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FLUORESCENCE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF FREE AND CONJUGATED BILE ACIDS IN SERUM AND BILE USING 1-BROMOACETYLPYRENE AS A PRE-LABELING REAGENT

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SUMMARY

A fluorescence high-performance liquid chromatographic method is described for the determination of free and conjugated bile acids in serum and bile. Free and conjugated bile acids are extracted from serum or bile using a Sep-Pak C_{18} cartridge and then fractionated on a piperidinohydroxypropyl Sephadex LH-20 column. Free and glycine-conjugated bile acids are labeled with 1-bromoacetylpyrene in acetonitrile using dicyclohexyl-18-crown-6-ether as catalyst. Taurine-conjugated bile acids are hydrolyzed by cholylglycine hydrolase and then derivatized by the same reagent. Derivatized bile acids are separated stepwise on a reversed-phase column (Radial Pak A) using acetonitrile—methanol—water (A) (100 : 50 : 40) and (B) (100 : 50 : 20) as mobile phase. The eluate is monitored by a fluorophotometer at 370 nm (excitation) and 440 nm (emission). Linearities of fluorescence intensities (peak heights) with the amounts of free and conjugated bile acids were obtained between 50 pmol and 200 pmol for free bile acids and between 25 pmol and 100 pmol for glycine-conjugated bile acids, respectively. Recoveries from serum and bile samples are not less than 90%. This method is sensitive, reliable and useful for the simultaneous determination of free and conjugated bile acids in serum and bile.

INTRODUCTION

The identification and determination of individual bile acids and their conjugates in serum and bile are of value in the diagnosis of liver disease [1]. A variety of methods for the simultaneous determination of individual free and conjugated bile acids in human serum and bile has been described, in which thin-layer chromatography [2,3], gas chromatography [4,5], and gas chromatography—mass spectrometry [6—8] were used. In recent years, high-

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performance liquid chromatography (HPLC) [9—17] has been developed, but the most common bile acids, which have no UV light-absorbing properties, could not be detected with high sensitivity by a photometric detector. Therefore, bile acids should be derivatized before chromatographic separation with a UV-absorbing prelabeling reagent, such as 1-*p*-nitrobenzyl-3-*p*-tolyltriazine [12], phenacyl bromide [13,14], *p*-chlorobenzoyl chloride [15], O-(*p*-nitrobenzyl)-N,N-diisopropylisourea [15,16], and 1-naphthyldiazomethane [17]. These labeling reagents are the derivatization reagents for carboxylic acids. Though these methods [12—17] are useful for the assay of bile acids in bile, their sensitivities are not enough to determine small amounts of bile acids in human serum samples. Therefore, in order to improve the detectability for bile acids, fluorescent derivatization reagents such as 4-bromomethyl-7-methoxycoumarin [18] and 9,10-diaminophenanthrene [19] have been used for the assay of bile acids by HPLC. Baba et al. [20] reported a different fluorescence HPLC system in which bile acids and their conjugates eluted from the column were converted to 3-oxo bile acids and NADH using 3α-hydroxysteroid dehydrogenase (3α-HSD) and NAD^+ solution, and NADH was monitored by a fluorophotometric detector. However, this method consumes considerable amounts of expensive enzyme. Okuyama et al. [21] and Arisue et al. [22] devised a modified method using an immobilized enzyme column instead of the enzyme solution. In the previous paper [23], we used a voltammetric detector to measure NADH instead of a fluorophotometric detector in an HPLC method for the determination of bile acids using an immobilized 3α-HSD column and NAD^+ solution. Though each bile acid was measurable at the 20 pmol level by this method, a more highly sensitive HPLC method was required because the amounts of bile acids in human serum samples are as low as a few picomoles. In this paper, we have attempted to develop a new fluorescent derivatizing reagent for a highly sensitive method for determining free and conjugated bile acids.

EXPERIMENTAL

Materials

Cholic acid, chenodeoxycholic acid, deoxycholic acid, ursodeoxycholic acid, lithocholic acid, and their glycine and taurine conjugates were purchased from Sigma (St. Louis, MO, U.S.A.), Wako (Osaka, Japan) and PL Biochemicals (Milwaukee, WI, U.S.A.). Dicyclohexyl-18-crown-6-ether was obtained from Nippon Soda (Tokyo, Japan), and Sep-Pak C_{18} cartridge from Waters Assoc. (Milford, MA, U.S.A.). 1-Bromoacetylpyrene and piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) were gifts from Dr. Y. Kawahara (Product development laboratories of Sankyo, Tokyo, Japan) and from Professor T. Nambara (Pharmaceutical Institute, Tohoku University, Sendai, Japan), respectively. Cholylglycine hydrolase (EC 3.5.1.24) was purchased from Sigma. All the other reagents employed were of analytical grade. Solvents were purified by distillation prior to use.

Reagent solutions

Stock solutions of bile acids: each bile acid was dissolved in methanol and made up to 10 μmol/ml with methanol.

Standard mixture solution of bile acid: standard mixture solutions of free, glycine-conjugated, and taurine-conjugated bile acids were prepared by mixing the bile acid stock solutions, and their concentrations were adjusted to 100 nmol/ml (free acids), 50 nmol/ml (glycine conjugates) and 100 nmol/ml (taurine conjugates).

Lauric acid standard solution (internal standard); lauric acid was used as internal standard. Purified lauric acid was dissolved in methanol and made up to 100 nmol/ml with methanol.

1-Bromoacetylpyrene solution: this solution was prepared by dissolving 1-bromoacetylpyrene in acetonitrile (25 μmol/ml).

Dicyclohexyl-18-crown-6-ether solution: this solution was prepared by dissolving dicyclohexyl-18-crown-6-ether in acetonitrile (1 mg/ml).

KOH–methanol solution (0.01%): this solution was prepared by dissolving KOH in methanol (0.1 mg/ml).

Instruments

A Shimadzu (Kyoto, Japan) Model LC-3A high-performance liquid chromatograph equipped with a Waters Assoc. Model RCM-100 Radial-Pak A column (100 mm × 8 mm I.D.; particle size 10 μm) was utilized for HPLC. A Shimadzu Model RF-500 spectrofluorophotometer equipped with an 8-μl micro flow cell was used as a monitor.

Derivatization procedure

A standard mixture solution of bile acids or sample solution (50 μl), a lauric acid standard solution (internal standard) (50 μl) and 0.01% KOH–methanol solution (50 μl) were mixed in a vial and evaporated to dryness under a stream of nitrogen gas. A dicyclohexyl-18-crown-6-ether solution (100 μl) and 1-bromoacetylpyrene solution (100 μl) were then added to the tube. The reaction mixture was heated at 40°C for 30 min. After cooling, an aliquot of 8 μl of the solution was injected into the chromatograph.

Chromatographic conditions

Separation of free and glycine-conjugated bile acids. After derivatization with 1-bromoacetylpyrene, the separation of derivatives of bile acids was carried out on a Radial-Pak A column at room temperature using acetonitrile–methanol–water [100 : 50 : 40 (A) and 100 : 50 : 20 (B)] as the mobile phase at a flow-rate of 2 ml/min. The mobile phase was changed by a solvent exchanger from A to B at 30 min after injection of the sample solution. The effluent from the column was monitored by a fluorophotometric detector at an excitation wavelength of 370 nm and an emission wavelength of 440 nm.

Taurine-conjugated bile acids. After derivatization of enzymatically hydrolyzed taurine-conjugated bile acids, the separation was carried out by the same column described above using acetonitrile–methanol–water (100 : 50 : 22) as the mobile phase at a flow-rate of 2 ml/min.

Procedure for the determination of free and glycine-conjugated bile acids in human serum

A serum sample (100 or 200 μl) was mixed with methanol (1 ml) and ultrasonicated for 15 min. The supernatant (600 μl) was transferred into a micro test tube and evaporated to dryness under nitrogen gas stream. The residue was dissolved by adding 0.05 *M* phosphate buffer (pH 7.0) (1 ml), and then percolated through a Sep-Pak C_{18} cartridge. The cartridge was washed with 20% (v/v) methanol (2 ml), and bile acids were then eluted with 80% (v/v) methanol (4 ml). After evaporation of methanol under reduced pressure at 40°C, the residue was redissolved by addition of methanol (1 ml), and an aliquot of 500 μl of the resultant solution was then used for the derivatization step described above.

Procedure for the determination of free and glycine-conjugated bile acids in human bile

A bile sample (10 μl) was diluted with 0.05 *M* phosphate buffer (pH 7.0) (10 ml) and the diluted solution (1 ml) was then applied to a Sep-Pak C_{18} cartridge and assayed as described above.

Procedure for the determination of taurine-conjugated bile acids in human serum

The bile acid fraction was extracted from the serum sample (100 or 200 μl) by a Sep-Pak C_{18} cartridge as described above. The evaporated bile acid fraction was dissolved in 90% (v/v) ethanol (1 ml) and applied gently to a column (115 × 7.5 mm I.D.) of PHP-LH-20 (acetate) (ca. 100 mg) at a flow-rate of 7—8 drops/min. After washing with 90% (v/v) ethanol (4 ml), free, glycine-conjugated and taurine-conjugated bile acids were eluted stepwise with 0.1 *M* acetic acid in 90% (v/v) ethanol (4 ml), 0.2 *M* formic acid in 90% (v/v) ethanol (4 ml), and 0.3 *M* acetic acid—potassium acetate in 90% (v/v) ethanol (pH 6.3—6.5) (4 ml). The fraction of taurine-conjugated bile acids was evaporated to dryness under a nitrogen gas stream. The residue was dissolved in water (1 ml) and percolated through a Sep-Pak C_{18} cartridge. After washing the column with 2% (v/v) methanol (2 ml), taurine-conjugated bile acids were eluted with 80% (v/v) methanol (4 ml). After evaporation of methanol, the residue was redissolved by the addition of water (500 μl) and 0.025 *M* phosphate buffer (200 μl), and then mixed with cholylglycine hydrolase solution (30 units/ml) (100 μl). After incubation at 37°C for 15 min, the reaction mixture was cooled in an ice bath and mixed with 0.25 *M* phosphate buffer (pH 7.0) (200 μl). The free bile acids in the resultant solution were extracted using a Sep-Pak C_{18} cartridge and then derivatized with 1-bromoacetylpyrene as mentioned above.

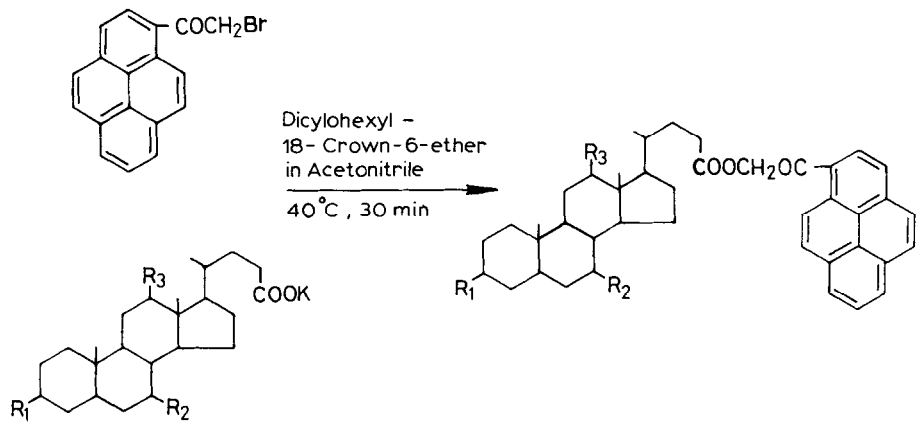
Recovery test

A synthetic mixture of 50 nmol of each free bile acid and 25 nmol of each glycine-conjugated bile acid was added to 1.0 ml of serum and 1 μl of bile, respectively, and then assayed by the procedure.

RESULTS AND DISCUSSION

Derivatization

Phenacyl bromide type derivatives are the favorite reagents for the formation of carboxylic acid esters because of their high reactivity under mild conditions. Therefore, 1-bromoacetylpyrene was synthesized as a fluorescent derivatization reagent [24]. It was relatively easy to synthesize this reagent from readily available compounds. The free and glycine-conjugated bile acids were esterified with 1-bromoacetylpyrene using dicyclohexyl-18-crown-6-ether as catalyst in acetonitrile after converting them to their potassium salts, as shown in Fig. 1.



R_1	R_2	R_3	
OH	OH	OH	Cholic acid
OH	H	OH	Deoxycholic acid
OH	OH	H	Chenodeoxycholic acid
OH	OH(β)	H	Ursodeoxycholic acid
OH	H	H	Lithocholic acid

Fig. 1. Esterification of bile acids with 1-bromoacetylpyrene.

Taurine-conjugated bile acids were separated from free and glycine-conjugated bile acids by column chromatography with PHP-LH-20 according to the method of Goto et al. [25]. After collecting the taurine-conjugated bile acid fraction and enzymatic hydrolysis with cholylglycine hydrolase, esterification was conducted with 1-bromoacetylpyrene as well as the free and glycine-conjugated acids.

As shown in Fig. 2, the reaction of all bile acids tested with the reagent at 40°C was completed within 20 min. When heated at 80°C, the reaction was completed within about 10 min and the fluorescence intensity increased slightly, but unknown subpeaks appeared and overlapped some bile acid peaks.

The peak height of bile acids increased with increasing the amount of reagent added and reached a constant value at 10 μmol/ml. Though bile acids were esterified in the absence of dicyclohexyl-18-crown-6-ether, the peak height of free bile acids was very low compared with that of glycine-conjugated bile

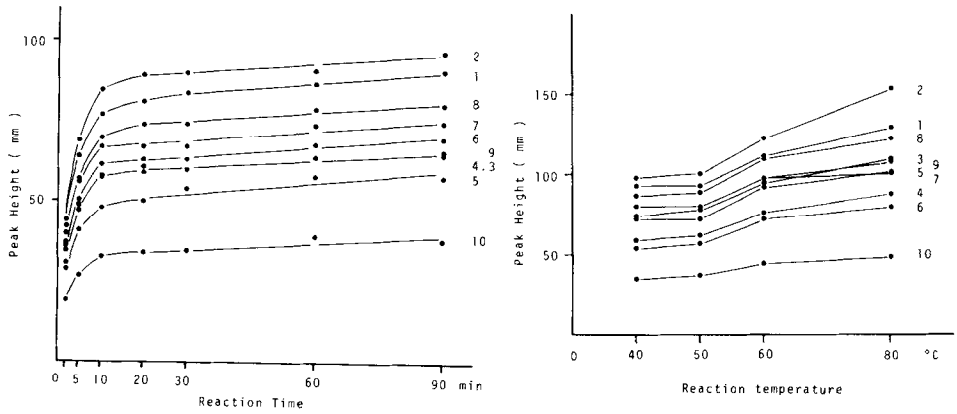


Fig. 2. Reaction conditions of derivatization. 1 = Glycoursodeoxycholic acid, 2 = glycocholic acid, 3 = glycochenodeoxycholic acid, 4 = glycodeoxycholic acid, 5 = ursodeoxycholic acid, 6 = cholic acid, 7 = glycolithocholic acid, 8 = chenodeoxycholic acid, 9 = deoxycholic acid, 10 = lithocholic acid.

acids. By adding 50 μl of 0.01% KOH–methanol solution the peak height of free bile acids increased and the peak height ratio with the internal standard peak of free and glycine-conjugated bile acids became nearly equal to each other.

From these results, the derivatization procedure was decided as described in Experimental.

Hydrolysis of taurine conjugated bile acids

Taurine-conjugated bile acids are unable to be directly esterified with 1-bromoacetylpyrene. Therefore, prior to derivatization, an hydrolysis step is inevitable. In a preliminary experiment, we examined alkaline hydrolysis of taurine-conjugated bile acids but could not obtain satisfactory reproducibility. Enzymatic hydrolysis with cholylglycine hydrolase from *Clostridium perfringens* (EC 3.5.1.24) was examined according to the literature reported by Karlaganis et al. [6]. In order to obtain quantitative hydrolysis conditions, the amount of enzyme, pH of buffer and incubation time were examined. From the results, the enzymatic hydrolysis conditions were decided as the procedure described in Experimental.

Chromatographic separation

The chromatographic separation of the derivatized free and glycine-conjugated bile acids was carried out under a variety of conditions. The reversed-phase type column, Waters Radial-Pak A, and the stepwise elution system using acetonitrile–methanol–water mixtures (A) and (B) were used as described in Experimental. A typical chromatogram obtained by synthetic mixture of free and glycine-conjugated bile acids is shown in Fig. 3. Though glycoursodeoxycholic acid was not separated completely from the peaks of excess or decomposed reagent, baseline separation was obtained between other bile acids.

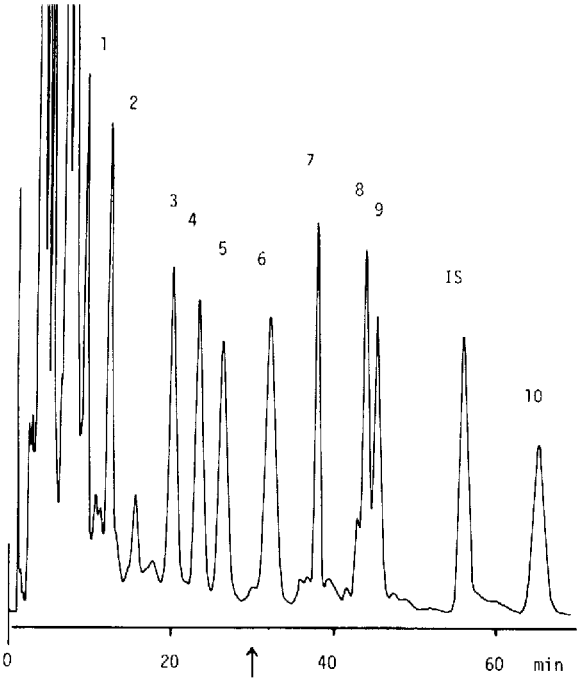


Fig. 3. Chromatogram of standard free and glycine conjugated bile acids. Peaks: 1 = glycoursodeoxycholic acid, 2 = glycocholic acid, 3 = glycochenodeoxycholic acid, 4 = glycodeoxycholic acid, 5 = ursodeoxycholic acid, 6 = cholic acid, 7 = glycolithocholic acid, 8 = chenodeoxycholic acid, 9 = deoxycholic acid, 10 = lithocholic acid.

In the assay of taurine-conjugated bile acids, derivatization was carried out after enzymatic hydrolysis and Sep-Pak C_{18} cartridge clean-up. As shown in Fig. 4, the derivatives of liberated free bile acids were satisfactorily separated from each other when acetonitrile—methanol—water (100 : 50 : 22, v/v) was used as the mobile phase.

Pyrenacyl esters of bile acids were eluted in the same definite order as underivatized bile acids. This suggests that the pyrene group bound to the side-chain carboxylic acid would not affect the elution pattern of bile acids.

Calibration graphs

Calibration graphs were constructed by plotting the ratio of the peak height of bile acid to that of an internal standard, lauric acid, against the amount of the bile acid. Typical calibration graphs are shown in Fig. 5. Linearity of the relationship between peak height ratio and the amount of bile acids was obtained in the range 50—200 pmol for free bile acids and 25–100 pmol for glycine-conjugated bile acids. From these graphs the detection limits were 10 pmol for free bile acids, 5 pmol for glycine-conjugated bile acids and 10 pmol for taurine-conjugated bile acids, depending on the efficiency of the detector and the final volume of sample solution.

As shown in Fig. 5, the responses of free bile acids were about half those of glycine-conjugated bile acids. In order to elucidate the reason for this

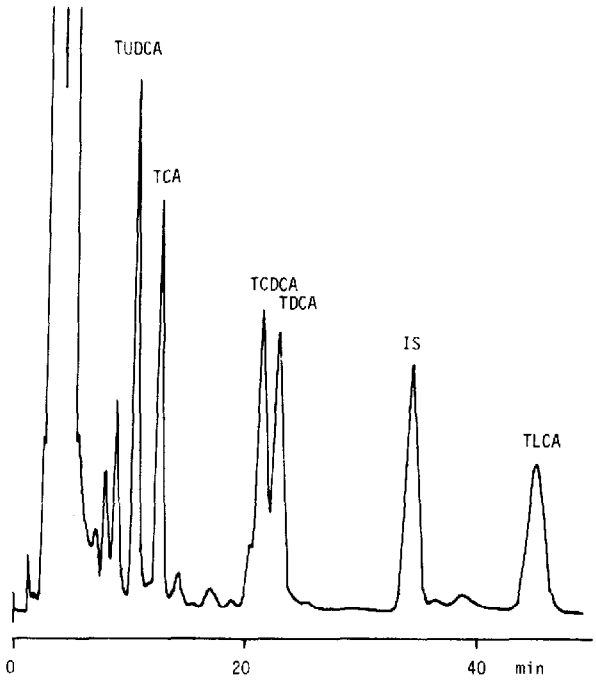


Fig. 4. Chromatogram of taurine-conjugated bile acids after enzymatic hydrolysis. TUDCA = tauroursodeoxycholic acid, TCA = taurocholic acid, TCDCA = taurochenodeoxycholic acid, TDCA = taurodeoxycholic acid, IS = internal standard, TLCA = taurolithocholic acid.

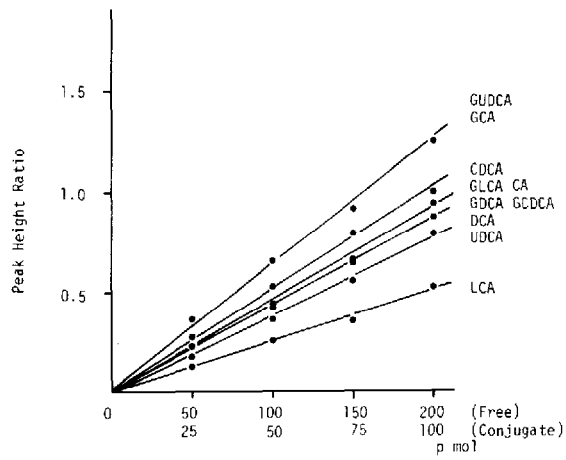


Fig. 5. Calibration curves obtained by standard bile acid mixture. GUDCA = glycoursodeoxycholic acid, GCA = glycocholic acid, CDCA = chenodeoxycholic acid, GLCA = glycolithocholic acid, CA = cholic acid, GDCA = glycodeoxycholic acid, GCDCA = glycochenodeoxycholic acid, DCA = deoxycholic acid, UDCA = ursodeoxycholic acid, LCA = lithocholic acid.

phenomenon, [^{3}H]glycochenodeoxycholic acid and [^{14}]cholic acid were diluted with carrier. After derivatization with 1-bromoacetylpyrene, the resultant reaction solution was divided into halves, one half being separated by HPLC as described in Experimental. The radioactivities of the separated fractions of cholic acid and glycochenodeoxycholic acid were measured and compared with those of the original derivatized reaction solution. After separation by HPLC, the recovery of [^{3}H]glycochenodeoxycholic acid was about 70%, and the recovery of [^{14}C]cholic acid was about 35%. Therefore, the derivatization yield of free bile acids appeared to be half that of the glycine-conjugated bile acids.

Recovery tests

In order to determine the recoveries, the standard bile acid mixture of each five free and glycine-conjugated bile acids was added to human serum and bile, and then subjected to the HPLC method after extraction and derivatization as described in Experimental. As shown in Table I, the recoveries of free and glycine-conjugated bile acids were satisfactory. The mean recoveries and coefficients of variation (C.V.) from serum samples ranged from 90.3 to 104.7% (C.V. = 2.9—18.0%) for intra-assay and from 96.8 to 108.5% (C.V. = 3.4—11.1%) for inter-assay. In the case of bile, the mean recoveries and C.V., intra-assay and inter-assay, ranged from 88.0 to 106.4% (C.V. = 1.0—2.5%) and from 94.7 to 107.7% (C.V. = 1.7—9.8%), respectively.

The recoveries of taurine-conjugated bile acids from serum were also determined. The results are shown in Table II. The recoveries and coefficients of variation were not good compared with those of free and glycine-conjugated bile acids.

TABLE I

RECOVERIES OF FREE AND GLYCINE-CONJUGATED BILE ACIDS FROM HUMAN SERUM AND BILE

Bile acids*	Serum				Bile			
	Intra-assay (n=4)		Inter-assay (n=4)		Intra-assay (n=4)		Inter-assay (n=4)	
	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)
GUDCA	95.9	7.3	96.8	9.4	105.2	2.4	107.7	6.2
GCA	90.3	18.0	97.9	11.1	98.4	2.5	103.6	9.7
GCDCA	98.1	10.8	98.3	3.9	104.4	1.3	104.4	5.6
GDCA	98.1	10.9	101.9	7.7	106.4	1.0	103.8	6.6
UDCA	104.7	9.7	109.1	8.0	103.4	2.2	103.2	3.6
CA	99.1	6.1	108.5	5.6	106.4	1.4	101.1	3.9
GLCA	102.0	5.9	98.4	3.4	103.0	1.6	98.6	3.6
CDCA	91.7	5.1	105.4	5.1	99.2	1.1	97.9	1.7
DCA	102.9	2.9	109.4	5.5	100.6	1.4	98.6	2.8
LCA	96.0	5.8	104.0	10.0	88.0	1.4	94.7	9.8

*For abbreviations see legend to Fig. 5.

TABLE II

RECOVERIES OF TAURINE-CONJUGATED BILE ACIDS FROM HUMAN SERUM

Bile acids*	Intra-assay ($n=5$)		Inter-assay ($n=5$)	
	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)
TUDCA	73.9	9.0	85.9	7.2
TCA	75.7	6.8	88.9	11.2
TCDCA	80.2	9.7	85.3	4.4
TDCA	78.7	11.5	82.5	4.3
TLCA	74.6	9.7	73.8	11.3

*For abbreviations see legend to Fig. 4.

The peak height ratios of the same amount of bile acids varied between different days and columns; therefore, proper calibration curves are required for each day and each column.

Application

In order to investigate the applicability of the present method the simultaneous determination of free and glycine-conjugated bile acids in serum and bile was carried out on eleven sera of patients and one bile sample from a normal subject. The results are given in Table III. The typical chromatograms of normal human serum and bile are shown in Fig. 6. The chromatograms of patients with liver cirrhosis and primary biliary cirrhosis are shown in Fig. 7. Appreciable differences were found among individual bile acids with each sample. Further studies on a large number of samples are necessary to elucidate

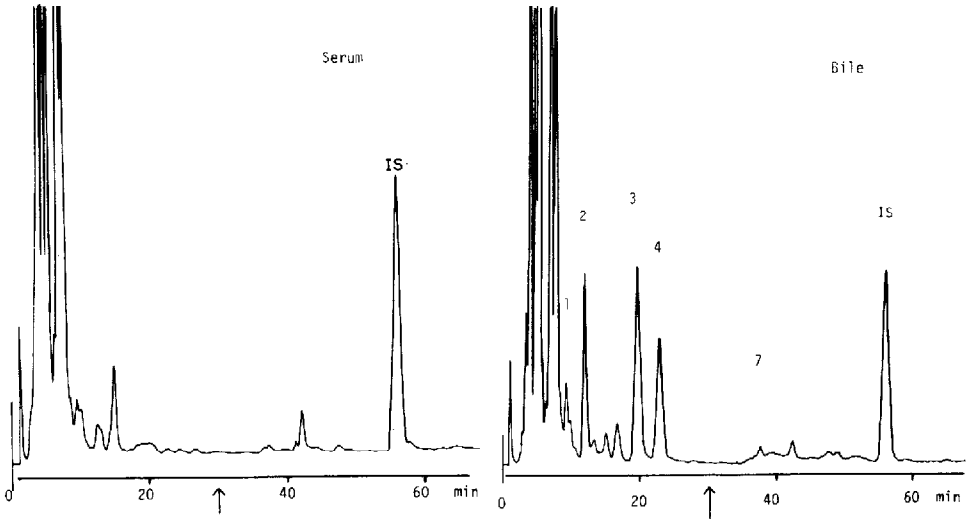


Fig. 6. Typical chromatograms of normal human serum and bile. Peaks: 1 = glycoursodeoxycholic acid, 2 = glycocholic acid, 3 = glycochenodeoxycholic acid, 4 = glycodeoxycholic acid, 7 = glycolithocholic acid, IS = internal standard.

TABLE III

INDIVIDUAL BILE ACIDS* IN SERUM AND BILE OF ONE HEALTHY SUBJECT AND PATIENTS BY FLUORESCENCE HPLC

Values are expressed as μmol/dl for serum and μmol/ml for bile.

Disease		Glycine-conjugated					Free				
		UDCA	CA	CDCA	DCA	LCA	UDCA	CA	CDCA	DCA	LCA
Liver cirrhosis		0.71	1.34	3.53	0.10	0.20	0.22	0.33	ND	0.25	0.54
Acute hepatitis	(1)	0.75	2.70	3.34	0	ND**	0	0	0	0.33	ND
	(2)	0.54	0.21	1.03	0	0.15	0	1.02	0.70	0.42	0.49
Primary biliary cirrhosis	(1)	0.33	7.10	5.51	0.14	0.25	ND	0.30	ND	13.32	3.98
	(2)	0.58	5.31	5.28	0.12	0.22	0.14	0.42	0.07	24.95	3.18
Fulminant		2.37	1.00	5.98	0.48	ND	1.25	ND	0	0	0.80
Pancreas cancer	(1)	1.17	0.33	0.21	0.09	0	ND	0.24	ND	0.23	0.39
	(2)	0.57	1.01	0.99	0.10	0.22	ND	ND	0	0.76	0.48
Breast cancer		0.61	0.13	0.41	0.18	0.32	0.19	0	0	0.70	7.36
Uterine cancer		0.93	0	0.19	0.12	0	0.23	0.64	ND	0.26	1.47
Hepatitis ?		0	0.81	1.73	0.24	0	0	0	0	0.39	0
Normal bile		2.33	8.35	10.00	7.68	0.88	ND	0	0	0	ND

*For abbreviations see legend to Fig. 5.
**ND = not detectable.

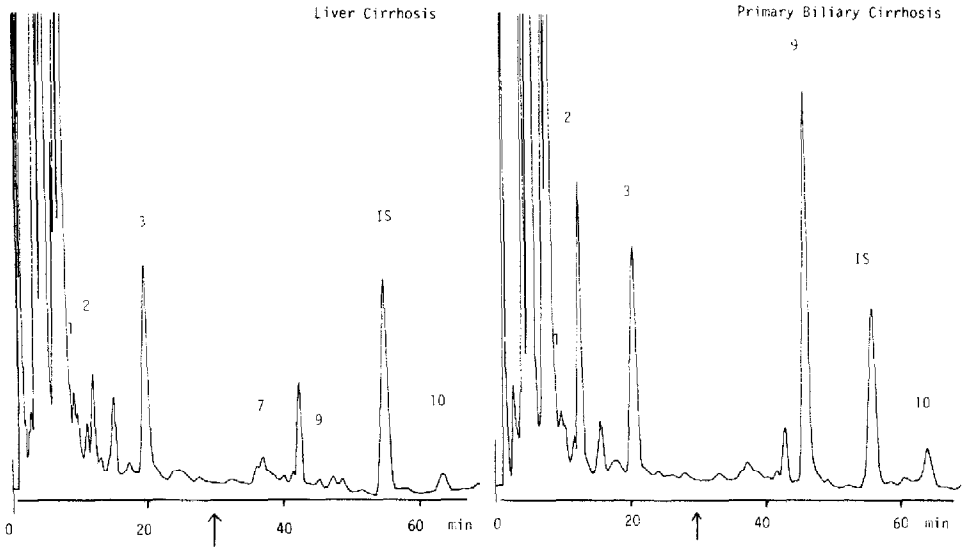


Fig. 7. Typical chromatograms of serum from patients. Peaks: 1 = glycoursodeoxycholic acid, 2 = glycocholic acid, 3 = glycochenodeoxycholic acid, 7 = glycolithocholic acid, 9 = deoxycholic acid, IS = internal standard, 10 = lithocholic acid.

this biological variability and to gain information in relation to physiological and/or pathological events. In this connection, the present method facilitates a much more accurate study of bile acids, allowing the investigation of a possible relationship between states of liver function and bile acid patterns.

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REFERENCES

1 M.A. Goldman, C.C. Schwartz, L. Swell and Z.R. Vlahcevic, in H. Popper and F. Schaffner (Editors), Progress in Liver Disease, Vol. 6, Grune and Stratton, New York, San Francisco, London, 1979, p. 225.
2 R.W. Shepherd, P.S. Bunting, M. Khan, J.G. Hill, S.J. Soldin and D.G. Gall, Clin. Biochem., 11 (1978) 106.
3 A.K. Batta, G. Salen and S. Shefer, J. Chromatogr., 168 (1979) 557.
4 G. Karlaganis and G. Paumgartner, Clin. Chim. Acta, 92 (1979) 19.
5 P.T. Clayton and D.P.R. Muller, Clin. Chim. Acta, 105 (1980) 401.
6 G. Karlaganis, R.P. Schwarzenbach and G. Paumgartner, J. Lipid Res., 21 (1980) 377.

7 J. Yanagisawa, M. Itoh, M. Ishibashi, H. Miyazaki and F. Nakayama, Anal. Biochem., 104 (1980) 75.
8 M. Tohma, Y. Nakata, Y. Yamada, T. Kurosawa, I. Makino and S. Nakagawa, Chem. Pharm. Bull., 29 (1981) 137.
9 K. Shimada, M. Hasegawa, J. Goto and T. Nambara, J. Chromatogr., 152 (1978) 431.
10 J. Goto, H. Kato, Y. Saruta and T. Nambara, J. Liquid Chromatogr., 3 (1980) 991.
11 G. Mingrone, A.V. Greco and S. Passi, J. Chromatogr., 183 (1980) 277.
12 S. Okuyama, D. Uemura and Y. Hirata, Chem. Lett., (1976) 679.
13 F. Stellaard, D.L. Hachey and P.D. Klein, Anal. Biochem., 87 (1978) 359.
14 G. Mingrone, A.V. Greco and S. Passi, J. Chromatogr., 183 (1980) 277.
15 B. Shaikh, N.J. Pontzer, J.E. Molina and M.I. Kelsey, Anal. Biochem., 85 (1978) 47.
16 S. Okuyama, D. Uemura and Y. Hirata, Bull. Chem. Soc. Jap., 52 (1979) 124.
17 D.P. Matter and W.C. Purdy, Anal. Chim. Acta, 109 (1979) 61.
18 S. Okuyama, D. Uemura and Y. Hirata, Chem. Lett., (1979) 461.
19 J.B.F. Lloyd, J. Chromatogr., 189 (1980) 359.
20 S. Baba, R. Uenoyama, K. Suminoe, F. Takeda, S. Hasegawa and Y. Kameno, Kobe J. Med. Sci., 26 (1980) 89.
21 S. Okuyama, N. Kokubun, S. Higashidate, D. Uemura and Y. Hirata, Chem. Lett., (1979) 1443.
22 K. Arisue, Z. Ogawa, K. Kohda, C. Hayashi and Y. Ishida, Jap. J. Clin. Chem., 9 (1980) 104.
23 S. Kamada, M. Maeda, A. Tsuji, Y. Umezawa and T. Kurahashi, J. Chromatogr., 239 (1982) 773.
24 Y. Kawahara, A. Inage, T. Morioka, Y. Shibano and N. Kasuga, The 101th Annual Meeting of Pharmaceutical Society of Japan, 1980, Abstract p. 568.
25 J. Goto, M. Hasegawa, H. Kato and T. Nambara, Clin. Chim. Acta, 87 (1978) 141.