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Simultaneous determination of bile acids in rat bile and serum by high-performance liquid chromatography

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ABSTRACT

A method for the simultaneous determination of bile acids in rat bile and serum by high-performance liquid chromatography with a post-column enzymic reaction and fluorescence detection has been developed. Without prior fractionation and alkaline hydrolysis, 26 unconjugated, glycine- and taurine-conjugated bile acids were determined. They were separated on a reversed-phase column using a linear gradient solvent system of 200 m*M* dibasic ammonium phosphate buffer (pH 7.9)–acetonitrile–methanol (73:19:8, v/v/v) and 20 m*M* dibasic ammonium phosphate buffer (pH 7.9)–acetonitrile–methanol (2:1:2, v/v/v). The limits of detection were 1–5 pmol, and calibration curves were linear for concentrations between 10 and 4000 pmol. This rapid and reliable method is effective for measuring bile acid levels in the bile and serum not only of rats but also of patients with hepatobiliary and other diseases.

INTRODUCTION

The determination of individual bile acids in biological fluids is important for studying their metabolism in hepatobiliary and other diseases. Concentrations of individual bile acids can be measured by various means, including radioimmunoassay [1], gas chromatography [2–4], gas chromatography–mass spectrometry [5–7], high-performance liquid chromatography (HPLC) with UV [8–11], refractive index [12–14] and other [15,16]. A combination of HPLC separation with a post-column enzymic reaction and fluorescence detection [17–20] has been developed, which can be used to 3α-hydroxylated bile acid levels in bile and serum with a sensitivity and specificity that is comparable with or better than most other methods [1–16].

Bile acid metabolism in human hepatobiliary diseases has been widely studied using rats. However, individual bile acids are inseparable by the methods described above, because rats have several peculiar bile acids, such as α-muricholic acid (α-MCA; 3α,6β,7α-trihydroxy-5β-cholan-24-oic acid) and β-muricholic acid (β-MCA; 3α,6β,7β-trihydroxy-5β-cholan-24-oic acid), hyodeoxycholic acid (HDCA; 3α,6α-dihydroxy-5β-cholan-24-oic acid) and hyocholic acid (HCA; 3α,6α,7α-trihydroxy-5β-cholan-24-oic acid). Therefore, the analysis of individual bile acids in rat bile and serum requires prior fractionation [16,20,21] and alkaline hydrolysis, which tend to form artifacts [22,23].

We developed a reversed-phase HPLC technique for the simultaneous determination of 26 bile acids in rat bile and serum.

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EXPERIMENTAL

Reagents

β-Nicotinamide adenine dinucleotide (β-NAD) was obtained from Oriental Yeast (Tokyo, Japan). Methanol and acetonitrile were of HPLC-grade, and the other reagents were of analytical grade. A Bond Elut C_{18} cartridge (200 mg, Varian Sample Preparation Products, Harbor City, CA, USA) was washed successively with methanol (3 ml × 2) and water (3 ml × 2) prior to use.

Bile acids

Ursodeoxycholic acid (UDCA; 3α,7β-dihydroxy-5β-cholan-24-oic acid) and chenodeoxycholic acid (CDCA; 3α,7α-dihydroxy-5β-cholan-24-oic acid) were obtained from Tokyo Tanabe (Tokyo, Japan). The following unconjugated bile acids were purchased from Steraloids (Wilton, NH, USA): α-MCA, β-MCA, HCA, HDCA, cholic acid (CA; 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid), deoxycholic acid (DCA; 3α,12α-dihydroxy-5β-cholan-24-oic acid), lithocholic acid (LCA; 3α-hydroxy-5β-cholan-24-oic acid) and 23-nordeoxycholic acid (3α,12α-dihydroxy-23-nor-5β-cholan-24-oic acid).

The following glycine (G)- and taurine (T)-conjugated bile acids were synthesized from the respective unconjugated bile acids in our laboratory according to the method reported by Kanazawa *et al.* [24]: G-α-MCA, T-α-MCA, G-β-MCA, T-β-MCA, GHCA, GUDCA, TUDCA, GHDCA, THDCA, GCA, TCA, GCDCA, TCDCA, GDCA, TDCA, GLCA, TLCA and tauro-23-nordeoxycholic acid. Tauro-23-nordeoxycholic acid was used as the internal standard (I.S.), dissolved in methanol at a concentration of 80 μmol/l.

Chromatographic apparatus

The HPLC system was a JASCO Trirotar-VI equipped with a JASCO FP-210 spectrofluorometer (Japan Spectroscopic, Tokyo, Japan). Inertsil ODS-2 (150 × 4.6 mm I.D., particle size 5 μm, pore size 150 Å; GL Sciences, Tokyo, Japan) was used for the separation of bile acids. For the immobilized 3α-hydroxysteroid dehydrogenase (3α-HSD) column, an E-3α-HSD column (20 × 4.0 mm I.D.; Sekisui Chemical, Osaka, Japan) was used.

Chromatographic conditions

Individual bile acids were eluted by reversed-phase HPLC with a linear gradient at a flow-rate of 1.0 ml/min. Mobile phase A was 200 m*M* dibasic ammonium phosphate buffer (pH 7.9)–acetonitrile–methanol (73:19:8, v/v/v) and mobile phase B was 20 m*M* dibasic ammonium phosphate buffer (pH 7.9)–acetonitrile–methanol (2:1:2, v/v/v).

The samples were eluted with mobile phase A for an initial 60 min after injection, then with a linear gradient of mobile phase B from 0% to 100% over 70 min, followed by continued elution with mobile phase B for 20 min. Before injection of the next sample, the column was equilibrated with 100% mobile phase A for 30 min.

Bile acids, separated on a reversed-phase column, were mixed with β-NAD pumped at a flow-rate of 1.0 ml/min. The mixture migrated through the 3α-HSD immobilized enzymic column, forming β-NADH and the corresponding 3-keto bile acids [17]. The separating and enzymic columns were maintained at 30°C. The β-NADH generated by the enzymic reaction was determined by means of fluorescence detection (excitation at 340 nm, emission at 460 nm). The β-NAD reagent for the enzymic reaction contained 0.3 m*M* β-NAD, 1 m*M* disodium EDTA and 0.05% 2-mercaptoethanol in 10 m*M* phosphate buffer (pH 7.8). Quantitation was based on peak-height measurements.

Procedure for determination of bile acids

Male Wistar rats (Charles River Japan, Kanagawa, Japan), weighing 200–250 g and 10 weeks old were housed in an air-conditioned room (25 ± 1°C, 50–60% humidity) lighted 12 h a day (8.00 a.m. to 8:00 p.m.) and fed a commercial balanced stock diet (CLEA CE-2, Clea Japan, Tokyo, Japan) and water *ad libitum*. Rats were abdominally dissected under ether anaesthesia,

and a polyethylene tube (0.28 mm I.D.) was cannulated into the common bile duct for the continuous collection of bile. After closure and suturing, the animal was immediately housed in a Bollman cage. Bile was collected for 1 h, then blood was withdrawn by heart puncture.

Serum (500 μl) was diluted with 5 ml of 0.1 *M* phosphate buffer (pH 7.2) [25], mixed with the I.S. (100 μl), then passed through the Bond Elut C_{18} cartridge. After washing with 3 ml of water, bile acids were eluted with 3 ml of methanol. The eluate was evaporated under reduced pressure. The residue was dissolved in 200 μl of mobile phase A. This solution was centrifuged at 1600 *g* for 10 min at 5°C, and 10 μl of the supernatant were injected into the HPLC column.

Bile (10 μl) was diluted with 2 ml of ethanol, deproteinized for 30 min, then centrifuged at 1600 *g* for 10 min at room temperature. The I.S. (100 μl) was added to 100 μl of diluted bile. This mixture was dried under reduced pressure and redissolved in 200 μl of mobile phase A, and 10 μl of this solution were injected into the HPLC column.

Analytical recovery

In order to determine the analytical recoveries and reproducibilities of the 26 bile acids, 2 or 20 μmol of standard bile acids were added to 1.0 ml of bile, and 5 or 50 nmol of standard bile acids were added to 1.0 ml of serum, respectively. These samples were submitted to the clean-up procedure described above.

RESULTS AND DISCUSSION

Chromatographic conditions

The best chromatographic conditions were achieved by optimizing the separation column and pH (salt), ionic strength, precentage of organic solvents in the mobile phase and column temperature as described in Experimental.

The bile acids were efficiently separated in a weakly alkaline mobile phase rather than in an acidic or neutral mobile phase. Consequently, among various salts added to the mobile phase, the dibasic ammonium phosphate buffer (pH 7.9) improved both the separation of the bile acids and the shape of the eluted peaks by reducing tailing. This weak alkalinity (pH 7.9) of the mobile phase increased the sensitivity of the enzymic reaction in the detection system.

The ionic strength of the mobile phase also played an important role in the separation. With 20 m*M* dibasic ammonium phosphate, all bile acids except α-MCA, β-MCA and their conjugates were satisfactory separated. When the salt concentration of the initial mobile phase was 200 m*M*, α-MCA, β-MCA and their conjugate peaks became more symmetrical, confirming the efficient separation of these bile acids.

Separation of bile acids

Fig. 1 shows a typical elution profile of the 26 bile acids and the I.S., representing 100 pmol of individual bile acids.

Separation into 27 peaks was achieved within 2.5 h in a gradient elution run. With this method, the bile acids were eluted in a definite order depending upon the number, position and configuration of the hydroxyl groups on the steroid nucleus [26,27]. Trihydroxylated (α-MCA, β-MCA, HCA, and CA), dihydroxylated (UDCA, CDCA, and DCA) and monohydroxylated (LCA) bile acids eluted in hydrophilic order. The trihydroxylated bile acids, *i.e.* α-MCA, β-MCA, HCA and CA also eluted likewise. However, the dihydroxylated bile acids such as HDCA and UDCA, had elution times similar to those of trihydroxylated bile acids (CA and HCA) because HDCA and UDCA, with a 6α- and a 7β-hydroxyl group, respectively, were more hydrophilic than other dihydroxylated bile acids, such as CDCA and DCA. The unconjugated form of each bile acid eluted before the glycine conjugate, which, in turn, eluted before the taurine conjugate.

Assay evaluation

Reproducibility. Table I shows the reproducibility of the retention times and the relative peak heights of each bile acid to that of the I.S. for 150 pmol of the individual bile acids.

The coefficients of variation (C.V.) for the re-

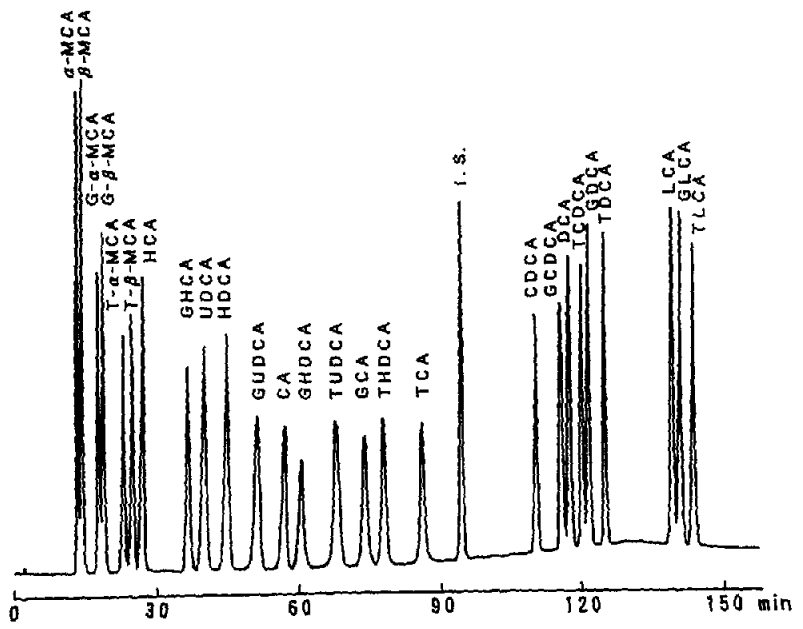

Fig. 1. Typical elution profile of the 26 bile acids 100 pmol of each acid were injected. Conditions as described in Experimental.

tention times and the relative peak heights were less than 0.9% and 1.3%, respectively.

Linearity and sensitivity. The calibration curves shown in Fig. 2 were constructed by plotting the relative peak heights against the amount of each bile acid injected. The linear regression equations and the correlation coefficients for each bile acid are listed in Table II.

Between the relative peak heights and the concentrations from 10 to 4000 pmol, the correlation

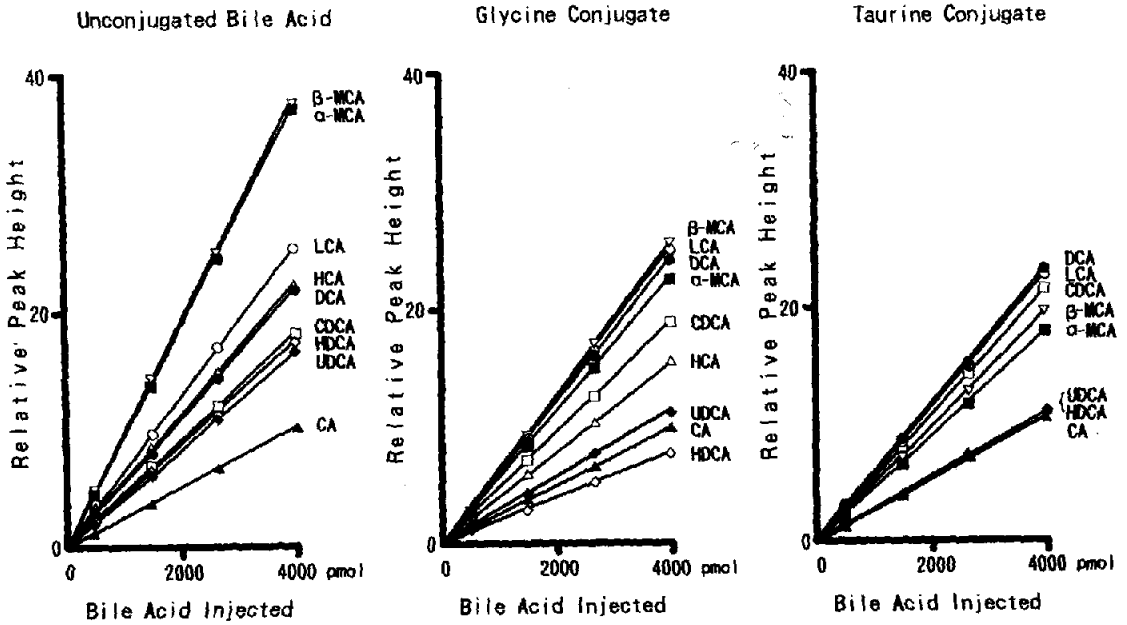

Fig. 2. Calibration curves for each bile acid. Each point represents the mean for three determination of each bile acid.

TABLE I

REPRODUCIBILITY OF THE RETENTION TIMES AND RELATIVE PEAK HEIGHTS

Bile acid	Retention time		Relative peak height[a]	
	Mean[b] (min)	C.V. (%)	Mean[b]	C.V. (%)
α-MCA	12.82	0.8	1.3470	1.0
β-MCA	13.72	0.9	1.3809	0.8
G-α-MCA	16.82	0.9	0.8298	0.9
G-β-MCA	17.84	0.8	0.9434	0.9
T-α-MCA	23.02	0.7	0.6551	1.2
T-β-MCA	24.88	0.8	0.7219	1.0
HCA	26.70	0.9	0.8355	1.1
GHCA	36.12	0.8	0.5633	1.0
UDCA	39.34	0.8	0.6235	1.2
HDCA	44.28	0.9	0.6525	0.8
GUDCA	50.12	0.8	0.4107	0.5
CA	56.62	0.7	0.3765	0.5
GHDCA	59.98	0.6	0.2864	0.3
TUDCA	68.09	0.7	0.3956	1.0
GCA	73.80	0.7	0.3562	0.8
THDCA	79.10	0.5	0.4015	1.3
TCA	86.57	0.8	0.3862	0.5
I.S.[c]	95.64	0.9	1.0000	
CDCA	111.85	0.5	0.6696	0.8
GCDCA	118.09	0.4	0.6899	0.9
DCA	119.76	0.5	0.8109	1.0
TCDCA	122.85	0.5	0.7856	0.9
GDCA	124.13	0.4	0.8875	1.0
TDCA	127.56	0.3	0.8646	0.8
LCA	140.82	0.4	0.9355	0.9
GLCA	142.23	0.4	0.9252	1.0
TLCA	144.50	0.4	0.8501	1.1

[a] Relative peak height was defined as the ratio of the peak height of individual bile acids to the peak height of the I.S.

[b] $n = 5$.

[c] Internal standard.

TABLE II

LINEAR REGRESSION EQUATIONS AND CORRELATION COEFFICIENTS

Bile acid	Linear regression equation	Correlation coefficient
Unconjugated bile acid		
β-MCA	$8.62 \cdot 10^{-3} x + 0.181$	0.999
α-MCA	$8.54 \cdot 10^{-3} x + 0.162$	1.000
LCA	$6.08 \cdot 10^{-3} x + 0.118$	1.000
HCA	$5.38 \cdot 10^{-3} x + 0.098$	0.999
DCA	$5.19 \cdot 10^{-3} x + 0.076$	0.999
CDCA	$4.26 \cdot 10^{-3} x + 0.064$	1.000
HDCA	$4.15 \cdot 10^{-3} x + 0.098$	0.999
UDCA	$4.06 \cdot 10^{-3} x + 0.053$	0.999
CA	$2.41 \cdot 10^{-3} x + 0.066$	0.999
Glycine conjugate		
β-MCA	$6.09 \cdot 10^{-3} x + 0.095$	0.999
LCA	$5.97 \cdot 10^{-3} x + 0.090$	1.000
DCA	$5.82 \cdot 10^{-3} x + 0.093$	1.000
α-MCA	$5.43 \cdot 10^{-3} x + 0.087$	1.000
CDCA	$4.40 \cdot 10^{-3} x + 0.079$	1.000
HCA	$3.66 \cdot 10^{-3} x + 0.074$	0.999
UDCA	$2.44 \cdot 10^{-3} x + 0.072$	0.999
CA	$2.18 \cdot 10^{-3} x + 0.051$	1.000
HDCA	$1.77 \cdot 10^{-3} x + 0.040$	1.000
Taurine conjugate		
DCA	$5.67 \cdot 10^{-3} x + 0.084$	1.000
LCA	$5.58 \cdot 10^{-3} x + 0.097$	1.000
CDCA	$5.08 \cdot 10^{-3} x + 0.082$	0.999
β-MCA	$4.72 \cdot 10^{-3} x + 0.080$	0.999
α-MCA	$4.36 \cdot 10^{-3} x + 0.096$	1.000
UDCA	$2.60 \cdot 10^{-3} x + 0.066$	1.000
HDCA	$2.56 \cdot 10^{-3} x + 0.072$	0.999
CA	$2.47 \cdot 10^{-3} x + 0.063$	0.999

coefficients were all over 0.999. The limits of detection ranged from 1 to 5 pmol per 10 μl injection, with a signal-to-noise ratio of 2.

Analytical recovery. The recoveries of the 26 bile acids added to rat bile and serum (Tables III and IV) were in the ranges 96.1–100.9% (C.V. 0.7–3.4%, $n = 3$) and 94.2–101.1% (C.V. 0.5–4.5%, $n = 3$), respectively. This indicated that the bile acids were effectively extracted by the Bond Elut C_{18} cartridge. These results were more satisfactory than those of previous methods requiring prior fractionation [16,20,21] and alkaline hydrolysis [22,23].

Column performance. When the 3α-HSD column was used repeatedly, the peak heights of the bile acids gradually decreased, owing to deactivation of the enzyme. The decrease in peak heights was remarkable, especially among bile acids possessing a 7α-hydroxyl group, such α-MCA, HCA, CA and CDCA. Under the present conditions,

TABLE III

RECOVERY OF BILE ACIDS ADDED TO RAT BILE

Bile acid	2 μmol/ml[a]		20 μmol/ml[a]	
	Recovery[b] (%)	C.V. (%)	Recovery[b] (%)	C.V. (%)
α-MCA	98.9 ± 1.06	1.1	98.8 ± 1.30	1.3
β-MCA	98.6 ± 1.33	1.3	98.2 ± 1.17	1.2
G-α-MCA	100.4 ± 0.81	0.8	99.4 ± 1.43	1.4
G-β-MCA	99.8 ± 1.51	1.5	97.6 ± 1.35	1.4
T-α-MCA	99.2 ± 1.62	1.6	97.6 ± 1.79	1.8
T-β-MCA	97.6 ± 3.10	3.1	98.9 ± 1.55	1.6
HCA	98.1 ± 2.26	2.3	98.8 ± 2.65	2.7
GHCA	97.8 ± 1.97	2.0	96.9 ± 2.19	2.2
UDCA	98.3 ± 1.56	1.6	99.6 ± 1.25	1.3
HDCA	97.5 ± 1.21	1.2	98.5 ± 1.30	1.3
GUDCA	99.7 ± 1.31	1.3	100.3 ± 1.34	1.3
CA	98.9 ± 1.06	1.1	97.6 ± 1.23	1.2
GHDCA	97.8 ± 3.42	3.4	96.9 ± 1.75	1.8
TUDCA	97.6 ± 2.80	2.8	99.6 ± 1.36	1.4
GCA	98.8 ± 3.09	3.1	97.2 ± 1.57	1.6
THDCA	100.9 ± 2.01	2.0	98.5 ± 2.60	2.6
TCA	98.8 ± 1.32	1.3	96.1 ± 2.45	2.5
CDCA	99.5 ± 0.73	0.7	98.1 ± 1.31	1.3
GCDCA	97.2 ± 1.92	1.9	97.7 ± 1.81	1.8
DCA	100.5 ± 1.81	1.8	99.6 ± 1.39	1.4
TCDCA	100.2 ± 1.46	1.5	100.6 ± 1.27	1.3
GDCA	99.5 ± 2.82	2.8	98.8 ± 2.10	2.1
TDCA	99.1 ± 1.19	1.1	100.1 ± 1.10	1.1
LCA	97.5 ± 1.50	1.5	99.3 ± 2.27	2.3
GLCA	99.6 ± 1.22	1.2	98.2 ± 1.76	1.8
TLCA	98.8 ± 1.06	1.1	99.6 ± 1.27	1.3

[a] Amount of standard bile acids added to 1.0 ml of bile.

[b] Mean ± S.D. (n = 3).

TABLE IV

RECOVERY OF BILE ACIDS ADDED TO RAT SERUM

Bile acid	5 nmol/ml[a]		50 nmol/ml[a]	
	Recovery[b] (%)	C.V. (%)	Recovery[b] (%)	C.V. (%)
α-MCA	99.5 ± 1.00	1.0	98.5 ± 1.31	1.3
β-MCA	98.5 ± 2.23	2.2	97.3 ± 1.65	1.7
G-α-MCA	100.6 ± 1.27	1.3	100.8 ± 3.26	3.3
G-βMCA	101.0 ± 2.03	2.0	99.8 ± 3.35	3.4
T-α-MCA	94.2 ± 3.88	3.9	98.2 ± 3.80	3.8
T-β-MCA	95.7 ± 1.96	2.0	98.8 ± 3.88	3.9
HCA	94.7 ± 1.53	1.5	100.6 ± 3.02	3.0
GHCA	101.0 ± 2.03	2.0	98.6 ± 2.45	2.5
UDCA	100.1 ± 1.80	1.8	97.9 ± 3.96	4.0
HDCA	94.8 ± 1.39	1.4	100.6 ± 2.65	2.7
GUDCA	101.1 ± 1.62	1.6	98.3 ± 3.37	3.4
CA	98.7 ± 2.40	2.4	98.4 ± 0.46	0.5
GHDCA	100.3 ± 2.95	3.0	98.0 ± 4.47	4.5
TUDCA	98.1 ± 1.80	1.8	99.6 ± 1.06	1.1
GCA	98.7 ± 3.07	3.1	100.7 ± 0.51	0.5
THDCA	99.8 ± 2.46	2.5	97.8 ± 2.84	2.8
TCA	99.6 ± 1.08	1.1	100.4 ± 0.46	0.5
CDCA	100.6 ± 1.52	1.5	99.0 ± 1.13	1.1
GCDCA	100.5 ± 2.76	2.8	99.8 ± 1.99	2.0
DCA	98.8 ± 1.48	1.5	99.2 ± 1.42	1.4
TCDCA	99.7 ± 3.10	3.1	99.7 ± 0.95	1.0
GDCA	99.6 ± 1.42	1.4	98.8 ± 0.65	0.7
TDCA	98.7 ± 1.12	1.1	100.5 ± 1.78	1.8
LCA	97.6 ± 3.44	3.4	99.3 ± 2.06	2.1
GLCA	100.2 ± 3.09	3.1	99.7 ± 1.66	1.7
TLCA	100.4 ± 2.28	2.3	99.4 ± 1.33	1.3

[a] Amount of standard bile acids added to 1.0 ml of serum.

[b] Mean ± S.D. (n = 3).

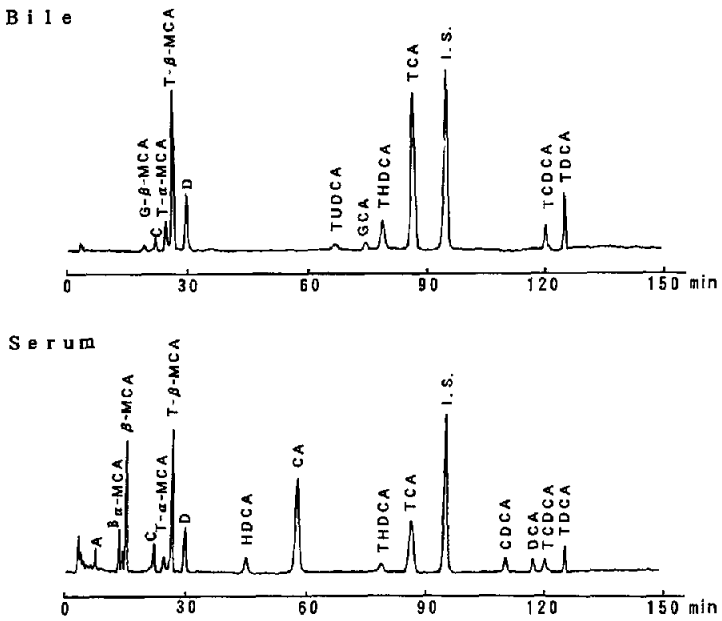

Fig. 3. Representative separation profiles of bile acids in rat bile and serum. Peaks A, B, C and D are unknowns.

however, the 3α-HSD column and separation column were stable for at least 1 month, enabling more than 200 analyses to be completed.

Sample analysis

Six normal rat bile and serum samples were analysed by HPLC, and representative results are shown in Fig. 3. The composition and the total amounts of bile acids are presented in Tables V and VI, respectively.

In bile, the total amounts of bile acids were 18.0 ± 3.53 μmol/ml. More than 95% of the total amount of bile acids consisted of taurine conjugates. Glycine conjugates were absent or present in low amounts, and unconjugated bile acids were at very low levels in the six samples. The major components were TCA (46.0%), T-β-MCA (24.3%) and THDCA (9.0%). TDCA, T-α-MCA, TCDCA, GCA, TUDCA and G-β-MCA were minor components, but LCA was not found in any sample. Appreciable inter-sample variations in total amount and composition of the bile acids were observed.

The total serum concentration of bile acids was 4.9 ± 6.46 nmol/ml, being mainly present as unconjugated bile acids (59.6%) and taurine conjugates (40.3%). Glycine conjugates were absent, or present at very low levels in the six samples. CA (21.8%), TCA (16.4%), β-MCA (15.1%), T-β-MCA (10.5%), HDCA (9.5%) and THDCA (4.4%) were the major components, whereas unconjugated bile acids and taurine conjugates of α-MCA, UDCA, CDCA and DCA were minor components. LCA was not detected. The total amount and composition of the bile acids varied considerably from sample to sample.

In both bile and serum, low levels of unidentified peaks (peak A, B, C, and D) appeared at 6, 12, 20 and 30 min, as shown in Fig. 3. They may be due to 3α-hydroxysteroids, because none of them appeared when the samples were analysed without β-NAD in the enzymic reaction. In consideration of the result described by Thompson *et al.* [28], the peaks B, C and D seemed to correspond to ω-muricholic acid (3α,6α,7β-trihydroxy-5β-cholan-24-oic acid), tauro-ω-murichol-

TABLE V

COMPOSITION AND TOTAL AMOUNT OF BILE ACIDS IN RAT BILE

Bile acid[a]		Bile acid composition (%)						
		1	2	3	4	5	6	Mean ± S.D.
α-MCA	F	–[b]	–	–	–	–	–	–
	G	–	–	–	–	–	–	–
	T	4.7	4.3	5.1	8.2	7.3	5.8	5.9 ± 1.54
β-MCA	F	0.3	–	–	–	–	–	0.1 ± 0.12
	G	0.6	–	0.7	–	0.8	0.8	0.5 ± 0.38
	T	22.8	33.8	27.4	16.9	16.7	28.0	24.3 ± 6.76
UDCA	F	–	–	–	–	–	–	–
	G	–	–	–	–	–	–	–
	T	1.7	1.4	2.6	1.8	1.5	1.5	1.8 ± 0.44
HDCA	F	–	–	–	–	–	–	–
	G	–	–		–	–	–	–
	T	5.8	1.5	8.9	12.6	13.4	11.8	9.0 ± 4.62
CA	F	0.8	–	–	–	–	–	0.1 ± 0.33
	G	2.5	1.5	2.5	2.7	2.8	2.1	2.4 ± 0.48
	T	52.0	47.9	41.6	47.4	46.4	40.8	46.0 ± 4.20
CDCA	F	–	–	–	–	–	–	–
	G	–	–	–	–	–	–	–
	T	2.5	2.8	3.0	4.9	3.9	3.6	3.5 ± 0.87
DCA	F	–	–	–	–	–	–	–
	G	–	–	–	–	–	–	–
	T	6.2	6.8	8.2	5.5	7.2	5.6	6.6 ± 1.03
LCA	F	–	–	–	–	–	–	–
	G	–	–	–	–	–	–	–
	T	–	–	–	–	–	–	–
Total[c]		24.1	17.1	17.6	15.0	14.5	19.7	18.0 ± 3.53

[a] F, unconjugated; G, glycine conjugated; T, taurine conjugated.
[b] Not detected.
[c] Total amount of bile acids is given in μmol/ml.

TABLE VI

COMPOSITION AND TOTAL AMOUNT OF BILE ACIDS IN RAT SERUM

Bile acid[a]		Bile acid composition (%)						
		1	2	3	4	5	6	Mean ± S.D.
α-MCA	F	1.9	2.0	2.9	7.1	6.4	–[b]	3.4 ± 2.78
	G	–	–	–	–	–	–	–
	T	3.0	2.7	4.4	1.7	2.0	9.1	3.8 ± 2.75
β-MCA	F	10.2	16.7	17.6	19.3	17.6	9.1	15.1 ± 4.31
	G	–	–	–	–	–	–	–
	T	16.2	20.6	5.9	2.4	2.0	15.9	10.5 ± 8.03
UDCA	F	0.7	–		2.0	–	–	0.5 ± 0.81
	G	–	–	–	–	–	–	–
	T	1.0	–	–	–	–		0.2 ± 0.41
HDCA	F	2.8	–	13.2	13.2	18.4	9.1	9.5 ± 6.96
	G	–	–	–	–	–	–	–
	T	4.3	–	–	–	4.0	18.3	4.4 ± 7.09
CA	F	15.3	17.6	28.0	35.4	34.4	–	21.8 ± 13.54
	G	1.3		–	–	–	–	0.2 ± 0.53
	T	33.1	29.1	11.8	5.4	5.6	13.6	16.4 ± 11.89
CDCA	F	1.9	2.7	8.8	8.1	4.8	4.5	5.2 ± 2.82
	G	–	–	–	–	–	–	–
	T	2.0	1.8	–	1.7	–	9.1	2.4 ± 3.39
DCA	F	1.7	2.5	7.4	3.7	4.8	4.5	4.1 ± 2.00
	G	–	–	–	–	–	–	–
	T	4.6	4.3	–	–	–	6.8	2.6 ± 2.99
LCA	F	–	–	–	–	–	–	–
	G	–	–	–	–	–	–	–
	T	–	–	–	–	–	–	–
Total[c]		17.6	4.9	0.7	3.0	2.5	0.4	4.9 ± 6.46

[a] F, unconjugated; G, glycine conjugated; T, taurine conjugated.
[b] Not detected.
[c] Total amount of bile acids is given in nmol/ml.

ic acid and the taurine conjugate of Δ^{22}-β-muricholic acid ($3\alpha,6\beta,7\beta$-trihydroxy-5β,22-cholen-24-oic acid), respectively. The major bile acids that we separated are similar to those reported previously [3,12,14,18,23,29].

It was proven that this method was reproducible, accurate and sensitive, allowing the 26 bile acids in rat bile and serum to be simultaneously determined, using a simple extraction procedure.

This rapid and reliable method should provide more precise knowledge on the metabolic profile of bile acids in bile and serum not only of the rat but also of the mouse, rabbit, dog, hamster, guinea-pig and pig. Moreover, this method will be useful in diagnosing hepatobiliary diseases, such as primary biliary cirrhosis and chronic hepatitis.

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