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CCXVII. SEPARATION AND CHARACTERIZATION OF BILE ACID 3-GLUCURONIDES IN HUMAN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The separation and characterization of unconjugated and conjugated bile acid 3-glucuronides in biological fluids without prior deconjugation by high-performance liquid chromatography (HPLC) are described. A urine sample from a patient with obstructive jaundice was passed through a Sep-Pak C_{18} cartridge and was separated into groups by ion-exchange chromatography on a lipophilic gel, piperidinohydroxypropyl Sephadex LH-20, providing the glucuronide fraction. Subsequent resolution into individual 3-glucuronides was attained by HPLC on μBondapak C_{18} and Shodex ODS Pak F-411 columns. The 3-glucuronides of cholate, deoxycholate, chenodeoxycholate, glycocholate, glycochenodeoxycholate and taurochenodeoxycholate were identified on the basis of their behaviour in HPLC using mobile phases of different pH. The enzymatic hydrolysis of these glucuronides and derivatization of deconjugated bile acids with 1-anthroyl nitrile followed by chromatographic separation on a Cosmosil $5C_{18}$ column with fluorescence detection were carried out for unequivocal characterization. The ratio of unconjugated, glyco- and tauro-conjugated bile acid 3-glucuronides excreted in urine was found to be ca. 2:3:1.

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INTRODUCTION

Since the first report on the occurrence of bile acid glucuronides in human urine [1], considerable attention has been drawn to the metabolic significance of glucuronidation of bile acids in hepatobiliary diseases. It has been clarified that the serum and urine levels of bile acid glucuronides are significantly elevated in patients with obstructive jaundice, whereas the amount of glucuronides relative to total bile acids decreases in patients with liver cirrhosis, probably due to hepatocellular dysfunction [2, 3]. It has also been reported that bile acid glucuronides in urine are present in the unconjugated form at C-24 [4, 5], although the glyco- and tauro-conjugates are predominant in the sulphate fraction. On the other hand, the glyco-conjugates exhibit equal or less affinity to bile acid UDP-glucuronyltransferase from human liver than the corresponding unconjugated bile acids, while the tauro-conjugates show equal or higher affinity [6]. These findings on glucuronidation of bile acids seem to be incompatible between in vivo and in vitro studies.

The methods commonly used for the determination of bile acid glucuronides in biological fluids involve prior hydrolysis with β-glucuronidase and then with alkali, followed by the chromatographic separation of deconjugated bile acids. These procedures, however, have inevitable disadvantages, such as the lack of reliability and the loss of information on the conjugated form. In previous studies of this series, we synthesized unconjugated and conjugated bile acid 3-glucuronides as the authentic specimens [7, 8] and established suitable conditions for the separation of these glucuronides by high-performance liquid chromatography (HPLC). This paper describes the HPLC separation and characterization of unconjugated and conjugated bile acid 3-glucuronides in urine of a patient with obstructive jaundice.

EXPERIMENTAL

High-performance liquid chromatography

The apparatus used for this work was a 6000A solvent delivery system (Waters Assoc., Milford, MA, U.S.A.) equipped with a Uvidec 100-II ultraviolet (UV) detector (205 nm) (Japan Spectroscopic, Tokyo, Japan) and a 650-10LC fluorescence spectrophotometer (excitation wavelength 370 nm; emission wavelength 470 nm) (Hitachi, Tokyo, Japan). The test samples were applied to the chromatograph by a U6K sample-loop injector (Waters Assoc.) with an effective volume of 2 ml. The μBondapak C_{18} (10 μm, 25 cm × 4.6 mm I.D.), Shodex ODS Pak F-411 (5 μm, 15 cm × 4.6 mm I.D.) and Cosmosil $5C_{18}$ (5 μm, 15 cm × 4.6 mm I.D.) columns were used at ambient temperature.

Materials

Bile acid 3-glucuronides were synthesized in these laboratories by the methods previously reported [7, 8]. Sephadex LH-20 was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). The β-glucuronidase preparations derived from *Escherichia coli* and beef liver were supplied by Sigma (St. Louis, MO, U.S.A.). All chemicals employed were of analytical-reagent grade. Solvents were purified by distillation prior to use. Piperidinohydroxypropyl

Sephadex LH-20 (PHP-LH-20) (acetate form, 0.6 mequiv./g) [9] and 1-anthroyl nitrile [10] were prepared in the manner previously reported. The Sep-Pak C_{18} cartridge (Waters Assoc.) was washed successively with ethanol (10 ml) and water (10 ml) prior to use. All glassware used was silanized with trimethylchlorosilane.

Extraction of bile acid 3-glucuronides by Sep-Pak C_{18} cartridge

A synthetic mixture of 100 μg each of 3-glucuronides of tauro-conjugated cholate, chenodeoxycholate, deoxycholate and lithocholate were dissolved in 0.5 *M* phosphate buffer (pH 7.0, 5 ml) and applied to the cartridge. After successive washing with water (10 ml) and 1% ethanol (5 ml), the 3-glucuronide fraction was eluted with 50% ethanol. The effluent was fractionally collected and, after addition of glycocholate 3-glucuronide (50 μg) as an internal standard, subjected to HPLC according to the procedure in a preceding paper [11].

Separation and characterization of bile acid 3-glucuronides in human urine

A urine sample (5 ml) from a patient with obstructive jaundice was diluted with 0.5 *M* phosphate buffer (pH 7.0, 5 ml) and passed through a Sep-Pak C_{18} cartridge. After successive washing with water (10 ml) and 1% ethanol (5 ml), the bile acid 3-glucuronide fraction was eluted with 50% ethanol (5 ml). The eluate was applied to a column (18 mm × 6 mm I.D.) of PHP-LH-20 acetate (100 mg). After washing with 90% ethanol (4 ml) and 0.1 *M* acetic acid in 90% ethanol (4 ml), the glucuronide fraction was eluted with 0.5% ammonium carbonate in 70% ethanol (4 ml) and the eluate was evaporated in vacuo below 40°C. The residue obtained was applied to a Sep-Pak C_{18} cartridge in the manner described above for removal of inorganic salts. Further purification was performed by HPLC on μBondapak C_{18} using 0.5% ammonium phosphate buffer (pH 7.0)—acetonitrile (15:4) and 0.5% ammonium phosphate buffer (pH 3.5)—acetonitrile (15:6) as mobile phases. The eluate corresponding to each peak on the chromatogram was collected and, after addition of an internal standard, subjected to HPLC on Shodex ODS Pak F-411. Bile acid glucuronides were monitored by a UV detector at 205 nm.

Hydrolysis of bile acid 3-glucuronides with β-glucuronidase

A synthetic mixture of unconjugated, glyco- and tauro-conjugated bile acid 3-glucuronides (50 nmol of each) was incubated with the β-glucuronidase preparation (1000 Fishman units) derived from *E. coli* in 0.1 *M* phosphate buffer (pH 6.5, 1 ml) at 37°C. The incubation mixture was diluted with ice-cooled 0.5 *M* phosphate buffer (pH 7.0, 3 ml) and passed through a Sep-Pak C_{18} cartridge. After washing with water (4 ml) and 1.5% ethanol (4 ml), deconjugated bile acids were eluted with 90% ethanol (4 ml). The eluate was then applied to a column (18 mm × 6 mm I.D.) packed with PHP-LH-20 acetate (100 mg). Following the removal of neutral compounds with 90% ethanol (4 ml), unconjugated, glyco- and tauro-conjugated bile acids were eluted stepwise with 0.1 *M* acetic acid in 90% ethanol (4 ml), 0.2 *M* formic acid in 90% ethanol (4 ml) and 0.3 *M* acetic acid—potassium acetate in 90% ethanol (pH 6.3, 4 ml), respectively. Each fraction was evaporated in vacuo below 40°C and

the residue obtained was subjected to HPLC according to the procedure described in the previous paper [12].

Characterization of bile acids in hydrolysates

The eluate corresponding to each peak on the chromatogram was incubated with the β-glucuronidase preparation (1000 Fishman units) derived from *E. coli* in 0.1 *M* phosphate bufer (pH 6.5, 1 ml) at 37°C for 4 h. After addition of an internal standard, the incubation mixture was diluted with 0.5 *M* phosphate buffer (pH 7.0, 3 ml) and passed through a Sep-Pak C_{18} cartridge. Each hydrolysate thus obtained was subjected to derivatization with 1-anthroyl nitrile [13]. Bile acids were dissolved in acetonitrile and treated with 1-anthroyl nitrile in the presence of quinuclidine at 60°C for 20 min. The 3-(1-anthroyl) derivatives were separated on PHP-LH-20 into the unconjugated, glyco- and tauro-conjugated fractions. Each fraction was then subjected to HPLC on a Cosmosil $5C_{18}$ column using 0.3% potassium phosphate buffer—methanol as a mobile phase. The eluates were monitored by fluorescence detection (excitation wavelength 370 nm; emission wavelength 470 nm).

RESULTS AND DISCUSSION

Since bile acid 3-glucuronides are very polar and lack thermostability, the separation of these glucuronides is markedly influenced by the clean-up procedure employed. The cartridges packed with octadecyl-substituted silica, such as Sep-Pak C_{18} and Bond-Elut, are currently employed for extraction of bile acids and their sulphates in biological fluids [12—15]. In the present study, a Sep-Pak C_{18} cartridge was used for extraction of bile acid 3-glucuronides in urine. A synthetic mixture of tauro-conjugated bile acid 3-glucuronides dissolved in phosphate buffer was applied to the cartridge. The eluate with 50% ethanol was then separated and determined by HPLC. The 3-glucuronides were recovered at a rate of more than 90% in an initial 3 ml of effluent.

Previously, we demonstrated the group separation of bile acids into the unconjugated, glyco- and tauro-conjugated, and sulphated bile acid fractions on PHP-LH-20 according to their p*K* values [9, 12, 13, 15]. This method is effective for removal of neutral, basic and more acidic co-existing substances in urine. When the group separation was performed in the same manner, bile acid 3-glucuronides having two acidic functions at both C-3 (p*K* 3.5) and C-24 (p*K* 6.0, 4.5 or 1.5) were separated into the glyco-conjugate (ca. 30%), tauro-conjugate (ca. 65%) and sulphate (ca. 5%) fractions. Therefore, 0.5% ammonium carbonate in 70% ethanol was chosen as a suitable eluent for these glucuronides. A synthetic mixture of 3-glucuronides of unconjugated, glyco- and tauro-conjugated bile acids dissolved in 90% ethanol was applied to a PHP-LH-20 column. After removal of the neutral compounds and unconjugated bile acids by successive washing with 90% ethanol and 0.1 *M* acetic acid in 90% ethanol, the desired glucuronide fraction was eluted with 0.5% ammonium carbonate in 70% ethanol and subjected to HPLC. As illustrated in Fig. 1, each glucuronide was recovered at a rate of more than 80% in an initial 2 ml of effluent. Other 3-glucuronides also showed a similar elution pattern.

The separation and characterization of bile acid 3-glucuronides in urine were

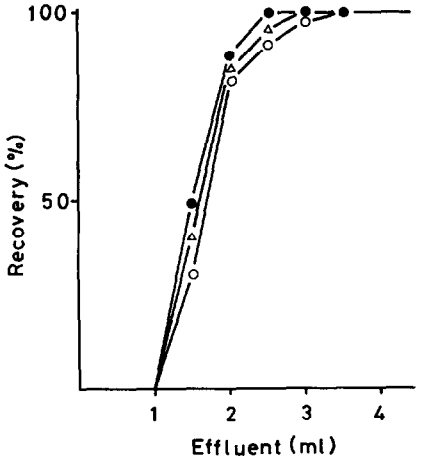

Fig. 1. Cumulative elution curves of bile acid 3-glucuronides on PHP-LH-20. (•) Chenodeoxycholate 3-glucuronide; (△) glycochenodeoxycholate 3-glucuronide; (○) taurochenodeoxycholate 3-glucuronide.

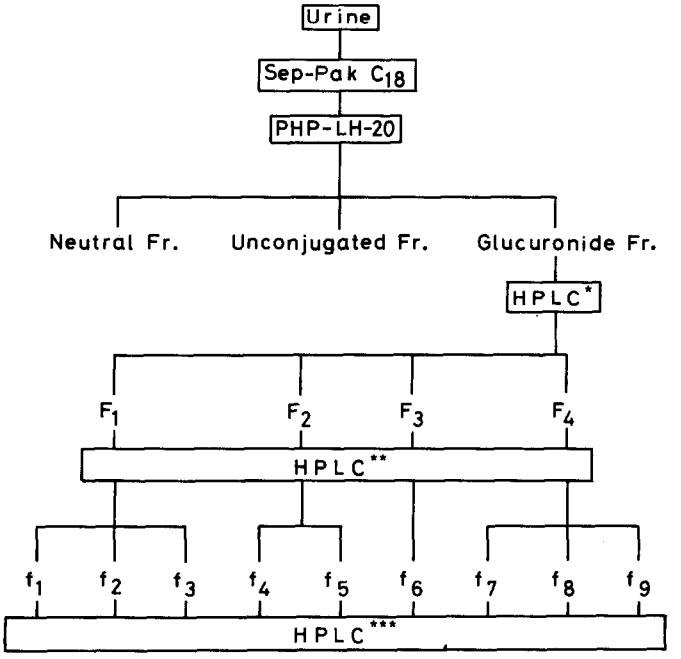

Fig. 2. General scheme for separation and characterization of bile acid 3-glucuronides in human urine. Each fraction corresponds to the following bile acid 3-glucuronides: (f_1) cholate 3-glucuronide; (f_2) glycocholate 3-glucuronide; (f_3) taurocholate 3-glucuronide; (f_4) deoxycholate and chenodeoxycholate 3-glucuronides; (f_5) glycodeoxycholate and glycochenodeoxycholate 3-glucuronides; (f_6) taurodeoxycholate and taurochenodeoxycholate 3-glucuronides; (f_7) lithocholate 3-glucuronide; (f_8) glycolithocholate 3-glucuronide; (f_9) taurolithocholate 3-glucuronide. Conditions: (*) μBondapak C_{18}, 0.5% ammonium phosphate buffer (pH 7.0)—acetonitrile (15:4); (**) μBondapak C_{18}, 0.5% ammonium phosphate buffer (pH 3.5)—acetonitrile (15:6); (***) Shodex ODS Pak F-411, 0.5% phosphate buffer—acetonitrile.

carried out according to the scheme shown in Fig. 2. The urine sample from a patient with obstructive jaundice was extracted with a Sep-Pak C_{18} cartridge and then subjected to group separation on PHP-LH-20. The glucuronide fraction thus obtained was still contaminated by glyco- and tauro-conjugated bile acids together with a small amount of sulphated bile acids, necessitating further purification.

In previous works, we investigated the chromatographic behaviour of bile

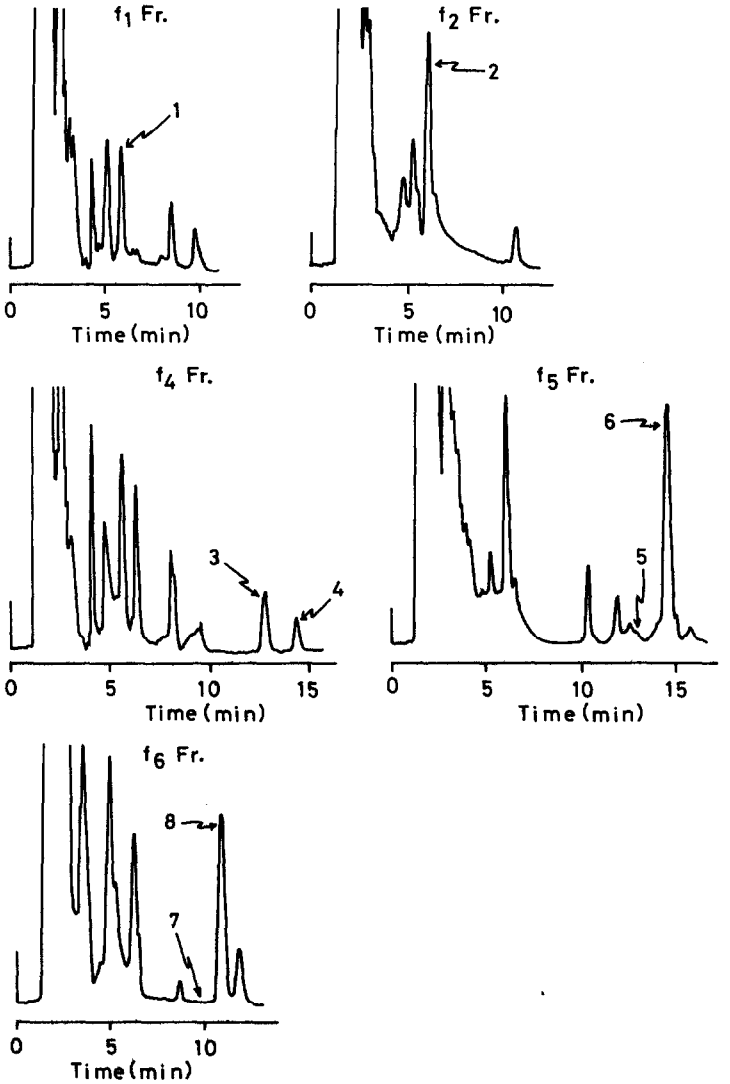

Fig. 3. Separation of bile acid 3-glucuronides in human urine by HPLC. Conditions: column, Shodex ODS Pak F-411; mobile phase, 0.5% ammonium phosphate buffer (pH 5.0)—acetonitrile, 100:45 (f_1 and f_4), 100:33 (f_2 and f_5), 100:34 (f_6), 1.0 ml/min; detection, 205 nm. Peaks: 1 = cholate 3-glucuronide; 2 = glycocholate 3-glucuronide; 3 = deoxycholate 3-glucuronide; 4 = chenodeoxycholate 3-glucuronide; 5 = glycodeoxycholate 3-glucuronide; 6 = glycochenodeoxycholate 3-glucuronide; 7 = taurodeoxycholate 3-glucuronide; 8 = taurochenodeoxycholate 3-glucuronide.

acids, their sulphates and glucuronides on the reversed-phase column with mobile phases of different pH [11—13, 16, 17]. In the higher pH region, the 3-glucuronides all exhibited nearly identical k' values and smaller k' values than sulphated and unsulphated bile acids. Accordingly, the glucuronide fraction was subjected to HPLC on a μBondapak C_{18} column using 0.5% ammonium phosphate buffer (pH 7.0)—acetonitrile as a mobile phase. The eluates corresponding to the capacity ratios (k') of 3.0—7.0 (F_1), 8.0—13.6 (F_2), 13.6—17.0 (F_3) and 18.0—22.0 (F_4) were collected and subjected to rechromatography with 0.5% ammonium phosphate buffer (pH 3.5)—acetonitrile.

It has been pointed out that the HPLC method has a disadvantage in structural elucidation. In this method, however, various combinations of stationary and mobile phases are available. In the preceding study, we disclosed that the chromatographic behaviour of bile acid 3-glucuronides was dependent upon the number and position of the hydroxyl group on the steroid nucleus, as well as the structure of the side-chain at C-17 [11]. Therefore, inspection of chromatographic behaviour under different conditions was performed for the characterization of bile acid 3-glucuronides in urine. The eluate corresponding to each peak on the chromatogram (Fig. 3) was collected and, after addition of an internal standard, subjected to HPLC on Shodex ODS Pak F-411, employing three mobile phases of different pH. It is evident from the data in Table I that the relative k' values of six bile acid 3-glucuronides in urine were identical with those of authentic cholate, deoxycholate, chenodeoxycholate, glycocholate, glycochenodeoxycholate and taurochenodeoxycholate 3-glucuronides.

The structures of bile acid 3-glucuronides were unambiguously characterized by hydrolysis with β-glucuronidase. In the previous paper, we reported that the

TABLE I

RELATIVE k' VALUES OF BILE ACID 3-GLUCURONIDES IN HUMAN URINE

The figures express the k' values relative to the internal standard. Conditions: column, Shodex ODS Pak F-411; mobile phase, 0.5% ammonium phosphate buffer—acetonitrile, 1.0 ml/min.

Bile acid 3-glucuronide	Internal standard	k'		
		pH 3.5	pH 5.0	pH 7.5
Cholate 3-G*	Chenodeoxycholate 3-G	0.39	0.35	0.39
Urine		0.40	0.35	0.40
Deoxycholate 3-G	Cholate 3-G	2.65	2.83	2.43
Urine		2.66	2.82	2.42
Chenodeoxycholate 3-G	Cholate 3-G	2.48	2.50	2.57
Urine		2.47	2.49	2.57
Glycocholate 3-G	Glycochenodeoxycholate 3-G	0.42	0.39	0.39
Urine		0.41	0.39	0.39
Glycochenodeoxycholate 3-G	Glycocholate 3-G	2.41	2.55	2.54
Urine		2.41	2.56	2.53
Taurochenodeoxycholate 3-G	Taurocholate 3-G	2.46	2.47	2.49
Urine		2.46	2.47	2.48

*G = Glucuronide.

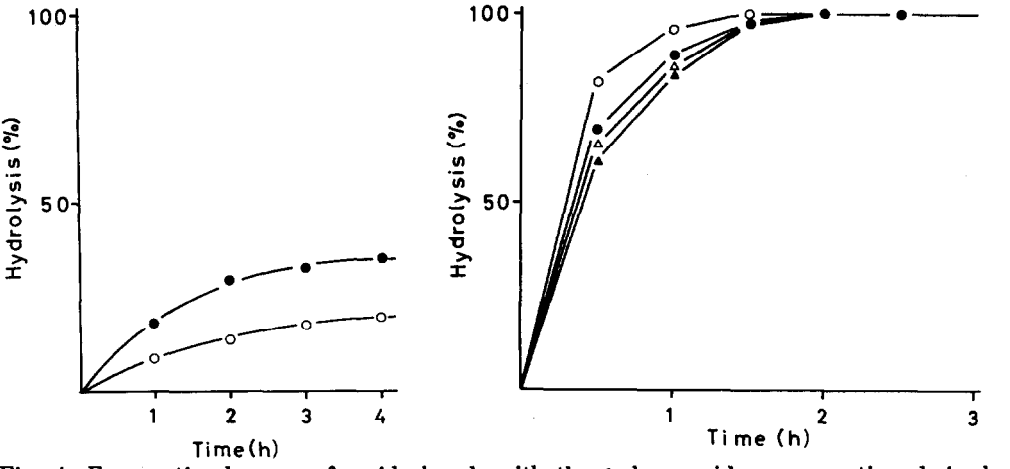

Fig. 4. Enzymatic cleavage of amide bonds with the β-glucuronidase preparation derived from beef liver. Glyco- or taurochenodeoxycholate (100 nmol) was incubated with the enzyme preparation (1000 Fishman units) in 0.1 *M* acetate buffer (pH 4.5, 1 ml) at 37°C. (●) Glycochenodeoxycholate; (○) taurochenodeoxycholate.

Fig. 5. Enzymatic hydrolysis of bile acid 3-glucuronides with the β-glucuronidase preparation derived from *E. coli*. (○) Lithocholate 3-glucuronide; (●) chenodeoxycholate 3-glucuronide; (△) glycochenodeoxycholate 3-glucuronide; (▲) taurochenodeoxycholate 3-glucuronide.

optimal pH for enzymatic hydrolysis of bile acid 3-glucuronides with β-glucuronidase preparations derived from *Helix pomatia* and *Patella vulgata* was dependent upon the number of hydroxyl groups on the steroid nucleus [18]. Moreover, when incubated with the β-glucuronidase preparation derived from beef liver, glyco- and tauro-conjugated chenodeoxycholates underwent cleavage of the amide bonds to provide chenodeoxycholate (Fig. 4). Accordingly, in the present study the β-glucuronidase preparation derived from *E. coli* was used for selective hydrolysis of the glucuronoside linkage. A mixture of unconjugated and conjugated bile acid 3-glucuronides was incubated with the enzyme preparation, and the bile acids liberated were separated and determined by the HPLC method [12]. As illustrated in Fig. 5, the reaction rate increased with the incubation time up to 2 h, resulting in quantitative hydrolysis of the 3-glucuronides.

In the previous paper, we developed a novel method for the determination of serum bile acids by HPLC with pre-column fluorescence labelling [13]. The new fluorescence-labelling reagent, 1-anthroyl nitrile, reacts selectively with the equatorial 3α-hydroxyl group, and derivatized bile acids are efficiently separated on the reversed-phase column with a detection limit of 20 fmol. The identities of bile acid 3-glucuronides were further confirmed by degradative means. The eluate corresponding to each peak on the chromatogram was incubated with β-glucuronidase. After addition of an internal standard, the bile acids liberated were treated with 1-anthroyl nitrile. The 3-(1-anthroyl) derivatives were resolved into the unconjugated, glyco- and tauro-conjugated fractions on PHP-LH-20, and each fraction was subjected to HPLC on Cosmosil $5C_{18}$, with three mobile phases. As listed in Table II, the relative k' values of the bile acids liberated were identical with those of authentic samples.

It is evident from these results that unconjugated, as well as glyco- and tauro-

TABLE II

RELATIVE k' VALUES OF BILE ACIDS IN HYDROLYSATES OBTAINED FROM BILE ACID 3-GLUCURONIDES

The figures express the k' values relative to the internal standard. Conditions: column, Cosmosil $5C_{18}$; mobile phase, 0.5% potassium phosphate buffer—methanol, 1.6 ml/min.

Bile acid	Internal standard	k'		
		pH 3.0	pH 6.0	pH 7.5
Cholate	Ursodeoxycholate	0.39	0.50	0.51
Urine		0.39	0.50	0.51
Deoxycholate	Ursodeoxycholate	1.20	1.47	1.50
Urine		1.20	1.48	1.50
Chenodeoxycholate	Ursodeoxycholate	1.13	1.29	1.31
Urine		1.14	1.28	1.31
Glycocholate	Glycoursodeoxycholate	0.50	0.53	0.53
Urine		0.50	0.54	0.52
Glycochenodeoxycholate	Glycoursodeoxycholate	1.25	1.33	1.35
Urine		1.24	1.34	1.35
Taurochenodeoxycholate	Tauroursodeoxycholate	0.84	0.85	0.86
Urine		0.84	0.85	0.85

conjugated, bile acids are present in urine as the 3-glucuronides. The principal component of the 3-glucuronide fraction was identified as glycochenodeoxycholate 3-glucuronide, and the ratio of unconjugated, glyco- and tauro-conjugated bile acid glucuronides was found to be ca. 2:3:1. Matern et al. [6] reported that the affinity of bile acids to UDP-glucuronyltransferase from human liver decreases with increasing number of hydroxyl groups on the steroid nucleus. This finding appears to be inconsistent with our result that cholate and chenodeoxycholate are predominant in the glucuronide fraction. It may be ascribable to the fact that the relative amount of primary bile acids in urine increases significantly in patients with obstructive jaundice.

In blood plasma of patients with intrahepatic cholestasis, chenodeoxycholate 3-glucuronide has been identified as the acetate-methyl ester by thin-layer chromatographic and mass spectral comparison with the authentic specimen [19]. However, the conjugated form of the side-chain has not been clarified. Almé and co-workers [4, 5] developed a gas chromatographic—mass spectrometric method for the separation and determination of bile acid glucuronides in urine specimens from healthy subjects and patients with mild liver affection. They indicated that urinary bile acid glucuronides are present exclusively in the unconjugated form, with the exception of tauro-conjugated $3\alpha,6\alpha,12\alpha$-trihydroxy-5β-cholanoate 6-glucuronide.

Further studies on the metabolism of bile acid glucuronides in hepatobiliary diseases are being conducted in these laboratories, and the results will be reported elsewhere.

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