio of Bile Acids

| | Free | | | Glyco-conjugate | | | Tauro-conjugate | | |
|---|---|---|---|---|---|---|---|---|---|
| | a | b | c | a | b | c | a | b | c |
| Ursodeoxycholate | 2.3 | 6.8 | — | 2.1 | 6.1 | — | 2.4 | 7.0 | — |
| Cholate | 2.7 | 8.0 | — | 2.5 | 7.4 | — | 2.8 | 8.3 | — |
| Chenodeoxycholate | 7.5 | — | — | 6.8 | — | — | 8.1 | — | — |
| Deoxycholate | 9.2 | — | 4.9 | 8.6 | — | 4.6 | 9.9 | — | 5.3 |
| Lithocholate | — | — | 14.1 | — | — | 13.3 | — | — | 14.9 |
| Estriol (IS) | 5.2 | 9.4 | 7.1 | 5.2 | 9.4 | 7.1 | 5.2 | 9.4 | 7.1 |

Mobile phase: 0.3% ammonium phosphate (pH 7.7)/acetonitrile, a) 19:8, b) 23:8, c) 16:8, 2 ml/min. $t_0$=0.9 min.



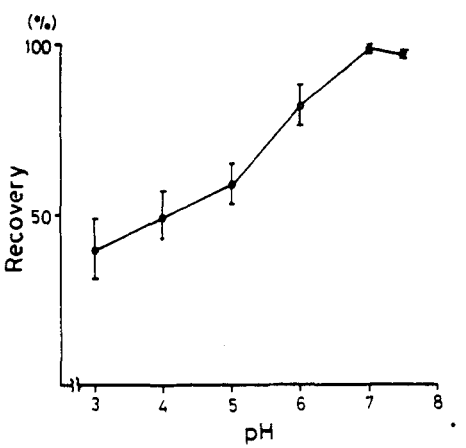


FIGURE 2 Effect of pH on Recovery of Chenodeoxycholate added to Human Bile in Clean-Up by Sep-Pak $C_{18}$
Five micrograms of chenodeoxycholate was added to bile acid-free human bile (100 µl) and applied to a Sep-pak $C_{18}$ cartridge. An initial 8 ml of the effluent was subjected to enzymatic determination with 3α-HSD.

in respect of reproducibility and simplicity. A synthetic mixture of 5 μg each of chenodeoxycholate, its glyco- and tauro-conjugate was added to bile acid-free human bile, diluted with a buffer solution (pH 3.0-7.8) and applied to the cartridge. After elimination of co-existing inorganic salts and other polar substances by washing with water and 1.5% ethanol, bile acids were eluted with 90% ethanol and then determined by the enzymatic method using 3α-HSD (6,7). As shown in Fig. 2, the recovery rate of bile acids added to human bile increased along with raising pH of the buffer solution and was almost quantitative at pH 7.0. The elution patterns obtained at pH 7.0 for uncojugated and conjugated chenodeoxycholates are illustrated in Fig. 3. Bile acids were recovered at a rate of more than 90% in an initial 1 ml effluent, and a similar elution pattern was observed among the three. It



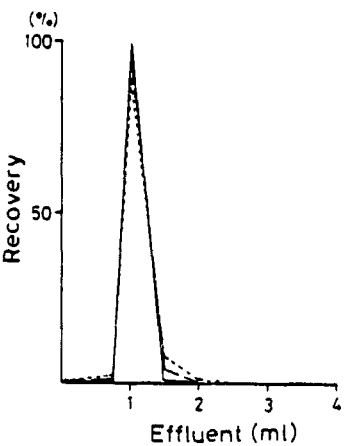


FIGURE 3 Recovery of Unconjugated and Conjugated Chenodeoxycholates added to Human Bile in Clean-Up by Sep-Pak $C_{18}$ A mixture of 5 μg each of chenodeoxycholate (——), glycochenodeoxycholate (— —) and taurochenodeoxycholate (---) was added to bile acid-free human bile (100 μl), diluted with phosphate buffer (pH 7.0, 1 ml) and applied to a Sep-pak $C_{18}$ cartridge. Each fraction (0.5 ml) was subjected to enzymatic determination with 3α-HSD.

is evident from the data that the present method with a Sep-pak $C_{18}$ cartridge is much more efficient for separation and purification of bile acids in biological fluids than the method with Amberlite XAD-2 resin.

Applying the standard procedure to human gallbladder bile, glycine- and taurine-conjugated bile acids were determined with standard deviations of 1.6-2.7% (Table 2). A known amount of representative unconjugated and conjugated bile acids was added to human bile, and their recovery rates were estimated. As listed in Table 3, each bile acid was recovered at a rate of more than 90%.

The separation of conjugated bile acids in human bile which has been previously processed in the manner described above is illustrated in Fig. 4. The chromatogram with a stable base line and without any interferences is favorable for determination of bile acids in biological fluids. Moreover, the present method is much more suitable for the resolution of ursodeoxycholate and cholate than the known methods.

Simultaneous determination of glycine- and taurine-conjugated bile acids in bile was carried out for forty patients with hepato-



TABLE 2

Reproducibility of the Proposed Method for Determination of Bile Acids in Human Bile

| Bile acid | Found ± S.D. (μg/0.01 ml) |
|---|---|
| Glycocholate | 55.4 ± 0.9 |
| Glycochenodeoxycholate | 38.3 ± 0.8 |
| Glycodeoxycholate | 31.5 ± 0.7 |
| Taurocholate | 31.5 ± 0.7 |
| Taurochenodeoxycholate | 28.6 ± 0.7 |
| Taurodeoxycholate | 18.3 ± 0.5 |

n=8

TABLE 3

Recovery of Free and Conjugated Bile Acids added to Human Bile

| Bile acid | Bile | Added | Expected | Found | Recovery |
|---|---|---|---|---|---|
| | | μg/0.01 ml | | | ± S.D.(%) |
| Cholate | 0 | 4.0 | 4.0 | 3.6 | 90.8 ± 6.3 |
| | | 20.0 | 20.0 | 19.4 | 96.9 ± 4.4 |
| Chenodeoxycholate | 0 | 4.0 | 4.0 | 3.6 | 90.4 ± 6.2 |
| | | 20.0 | 20.0 | 19.5 | 97.7 ± 4.2 |
| Deoxycholate | 0 | 4.0 | 4.0 | 3.6 | 90.4 ± 5.3 |
| | | 20.0 | 20.0 | 19.2 | 96.0 ± 3.7 |
| Glycocholate | 58.6 | 20.0 | 78.6 | 74.1 | 94.2 ± 3.6 |
| | | 100.0 | 158.6 | 146.9 | 92.6 ± 3.1 |
| Glycochenodeoxycholate | 44.0 | 20.0 | 64.0 | 61.6 | 96.2 ± 2.0 |
| | | 100.0 | 144.0 | 140.0 | 97.2 ± 3.0 |
| Glycodeoxycholate | 39.8 | 20.0 | 59.8 | 57.2 | 95.7 ± 4.3 |
| | | 100.0 | 139.8 | 130.6 | 93.4 ± 3.1 |
| Taurocholate | 39.8 | 20.0 | 59.8 | 58.4 | 97.6 ± 0.9 |
| Taurochenodeoxycholate | 35.8 | 20.0 | 55.8 | 54.3 | 97.3 ± 0.7 |
| Taurodeoxycholate | 26.1 | 20.0 | 46.1 | 45.7 | 99.3 ± 0.5 |

n=6

biliary diseases. The results obtained are collected in Table 4. In all the cases lithocholate and ursodeoxycholate were found in small amounts, corresponding to 1% and 1.7-3.6% of total bile acids, respectively. The cholesterol stone patient group shows somewhat lower G/T values (ratio of glyco- to tauro-conjugate) than other groups. It is also of particular interest that there is seen a

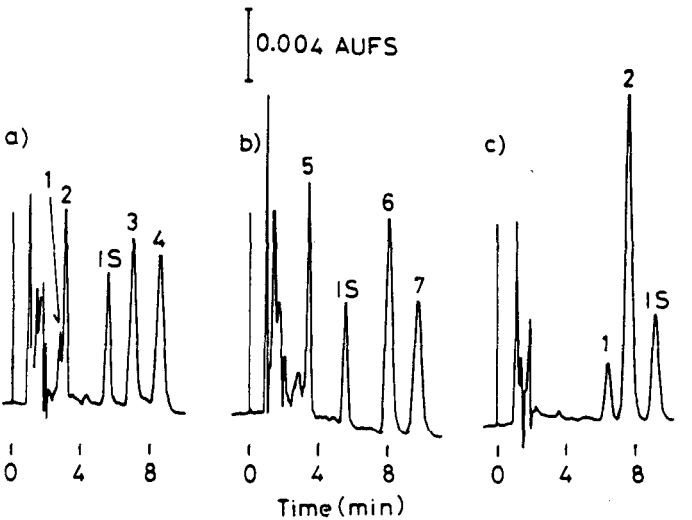


FIGURE 4 Separation of Conjugated Bile Acids in Human Bile by HPLC
1: glycoursodeoxycholate, 2: glycocholate, 3: glycochenodeoxycholate, 4: glycodeoxycholate, 5: taurocholate, 6: taurochenodeoxycholate, 7: taurodeoxycholate, IS: estriol. Conditions: column, Radial-PAK A; mobile phase, 0.3% ammonium phosphate, a) and b) 19:8 (v/v), c) 23:8 (v/v), 2.0 ml/min; detection, 205 nm.

marked difference in the ratio of cholate to chenodeoxycholate between the glyco- and tauro-conjugate for cholesterol stone patients but not for other groups.

The availability of an excellent method for separation and determination of bile acids without prior hydrolysis may provide more precise knowledge on the metabolic profile of bile acids in patients with hepatobiliary diseases.

TABLE 4

Per Cent Composition of Bile Acids in Bile of Patients with Hepatobiliary Diseases

| Bile | CA | | CDCA | | DCA | | LCA | | UDCA | |
|---|---|---|---|---|---|---|---|---|---|---|
| | G | T | G | T | G | T | G | T | G | T |
| A (9) | 32.0 | 10.2 | 25.5 | 10.8 | 15.3 | 4.9 | 0.8 | n.d. | 1.8 | n.d. |
| B (10) | 32.3 | 7.5 | 31.6 | 7.4 | 15.2 | 2.1 | 0.5 | n.d. | 3.6 | n.d. |
| C (5) | 29.6 | 4.3 | 34.6 | 7.3 | 12.7 | 5.7 | 1.1 | n.d. | 3.1 | n.d. |
| C (6) | 33.7 | 7.4 | 28.7 | 7.0 | 14.1 | 3.6 | 1.4 | n.d. | 1.9 | n.d. |
| D (10) | 27.9 | 5.8 | 30.5 | 7.1 | 22.8 | 3.2 | 1.1 | n.d. | 1.7 | n.d. |

CA: cholate, CDCA: chenodeoxycholate, DCA: deoxycholate, LCA:lithocholate, UDCA: ursodeoxycholate, G: glyco-conjugate, T: tauro-conjugate. A: cholesterol stone, B: mixed stone, C: bilirubin stone, D: pigment stone, E: stomach cancer. Number of patients is given in parentheses. n.d.: not detectable.



## ACKNOWLEDGEMENTS

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