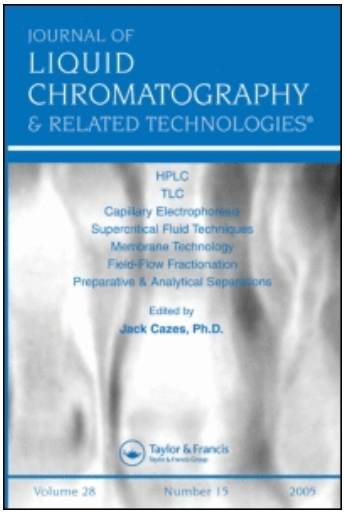

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A Fully Automatic Method for Analysis of Individual Bile Acids in the Serum Using High-Performance Liquid Chromatography for Clinical Use

S. Hasegawa[a]; R. Uenoyaraa[a]; F. Takeda[a]; J. Chuma[a]; K. Suzuki[a]; F. Kamiyama[b]; K. Yamazaki[b]; S. Baba[a]
[a] The Second Department of Internal Medicine, Kobe University School of Medicine, Kobe, Japan [b] Central Research Laboratory, Sekisui Chemical Co., Ltd., Osaka, Japan

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A FULLY AUTOMATIC METHOD FOR ANALYSIS OF INDIVIDUAL BILE ACIDS IN THE SERUM USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR CLINICAL USE

S. Hasegawa, R. Uenoyama, F. Takeda,
J. Chuma, K. Suzuki, F. Kamiyama*,
K. Yamazaki* and S. Baba.

The Second Department of Internal Medicine,
Kobe University School of Medicine, Kobe, Japan.
*Central Research Laboratory, Sekisui Chemical
Co., Ltd. Osaka, Japan.

ABSTRACT

We have reported highly sensitive methods for the analysis of individual bile acids in the serum using high-performance liquid chromatography coupled with an enzymatic fluorometric system. In this report, a new system equipped with a sample treatment mechanism for chromatographic analysis of serum bile acids is detailed. Most of the protein and other hydrophilic components of the injected serum sample are removed in the pretreatment system, so that only the remaining bile acids are introduced into the chromatographic system and eluted with irrigants containing a coenzyme (NAD) for fluorometric detection. With this method, we are able to simultaneously determine 15 different serum bile acids in an hour without the tedious manual sample processing steps. This system opens up an approach to fully automated analysis of bile acids in the blood.

INTRODUCTION

Bile acids are amphipathic molecules synthesized from cholesterol and conjugated with glycine or taurine in the liver cells. Conjugated bile acids are excreted into the small intestine, where about 30% of them are deconju-

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gated and converted into secondary bile acids by intestinal micro-organisms. Most bile acids are reabsorbed from the terminal ileum by a process of active transport and are returned to the liver via the portal vein. This constitutes the enterohepatic circulation. Disturbances of the enterohepatic circulation can occur secondary to disorders affecting hepatic, biliary, or intestinal function, but may also be of primary relevance in the pathogenesis of clinically important conditions, such as cholelithiasis. Therefore, detailed information on the enterohepatic circulation of bile acids in man is potentially of great clinical importance. For determining serum bile acids in particular, it would be most advantageous to be able to ascertain not only the total concentration, but also the concentrations of each individual bile acid, since these patterns may yield clinical information in hepatobiliary or gastrointestinal diseases. In 1978, we reported a highly sensitive method for the analysis of individual (3α)-hydroxy bile acids in the serum using high-performance liquid chromatography (HPLC) coupled with an enzymatic fluorometric system (1,2); in 1981, we succeeded in combining our original method with a new immobilized enzyme column suitable for use at the alkaline pH that is optimum for (3α)-hydroxysteroid dehydrogenase (3α HSD) enzyme activity (3); and , in 1982, we reported a new column of polymer reversed phase gel exclusively for the analysis of bile acids (4,5). In the present paper, we are presenting a new system for the chromatographic analysis of bile acids.

The system consists of a sample treatment system, a high-performance liquid chromatographic pump with a step-wise gradient programmer, a fractionating column of hydrophilic polymer gel, an immobilized enzyme column and a fluorometer. A serum sample is injected into the sample-treatment-system, through which most of the protein and other hydrophilic components are removed. Then, by a simple valve operation, the remaining bile acids are introduced into the fractionating column and eluted with irrigants containing a coenzyme (NAD). Each fraction of bile acid flows into the immobilized enzyme column where NAD irrigants are transformed into NADH, which is detected fluorometrically. The fractionating step is completed within about 60 minutes. Thus, this system has eliminated the tedious manual sample processing steps and can be controlled fully automatically.

MATERIALS AND METHODS

Materials Used for the Fractionating Column and the Short Column of the Sample Treatment System

As the monomers, tetraethyleneglycol di-acrylate (Sin Nakamura Chem. Co., Ltd. Wakayama, Japan) and tetramethylolmethane tri-acrylate (Sin Nakamura Chem. Co., Ltd. Wakayama, Japan) were prepared. Toluene and benzoyl peroxide were used for the polymerization.

Materials Used for the Immobilized Enzyme Column

Beaded cellulose (Cellulofine GC-200m. Seikagaku Kogyo Co., Ltd. Tokyo, Japan) was adopted as solid support because of its chemical stability at alkaline pH and its superior

mechanical properties. The enzyme was (3α)-hydroxy steroid dehydrogenase extracted from Pseudomonas Testosterony and purified (Sekisui Chem. Co., Ltd. Osaka, Japan)(6).

Eluent Containing a Coenzyme

Triammonium phosphate was dissolved in distilled water to make 0.5% and 1.5% (W/V) triammonium phosphate solution. The 0.5% solution was adjusted to pH 9.1 and the 1.5% solution to pH 9.7 with ammonium hydroxide solution. Each solution was mixed with acetonitrile. Three kinds of eluent were prepared.

The volume ratio (V/V) of Eluent I was 13:87 (acetonitrile: 0.5% triammonium phosphate solution), that of Eluent II was 15:85 (acetonitrile:1.5% solution) and that of Eluent III was 24:76 (acetonitrile:0.5% solution). Each eluent contained 200 mg/l of β-NAD^+ (Sigma Chem. Co., St. Louis, U.S.A.).

Standard Material

Sodium salts of cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA) and lithocholic acid (LCA) were purchased from Calbiochem-Behring Co. (San Diego, U.S.A.). Sodium salts of glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA), glycolithocholic acid (GLCA), taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), taurodeoxycholic acid (TDCA) and taurolithocholic acid (TLCA) were obtained from P-L Biochemicals Inc. (Milwaukee, U.S.A.). Sodium salts of ursodeoxycholic acid (UDCA), glycoursodeoxycholic acid (GUDCA) and tauroursodeoxycholic acid (TUDCA) were supplied

gratis from Tokyo Tanabe Pharmaceutical Co. (Tokyo, Japan). As an internal standard (I.S.), we used 5β,-pregnan-3α, 17α, 20α-triol (Sigma Chem. Co., St. Louis, U.S.A.).

Instruments

A schematic diagram of the method is shown in Fig. 1. As for the pretreatment system, which is equipped with an injector, we will touch on this later. The high-performance liquid chromatography was carried out on an ALC 204 type system (Waters Associates Instrument, Waltham, U.S.A.). The detector used was a fluorochrome fluorescent detector (Shimadze Seisakusho Ltd. Kyoto, Japan) with an exciting wavelength 350 nm and an emitting wavelength of 460 nm.

Pretreatment System

The sample treatment system consists of a constant flow pump, a short column of polymer reversed phase gel and an injector. The polymer gel is the same copolymer hydrophilic bead packed in the fractionating column mentioned later. The principle of this system is shown schematically in Fig. 2. A serum sample is first injected into the short column, where triammonium phosphate solution (pH 7) from the constant flow pump eliminates most of the protein and other hydrophilic components; then, by a simple valve operation, the remaining bile acids in the short column are introduced into the fractionating column (HPLC column) and are eluted with irrigants containing a coenzyme. This system was devised by the staff of Sekisui Laboratory.

Preparation of the HPLC Column

Copolymer beads were synthesized by a standard suspension

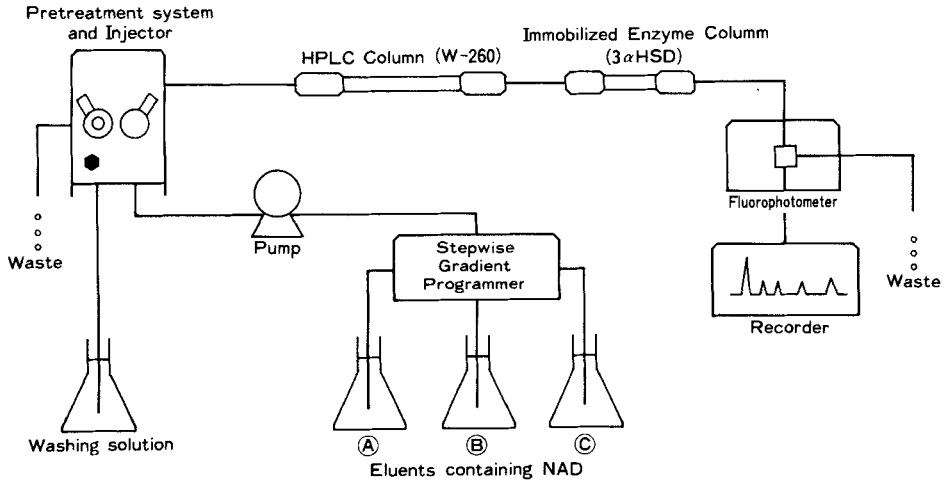

Fig. 1 : Flow diagram of the system

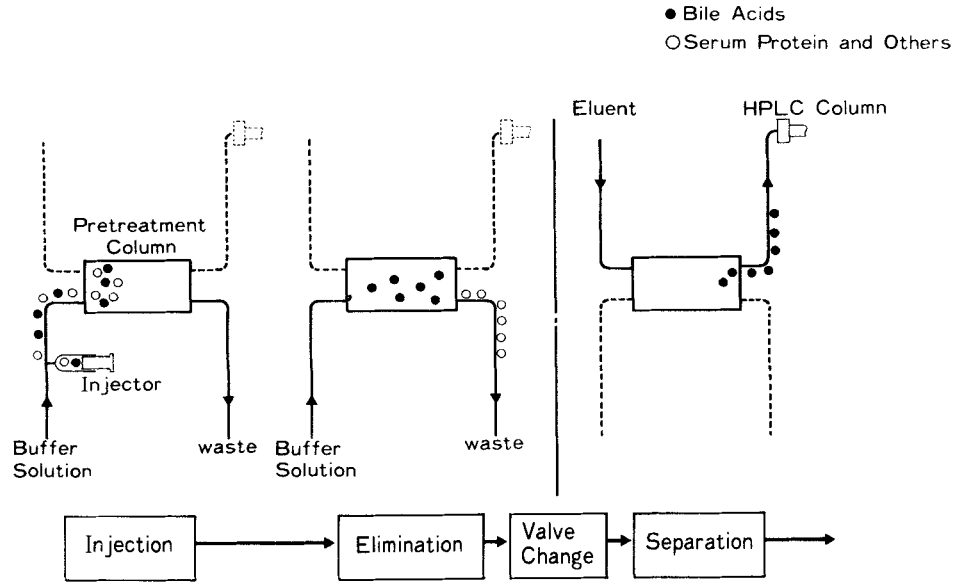

Fig. 2 : Principle of the pretreatment system

polymerization method; the details of the procedure have been mentioned in our recent report (5). The porous beads thus obtained were packed into a stainless steel column (0.6ϕ x 25cm), which was made 20% smaller than that described in our recent report. This was to shorten the retention time of each bile acid.

Preparation of the Immobilized Enzyme Column

Activation of the beaded cellulose by the addition of CNBr was followed by the coupling reaction with (3α)-hydroxy steroid dehydrogenase. The reactions were performed according to the procedure described by S.C. March et al. (7). The enzyme-bound beads were packed into a stainless steel column (0.4ϕ x 10cm), and this was referred to as the immobilized enzyme column.

Preparation of Serum Material

Human sera were collected from the peripheral veins of healthy volunteers and patients with hepatobiliary diseases after overnight fasting and were frozen (-20°C) until analyzed for bile acids. For the analysis, 5,000 ng of 5β-pregnan-3α, 7α, 20α-triol (dissolved in 10 μl of methanol) were added to 1 ml of serum as I.S.

Measurement Procedure

Twenty-five μl of the samples were applied to the system with a microsyringe (Hamilton Co. Reno, U.S.A.). Eluent was passed through at the rate of 1 ml per minute. In order to shorten the retention time, the mobile phase was changed from Eluent I to Eluent II four minutes after the operation of a valve attached to the pretreatment system

(this corresponds to six minutes after injection). Then Eluent II was replaced by Eluent III 35 minutes later. Each fraction of bile acids flowed into the immobilized enzyme column where the NAD irrigants were transformed into NADH, which was detected fluorometrically. This reaction was carried out at 27°C in a water bath. The chart speed was 2.5 mm per minute. Fig. 3 shows a standard chromatogram. This fractionating step was completed in 68 minutes.

Standard Curves

Five concentrations of a mixture of each of the standard bile acids were prepared 10 μmol/l, 7.5 μmol/l, 5 μmol/l, 2.5 μmol/l and 1.25 μmol/l. These samples were dissolved in distilled water, and I.S. was added as the sera were treated. The standard curves were established by plotting the peak height against the amount of each bile acid.

Recovery Experiments

Recovery experiments were performed by adding a mixture of standard samples to the sera of healthy subjects. This mixture contained 5 μmol/l of each bile acid.

Reproducibility

As retention times, several samples were analyzed 20 times continuously. The %CV of each bile acid's retention times was calculated. As an intra-assay, the same samples were determined five times during a day, and, for interassay, the same samples were determined during another six-day period. The %CV values were calculated from the mean values and SD values.

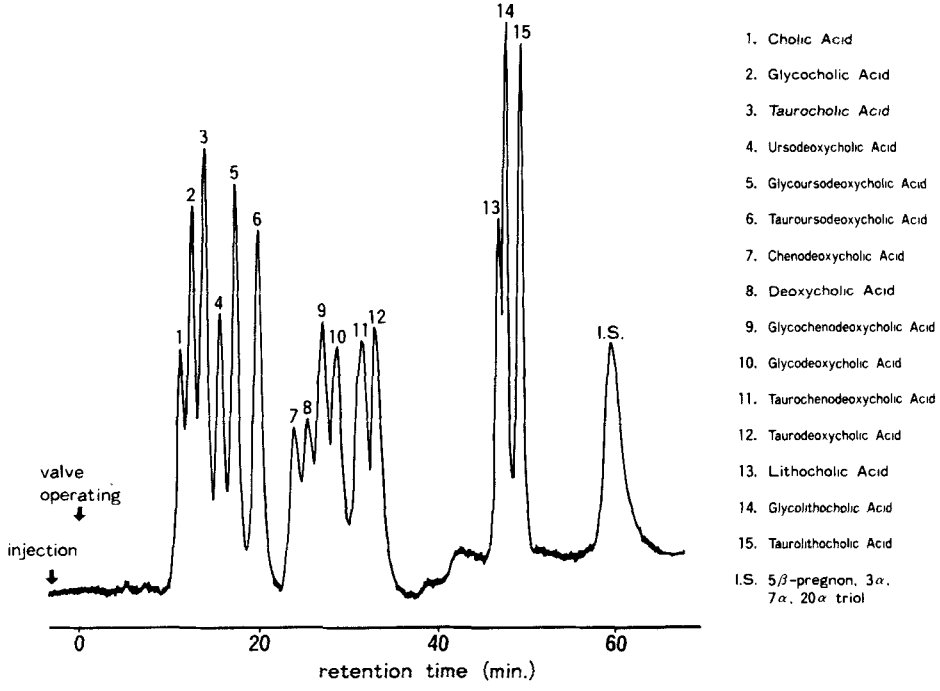

Fig. 3 : Standard chromatograms of individual bile acids (2.5 μmol/l each)

Influences of Bilirubin, Hemoglobin and Lactate Dehydrogenase (LDH)

These tests were performed by adding these substances to the serum of a patient with hepatobiliary disease. The control concentration of bilirubin ranged from 0 to 20 mg/dl, of hemoglobin from 0 to 500 mg/dl and of LDH from 0 to 2500 IU/dl. The concentrations of 15 kinds of bile acids were compared with the sample containing no added substances.

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 diagnosis of each patient was based on clinical, biochemical and histological findings.

RESULTS

Fig. 3 shows a chromatogram of a mixture of free, glycine- and taurin-conjugates of each of five bile acids. The separation of those bile acids was satisfactorily obtained in the order of CA, GCA, TCA, UDCA, GUDCA, TUDCA, CDCA, DCA, GCDCA, GDCA, TCDCA, TDCA, LCA, GLCA and TLCA. The resolution value of TCDCA and TDCA, in which separation was most difficult, was 0.7. Fig. 4 shows the standard curve of each bile acid. Linear correlations were obtained between the peak height and the amount of each bile acid. Table 1 indicates the reproducibility of each retention time. Each %CV was satisfactory. Table 2 indicates the recovery ratios, reproducibility and minimum detectable range. Satisfactory recovery ratios of between 91.9% and 104.5% were obtained. The intra-assay CV value of each bile acid ranged from 1.6 to 8.7% in replicated determinations and from 2.4 to 9.4 in six replicated determinations for interassay CV. The sensitivity limits of each bile acid by this method were between 0.042 and 0.116 μmol/l. There was no influence of bilirubin ranging from 0 to 20 mg/dl added to human sera as shown in Fig. 5, no

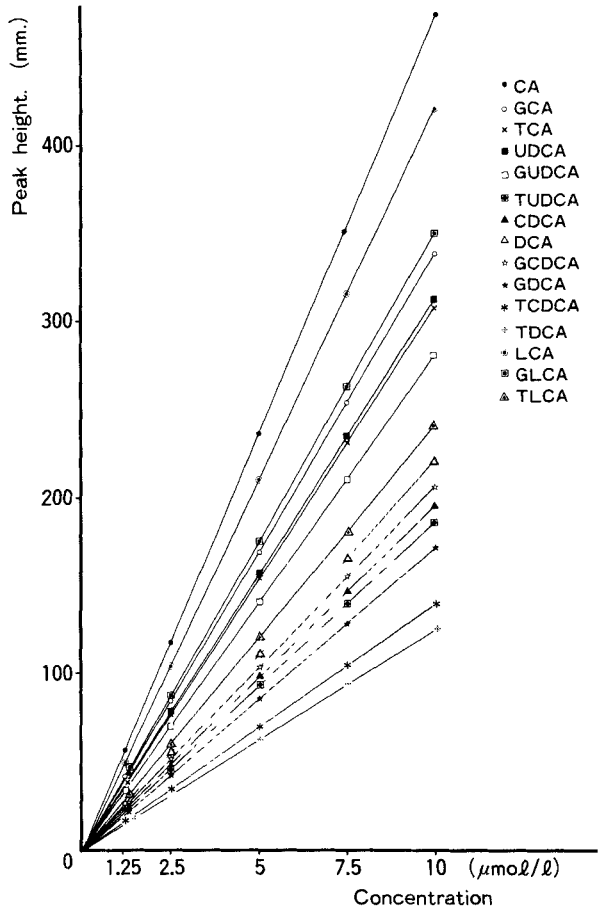

Fig. 4 : Standard curves of individual bile acid

influence of hemoglobin from 0 to 500 mg/dl as shown in Fig. 6 and no influence of LDH from 0 to 2500 IU/dl as shown in Fig. 7. A normal serum analysis by this method is presented in Fig. 8. In addition, an example of serum analysis in a patient with acute hepatitis is shown in Fig. 9. A typical bile acid pattern of acute liver cell

Table 1: Reproducibility of each retention time

Bile Acids	C.V. (%)
CA	0.75
GCA	1.07
TCA	0.13
UDCA	0.13
GUDCA	0.29
TUDCA	0.45
CDCA	0.42
GCDCA	0.68
TCDCA	0.52
DCA	0.72
GDCA	0.50
TDCA	0.65
LCA	0.34
GLCA	0.20
TLCA	0.24

damage was obtained. Normal values analyzed in this system are compared with those from other methods, including our previous HPLC method using 3α-HSD reagent solution in Table 3 (2,5,8,9,10). Table 4 shows mean concentrations of individual bile acids in the sera of six healthy volunteers and 82 patients with hepatobiliary diseases. Characteristic patterns of individual serum bile acids were found in patients with various hepatobiliary diseases.

Table 2: Recovery, reproducibility and minimum detectable range

Bile Acids	Reproducibility of Each Bile Acid Assay		Recovery Rate (%)	Minimum Detectable Range (μmol/ℓ) S/N=2
	Intra asasy C.V. (%) n=5	Inter assay C.V. (%) n=6		
CA	2.4	5.3	102.3	0.042
GCA	3.7	8.9	101.5	0.060
TCA	8.7	2.4	104.5	0.071
UDCA	5.3	4.6	99.7	0.065
GUDCA	7.3	8.7	98.8	0.065
TUDCA	1.6	9.4	99.2	0.104
CDCA	8.8	4.9	99.7	0.102
GCDCA	7.6	5.2	100.2	0.097
TCDCA	2.4	8.9	100.2	0.140
DCA	5.3	8.3	101.0	0.092
GDCA	2.9	5.8	101.2	0.116
TDCA	5.7	2.8	100.7	0.162
LCA	2.2	8.7	94.2	0.049
GLCA	2.7	3.7	94.0	0.057
TLCA	5.3	2.8	91.9	0.083

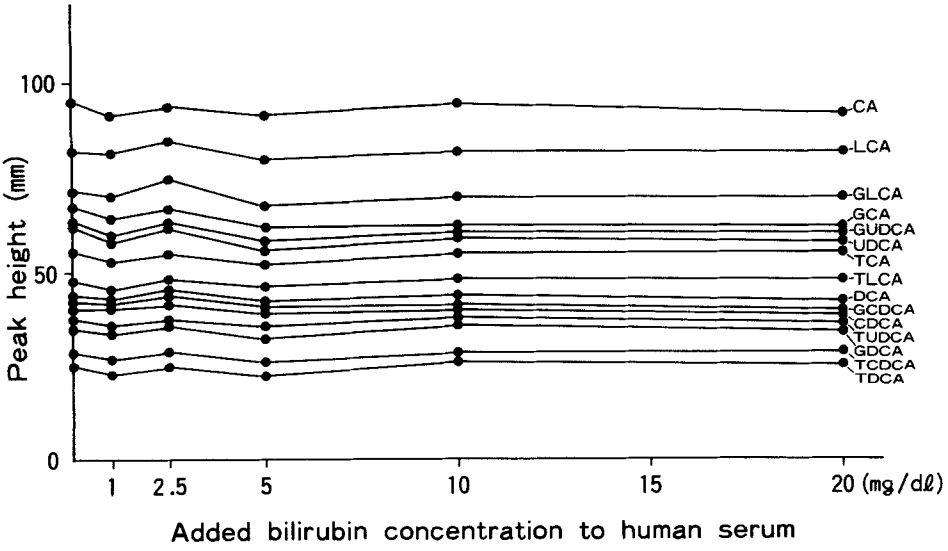

Fig. 5 : Influence of bilirubin

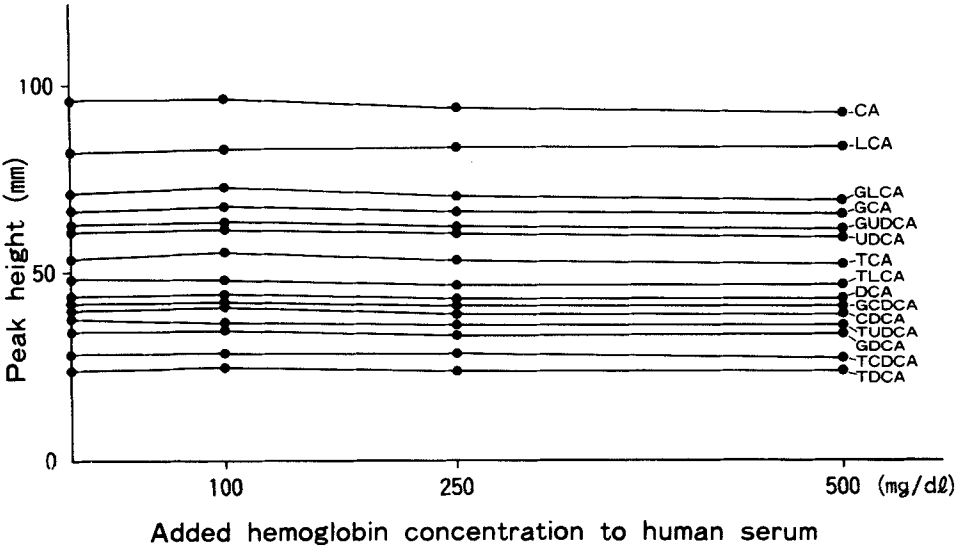

Fig. 6 : Influence of hemoglobin

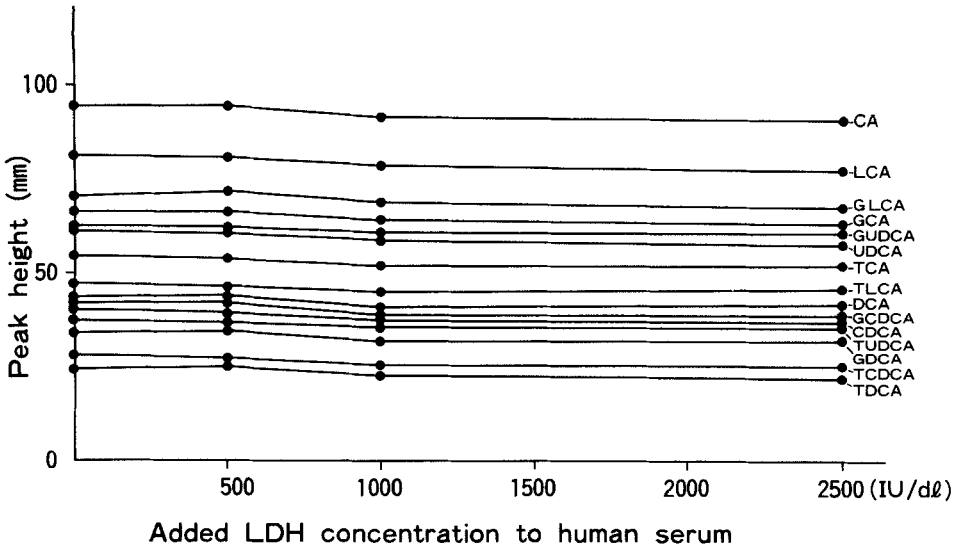

Fig. 7 : Influence of LDH

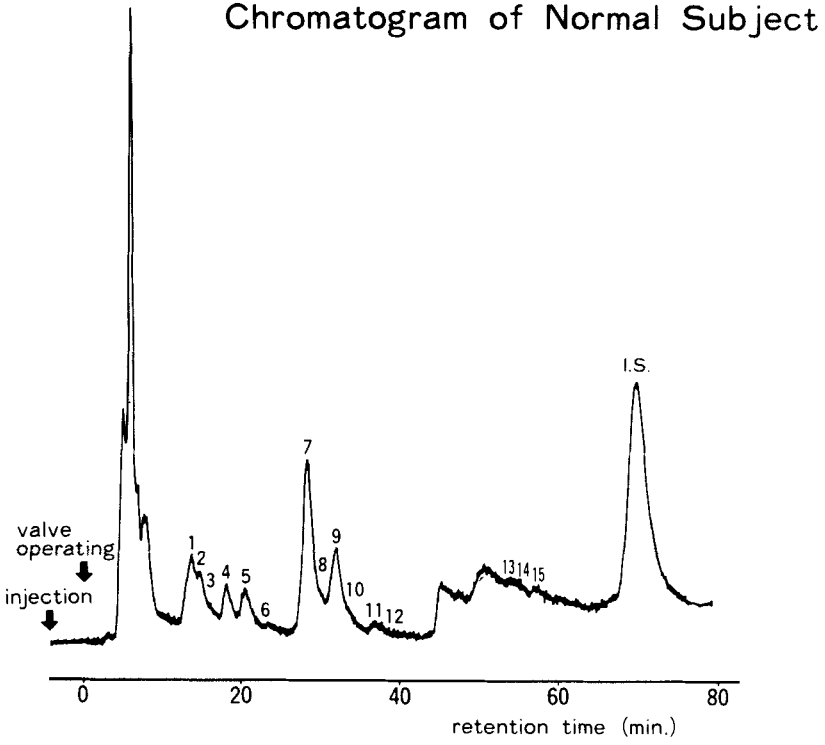

Fig. 8 : A bile acid pattern of normal serum

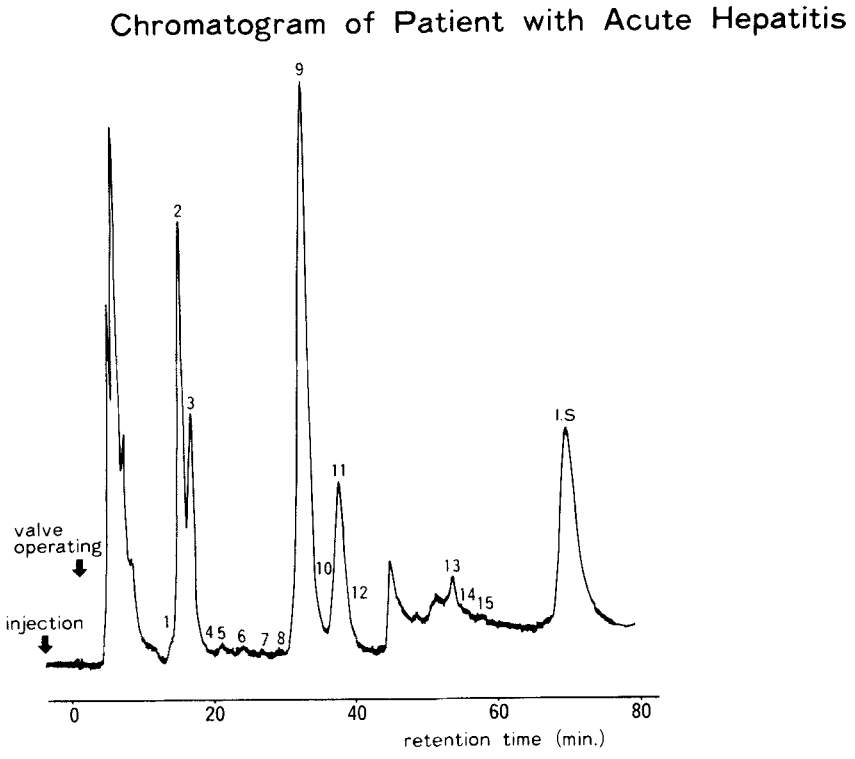

Fig. 9 : A bile acid pattern of serum in a patient with acute hepatitis

The number of peaks on these examples indicates the same substance shown in the standard chromatogram

DISCUSSION

This method allows analysis of 15 kinds of (3α)-hydroxy bile acids in the serum without tedious extraction steps and can be completed within only an hour. The strong points of this method are: firstly, adequate separation of each bile acid with neither chemical modification nor disadvantages

Table 3: Normal values of serum bile acids. Values of HPLC and RIA are given in μmol/l (mean ± S.E.M.). Ranges of GLC are given in μmol/l. Mean values of GC-MS are given in μmol/l. N.D. = not determined.

Bile acid μmol/ℓ	No.	Total	UDCA group			CA group			CDCA group			DCA group			LCA group		
Author and Method			UDCA	GUDCA	TUDCA	CA	GCA	TCA	CDCA	GCDCA	TCDCA	DCA	GDCA	TDCA	LCA	GLCA	TLCA
Present Method HPLC (Equipped with pretreatment system)	6	3.32 ±0.37	0.16 ±0.04	0.28 ±0.13	0.14 ±0.06	0.16 ±0.04	0.30 ±0.07	0.12 ±0.03	0.42 ±0.19	0.59 ±0.15	0.27 ±0.06	0.23 ±0.05	0.34 ±0.10	0.27 ±0.06	N.D.	0.04 ±0.01	0.08 ±0.03
Baba (4) HPLC (Using immobilized enzyme)	12	3.90 ±0.85	0.08 ±0.03	0.14 ±0.05	0.07 ±0.02	0.26 ±0.05	0.30 ±0.08	0.21 ±0.03	0.75 ±0.20	0.97 ±0.38	0.45 ±0.13	0.20 ±0.06	0.23 ±0.04	0.23 ±0.05	0.02 ±0.01	0.01 ±0.00	0.01 ±0.00
Baba (2) HPLC (Using enzyme solution)	8	2.88 ±0.74	0.07 ±0.04			0.14 ±0.05	0.16 ±0.07	0.16 ±0.05	0.42 ±0.16	0.47 ±0.05	0.36 ±0.08	0.30 ±0.05	0.46 ±0.26	0.36 ±0.08	N.D.	N.D.	N.D.
Demers (8) RIA	25						0.27 ±0.03		0.20 ±0.03				0.06 ±0.01				
Sandberg (9) GLC	18	0.9 ∿ 6.9		N.D.			0.09∿1.95			0.15∿3.9			0.18∿1.35			N.D.	
Shino (10) GC-MS	4			1.02			0.55			1.81			1.12			0.01	

Table 4: Individual bile acids in the serum of healthy subjects and patients with hepatobiliary diseases. Acute H. = Acute hepatitis. Chronic inact. = Chronic inactive hepatitis. Chronic act. = Chronic active hepatitis. Comp. = compensative stage. I.H.C. = Intrahepatic cholestasis. E.H.C. = Extrahepatic cholestatis

Disease	μmol /ℓ No.	T.B.A	UDCA	CA	CDCA	DCA	LCA	GUDCA	GCA	GCDCA	GDCA	GLCA	TUDCA	TCA	TCDCA	TDCA	TLCA
Normal	6	3.22 ±0.37	0.16 ±0.04	0.16 ±0.04	0.42 ±0.19	0.23 ±0.05	N.D.	0.28 ±0.13	0.30 ±0.07	0.59 ±0.15	0.34 ±0.10	0.04 ±0.01	0.14 ±0.06	0.12 ±0.03	0.27 ±0.06	0.27 ±0.06	0.08 ±0.03
Acute H.	16	216.84 ±34.17	0.10 ±0.06	1.05 ±0.47	0.37 ±0.13	0.16 ±0.07	0.17 ±0.08	1.09 ±0.45	46.02 ±8.70	77.38 ±14.22	2.21 ±1.81	0.41 ±0.15	0.55 ±0.17	37.40 ±6.39	48.25 ±9.89	1.38 ±0.88	0.30 ±0.14
Fatty liver	3	5.70 ±1.65	1.16 ±0.41	0.67 ±0.27	0.22 ±0.13	0.08 ±0.08	0.05 ±0.05	0.23 ±0.06	0.41 ±0.05	1.72 ±0.66	0.42 ±0.28	0.06 ±0.04	0.12 ±0.09	0.16 ±0.11	0.40 ±0.00	N.D.	N.D.
Chronic inact	16	6.51 ±0.93	0.13 ±0.03	0.15 ±0.06	0.31 ±0.09	0.38 ±0.09	0.01 ±0.01	0.26 ±0.05	0.78 ±0.16	1.65 ±0.44	0.64 ±0.17	0.04 ±0.01	0.20 ±0.04	0.44 ±0.11	1.15 ±0.43	0.21 ±0.04	0.10 ±0.06
Chronic act.	9	22.55 ±5.25	0.16 ±0.04	0.98 ±0.34	1.20 ±0.30	1.20 ±0.56	0.05 ±0.02	0.88 ±0.54	3.55 ±1.59	11.84 ±4.56	3.32 ±1.64	0.19 ±0.07	0.30 ±0.11	1.36 ±0.54	4.62 ±1.79	0.51 ±0.30	0.10 ±0.05
Cirrhosis comp.	9	26.36 ±3.86	0.91 ±0.49	0.54 ±0.19	1.22 ±0.31	0.82 ±0.26	0.03 ±0.02	0.24 ±0.05	2.12 ±0.62	7.86 ±2.12	1.43 ±0.56	0.11 ±0.03	0.20 ±0.07	1.94 ±0.51	7.47 ±1.83	1.33 ±0.42	0.14 ±0.09
Cirrhosis non-comp.	12	101.96 ±21.85	0.12 ±0.07	1.00 ±0.43	1.31 ±0.86	0.19 ±0.08	0.03 ±0.01	1.81 ±0.67	16.34 ±2.80	46.51 ±12.36	0.26 ±0.26	0.21 ±0.06	0.48 ±0.19	10.90 ±2.15	24.40 ±7.09	0.53 ±0.28	0.09 ±0.04
I.H.C	8	149.92 ±35.10	0.02 ±0.02	1.77 ±0.30	0.45 ±0.20	0.09 ±0.06	0.23 ±0.14	0.26 ±0.08	32.95 ±8.34	34.29 ±15.36	0.51 ±0.51	0.23 ±0.07	0.30 ±0.10	47.88 ±15.30	29.68 ±7.67	1.09 ±0.90	0.18 ±0.08
E.H.C.	5	377.94 ±182.85	N.D.	0.04 ±0.04	0.09 ±0.09	N.D.	0.03 ±0.02	26.47 ±23.49	73.40 ±33.65	107.88 ±79.41	8.53 ±8.53	0.47 ±0.20	1.73 ±1.35	71.56 ±30.20	79.32 ±50.53	8.15 ±8.15	0.29 ±0.29
Silent Stone	4	6.31 ±2.33	0.25 ±0.21	0.42 ±0.22	0.67 ±0.24	0.29 ±0.15	N.D.	0.29 ±0.17	0.68 ±0.32	2.08 ±0.93	0.47 ±0.27	0.04 ±0.02	0.19 ±0.09	0.11 ±0.04	0.50 ±0.19	0.93 ±0.09	N.D.

in the preparation; secondly, high sensitivity is obtained by high-performance liquid chromatography combined with fluorometric techniques using immobilized enzymes, which enables this measurement to be made in only 25 µl of even healthy human serum. There are several recent approaches to the component analysis of serum bile acids. Gas-liquid chromatography is the standard method. It allows assay ot not only nonsulphated but also sulphated bile acids that are not detectable by our method (9). However, gas-liquid chromatography (GLC) suffers from such disadvantages as extraction, nonconjugation, final derivation prior to injection and substantial loss of bile acids during these procedures. All this results in a failure to identify minor components, such as lithocholic acid. Thus, it is inconvenient for routine clinical use. Gas chromatography-mass spectrometry (GC-MS) is the most advantageous of these techniques because no other method allows identification of unknown bile acids (10). However, this technique has the same disadvantages in the pretreatment procedure and is also rather costly. As a result, we must now consider GC-MS as the last resort technique for clinical use.

We can point to radioimmunoassay (RIA) as one of the approaches well suited to routine clinical use, provided the antibody specificity is carefully determined (8). An enzyme immunoassay is an improvement on radioimmunoassay, with respect to safety, because it does not use radioisotopes (11), though it still has many of the same disadvantages as the latter. Several ambitious trials involving high-

performance liquid chromatographic analysis have been reported (12,13,14). However, without exception, they require a tedious extraction step. Some of them have still more complicated procedures, such as a separating step prior to subjecting the bile acids to the high-performance chromatography (14).

In the present method, we made 200 analyses without a significant change in operating characteristics. Thus, this system can be fully automated quite easily. We hope that the ability to analyze individual circulating bile acid species quickly, easily and specifically will become clinically useful in differentiating various kinds of liver disease. We have shown the reproducibility of this method and have defined its practicality in clinical use.

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