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BABA, Shigeaki

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# A MEASUREMENT OF INDIVIDUAL BILE ACIDS IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR CLINICAL DIAGNOSTIC INFORMATION OF HEPATOBILIARY DISEASES

Shigeaki BABA, Rinzo UENOYAMA, Keiko SUMINOE,
Fumihiko TAKEDA, Shu HASEGAWA and Yasuro KAMENO

The Second Department of Internal Medicine
Kobe University School of Medicine

## INDEXING WORDS

serum bile acid; individual bile acid; 3α-hydroxysteroid dehydrogenase; high-performance liquid chromatography; free bile acid; glycine-conjugated bile acid; taurine-conjugated bile acid

## SYNOPSIS

A highly sensitive method for the simultaneous meausrement of individual 3α-hydroxy bile acids in serum using high-performance liquid chromatography combined with enzymatic fluorometric measurement is discribed.

The satisfactory separation of thirteen kinds of bile acid, ursodeoxycholic acid and glycine- or taurine-conjugated cholic acid, chenodeoxycholic acid, deoxycholic acid and lithocholic acid, was attained at two stages on a μ-Bondapak phenyl column. Firstly, 0.3% ammonium carbonate-acetonitrile (14:4) was used as a mobil phase, following free and conjugated lithocholic acids were resolved by chromatography in 0.3% ammonium carbonate-acetonitrile (8:4). The reagent used was 3α-hydroxysteroid dehydrogenase.

The sensitivity limit of each bile acid as determined by this method was in the range of 6.3 - 31.0 ng on the column. The inter-assay coefficiency values were in the range of 0.68 to 10.0% and those of intra-assay 0.70 to 9.09%. The recovery rate of each bile acid added to human serum ranged from 81.7 to 106.6%.

This new method enables the measurement of thirteen kinds of bile acids in 1 ml of a healthy serum, and provides useful diagnostic information in cases of hepatobiliary disease.

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Author's name in Japanese: 馬場 茂明　上野山林造　住之江啓子　竹田 文彦　長谷川 修　亀野 靖郎

## INTRODUCTION

Bile acids are synthesized from cholesterol and conjugated with glycine or taurine in liver cells. Conjugated bile acids are excreted into the small intestine, where about 30% of them are deconjugated and converted into secondary bile acids by intestinal bacteria. Most of them, however, are reabsorbed mainly from terminal ileum by an active transport system, and then return to the liver through the potal vein. Namely the metabolic circulating system of bile acids forms a closed enterohepatic circulation. Thus, it is important to determine not only the total volume of serum bile acids, but also individual bile acids.

In this paper, we describe a new and simpler method of measuring individual 3$\alpha$-hydroxy bile acids in serum, using a high-performance liquid chromatography combined with enzymatic fluorometric method.

The first strong point of this study is an adequate separation of thirteen kinds of serum bile acids without any chemical modification or difficulties in preparation. The second point is that high sensitivity was obtained by combination of enzymatic fluorometric measurement, thus making it possible to measure the individual bile acids even in healthy human serum. The third point is the application of this method to provide useful clinical information in cases of hepatobiliary disease.

## MATERIALS AND METHODS

### Instrument

High-performance liquid chromatography was carried out by Waters Associates, Inc.(Mass), Type ALC-204. The FLORICHROM fluorescence detector (Varian Associates, Inc., California) was used, set at 350 nm for excitation and 460 nm for emission. Samples were analyzed on a $\mu$-Bondapak phenyl column ( 1ft. x ¼ in. I.D.) (Waters Associates, Inc., Mass). The flow diagram of this method is shown in Fig. 1.

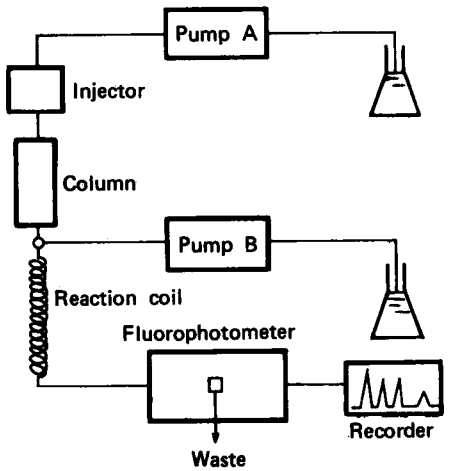


Fig. 1 Flow diagram of simultaneous measurement of bile acids by high-performance liquid chromatography combined with enzymatic fluorometric method.

Pump A : Eluent 1.0 ml/min.
Pump B : Reagent 1.0 ml/min.
Column : $\mu$-Bondapak phenyl ( 1 ft. x¼ in. I.D.),
Reacting coil : teflon tube (0.5mm I.D. x 10m ),
Fluorophotometer: EX = 350 nm, EM = 460 nm,
cell volume = 2.5 $\mu$l.

Materials:

Standard materials: Cholic acid (CA, Lot No. 126C-0075), chenodeoxycholic acid (CDCA, Lot No. 113C-3170), sodium salts of deoxycholic acid (DCA, Lot No. 96C-0474) and lithocholic acid (LCA, Lot No. 25C-0011) were purchased from Sigma Chemical Co. (St. Louis). Sodium salts of glycocholic acid (GCA, Lot No. 880062), glycochenodeoxycholic acid (GCDCA, Lot No. 860062), glycodeoxycholic acid (GDCA, Lot No. 860082), glycolithocholic acid (GLCA, Lot No. 860161), taurocholic acid (TCA, Lot No. 860181), taurochenodeoxycholic acid (TCDCA, Lot No. 860201), taurodeoxycholic acid (TDCA, Lot No. 860221) and taurolithocholic acid (TLCA, Lot No. 860251) were purchased from P-L Biochemicals Inc. (Milwaukee). Usrodeoxycholic acid (UDCA, Lot No. 90415) was offered by Tokyo Tanabe Pharmaceutical Co.. As an internal standard (I.S.), we used 5 $\beta$-pregnan- 3$\alpha$, 17$\alpha$, 20$\alpha$-triol (Lot No. 107C-0091, Sigma Chemical Co.). All standard materials were dissolved individually in methylalcohol (for liquid chromatography, Lot No. KLN 2214, Wako Pure Chemical Industries, Ltd., Osaka).

Eluent: Distilled water was passed through a $\mu$-Bondapak $C_{18}$ column (2ft. x ½ in. I.D.) (Waters Associates, Inc.). Ammonium carbonate was dissolved in this distilled water to make a 0.3% (W/V) ammonium carbonate solution. Acetonitrile was percolated through a millipore filter (FH 0.5 $\mu$m, Lot No. CBC 44009A) and degassed with supersonic wave. The 0.3% ammonium carbonate solution was mixed with the acetonitrile. The volume ratio (v/v) of Eluent I (pH 8.70) was 14:4 (0.3% ammonium carbonate solution: acetonitrile), and that of Eluent II (pH 8.68) 8:4.

Reagent : As reagent, we used Sterognost-3 $\alpha$ (Lot No. 58 and 59, Nyegaad Co., Oslo, Norway) enzymatic kits. One vial of Sterognost-3$\alpha$ contains: 1) 1.75 I.U. of 3$\alpha$- hydroxysteroid dehydrogenase (3$\alpha$-HSD, EC. 1.1.1.50, Nyegaad Co.). 1 I.U. is the amount of enzyme that will convert 1 $\mu$mol of deoxycholic acid per minute at 25° C and pH = 9.5. 2) 10 $\mu$mol of NAD (Sigma Chemical Co.). 3) 33 $\mu$mol of EDTA. 4) 0.5 $\mu$mol of dithiothreitol. 5) 1.3 $\mu$mol of sodium pyrophosphate. 6) 2 $\mu$mol of sucrose. The content of one vial of Sterognost-3$\alpha$ is reconstituted in 50 ml of 0.1 mol/l (v/v) hydrazine hydrate, pH = 9.5.

Preparation of serum materials: Human serum was collected from peripheral veins of healthy volunteers and patients with hepatobiliary diseases after overnight fasting. 1 ml of serum was diluted with 9 ml of 0.9% NaCl containing 0.1 N of NaOH. This solution was applied to a column of Amberlite XAD-2 resin. After the serum solution was applied, the column was washed with 10 ml of ethylalcohol at the rate of 5 drops a minute. Then the elute was evaporated to dryness and the residue was redissolved in 0.25 to 1 ml of methylalcohol containing 1250 ng/ml of I.S.. The volume used for resolution of the residue varied according to the expected concentration of bile acids in serum sample.

Measurement procedure: The flow rate of the eluent was 1 ml a minute (600 PSI) as was that of the reagent (420-520 PSI). 100 $\mu$l of samples were applied to $\mu$-Bondapak phenyl column from the injector with microsyringe (Hamillton Co., Ltd., Nevada). All components analyzed on the column were carried into the mixing coil (made of teflon, 10m× 0.5mm I.D.), where 3$\alpha$-hydroxysteroids were converted to their corresponding 3-ketosteroids and NAD was converted into NADH in the presence of the reagent. This reaction took place at room temperature and lasted for about 1.0 minute. The NADH produced by this reaction was simultaneously measured by a fluorophotometer connected to the mixing coil. In order to shorten the retention time of three kinds of lithocholic acids, mobile phase was changed from Eluent I to Eluent II at 48 minutes after the injection. The chart speed was 2.5 mm a minute.

Standard curves : Four kinds of standard sample mixture were made. The first mixture contained 125 ng of UDCA, CA, CDCA, DCA and LCA (free bile acid), 187.5 ng of GCA, GCDCA, GDCA and GLCA (glycine-conjugated bile acid) and 250 ng of TCA, TCDCA, TDCA and TLCA (taurine-conjugated bile acid). The second mixture contained 62.5 ng of free bile acid, 93.75 ng of glycine-conjugated bile acid and 125 ng of taurine-conjugated bile acid. The third mixture contained 31.25 ng of free bile acid, 46.875 ng of glycine-conjugated bile acid and 62.5 ng of taurine-conjugated bile acid. The fourth contained 15.625 ng of free bile acid, 23.4875 ng of glycine-conjugated bile acid and 31.25 ng of turine-conjugated bile acid. These mixtures were treated with Amberlite XAD-2 resin. The standard curves were constructed by plotting the peak height and the amount of each bile acid.

Recovery experiments: Recovery experiments were performed by adding the four kinds of standard sample mixture to the serum of healthy men and patients with hapatobiliary disease.

Reproducibility : The same samples were subjected to inter-assay and intra-assay.

Subjects: The subjects studied are listed in Table 4. The control group consisted of asymptomatic, clinically and biochemically normal volunteers, who were not receiving any drugs. The patient's diagnosis were based on clinical, biochemical and histological findings. The diagnosis of chronic hepatitis and liver cirrhosis was based on liver biopsy findings. Among patients with intra-hepatic cholestasis, those who did not show typical findings of acute or chronic hepatitis and demonstrated long-term cholestasis without any findings of obstruction in choledochus, were selected in accordance with the criteria of intra-hepatic cholestasis as set forth by the Research Grant for Intractable Diseases Division, Public Health Bureau, Japanese Ministry of Health and Welfare. Among patients with extra-hepatic cholestasis, six cases of cholangioma and four cases of gallstone were included. Regarding patients with liver cirrhosis, we distinguished between non-compensative and compensative stages in our clinical findings. Serum from these subjects was frozen (-20° C) until analyzed for bile acids.

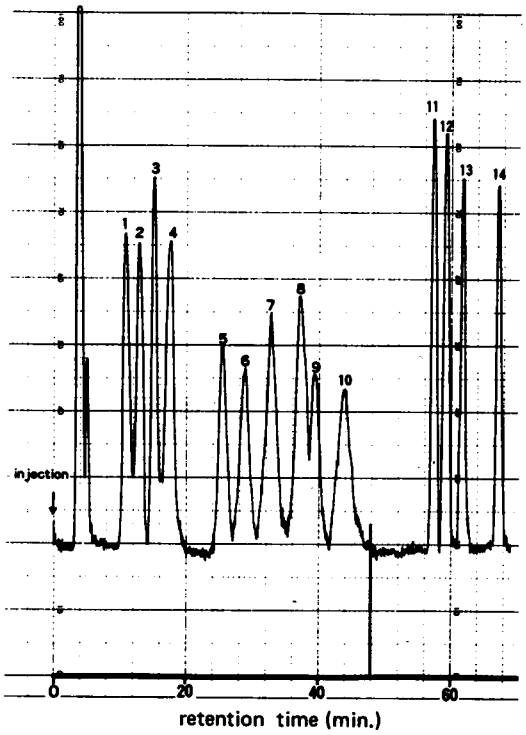


Fig. 2 Chromatogram of a mixture of free, glycine- and taurine-conjugated bile acids.

1) ursodeoxycholic acid, 2) cholic acid,
3) glycocholic acid, 4) taurocholic acid,
5) chenodeoxycholic acid, 6) deoxycholic acid,
7) glycochenodeoxycholic acid, 8) glycodeoxycholic acid,
9) taurochenodeoxycholic acid, 10) taurodeoxycholic acid,
11) lithocholic acid, 12) glycholithocholic acid,
13) taurolithocholic acid, 14) 5β-pregnan- 3α, 17α , 20α -triol.

| Column<br>0.3% $(NH_4)_2CO_3$ : $CH_3CN$ | μ Bondapakphenyl<br>8 : 4 | <br>14:4 |
|---|---|---|
| Ursodeoxycholic acid | | 10.8 |
| Cholic acid | | 12.8 |
| Chenodeoxycholic acid | | 24.8 |
| Deoxycholic acid | | 28.8 |
| Glychocholic acid | | 15.2 |
| Glycochenodeoxycholic acid | | 32.4 |
| Glycodeoxycholic acid | | 36.8 |
| Taurocholic acid | | 17.8 |
| Taurochenodeoxycholic acid | | 39.2 |
| Taurodeoxycholic acid | | 43.6 |
| Lithocholic acid | 9.4 | ( 57.2 ) |
| Glycolithocholic acid | 11.7 | ( 59.6 ) |
| Taurolithocholic acid | 13.7 | ( 62.0 ) |
| Internal standard | 21.3 | ( 67.2 ) |

( ) is the time after changing the mobile phase.

Table 1 Retention time of each bile acid.

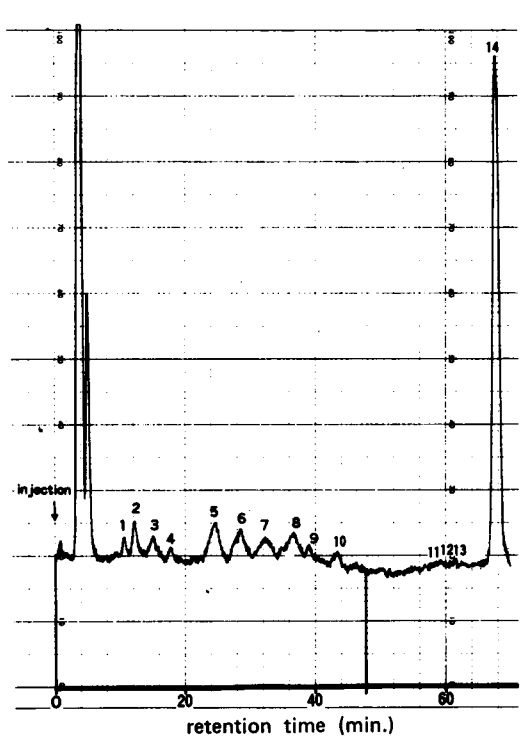


Fig. 3 Chromatogram of individual serum bile acid acids of healthy male subject.

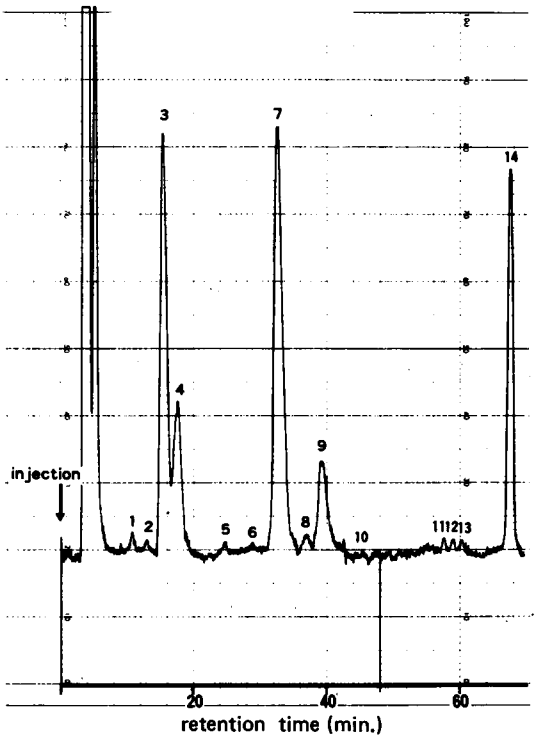


Fig. 4 Chromatogram of individual serum bile acids of a patient with acute hepatitis.

1) ursodeoxycholic acid, 2) cholic acid, 3) glycocholic acid,
4) taurocholic acid, 5) chenodeoxycholic acid, 6) deoxycholic acid,
7) glycochenodeoxycholic acid, 8) glycodeoxycholic acid, 9) taurochenodeoxycholic acid,
10) taurodeoxycholic acid, 11) lithocholic acid, 12) glycolithocholic acid,
13) taurolithocholic acid, 14) I. S.

## RESULTS

Separation: The analytical measurement of a mixture of fourteen kinds of standard bile acids containing 125 ng of I.S. was satisfactorily obtained in the order of UDCA, CA, GCA, TCA, CDCA, DCA, GCDCA, GDCA, TCDCA, TDCA, LCA, GLCA, TLCA and I. S.. Fig. 2 shows the chromatogram of 125 ng of free bile acid, 250 ng of glycine- and taurine-conjugated bile acid and 125 ng of I.S. Table 1 showed the retention time of each bile acid. Fig. 3 shows a chromatogram of serum from a healthy male (total serum bile acid level was 2.53 $\mu$mol/l) and Fig. 4 the chromatogram of serum from a patient with acute hepatitis (total serum bile acid level was 82.54 $\mu$mol/l).

Standard curve: Fig. 5 shows the standard curve of each bile acid. Linear correlations were obtained between the peak height and the amount of each bile acid.

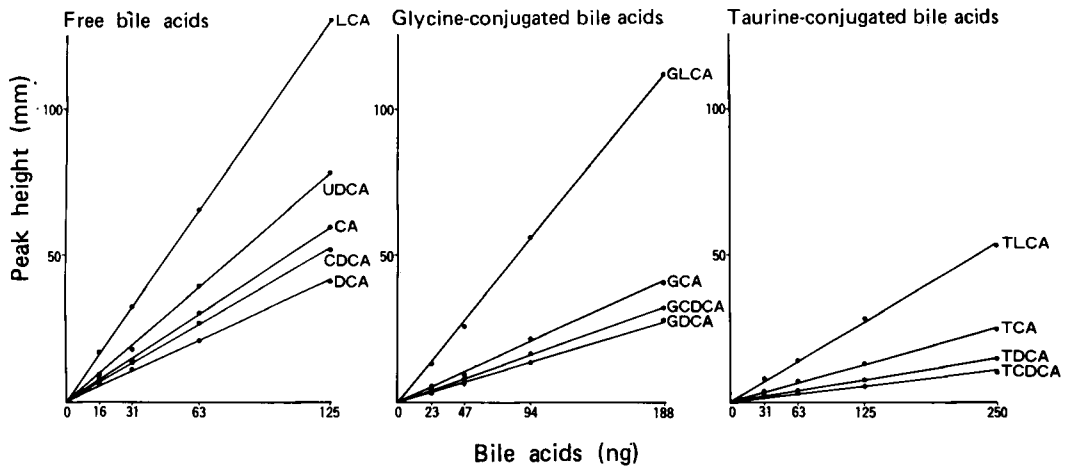


Fig. 5 Standard curves of free, glycine-conjugated, and taurine-conjugated bile acids

The sensitivity limits of each bile acid as determined by this method ranged from 6.3 to 31.0 ng.

Recovery test: Table 2 indicated the recovery rate of each bile acid. Satisfactory results were obtained in every concentration of bile acid were in a range of 81.7 to 106.6%.

Table 2 The recovery rate of each bile acid

| Bile Acids | Added (ng/ml) | Recovered (ng/ml) | Recovery % | Added (ng/ml) | Recovered (ng/ml) | Recovery % | Added (ng/ml) | Recovered (ng/ml) | Recovery % | Added (ng/ml) | Recovered (ng/ml) | Recovery % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ursodeoxycholic acid | 1250 | 1120 | 89.6 | 625.0 | 510.3 | 81.7 | 312.5 | 285.3 | 91.3 | 156.25 | 140.00 | 89.6 |
| Cholic acid | 1250 | 1104 | 88.3 | 625.0 | 549.1 | 87.9 | 312.5 | 295.9 | 94.7 | 156.25 | 156.05 | 99.9 |
| Chenodeoxycholic acid | 1250 | 1096 | 87.7 | 625.0 | 545.3 | 87.2 | 312.5 | 255.8 | 81.9 | 156.25 | 138.85 | 88.9 |
| Deoxycholic acid | 1250 | 1119 | 89.4 | 625.0 | 559.9 | 89.6 | 312.5 | 287.7 | 92.1 | 156.25 | 156.75 | 100.3 |
| Lithocholic acid | 1250 | 1080 | 86.4 | 625.0 | 509.9 | 81.5 | 312.5 | 260.4 | 83.3 | 156.25 | 133.65 | 85.5 |
| Glycocholic acid | 1875 | 1956 | 104.3 | 937.5 | 868.5 | 92.6 | 468.75 | 447.80 | 95.5 | 234.375 | 238.25 | 101.7 |
| Glycochenodeoxycholic acid | 1875 | 1936 | 103.2 | 937.5 | 826.3 | 88.1 | 468.75 | 442.70 | 94.4 | 234.375 | 220.90 | 94.3 |
| Glycodeoxycholic acid | 1875 | 1853 | 98.8 | 937.5 | 861.1 | 91.9 | 468.75 | 402.50 | 85.9 | 234.375 | 248.70 | 106.1 |
| Glycolithocholic acid | 1875 | 1653 | 88.2 | 937.5 | 790.8 | 84.4 | 468.75 | 397.25 | 84.8 | 234.375 | 202.90 | 86.6 |
| Taurocholic acid | 2500 | 2638 | 105.5 | 1250 | 1165 | 93.2 | 625.0 | 519.2 | 83.1 | 312.50 | 333.20 | 106.6 |
| Taurochenodeoxycholic acid | 2500 | 2655 | 106.2 | 1250 | 1297 | 103.8 | 625.0 | 562.5 | 90.0 | 312.50 | 298.45 | 95.5 |
| Taurodeoxycholic acid | 2500 | 2431 | 97.2 | 1250 | 1197 | 95.7 | 625.0 | 593.5 | 95.0 | 312.50 | 272.85 | 87.3 |
| Taurolithocholic acid | 2500 | 2457 | 98.3 | 1250 | 1055 | 87.6 | 625.0 | 543.8 | 87.0 | 312.50 | 260.15 | 83.2 |

Table 3 Reproducibility of each individual serum bile acid by inter-assay and intra-assay.

| Bile acids | ( Inter - assay ) Found ± S.D. (μmol/l) | | ( Intra - assay ) Found ± S.D. (μmol/l) | |
|---|---|---|---|---|
| Ursodeoxycholic acid | 0.10 ± 0.01 | ( 10.0 ) | 0.11 ± 0.01 | ( 9.09 ) |
| Cholic acid | 0.68 ± 0.01 | ( 1.47 ) | 0.50 ± 0.01 | ( 2.00 ) |
| Chenodeoxycholic acid | 0.26 ± 0.01 | ( 3.85 ) | 0.25 ± 0.01 | ( 4.00 ) |
| Deoxycholic acid | 0.45 ± 0.01 | ( 2.22 ) | 0.44 ± 0.03 | ( 6.82 ) |
| Lithocholic acid | N. D. | | N. D. | |
| Glycocholic acid | 1.44 ± 0.02 | ( 1.39 ) | 1.25 ± 0.03 | ( 2.40 ) |
| Glycochenodeoxycholic acid | 2.04 ± 0.05 | ( 2.45 ) | 1.92 ± 0.05 | ( 2.60 ) |
| Glycodeoxycholic acid | 1.46 ± 0.01 | ( 0.68 ) | 1.43 ± 0.02 | ( 1.40 ) |
| Glycolithocholic acid | N. D. | | N. D. | |
| Taurocholic acid | 0.72 ± 0.04 | ( 5.56 ) | 0.63 ± 0.03 | ( 4.76 ) |
| Taurochenodeoxycholic acid | 1.34 ± 0.01 | ( 0.75 ) | 1.38 ± 0.01 | ( 0.70 ) |
| Taurodeoxycholic acid | 1.40 ± 0.02 | ( 1.43 ) | 1.32 ± 0.02 | ( 1.52 ) |
| Taurolithocholic acid | N. D. | | N. D. | |

n = 5 ( ) % = CV

Reproducibility : As shown in Table 3, the inter-assay and intra-assay coefficiency values of each bile acid ranged from 0.68 to 10.0% and from 0.70 to 9.09%, respectively.

Individual serum bile acids: Table 4 shows mean concentrations of individual bile acid in the sera of 8 healthy volunteers and 76 patients with hepatobiliary diseases. All individual bile acids were detected in sera of healthy subjects, though lithocholic acid was not detected. Conjugated cholic acids increased in the patients with intra- and extra-hepatic cholestasis. Free cholic acid was significantly elevated in patients with intra-hepatic cholestasis as compared with extra-hepatic cholestasis. In patients with liver cirrhosis, every individual bile acid showed significant increase as compared with normal subjects, with a particular increase in the value of conjugated chenodeoxycholic acid. The value of glycine conjugated primary bile acid was relatively higher in the non-compensative stage. In chronic hepatitis, conjugated chenodeoxycholic acids showed slight rises as compared with normal values, but with higher values in the active type than in the inactive type. In the cases of silent stone the chromatogram showed an almost normal pattern, except for slight traces of lithocholic acids.

## DISCUSSION

There are three recent approaches to the measurement of bile acids in serum. The first is gas-liquid chromatography[5)6)]. This method, however, suffers from such disadvan-

Table 4 Individual bile acids in serum of healthy subject and patients with hepatobiliary diseases.

| Disease | μmol/l / No. | T.B.A. | UDC | C | CDC | DC | LC | GC | GCDC | GDC | GLC | TC | TCDC | TDC | TLC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Normal | 8 | 2.88 ±0.74 | 0.07 ±0.04 | 0.14 ±0.05 | 0.42 ±0.16 | 0.30 ±0.05 | N.D. | 0.16 ±0.07 | 0.47 ±0.05 | 0.46 ±0.26 | N.D. | 0.16 ±0.05 | 0.36 ±0.08 | 0.34 ±0.13 | N.D. |
| Acute H. | 5 | 90.82 ±13.41 | 0.88 ±0.29 | 0.71 ±0.11 | 0.90 ±0.31 | 0.78 ±0.29 | 0.29 ±0.14 | 24.70 ±4.29 | 29.09 ±6.23 | 2.53 ±1.00 | 0.75 ±0.29 | 17.96 ± 5.48 | 9.67 ±4.44 | 1.47 ± 0.51 | 1.10 ±0.33 |
| Chronic inact. | 11 | 4.76 ± 0.96 | 0.09 ± 0.04 | 0.22 ± 0.06 | 0.38 ±0.09 | 0.26 ±0.05 | 0.04 ±0.02 | 0.58 ±0.24 | 0.94 ±0.24 | 0.40 ± 0.06 | 0.07 ±0.02 | 0.42 ±0.17 | 0.91 ±0.19 | 0.36 ± 0.08 | 0.09 ±0.03 |
| Chronic act. | 14 | 14.10 ± 2.77 | 0.34 ±0.08 | 0.97 ± 0.26 | 1.08 ±0.20 | 0.46 ±0.10 | 0.17 ±0.05 | 2.00 ±0.45 | 3.27 ±0.84 | 1.32 ± 0.43 | 0.35 ±0.13 | 1.39 ±0.43 | 1.69 ±0.54 | 0.64 ±0.20 | 0.43 ±0.12 |
| Cirrhosis comp. | 12 | 45.14 ± 8.61 | 0.81 ±0.24 | 2.44 ± 0.81 | 3.67 ±1.32 | 1.31 ±0.40 | 0.14 ±0.04 | 4.32 ±1.24 | 15.46 ±5.10 | 2.72 ±0.88 | 0.31 ±0.08 | 2.68 ±0.90 | 9.53 ±3.99 | 1.28 ±0.26 | 0.47 ±0.20 |
| Cirrhosis non-comp. | 11 | 82.36 ±12.99 | 0.33 ±0.12 | 2.17 ± 0.69 | 4.74 ±3.26 | 0.98 ±0.25 | 0.17 ±0.06 | 7.34 ±1.18 | 31.49 ±5.60 | 3.59 ±1.30 | 0.31 ±0.12 | 6.39 ±1.55 | 23.47 ±6.89 | 0.90 ±0.22 | 0.46 ±0.20 |
| I. H. C. | 7 | 79.40 ±12.80 | 0.56 ±0.26 | 6.55 ± 1.89 | 1.04 ±0.36 | 0.36 ±0.17 | 0.43 ±0.10 | 21.62 ±4.73 | 19.18 ±4.40 | 1.57 ±0.46 | 0.84 ±0.37 | 16.93 ±4.61 | 7.88 ±1.75 | 1.14 ±0.41 | 1.30 ±0.39 |
| E. H. C. | 10 | 86.81 ±19.19 | 0.40 ±0.12 | 0.84 ± 0.23 | 0.44 ±0.13 | 0.38 ±0.14 | 0.22 ±0.08 | 27.62 ±7.69 | 17.26 ±4.58 | 2.31 ±0.84 | 0.34 ±0.13 | 23.37 ±4.57 | 12.04 ±2.90 | 0.65 ±0.19 | 0.79 ±0.24 |
| Silent stone | 6 | 4.64 ± 1.12 | 0.15 ±0.04 | 0.16 ± 0.03 | 0.43 ±0.08 | 0.49 ±0.18 | 0.10 ±0.02 | 0.57 ±0.26 | 0.67 ±0.18 | 0.55 ±0.31 | 0.22 ±0.06 | 0.40 ±0.11 | 0.33 ±0.10 | 0.33 ±0.12 | 0.25 ±0.10 |

(Mean±SEM)

Acute H = acute hepatitis, Chronic inact. = chronic inactive hepatitis,
Chronic act. = chronic active hepatitis, IHC = intra-hepatic cholestasis,
EHC = extra-hepatic cholestasis, comp. = compensative stage.

tages as extraction, nonconjugation and finally derivation necessary prior to injection of the sample into the chromatograph, and therefore is inconvenient for routine clinical use.

The second approach is radioimmunoassay. As this method has certain methological advantages, it is well suited for routine use provided that the antibody specificity is carefully determined. Recently, Ozaki et al.[8] introduced an enzyme immunoassay for the measurement of ursodeoxycholic acid. This was an improvement on radioimmunoassay in respect of safety because it does not use radioisotopes, but it still has the same disadvantages as the latter.

The third approach is the enzymatic method. It is based on the enzyme 3α -hydroxysteroid dehydrogenase. Recently, Mashige et al.[3] modified this method and avoided prior extraction. It is a simple, rapid and sensitive method, but does not discriminate between individual different bile acids and measure only the total amount of them except for the bile acids, which are sulfated or glucronidated at the 3α -position.

With introduction of efficient high-performance liquid chromatography, such difficulties can be circumbented. Furthermore, there are some encouraging reports on the use of a reverse-phase system coupled to an ultraviolet (UV) detector for the measurement of bile acids. Okuyama[4] reported analysis of the carboxylic group of bile acids with l-p-nitrobenzyl-3-tolyltriazene. Show et al.[7] made it possible to measure taurine- and glycine-conjugated bile acids of rat bile. Goto et al.[2] developed a new exchange chromatography (PHP-LH 20), and separated bile acids into three groups, namely free, glycine- and taurine-conjugated bile acids prior to subjecting them to HPLC. These methods, however, require large volume serum samples because of their low sensitivity and are not utilized for clinical examination due to their unavoidable disadvantages in preparation. Therefore, the development of a more convenient and sensitive method was expected.

We have previously reported a new method by HPLC combined with enzymatic fluorometric measurement.[1] In this study we investigated a method of simultaneously measuring thirteen kinds of individual bile acids in serum utilizing HPLC combined with enzymatic fluorometry and tried the fundamental examinations about this method.

The strong point of this method consists firstly in adequate separation of free, glycine- and taurine-conjugated bile acids with neither chemical modification nor disadvantages in the preparation. Secondly, high sensitivity was obtained by high-performance liquid chromatography combined with enzymatic fluorometric method and enables measurement of thirteen kinds of bile acids in only 1 ml of even healthy human serum. Elution time was shortened by a stepwise gradient procedure. This method coefficiant resulted in from 0.68 to 10.0 and from 0.70 to 9.09% in inter- and intra-assay respectively. The recovery test was in the range of 81.7 to 106.6%. The sensitivity limits of each bile acid ranged from 6.3 to 31.0 ng. Characteristic patterns of individual serum bile acids were found in patients with various hepatobiliary disease. These results suggest that this method provided much more information on the metabolism of bile acids and might be utilized for clinical diagnosis of hepatobiliary diseases.

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