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SEPARATION AND DETERMINATION OF BILE ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING IMMOBILIZED 3α-HYDROXYSTEROID DEHYDROGENASE AND AN ELECTROCHEMICAL DETECTOR

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SUMMARY

A high-performance liquid chromatographic method using an immobilized 3α-hydroxysteroid dehydrogenase column and an electrochemical detector was developed for the determination of individual bile acids in serum and bile. Bile acids in the eluate from a Radial-Pak A column reacted with NAD in the enzyme column to generate NADH, which was monitored by a voltammetric detector after mixing with phenazine methosulphate solution. Each bile acid was measurable at the 20 pmole level at the highest sensitivity of the detector. The mean recoveries and reproducibilities of bile acids were 86.7–104.6% [coefficient of variation (C.V.) = 0.3–8.7%] within-assay and 83.8–103.4% (C.V. = 1.6–7.0%) between-assay.

INTRODUCTION

Methods for the simultaneous determination of individual free and conjugated bile acids in serum and bile have usually been based on gas–liquid chromatography[1], gas chromatography–mass spectrometry[2,3], mass fragmentography[4] or thin-layer chromatography[5,6]. In recent years, high-performance liquid chromatography (HPLC)[7–12] has been developed, but the most common bile acids have no ultraviolet light-absorbing properties, which are necessary with the photometric detector of the liquid chromatograph. Therefore, bile acids have to be derivatized before column separation with a UV-absorbing reagent[13–16]. A fluorescence derivatizing reagent, 4-bromomethyl-7-methoxycoumarin[17], has also been used in the fluorescent liquid chromatography of bile acids in order to improve their detectability.

Baba and co-workers[18,19] reported a highly sensitive and selective fluorescence HPLC determination of bile acids and their conjugates using 3α-hydroxysteroid de-

hydrogenase and cofactor. However, this method consumes considerable amounts of expensive enzyme. Okuyama *et al.*[20] and Arisue *et al.*[21] developed a modified method using an immobilized enzyme column instead of the enzyme solution. Since an electrochemical detector was used in the HPLC analysis of catecholamines by Kissinger *et al.*[22], this novel detector has been widely applied in the HPLC analysis of biological substances. Although this type of detection is more selective than UV detection, non-redox compounds not be detected.

Recently, derivatization to an electrochemically active form was reported by Shimada *et al.*[23] and Ikenoya *et al.*[24]. In this work, we have attempted to develop a method for the determination of bile acids and their conjugates using immobilized 3α-hydroxysteroid dehydrogenase combined with an electrochemical detector.

EXPERIMENTAL

Materials

Cholic acid, chenodeoxycholic acid, deoxycholic acid, ursodeoxycholic acid, lithocholic acid, glycine conjugates and taurine conjugates were purchased from Sigma (U.S.A.), Wako (Japan) and PL Biochemical Co. (U.S.A.), respectively. NAD, NADH, phenazine metosulphate and sodium pyrophosphate were obtained from Wako and 3α-hydroxysteroid dehydrogenase (grade II) from Sigma. Amino glass beads used as the solid phase of the immobilized enzyme were Amino Propyl-CPG 180 Å from Electro-Nucleonics (U.S.A.). PHP-LH-20 was a gift from Professor T. Nambara (Pharmaceutical Institute, Tohoku University, Sendai, Japan).

Reagent solutions

Bile acid stock solutions. Each bile acid was dissolved in methanol and made up to 10 μmol/ml with methanol.

Bile acid mixture solutions. Each mixture of free bile acids, glycine conjugates and taurine conjugates was prepared by mixing the bile acid stock solutions.

NAD solution. A 0.5 mM NAD solution was prepared by dissolving NAD in 0.1 M pyrophosphate buffer (pH 9.0).

Phenazine methosulphate solution. 1.0 mM phenazine methosulphate solution was prepared by dissolving phenazine methosulphate in 0.1 M pyrophosphate buffer (pH 7.0).

Immobilized 3α-HSD column. 3α-hydroxysteroid dehydrogenase was coupled to amino glass beads (120–200 mesh) by the glutaraldehyde method[25] and packed in a stainless-steel tube (25 × 4.6 mm I.D.).

Apparatus

The apparatus consisted of a Model LC-3A chromatograph (Shimadzu, Kyoto, Japan), a Waters Radial-Pak A column (10 μm, 5 × 100 mm) (Waters Assoc., Milford, MA, U.S.A.), a Model VMD-101 voltammetric detector (Yanagimoto Seisakusho, Japan), a solvent exchanger (Kyowaseimitsu Co., Japan), a Model KSU-16 reciprocating pump (Kyowaseimitsu Co.) and a Model M-4 thermostated water bath (Thermonics Co., Japan).
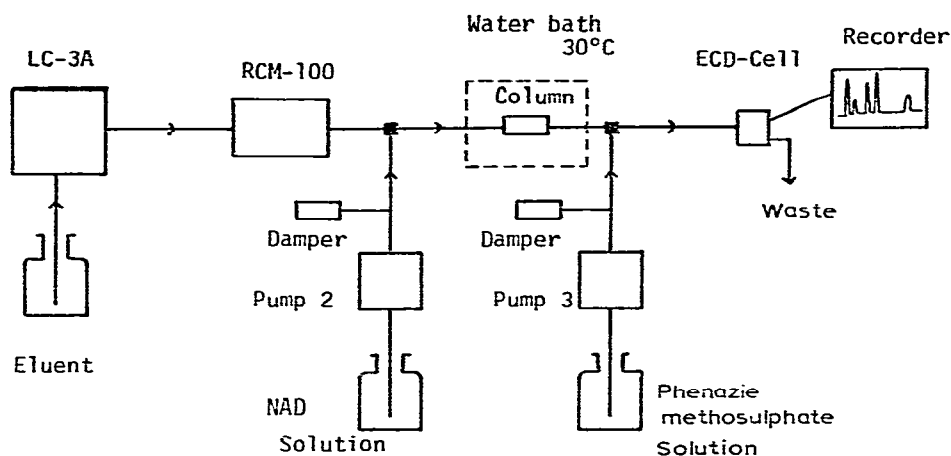
Fig. 1. Flow diagram of the HPLC system.

Chromatographic conditions

The HPLC system shown in Fig. 1 was used. The separation of individual bile acids was carried out on a Radial-Pak A column at room temperature using 0.3 % ammonium phosphate (pH 7.3)–acetonitrile–methanol [100:35:15 (A) and 100:45:15 (B)] as the mobile phase at a flow-rate of 1 ml/min. The mobile phase was changed by a solvent exchanger from A to B 4 min after injection of the sample solution. The eluate from the column was mixed with the NAD solution pumped at the rate of 0.64 ml/min just before the enzyme reactor column maintained at 30°C. After the enzyme reaction in column, the eluate was mixed with the phenazine methosulphate solution delivered with a pump at the rate of 0.42 ml/min. The mixture was monitored by using a Model VMD-101 voltammetric detector, the potential of which was set at +0.10 V vs. a silver–silver chloride reference electrode.

Assay procedure

Extraction of free and conjugated bile acids from serum. A 0.5-ml serum sample was mixed with 2.5 ml of methanol and ultrasonicated for 15 min. A 1.5-ml volume of the supernatant was transferred into a test-tube and evaporated to dryness under nitrogen. The residue was dissolved in 1.0 ml of 0.05 M phosphate buffer (pH 7.0) and applied to a Sep-Pak C_{18} column. After washing the column with 2 ml of 2% methanol, bile acids were eluted with 4 ml of 80% methanol, and evaporated under reduced pressure at 40°C.

Extraction of free and conjugated bile acids from bile. A 20-µl bile sample was diluted with 5.0 ml of 0.05 M phosphate buffer (pH 7.0). A 1-ml volume of the diluted bile sample was applied to a Sep-Pak C_{18} column and extracted as described above.

Fractionation of free and conjugated bile acids. The fractionation of bile acids was carried out as described by Goto et al.[26]. The bile acids and their conjugates extracted from serum or bile were dissolved in 1 ml of 90% ethanol and applied to a PHP-LH-20 column (13 × 7.5 mm I.D.). After washing the column with 3 ml of 90% ethanol, free acids, glycine conjugates and taurine conjugates were fractioned by stepwise elution with 4-ml volumes of 0.1 M acetic acid, 0.2 M formic acid and 0.3 M

acetic acid–potassium acetate buffer (pH 6.3) in 90 % ethanol solution. Each fraction was evaporated to dryness under reduced pressure. In order to remove salts, the taurine fraction was redissolved in 1 ml of water, applied to a Sep-Pak C_{18} column, eluted with 4 ml of 80 % methanol and then re-evaporated to dryness under nitrogen.

The residues were dissolved in 50–100 μl of methanol and applied to the HPLC system mentioned above.

RESULTS AND DISCUSSION

Several parameters were examined in order to determine the optimum conditions for the enzymatic reaction of bile acids by using the system without a separation column shown in Fig. 1.

Type of buffer

Various buffers, such as Tris–HCl, phosphate and pyrophosphate, were used for the reaction of 3α-hydroxysteroid dehydrogenase. The pyrophosphate buffer gave the highest response in this system and was therefore used for the following experiments and the assay procedure.

Concentration and pH of buffer

Fig. 2 depicts the peak heights plotted *versus* the concentration and pH of the pyrophosphate buffer. When the concentration of the buffer (pH 9.0) was increased from 0.01 to 0.05 M, the peak height reached plateaux at a concentration of buffer of 0.025 M and at pH of the buffer of 9.0.

Concentration of phenazine methosulphate and NAD solutions

Oxidation of NADH occurs only at high potentials, which considerably decreases the selectivity of the assay in eluates that contain other electrochemically
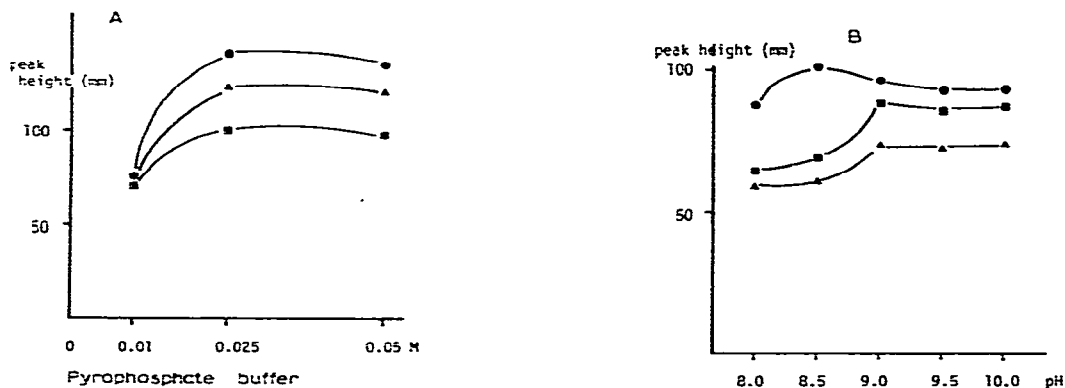

Fig. 2. (A) Peak height of 2-nmol samples of bile acids plotted against concentration of pyrophosphate buffer (pH 9.0). NAD, 0.2 mM (1 ml/min): phenazine methosulphate, 1.0 mM (0.3 ml/ml); detector potential, +0.1 V, 200 nA full-scale. (B) Peak height of 1-nmol samples of bile acids plotted against the pH of pyrophosphate buffer (0.05 M). NAD, 0.1 mM (1 ml/min); phenazine methosulphate, 0.5 mM (0.3 ml/min); detector potential, +0.32 V, 200 nA full-scale. ●, Cholic acid; ■, taurinecholic acid; ▲, glycinecholic acid.

active compounds. The rate of oxidation of NADH is slow and consequently electro-oxidation of NADH can limit the entire conjugated enzyme reaction process. To eliminate these problems, phenazine methosulphate was used as an electron-transfer intermediate in the enzyme electrode using oxidoreductase[27]. In absence of phenazine methosulphate in the present system, the potential of detector should be set at +0.33 V and the responses of all bile acids were low compared with those obtained by using the phenazine methosulphate solution. Therefore, phenazine methosulphate was used as a mediator for electrochemical regeneration of coenzyme in this system. The sequence of conjugated enzyme reactions occurring at the detection part in the system may be illustrated as follows:
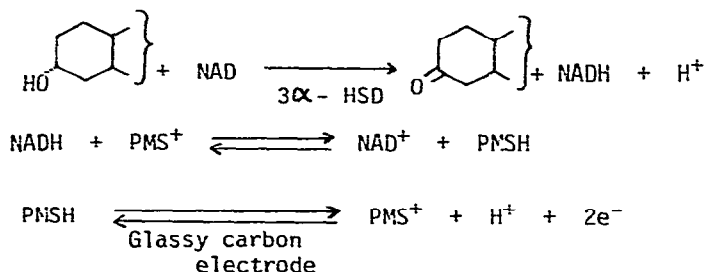
(where PMS = phenazine methosulphate and 3α-HSD = 3α-hydroxysteroid dehydrogenase).

As shown in Fig. 3. the peak height increased with increasing concentration of phenazine methosulphate, whereas the peak height reached a maximum at a concentration of NAD of 0.2 mM.

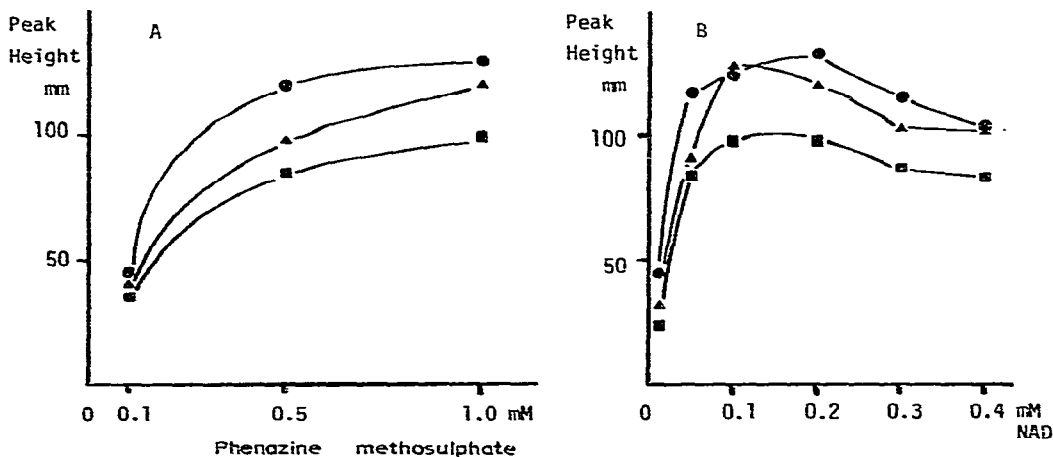From the above results and the mixing ratio of the reagent to the eluate from

Fig. 3. Effects of phenazine methosulphate and NAD concentration on the enzymatic reaction (0.05 M pyrophosphate buffer, pH 9.0). Detector potential, +0.1 V, 100 nA full-scale. (A) 0.2 mM NAD (1 ml/min); (B) 1.0 mM phenazine methosulphate (0.3 ml/min). ●, Cholic acid; ■, glycinecholic acid; ▲, taurinecholic acid.
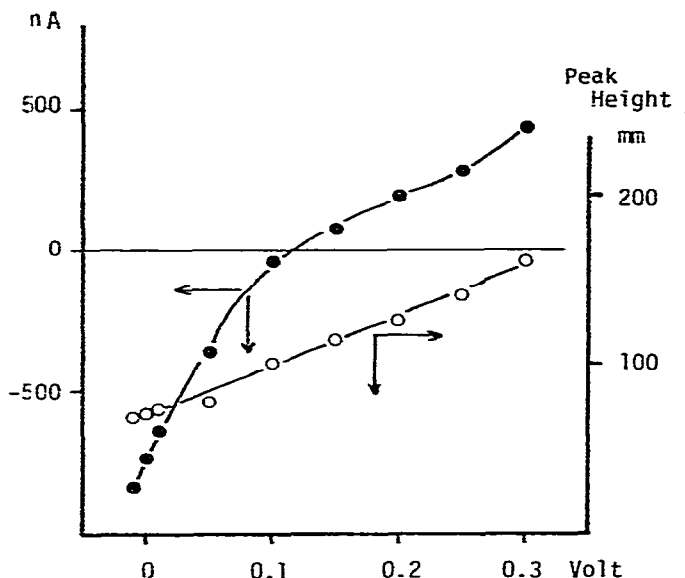
Fig. 4. Background current and peak height versus detector potential. NAD. 0.1 mM (1 ml/min); phenazine methosulphate. 0.5 mM (0.3 ml/min).

the column in the HPLC system, the concentration of phenazine methosulphate, NAD and pyrophosphate buffer were fixed at 1.0 mM, 0.5 mM and 0.1 M, respectively.

Potential of the detector

The peak height and background currents were in the potential range from −0.10 to +0.30 V using NADH solution (2.0 nmol/ml), 0.1 mM NAD solution (flow-rate 1 ml/min) and 0.5 mM phenazine methosulphate solution (flow-rate 0.3 ml/min). As the potential of the detector increased, the peak height increased and the background current also increased (Fig. 4). Therefore, the potential was fixed at +0.10 V. at which the background current was nearly zero.

Chromatographic separation

Various solvent systems for the separation of bile acids and their conjugates have been reported. In this study, the separation of bile acids should be carried out under nearly neutral conditions because enzymatic reaction was used in the detection system. On the basis of the separation system reported by Goto et al.[26], we examined a range of solvent systems using a Waters Radial-Pak A separation column. It was difficult to separate completely ursodeoxycholic acid from cholic acid or chenodeoxycholic acid from deoxycholic acid with 0.3% ammonium phosphate–acetonitrile as the solvent system. Addition of methanol to the above solvent system was effective for the separation of bile acids. Finally, two solvent systems, 0.3% ammonium phosphate (pH 7.3)–acetonitrile–methanol [100:35:15 (A) and 100:45:15 (B)] were found to be suitable for the separation of all of the bile acids in each group (free acids, glycine conjugates and taurine conjugates), but ursodeoxycholic acid and
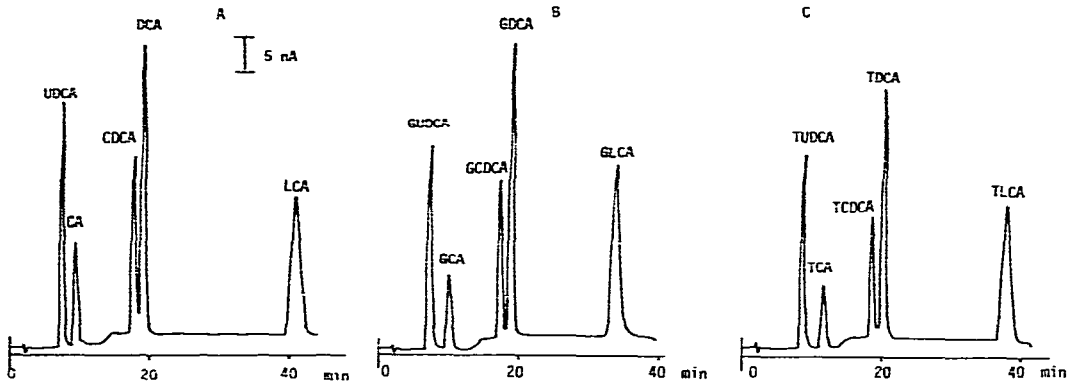
Fig. 5. Typical chromatograms of standard mixture of bile acids (1 nmol). (A) Free bile acids: UDCA = ursodeoxycholic acid, CA = cholic acid, CDCA = chenodeoxycholic acid, DCA = deoxycholic acid, LCA = lithocholic acid; (B) glycine (G) conjugates; (C) taurine (T) conjugates.

chenodeoxycholic acid could not be separated as their conjugates. Therefore, bile acids extracted from serum by Sep-Pak C_{18} were fractionated into three groups by using a PHP-LH-20 column[26] and then each fraction was subjected separately to the HPLC system. Stepwise elution using solvent systems A and B was adopted to shorten the retention times of lithocholic acid, glycinelithocholic acid and taurinelithocholic acid.

Calibration graphs

Typical chromatograms of bile acids and their conjugates obtained by using standard mixtures of free and conjugated bile acids in methanol are shown in Fig. 5. Calibration graphs were constructed from the chromatograms obtained by injecting 2, 4, 6, 8 and 10 μl of the standard mixtures of free bile acids and their conjugates (250 nmol/ml) in methanol.

Typical calibration graphs are shown in Fig. 6. Linearity of the relationship
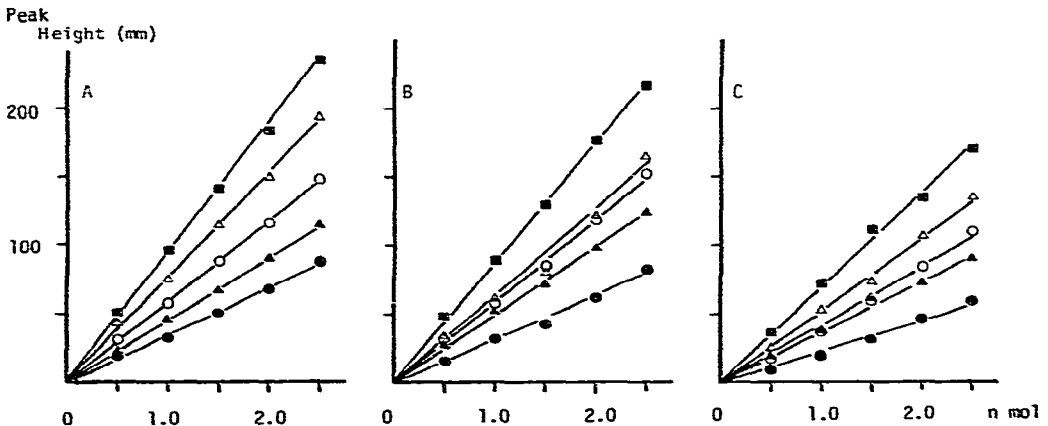

Fig. 6. Calibration graphs for bile acids. (A) Free bile acids; (B) glycine conjugates; (C) taurine conjugates. ●, Cholic acid; ▲, lithocholic acid; O, chenodeoxycholic acid; △, ursodeoxycholic acid; ■, deoxycholic acid.

TABLE I

RECOVERY AND REPRODUCIBILITY OF INDIVIDUAL BILE ACIDS IN HUMAN SERUM (WITHIN-ASSAY) ($n = 5$)

Compound	Added (nmol)	Free		Glycine		Taurine	
		Mean recovery (%)	C.V. (%)	Mean recovery (%)	C.V. (%)	Mean recovery (%)	C.V. (%)
Ursodeoxycholic acid	20	104.6	4.8	88.0	2.8	92.9	2.2
	200	102.1	2.9	90.4	1.9	93.6	3.6
Cholic acid	20	94.6	8.7	91.0	7.5	94.4	2.0
	200	98.6	0.3	93.5	1.4	94.9	3.9
Chenodeoxycholic acid	20	99.3	1.5	86.7	6.6	92.6	2.3
	200	90.5	4.8	94.4	1.4	97.9	1.4
Deoxycholic acid	20	99.7	2.4	90.0	4.5	93.0	1.2
	200	98.1	4.6	95.1	2.0	93.4	1.2
Lithocholic acid	20	98.2	2.0	88.5	2.8	88.4	1.8
	200	100.6	3.8	95.5	2.5	97.3	1.1

between peak height and amount of bile acids injected was obtained between 0.5 and 2.5 nmol. The detection limit for bile acids was about 0.2 nmol, depending on the efficiency of the detector and the final volume of sample. The lowest detection limit was 20 pmol, achieved by adjusting the detector sensitivity to 20 nA full-scale.

Recovery and reproducibility

In order to determine the recovery and reproducibility for each bile acid, standard bile acids (20 or 200 nmol) were added to 1.0 ml of human serum and assayed with the HPLC system after extraction and fractionation as described under Experimental. The results are summarized in Tables I and II. The mean recoveries and

TABLE II

RECOVERY AND REPRODUCIBILITY OF INDIVIDUAL BILE ACIDS IN HUMAN SERUM (BETWEEN-ASSAY) ($n = 5$)

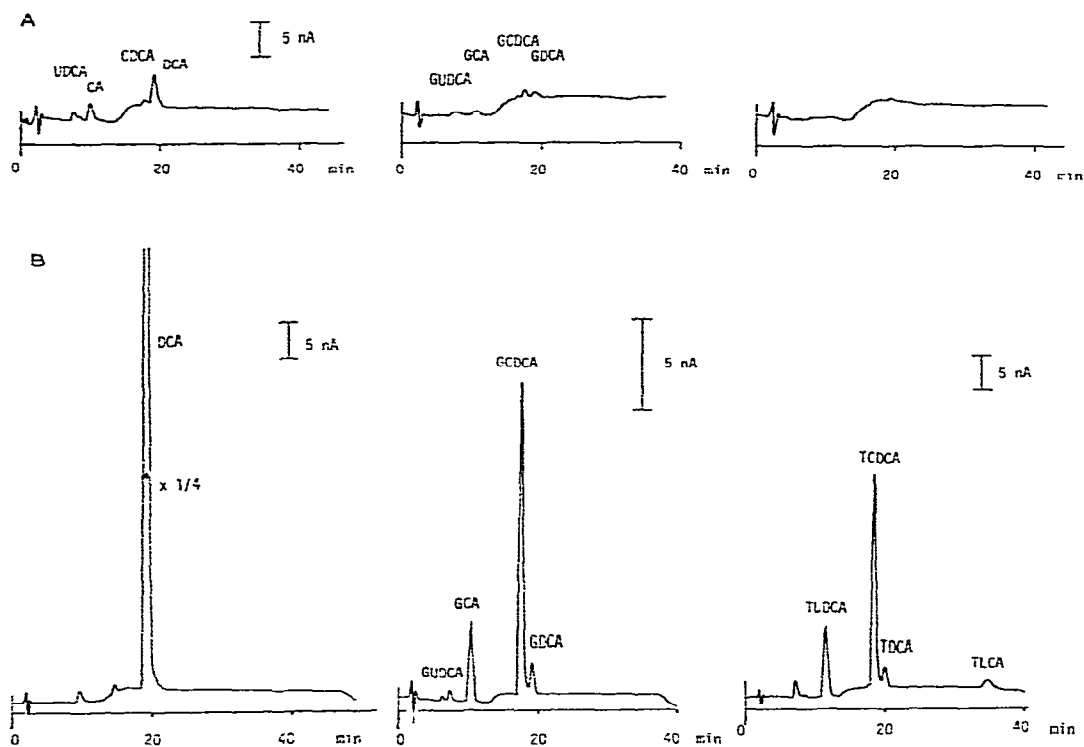| Compound	Added (nmol)	Free		Glycine		Taurine	
		Mean recovery (%)	C.V. (%)	Mean recovery (%)	C.V. (%)	Mean recovery (%)	C.V. (%)
Ursodeoxycholic acid	20	94.7	2.3	96.2	6.7	92.1	3.1
Cholic acid	20	94.2	3.1	98.0	3.1	94.5	3.4
Chenodeoxycholic acid	20	92.1	4.0	103.4	2.7	93.3	5.8
Deoxycholic acid	20	90.9	4.2	95.7	3.0	90.3	3.6
Lithocholic acid	20	92.3	7.0	92.2	1.6	83.8	2.2

Fig. 7. Typical chromatograms obtained from sera of (A) a normal healthy male and (B) a patient with acute hepatitis. For abbreviations, see Fig. 5.

coefficients of variation, within-assay and between-assay, ranged from 86.7 to 104.6 % [coefficient of variation (C.V.) = 0.3–8.7 %) and from 83.8 to 103.4 % (C.V. = 1.6–7.0 %), respectively.

Application

In order to investigate the applicability of the present method, the simultaneous determination of free and conjugated bile acids in serum and bile was carried out for 11 patients with various diseases and a healthy volunteer. The results are given in Table III and the typical chromatograms of a healthy male and a patient with liver cirrhosis are shown in Fig. 7. Although taurine conjugates, lithocholic acid and glycinelithocholic acid could not be detected in the case of Fig. 7, using 100 μl of serum sample, all other bile acids were detected in normal serum. Characteristic patterns of individual serum bile acids were observed in patients with various diseases. Free, conjugated cholic acid and chenodeoxycholic acid were significally elevated in patients with liver cirrhosis and hepatitis and liver carcinoma. It is also of particular interest that cholic acid, deoxycholic acid and taurinedeoxycholic acid in two breast-cancer patients showed a significant increase compared with normal serum, and a large unknown peak appeared near the peak of taurinedeoxycholic acid in serum of patients with breast cancer and lung cancer. The results suggest that the method may provide more precise information on the meta-

TABLE III

INDIVIDUAL BILE ACIDS IN SERUM AND BILE OF A HEALTHY SUBJECT AND PATIENTS

UDCA = Ursodeoxycholic acid; CA = cholic acid; CDCA = chenodeoxycholic acid; DCA = deoxycholic acid; LCA = lithocholic acid. 0 = No peak observed.

Sample	Disease	Free					Glycine					Taurine				
		UDCA	CA	CDCA	DCA	LCA	UDCA	CA	CDCA	DCA	LCA	UDCA	CA	CDCA	DCA	LCA
Serum*	Normal	0.03	0.08	0.02	0.10	0	0.02	0.02	0.03	0.02	0	0	0	0	0	0
	Hepatitis	0.04	0.55	0.13	0.97	0	0.08	0.18	0.13	0.07	0	0.02	0.55	ND	0.59	0
	Liver cirrhosis	ND***	0.27	ND	6.59	ND	0.07	1.03	1.79	0.13	0	0.01	1.12	1.72	0.08	0.05
	Liver cancer:															
	1	ND	0.13	0.02	0.10	0	0.04	13.30	0.29	13.85	0	ND	0.18	0.39	0.61	0
	2	0	0.07	0.10	0.10	0	0.02	0.36	0.13	0.09	0	ND	0.18	0.11	0.13	ND
	3	0.10	0.39	0.48	0.25	0	0.17	0.27	1.24	0.16	0.03	6.38	1.21	ND	1.79	ND
	Pancreas cancer	0.01	0.26	0	0.50	0	0.02	0.04	0.05	0	0	ND	ND	ND	0.25	0
	Breast cancer:															
	1	0.09	6.13	ND	7.80	0	0.13	2.36	1.69	0.13	0.08	0.09	6.82	4.46	1.79	0
	2	0.03	1.45	ND	1.48	0	0.02	0.09	0.02	0.78	0	ND	UP†	ND	4.62	0
	3	ND	0.52	0.02	0.31	0	0	0	0.05	0.03	0	ND	UP	0	1.39	0
	4	0	0.42	0.04	0.20	0	0.04	0.04	0.25	0.13	0	ND	UP	0.04	4.30	0
	Lung cancer	0	3.77	ND	2.42	0	0.04	0.44	0.05	0.24	0	0.02	UP	0.04	0.36	ND
Bile**	Normal	0.03	0.09	ND	1.18	0.07	1.00	8.71	10.00	7.28	0.66	0.26	5.30	6.49	3.90	0.49

* Serum: μmol/dl.
** Bile: μmol/ml.
*** ND = Non-detectable (peak height <1 mm).
† UP = Unknown peak.

bolic profile of bile acids in patients with various diseases, including hepatobiliary diseases.

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