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RAPID DETERMINATION OF GLYCINE- AND TAURINE-CONJUGATED BILE ACIDS IN HUMAN BILE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

With use of an anion-exchange packing, TSK Gel IEX 540 DEAE, for high-performance liquid column chromatography, glycine- and taurine-conjugated bile acids were separated in 10 min and detected with a differential refractometer. Human bile could be analyzed after a simple pretreatment. The purity of the peaks of glycine- and taurine-conjugated bile acids in human bile was confirmed by enzymatic determination using 3α-hydroxysteroid:NAD oxidoreductase. The molar ratios of the two forms of the conjugates (glycine/taurine ratios) in bile from normal subjects and from patients suffering from various hepatobiliary diseases were measured.

INTRODUCTION

The bile acids are synthesized from cholesterol, metabolized mainly to glycine conjugates (G) and/or taurine conjugates (T) in the liver, stored in the gallbladder, excreted intermittently into the duodenum, partially deconjugated by the anaerobes, reabsorbed from the ileum—proximal colon into the portal vein to reach the liver, and recirculated to reach the enterohepatic circulation.

The G/T ratio in the bile is changed in hepatobiliary diseases and intestinal disorders [1—7]. Patients with ileal disorders showed markedly high G/T ratios from 10 to 20, sometimes nearing 30, while the normal range is between 2 and 5 [3, 8]. This is supposedly due to a more rapid rate of conjugation for glycine than taurine in the liver to compensate for a deficiency in the conjugates caused by malabsorption in the intestine in ileal disorders. On the other hand, the majority of patients with tropical sprue showed

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G/T ratios lower than 1 [2]. These findings suggest the importance of measuring G/T ratios.

The separation of G and T groups has been attained on a column of silicic acid [9], ion-exchange resin [1], for example Dowex-1, and Sephadex PHP-LH-20 [10]. However, these procedures were not sufficiently simple or rapid for routine clinical use.

In this paper, a rapid assay of G and T by high-performance liquid chromatography (HPLC) using an anion-exchange packing, TSK Gel IEX 540 DEAE, was developed and applied to human bile samples.

EXPERIMENTAL

Chemicals

Glycocholic acid (GC), glycodeoxycholic acid (GDC), glycochenodeoxycholic acid (GCDC), taurocholic acid (TC), taurodeoxycholic acid (TDC) and taurochenodeoxycholic acid (TCDC) were of the highest purity obtained from Sigma (St. Louis, Mo., U.S.A.). Glycoursodeoxycholic acid (GUDC), glycolithocholic acid (GLC) and taurolithocholic acid (TLC) were the gift of Dr. Osuga, Faculty of Medicine, Tsukuba University. Cholic acid (C; extra pure reagent) and deoxycholic acid (DC) were obtained from Nakarai (Kyoto, Japan) and lithocholic acid (LC) was from Tokyo Kasei (Tokyo, Japan). Chenodeoxycholic acid (CDC) was kindly supplied by Yamanouchi (Tokyo, Japan) and ursodeoxycholic acid (UDC) from Tokyo Tanabe (Tokyo, Japan). Lithocholic acid-3α-sulfate (LC-3α-S) and glycochenodeoxycholic acid-3α-sulfate (GCDC-3α-S) were synthesized according to the method of Haslewood and Haslewood [11].

Ethanol, methanol, citric acid and sodium citrate were of reagent grade from Kanto Kagaku (Tokyo, Japan). 3α-Hydroxysteroid:NAD oxidoreductase (EC 1.1.1.50; 3α-HSD) from Nyegaard & Co. (Oslo, Norway) and diaphorase (EC 1.6.4.3) from Sigma were products of the highest purity. NAD was obtained from Boehringer (Mannheim, G.F.R.) and resazurin from Daiichi (Tokyo, Japan).

Packing for high-performance liquid chromatography

Recently, an anion-exchange packing, TSK Gel IEX 540 DEAE (Toyo Soda Manufacturing Co., Tokyo, Japan), for HPLC became available. The packing consists of spherical particles of silica gel coated covalently with polyethylene glycol to which diethylaminoethyl residues are attached.

Chromatographic system

The HPLC system used in this study was assembled from modular components and consisted of a Model SM8P10 synchronous motor pump (Japan Servo Co., Tokyo, Japan), a Kyowa KMH-6V sampling valve with a 112-μl loop (Kyowa Seimitsu Co., Tokyo, Japan), a glass column packed with TSK Gel IEX 540 DEAE (5 μm particle diameter, 80 mm × 8 mm I.D.), and a differential refractometer, Shodex RI SE-11 (Showa Denko, Tokyo, Japan). The column temperature was maintained at 45°. The eluent, 0.017 *M* sodium citrate buffer (pH 3.6)—ethanol (3:2, v/v) degassed prior to use by aspiration and ultrasonication, was pumped at a rate of 0.92 ml/min.

Human bile

Human bile samples from normal subjects and from patients suffering from various diseases were collected by intubation using magnesium sulfate mixture as a gallbladder constrictor.

Procedures

The standard bile acids were dissolved in the eluent. The bile was centrifuged at 650 *g* for 20 min and the supernatant was diluted several fold with the eluent. The solution was recentrifuged, if necessary, and was directly applied to HPLC.

For the enzymatic analysis the peak of G or T from HPLC was collected and subjected to enzymatic assay using 3α-HSD according to the method originally reported by Mashige et al. [12] but slightly modified as follows. To each of 0.2-ml portions of the fraction in two separate tubes, A and B, 1.9 ml of 0.05 *M* Tris—HCl buffer (pH 9.0), the mixture of 0.2 ml of resazurin (100 μ*M*)—NAD (6 mg/ml) and 0.5 ml of diaphorase solution (0.5 U/ml) were added. Then, 0.2 ml of 3α-HSD solution (0.024 I.U./ml) was added to tube A and 0.2 ml of 0.05 *M* Tris—HCl buffer (pH 9.0) without 3α-HSD was added to tube B. After incubation at 20° for 60 min, fluorescence was measured at 580 nm (excitation at 560 nm) using an Hitachi MPF-2A fluorescence spectrophotometer (Hitachi, Tokyo, Japan). The net fluorescence was obtained by subtracting the intensity of B from that of A.

RESULTS

Conditions for the separation of G and T groups

The free bile acids (F), G and T were not eluted from the packing with an aqueous solution containing no ethanol. In the presence of 40% ethanol the group separation of G from T was good at pH 3.6 (Table I). F were not retained under these conditions. However, at pH 3.3 and 4.0 separation was observed within the G or T group (Fig. 1). A small change in the ethanol content of the buffer greatly affected the capacity factor (k') of each bile acid (Fig. 2). The more hydrophilic bile acids have a tendency to elute earlier with a lower ethanol content in the buffer. Therefore the ethanol content should be fixed at 40% to allow the bile acids of each group to be eluted simultaneously. There

TABLE I

CAPACITY FACTOR (k')* OF THE FREE (F), GLYCINE (G) AND TAURINE (T) CONJUGATED BILE ACIDS ON TSK GEL IEX 540 DEAE COLUMN

Conditions: TSK Gel IEX 540 DEAE column (5 μm particle size; 80 × 8 mm I.D.); column temperature, 45°; detector, Shodex RI SE-11; eluent, 0.017 *M* sodium citrate buffer (pH 3.6)—ethanol (3:2, v/v); flow-rate, 0.92 ml/min (24—29 kg/cm²).

Bile acid	LC	DC	CDC	UDC	C
F	≈ 0	≈ 0	≈ 0	≈ 0	≈ 0
G	0.83 ±0.00	0.89 ±0.01	0.85 ±0.01	0.90 ±0.00	0.88 ±0.01
T	2.23 ±0.01	2.27 ±0.00	2.34 ±0.01	2.29 ±0.03	2.34 ±0.01

*$k' = (t_R - t_0)/t_0$, where t_R = retention time for solute molecules and t_0 = retention time for solvent molecules.

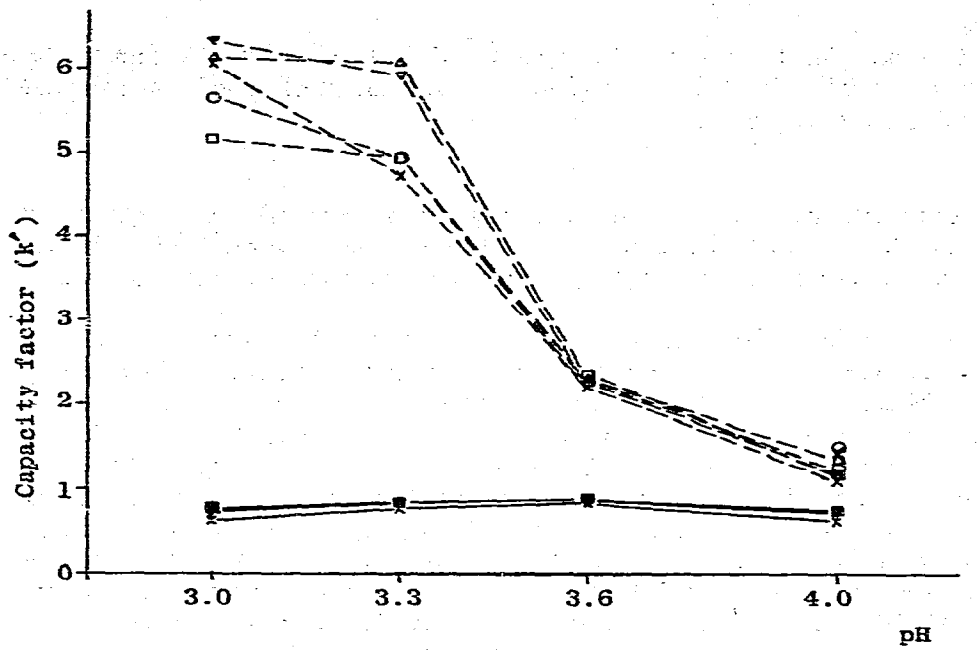

Fig. 1. Effect of buffer pH on the capacity factor (k') of the conjugated bile acids. The HPLC conditions are the same as in Table I except for the pH of the buffer. ×, GLC; •, GDC; ▲, GCDC; ▲, GUDC; ■, GC; ×, TLC; ○, TDC; △, TCDC; ▽, TUDC; □, TC.

were still small differences in the k' values of the bile acids within a group; thus calibration was performed using peak area instead of peak height.

Calibration of G and T

Since the relative peak area (the peak area was calculated by multiplying peak height (h) by peak width measured at 0.607 h) of each bile acid in each group to that of GDC or TDC was almost unity, as shown in Table II, the calibration curves for G and T were prepared using GDC and TDC, respectively, as representatives. The peak areas were proportional to the quantities of bile acids from 0.2 to 4 mM for G and from 0.2 to 2 mM for T.

Recovery and reproducibility of the HPLC method

Various amounts of GDC and TDC were added to a bile sample and analyzed. By the use of the calibration curves mentioned above, the recovery of these added standards was found to be quantitative. The reproducibility of the

TABLE II

RELATIVE RESPONSE OF GLYCINE (G) AND TAURINE (T) CONJUGATED BILE ACIDS

The HPLC conditions are the same as in Table I.

Bile acid	LC	DC	CDC	UDC	C
G	0.97 ±0.02	1	1.02 ±0.01	1.00 ±0.00	0.98 ±0.01
T	1.06 ±0.02	1	1.05 ±0.02	0.97 ±0.01	1.05 ±0.01

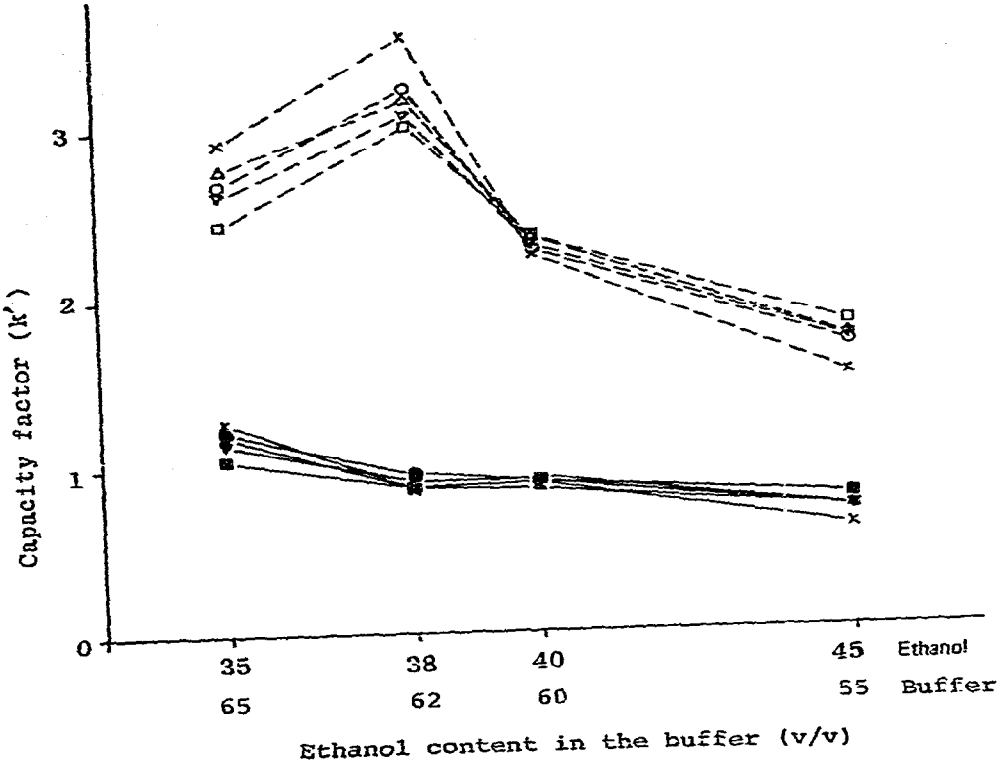

Fig. 2. Effect of the ethanol content of the buffer on the capacity factor (k') of the conjugated bile acids. The HPLC conditions are the same as in Table I except for the ethanol content of the buffer. Symbols as in Fig. 1.

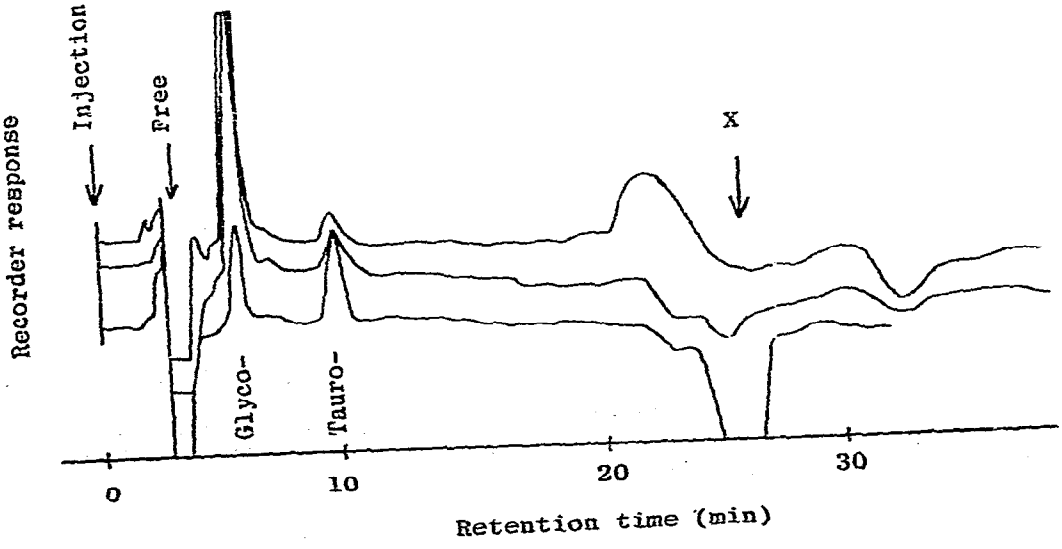

Fig. 3. HPLC of human bile samples from various hepatobiliary diseases. The chromatogram correspond to 28 µl of the original samples. The HPLC conditions are the same as in Table I

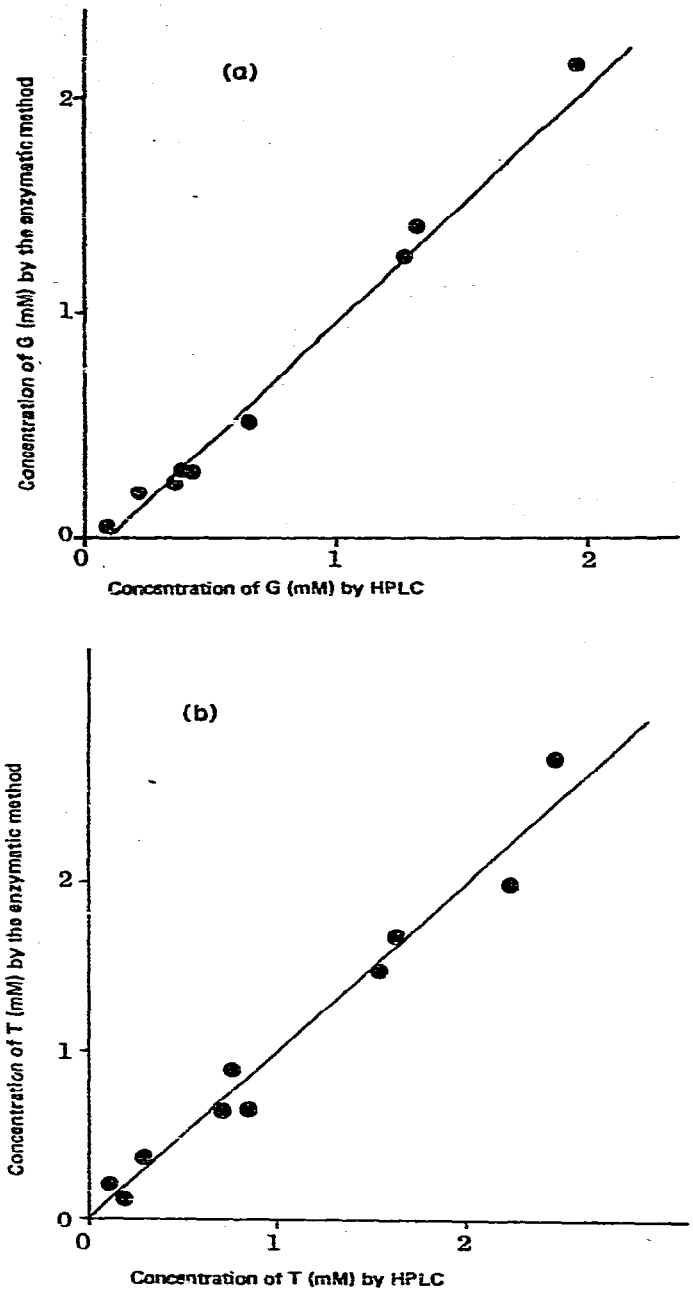

Fig. 4. Correlation of the values of the glycine (a) and taurine (b) conjugated bile acids obtained by the present method with those obtained by the enzymatic method. Peak fractions corresponding to G and T were treated as in the text.

TABLE III

CONCENTRATION AND RATIO OF THE CONJUGATED BILE ACIDS DETERMINED BY HPLC IN HUMAN BILE FROM NORMAL SUBJECTS AND FROM PATIENTS SUFFERING FROM VARIOUS HEPATOBILIARY DISEASES

Sample	Disease***	Concentration (m*M*)		G/T ratio
		G	T	
1*	Obstructive jaundice	0.53	0.20	2.7
2**	Obstructive jaundice[a]	10.72	4.88	2.2
3**	Obstructive jaundice[a]	1.50	2.20	0.7
4**	Cholelithiasis	16.00	3.20	5.0
5**	Cholelithiasis	1.63	0.82	2.0
6**	Cholelithiasis[b]	20.11	3.99	5.0
7*	Choledochus cyst	5.24	1.30	4.0
8*	Choledochus cyst	1.81	3.02	0.6
9**	Dilation of choledochus	10.21	1.51	6.8
10*	Carcinoma of the gallbladder[c]	63.25	8.25	7.7
	Normal subjects (n = 7)	—	—	2.2 ±0.8

*Hepatic bile.
**Gallbladder bile.
***a, During operation; b, under medication with CDC; c, post operation.

analysis was good (4.64 ± 0.13 m*M*, C.V. = 3%, for G and 1.17 ± 0.08 m*M*, C.V. = 4%, for T, n = 6).

Confirmation of the purity of the peaks of G and T

Typical chromatograms obtained with human bile are shown in Fig. 3. The values obtained by the enzymatic determination of bile acids in the fractions corresponding to G and T agreed well with those obtained using the present method ($y = 1.08x - 0.16$, $r = 0.990$ in Fig. 4a, and $y = 1.02x - 0.003$, $r = 0.988$ in Fig. 4b).

Application to human bile

The method was applied to human bile samples from normal subjects and from patients suffering from various hepatobiliary diseases. The values of G, T and the G/T ratio are shown in Table III. The normal value of the G/T ratio agreed with those in the literature; for example, 3.2 ± 1.0 (n = 20; Sjövall, 1960 [8]) and 3.4 ± 0.6 (n = 4; Abaurre et al., 1969 [3]). The values obtained from patients suffering from obstructive jaundice and cholelithiasis also agreed with those in the literature [8, 13, 14].

DISCUSSION

It seems reasonable that F, G and T were eluted in this order using the TSK Gel IEX 540 DEAE anion-exchange column because in the eluting buffer (pH 3.6) F, G and T are scarcely, partially and almost completely dissociated, respectively (pK_a of F, G and T: 5.0—6.5, 3.8—4.8 and 1.8—2.0, respectively [15]). Therefore, the mechanism of the separation by this system is mainly ion exchange. In addition, the fact that only a slight change in the ethanol content

of the buffer affects the k' of each bile acid suggests the secondary contribution of reversed-phase partition.

The group separation of G and T in human bile was possible in about 10 min after simple pretreatment of the bile samples. It would be suggested that each peak of G or T obtained from bile samples contained solely 3α-hydroxysteroids. Therefore, this method is simple, rapid and reliable in obtaining G/T ratios of bile. It is also useful for investigating the conjugation pattern of bile acids in hepatobiliary diseases.

The sensitivity of the method with a differential refractometer is about 0.1 m*M* for G and T. However, by using fluorescence detection with 3α-HSD, NAD and/or diaphorase–resazurin [16, 17], sufficient sensitivity will be obtained to analyze human serum.

The fairly good agreement between the values of the G/T ratios in human bile obtained by the present method and those in the literature would suggest the adequate reliability of this method.

The negative peak X in Fig. 3, which did not react in the enzymatic assay, was subjected to acid hydrolysis [18] and a portion was subjected to HPLC. As the result, new peaks appeared with retention times of 5.5 min and 10 min, which corresponds to those of G and T, respectively. The synthesized LC-3α-S and GCDC-3α-S were injected into the HPLC column and eluted at about 26 min. These data suggest that peak X contained 3α-sulfate(s) of bile acids.

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