Glucuronidation of 6α-Hydroxy Bile Acids by Human Liver Microsomes

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Abstract

The glucuronidation of 6-hydroxylated bile acids by human liver microsomes has been studied in vitro; for comparison, several major bile acids lacking a 6-hydroxyl group were also investigated. Glucuronidation rates for 6α-hydroxylated bile acids were 10–20 times higher than those of substrates lacking a hydroxyl group in position 6. The highest rates measured were for hyodeoxy- and hyocholic acids, and kinetic analyses were carried out using these substrates. Rigorous product identification by high-field proton nuclear magnetic resonance and by electron impact mass spectrometry of methyl ester/peracetate derivatives revealed that 6-*O*-β-D-glucuronides were the exclusive products formed in these enzymatic reactions. These results, together with literature data, indicate that 6α-hydroxylation followed by 6-*O*-glucuronidation constitutes an alternative route of excretion of toxic hydrophobic bile acids.

Introduction

Bile acids with a hydroxyl group in position 6 of the steroidal nucleus occur in several mammalian species. In pigs, 3α,6α-dihydroxycholanoic (hyodeoxycholic) acid constitutes the major biliary bile acid, with 3α,6α,7α-trihydroxycholanoic (hyocholic) acid occurring in smaller amounts; in the bile of rodents, several 6β-hydroxylated bile acids are present (1). Although 6α-hydroxylated bile acids can be identified in the urine of healthy human subjects (2–4), as well as in meconium (5), they do not occur in normal human bile or serum. Inasmuch as the site of bile acid 6-hydroxylation is the liver (6, but see below), the lack of detectable circulating 6-hydroxy bile acids attests to their very rapid renal clearance, in agreement with direct experimental evidence (7, 8). In pathological states, especially in cholestatic liver disease, the amount of excreted 6-hydroxylated bile acids increases, and they also become detectable in bile and serum (2–4, 9, 10). This correlation suggests (although it obviously does not prove) that the occurrence of 6-hydroxy bile acids is either a cause or a consequence of cholestasis. The former possibility is unlikely in that no adverse effects were reported upon long-term (11) or short-term (8) administration of hyodeoxycholate. We interpret the formation of large amounts of 6-hydroxylated bile acids in cholestasis as a response of the organism to the accumulation of cytotoxic mono- and dihydroxylated bile acids in this disease; details of this viewpoint will be discussed later.

A certain amount of disagreement exists in the literature concerning the presence of bile acid 6α-hydroxylase activity in human tissues. Whereas Björkhem et al. (12) could not detect any appreciable hydroxylation of lithocholate, taurolithocholate, and taurochenodeoxycholate by isolated human liver microsomes in the presence of NADPH, Trülzsch et al. (6) found conversion of taurolithocholate to taurohyodeoxycholate under similar conditions. The lack of a 6α-hydroxylase would be, however, inconsistent with the well-documented occurrence of 6α-hydroxylated bile acids in humans: no bacterial hydroxylation of bile acids is known to take place in the gut (13, 14), and dietary intake is likely to be negligible. Moreover, 6α-hydroxylase activity was reported to be inducible with phenobarbital in humans (15). Thus, the emerging consensus appears to be that a bile acid 6α-hydroxylase activity is present in the human liver (3, 10).

A major form of urinary excretion of many 6α-hydroxy bile acids is the glucuronide (4, 8) identified, in the case of hyodeoxycholic acid, as the 6-*O*-β-glucuronide (2). The formation of hyodeoxycholic acid glucuronide upon oral administration of either the free bile acid or of its glycine conjugate to human volunteers has been studied in vivo (7); in a subsequent communication, the same group of investigators reported that the reaction can be demonstrated in vitro using either human liver or kidney microsomes (16). The reaction product was assigned the structure of a 3-*O*-glucuronide (7), at variance with data previously published for the urinary metabolite (2) and with our results (this report).

The purpose of the present study was (*a*) to expand the in vitro studies of glucuronidation of 6-hydroxylated bile acids by human hepatic microsomes using several representative substrates of varying configuration of the molecule and number of hydroxyl groups, (*b*) to compare the glucuronidation of 6α-hydroxylated bile acids with that of the major primary and secondary human bile acids in which the glucuronyl moiety is attached to the hydroxyl group in position 3α (3α-OH), and (*c*) to perform rigorous spectral identification of enzymatically formed glucuronides of 6-hydroxylated bile acids.

Preliminary accounts of this work were presented at the 9th International Bile Acid Meeting, Basel, October 1986, and at the meeting of the American Association for the Study of Liver Diseases, Chicago, November 1986 (17).

Methods

Human liver samples and microsomes. Human liver samples were obtained from organ donors as part of an organ transplant program. Livers B and C were obtained from a 25-yr-old man and a 5-yr-old girl, respectively, killed in traffic accidents. Liver A was obtained from a 65-yr-old woman who died as a result of a cerebrovascular accident. This patient had a history of multiple drug treatment, including mannitol,

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dopamine, dexamethasone, methylprednisolone, vasopressin, hydralazine, and ampicillin. Histology was normal for all livers. Livers were perfused briefly (flushed) with ice-cold Collin solution, dissected, and frozen in dry ice. The livers were stored at −70°C until used for the preparation of microsomes. Microsomes were prepared as previously described (18), except that they were washed once and suspended in 0.25 M sucrose, 5 mM Tris-HCl, pH 7.5, and added to the incubation mixture in this buffer. The microsomes were stored at −70°C and, in respect to the activities studied, were stable for at least 12 mo.

Materials. Nonlabeled bile acids, including hyocholic acid (3α,6α,7α-trihydroxy-5β-cholanoic acid) and hyodeoxycholic acid (3α,6α-dihydroxy-5β-cholanoic acid), were obtained from Calbiochem-Behring Corp., La Jolla, CA. 6-Epi-hyodeoxycholic acid (3α,6β-dihydroxy-5β-cholanoic acid) was obtained from Research Plus, Bayonne, NJ. Allohyodeoxycholic acid (3α,6α-dihydroxy-5α-cholanoic acid) was obtained by sodium borohydride reduction of 3α-hydroxy-6-oxo-5α-cholanoic acid (Research Plus) and separation of the resulting epimers; the identity of the product was confirmed by nuclear magnetic resonance (NMR) and mass spectrometry (MS). The acetonide of hyocholic acid (3α-hydroxy-6α,7α-isopropylidenedioxy-5β-cholanoic acid) was synthesized using dimethoxypropane (19) and characterized by NMR and MS.

All compounds were checked for chemical purity by thin-layer chromatography (TLC).[1] Uridine diphosphate [U-^{14}C]glucuronic acid (225–325 mCi/mmol) was from New England Nuclear, Boston, MA, or from ICN, Irvine, CA. Uridine diphosphate glucuronic acid, ammonium salt, saccharolactone, ursodeoxycholic acid, and Brij 58 were from Sigma Chemical Co., St. Louis, MO. Bond-Elut cartridges (C_{18}, size: 6 cm^3) were from Analytichem International, Harbor City, CA.

Enzyme assays. Bile acid substrates were prepared in the form of mixed micelles with Brij 58, and microsomes were activated (permeabilized) with the same detergent as previously described (20). The basic reaction mixture for the determination of UDP-glucuronosyltransferase activity contained 100 mM Na-Hepes, pH 6.5, 5 mM $MgCl_2$, 0.05% Brij 58 (final concentration, adjusted when necessary with 0.12% Brij 58), 5 mM D-saccharic acid 1,4-lactone to inhibit β-glucuronidase, 3.3 mM uridine diphosphate [^{14}C]glucuronic acid (200–600 dpm/nmol), and the bile acid substrate (0.02–0.2 mM). The incubation mixture contained ~ 50 μg of microsomal protein in a total volume of 60 μl. After 10 min at 37°C, the reaction was stopped by addition of 5 ml of ice-cold 0.1 M glycine-trichloroacetic acid buffer, pH 2.8, and applied to Bond-Elut cartridges that were previously washed sequentially with 10 ml of methanol and 20 ml of 0.1 M glycine-trichloroacetic acid, pH 2.8. The C_{18} cartridge was then washed with 20 ml of the above buffer, and eluted with 3 ml of methanol to recover the radioactive bile acid glucuronide. The methanol eluate was mixed with 10 ml of Liquiscint (National Diagnostics, Inc., Somerville, NJ) and radioactivity was measured in a Mark III (Tracor Analytic Inc., Elk Grove Village, IL) scintillation counter equipped with an automatic quench correction program. To distinguish between hydroxyl- and carboxyl-linked bile acid glucuronides by TLC, the Bond-Elut cartridge was additionally washed with 10 ml of water before the elution with methanol (to remove the glycine-trichloroacetic acid buffer), the methanol eluate was evaporated, the radioactive bile acid glucuronide was applied to a TLC plate, and the plate was developed in the alkaline solvent system as described previously (20). The glucuronosyl transferase reaction was linear with time for 25 min and linear with protein concentration up to 1.2 mg/ml. Protein was measured by the method of Lowry et al. (21) in the presence of 1% sodium dodecyl sulfate. Specific activities of enzymes are expressed as nanomoles of bile acid glucuronide formed per milligram of protein per minute.

Large-scale preparation and structural identification of biosynthetic bile acid glucuronides. Preparative enzymatic reactions (volume 0.6–1.2 ml) were run under conditions similar to those used in kinetic studies. The bile acid substrates and protein concentrations were 0.2 mM and 1.5 mg/ml, respectively. The reaction was terminated by addition of an excess of ice-cold glycine-trichloroacetic acid buffer, pH 2.8, and applying to Bond-Elut cartridges as described above. The bile acid glucuronides were further purified on preparative TLC plates, eluted, and the corresponding methyl ester-acetates were prepared as described previously (20). The resulting derivatives of bile acid glucuronides were analyzed by TLC, MS, and ^{1}H-NMR.

MS and NMR spectrometry. Electron impact (70 and 20 eV, solid probe) mass spectra were obtained on Shimadzu QP1000 (Shimadzu Scientific Instruments, Inc., Columbia, MD) and Extrel ELQ-400 (Extrel, Pittsburgh, PA) mass spectrometers. Fourier-transformed ^{1}H-NMR spectra were measured at 300 MHz in $CDCl_3$ using a General Electric QE-300 (General Electric Co., Fremont, CA) instrument.

Statistical analysis. Enzyme activity measurements are reported as mean±standard deviation. Kinetic parameters were calculated using a numerical version of the direct linear plot method (22–24).

Results

Substrate specificity. The formation of bile acid glucuronides by detergent-activated hepatic microsomes was studied on liver samples obtained from three human subjects who are described in more detail in the Methods section. The conditions for the enzymatic reaction were as reported before (20), except that initially two pH values, 6.5 and 7.5, were used. Reaction rates were higher by ~ 30% at pH 6.5 for both deoxycholic and hyodeoxycholic acid, in agreement with previous findings for C_{24} bile acids (20, 25); consequently, this pH was used throughout the study. The results are summarized in Fig. 1. In the right-hand panels of the figure, bile acids that lack a hydroxyl group in position 6 are shown. The rates of their glucuronidation are generally low in human liver microsomes; our results agree well with those published previously (26, 27). The left-hand panels of Fig. 1 list bile acids that carry a hydroxyl group in position 6; the structures of these compounds are given in Fig. 2. Hyodeoxycholic acid has been shown previously to undergo glucuronidation by human liver homogenates and microsomes (16); our results confirm this finding. We extended the study to other 6-hydroxy bile acids and could show that they are also substrates for the glucuronidation reaction. At a concentration of 0.1 mM, hyocholic acid was utilized at 70% of the rate found for hyodeoxycholic acid. Hyocholic acid 6,7-acetonide, a derivative in which the hydroxyl groups in positions 6 and 7 are blocked, was not glucuronidated by any of the human livers tested. Allohyodeoxycholic acid, which differs from hyodeoxycholic acid in the configuration of the A/B ring junction, was conjugated at a lower rate than hyodeoxycholic acid. Finally, 6-epihyodeoxycholic acid was glucuronidated at a very low rate, and only by two of the three livers studied, thus documenting that the UDP-glucuronosyltransferase involved is highly specific for 6α (as compared with 6β) hydroxyl groups.

The substrate specificity profiles of microsomes isolated from the three human livers available for this study were qualitatively similar. Some quantitative differences were, however, discernible, especially the high activity of liver A for some bile acids, most notably for lithocholic acid whose rate of glucuronidation was ten times higher than in the other livers. Liver A was obtained from a patient who received multiple medications, including dexamethasone and methylprednisolone. Glucocorticoids are known to induce UDP-glucuronosyltransferase activity in fetal rat liver (28) and it has been suggested that they have a similar effect on the adult human liver (29). Irshaid and Tephly (30) have recently shown that the liver of a patient treated with glucocorticoids exhibits high glucuronosyltransferase activity for a

1. *Abbreviations used in this paper:* MS, mass spectrometry; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography.

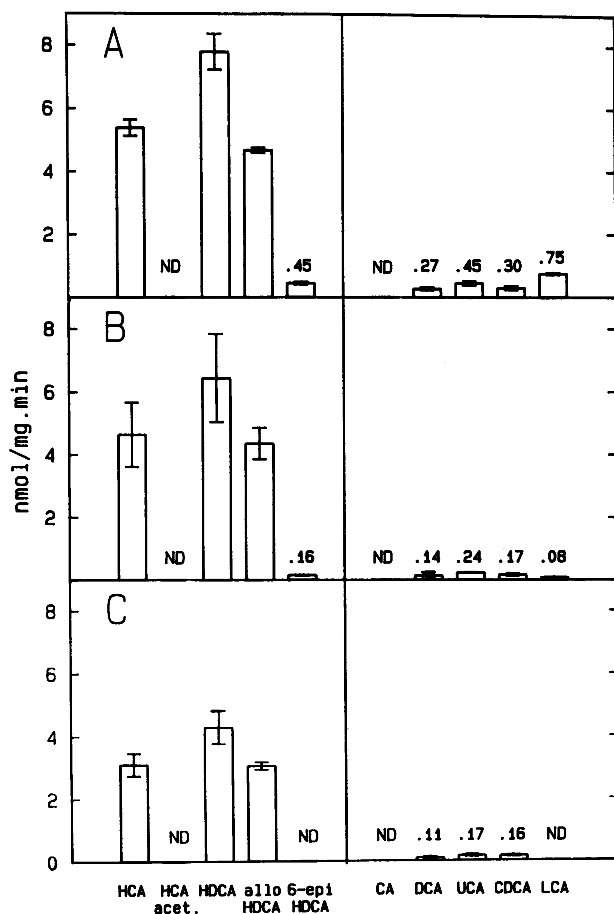


Figure 1. Specific activity of hepatic microsomal UDP-glucuronosyltransferase toward bile acids. Results obtained with microsomes from three different human subjects are shown in *A, B,* and *C.* Refer to the Methods section for a short description of the patients. Data are plotted as mean±SD (n = 3–8, with n denoting the number of separate experiments, each done in duplicate). For small values, the mean is listed above the bar. *ND* denotes activity below the level of detection (~ 0.03 nmol/mg · min). The substrate concentration was 0.1 mM for all bile acids. Abbreviations: HCA, hyocholic acid; HCA acet., hyocholic acid 6,7-acetonide; HDCA, hyodeoxycholic acid; allo HDCA, allohyodeoxycholic acid; 6-epi HDCA, 6-epihyodeoxycholic acid; CA, cholic acid; DCA, deoxycholic acid; UCA, ursodeoxycholic acid; CDCA, chenodeoxycholic acid; LCA, lithocholic acid.

variety of substrates. It is therefore possible that the high enzymatic activity of liver A is an effect of dexamethasone and methylprednisolone treatment of the patient.

Kinetic constants. The two physiologically most relevant 6-hydroxy bile acids, hyocholic and hyodeoxycholic, were selected for a kinetic characterization with two of the human liver microsomal samples. For both substrates, the reaction conformed to Michaelis-Menten kinetics, with substrate inhibition observed above 0.1 mM. The kinetic parameters are shown in Table I. Our values for hyodeoxycholic acid are similar to those published previously (16). It is interesting that hyocholic acid exhibits a similar V_{max} value to hyodeoxycholic acid but a significantly higher K_M; the possible significance of this finding will be discussed later.

Product identification. High-field proton NMR was the principal method of identification of enzymatic reaction products. The assignments for methyl ester/acetate derivatives of glucuronides of selected 6-hydroxy bile acids, synthesized in vitro using human liver microsomes, are summarized in Table II and are compared with data for methylated/acetylated but not glucuronidated parent bile acids. An example of a spectrum of the 6-*O*-glucuronide of hyodeoxycholic acid is shown in Fig. 3. The ratio of intensities of sugar signals to steroidal signals (integration not shown) indicates the presence of one glucuronic acid per bile acid molecule. This is important in view of our recent finding that certain bile acids undergo double glucuronidation (31). Two

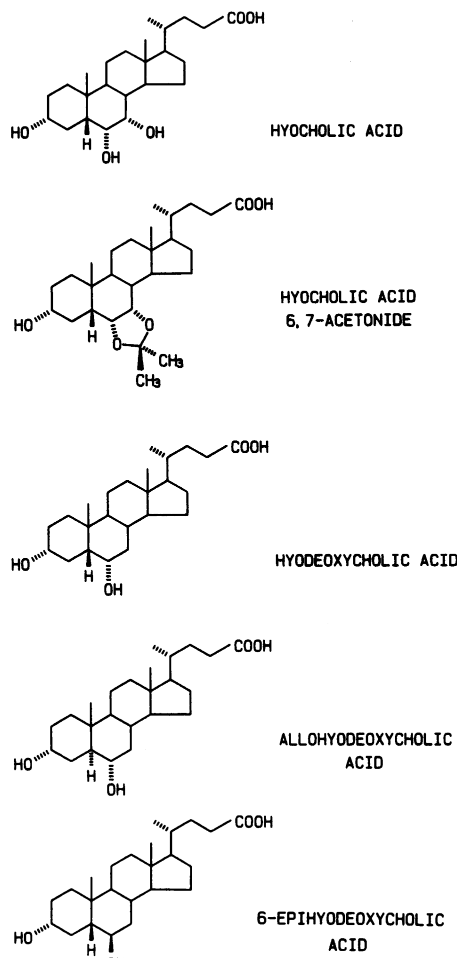


Figure 2. Structures of 6-hydroxy bile acids used in this work. Systematic names of the compounds are given in the Methods section.

Table I. Apparent Kinetic Constants for the Glucuronidation of Hyocholic and Hyodeoxycholic Acids Obtained with Human Liver Microsomes

Patient	Hyocholic acid K_M	Hyocholic acid V_{max}	Hyodeoxycholic acid K_M	Hyodeoxycholic acid V_{max}
	μM	*nmol/mg · min*	*μM*	*nmol/mg · min*
Female, 65 yr (liver A)	97±22	10.4±1.2	36±6	7.3±1.7
Female, 25 yr (liver C)	165±13	7.0±1.2	36±1	6.2±0.7

Table II. ¹H-NMR Spectra of 6-O-Glucuronides of Hyodeoxycholic and Hyocholic Acids and of the Parent Bile Acids (as Methyl Ester/Peracetates)

Proton	Hyodeoxycholic acid 6-*O*-glucuronide (methyl ester/peracetate)	Hyocholic acid 6-*O*-glucuronide (methyl ester/peracetate)	Hyodeoxycholic acid (methyl ester/peracetate)	Hyocholic acid (methyl ester/peracetate)
H-1′	4.60d (J = 7.6)	4.60d (J = 7.3)	—	—
H-2′	4.96dd ($J_{1',2'}$ = 7.6, $J_{2',3'}$ = 8.4)	4.98t (J = 7.3)	—	—
H-3′ } H-4′ }	5.15–5.30m	5.18–5.28m	—	—
H-5′	4.02d (J = 9.5)	4.02 overlapped	—	—
H-3	4.69br (H-3β)	4.6br (H-3β)	4.72tt (J = 11.3, 5.6)	4.60m (H-3β)
H-6	3.98br (H-6β)	4.0 overlapped (H-6β)	5.16dt ($J_{6\beta7\alpha}$ = 12.1, $J_{6\beta7\beta}$ = $J_{5\beta6\beta}$ = 4.5)	5.12t (H-6β) (J = 4.3)
H-7	—	5.27bs (H-7β)		5.22bt (H-7β) (J = 2.3)
Me-18	0.63s	0.63s	0.67s	0.66s
Me-19	0.90s	0.94s	1.00s	1.01s
Me-21	0.90d (J = 5.4)	0.90d (J = 6.3)	0.93d (J = 6.3)	0.93d (J = 6.5)
MeO	3.66s 3.76s	3.66s 3.76s	3.69s	3.69s
AcO	2.00s (one) 2.01s (two) 2.02s (one)	2.00–2.02 (five)	2.03s (one) 2.07s (one)	1.99s (one) 2.08s (one) 2.12s (one)

The glucuronides were isolated from large-scale enzymatic incubations that included human liver microsomes as the enzyme source, and were methylated and acetylated as described in the Methods section. Spectra were obtained on a Fourier-transform 300 MHz instrument. Protons of the sugar moiety carry a "prime" index, whereas steroidal protons are not indexed. Abbreviations used in signal description: s, singlet; d, doublet; t, triplet; m, multiplet; dd, doublet of doublets; tt, triplet of triplets; dt, doublet of triplets; br, broad; bs, broad singlet; bd, broad doublet; and bt, broad triplet.

signals of methoxyl groups and four and five signals of acetoxyl groups (for the glucuronides of hyodeoxy- and hyocholic acids, respectively) are compatible with the sugar being attached to a hydroxyl group of the bile acid; this conclusion is confirmed by chemical shifts of the glucuronic acid moiety characteristic for a hydroxyl-linked glucuronide (20, 31). The position of glucuronidation can be inferred from the chemical shifts of the steroidal protons H-3β and H-6β. In both compounds, H-3β is found at 4.6–4.7 parts per million (ppm) (Table II). This is a position characteristic for a proton adjacent to an acetylated 3α-

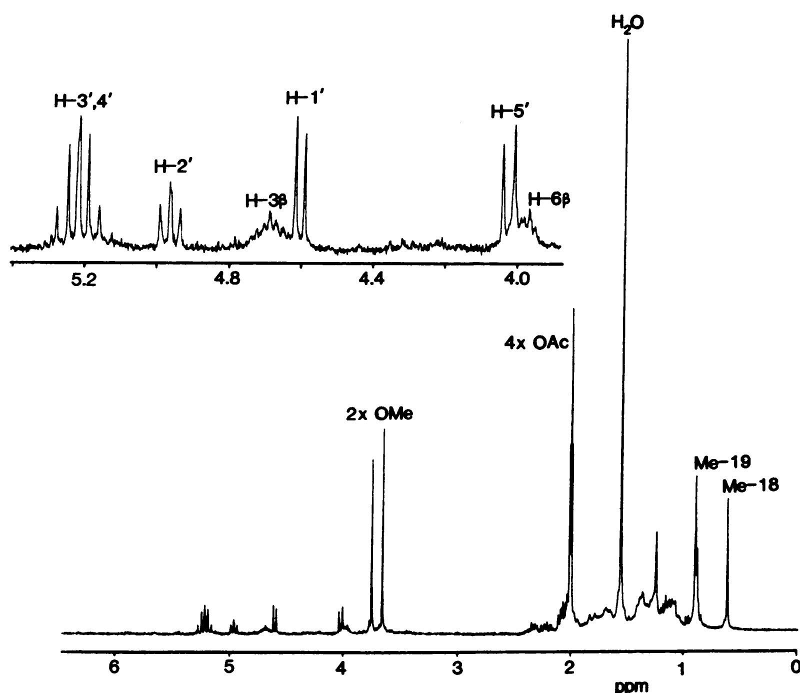


Figure 3. ¹H-NMR (300 MHz) spectrum of the 6-*O*-glucuronide of hyodeoxycholic acid (as the methyl ester/peracetate). See legend to Table II for an explanation of symbols.

hydroxyl group; if the group is free or carries a glucuronic acid moiety, the H-3β signal appears at ~ 3.6 ppm. The 3β (and, in the case of hyocholic acid glucuronide, also 7β) proton shows this characteristic downfield acylation shift and is found in the same position as in the parent acetylated acids (Table II), thus confirming that 3α-OH (and 7α-OH in hyocholic acid) is acetylated. On the other hand, H-6β exhibits no downfield shift. It can thus be concluded that the glucuronic acid molecule is attached to the 6-hydroxyl group of hyocholic and hyodeoxycholic acid (and, by inference, the other 6-hydroxy bile acids).

The chemical shift of the proton attached to the sugar carbon atom C-1′ is very sensitive to the substituent at this carbon. In the underivatized sugar, the position of H-1′ is at ~ 4.5 ppm. This value does not change appreciably upon formation of a glycosidic bond; for example, in hydroxyl-linked glucuronides of bile acids the H-1′ signal is found at 4.6–4.7 ppm (20, 32). On the other hand, acylation of the hydroxyl group at C-1′ causes a downfield shift of H-1′ to 5.8 ppm (32), as observed for carboxyl-linked bile acid glucuronides (20). In the glucuronides of hyocholic and hyodeoxycholic acids, the H-1′ signal is located at 4.6 ppm (Table II). This not only confirms that the latter bile acids formed hydroxyl-linked glucuronides, but also proves that the linkage to the sugar is via its C-1′, i.e., a glycosidic bond. This is important in light of the existence of positional isomers of certain glucuronides in which the aglycone has migrated from C-1′ to C-2′, C-3′, and C-4′ (see references 33 and 34 for reviews); in such compounds, H-1′ would be adjacent to an acetoxyl group and would be expected to give a resonance at a lower field. Finally, the large coupling constant of H-1′ and H-2′, J = 7.3–7.6 Hz, indicates an axial configuration of both protons and thus proves the existence of a β-glycosidic bond. The assigned structure is shown for the glucuronide of hyodeoxycholic acid in Fig. 4 *A*.

Electron impact ionization mass spectra of both derivatized glucuronides are shown in Fig. 5 and Fig. 6, respectively. In the case of the methyl/peracetyl hyodeoxycholic acid glucuronide, the molecular ion (764) is absent. Ions resulting from the sequential loss of the four acetic acid molecules (60 each) are present at a mass-to-charge ratio (m/z) of 704, 644, 584, and 524. The loss of the glucuronic acid moiety, with and without hydrogen migration, results in ions at m/z 430 and 431; both of these ions can lose the remaining acetic acid, resulting in ions at m/z 370 and 371 (base peak). Rupture of the bond between C-1′ of glucuronic acid and the oxygen of the steroidal 6-OH results in both possible ions, namely 317 (sugar part) and 447 (steroidal part). A series of ions at m/z < 317, including the prominent ion at 155, is derived from the glucuronic acid moiety.

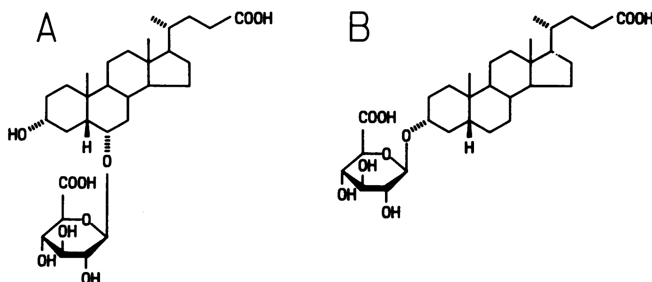


Figure 4. Structures of (*A*) hyodeoxycholic acid 6-*O*-glucuronide and (*B*) lithocholic acid 3-*O*-glucuronide.

The fragmentation pattern of the derivatized glucuronide of hyocholic acid (Fig. 6) is similar to that of hyodeoxycholic acid glucuronide. The molecular ion (822) is absent; the loss of acetic acid and water results in the ion at 744, and the loss of acetic acid and the $COOCH_3$ fragment yields the ion at 703. Ions at 702, 642, 582, and 522 are the result of the loss of two, three, four, and five acetic acid molecules. The cleavage of the C(3)-*O* bond gives rise to a fragment at m/z 489, followed by ions at m/z 429 (−acetic acid), 369 (−2 × acetic acid), 387 (−acetic acid−ketene). A parallel series of ions with masses smaller by 1 atomic mass unit, i.e., 428 and 386, results from the loss of glucuronic acid. Ions at m/z 317, 257, 197, and the base peak 155, as described above, correspond to the sugar moiety.

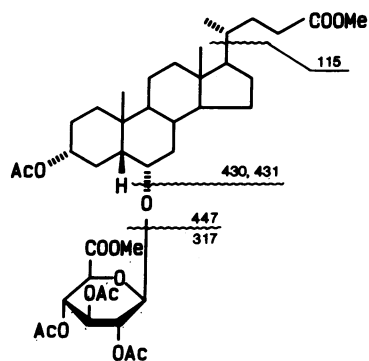
No structure elucidation was performed for glucuronides of bile acids that lack a 6-hydroxyl group (right-hand panels in Fig. 1), because the attachment of glucuronic acid to the 3-OH of the bile acids is known from the literature (2). The structure of the 3-*O*-glucuronide of lithocholate acid is shown, as an example, in Fig. 4 *B*.

Discussion

Determination of the substrate specificity profile of human microsomal UDP-glucuronosyltransferase with the major primary and secondary bile acids, including 6α-hydroxylated bile acids, revealed the fact that the latter are by far the superior substrates for the reaction: the glucuronidation of hyodeoxycholic acid is some 30 times more rapid than that of deoxycholic or lithocholic acids under the optimized experimental conditions used. This ratio of velocities is expected to hold at the lower bile acid concentrations likely to be encountered in liver cells: the apparent K_M for hyodeoxycholic acid (close to 30 μM, this communication) is similar or lower than the values reported for bile acids that lack a hydroxyl group in position 6 (26). Thus, at low substrate concentrations, the ratio of reaction velocities should be the same or even more favorable for hyodeoxycholic acid.

The high activity of the UDP-glucuronosyltransferase toward hyocholic acid is noteworthy. While its positional isomer, cholic acid, was not glucuronidated under our conditions and only weakly in the hands of other investigators (27), hyocholate showed, in this study, the highest V_{max} of the substrates tested, and at 0.1 mM it was reacting at a significantly higher rate than lithocholic acid or any of the dihydroxylated bile acids except hyodeoxycholate. On the other hand, the apparent K_M for hyocholic acid is high, markedly higher than that for hyodeoxycholic acid (Table I). This means that hyocholic acid is glucuronidated at high rates only when its concentration becomes high, perhaps sufficiently high to permit the expression of its detergent properties and, thus, toxicity; at low concentrations its glucuronidation should become slower than that of bile acids with a lower K_M. This regulation of the enzyme by the substrate concentration could have the physiological function of guarding against a potentially harmful buildup of hyocholic acid while preventing its glucuronidation at low concentrations, thus helping to conserve the limited pool of UDP-glucuronic acid in the liver (35).

As mentioned before, all 6α-hydroxylated bile acids tested were glucuronidated at rates significantly higher than the remaining substrates. This suggested to us the importance of the 6α-hydroxyl group, possibly as the target group for glucuronidation. In the case of a urinary glucuronide of a 6α-hydroxy bile acid, indirect evidence resulting from chemical degradation indicated that the glucuronyl moiety was attached to the 6-hydroxyl



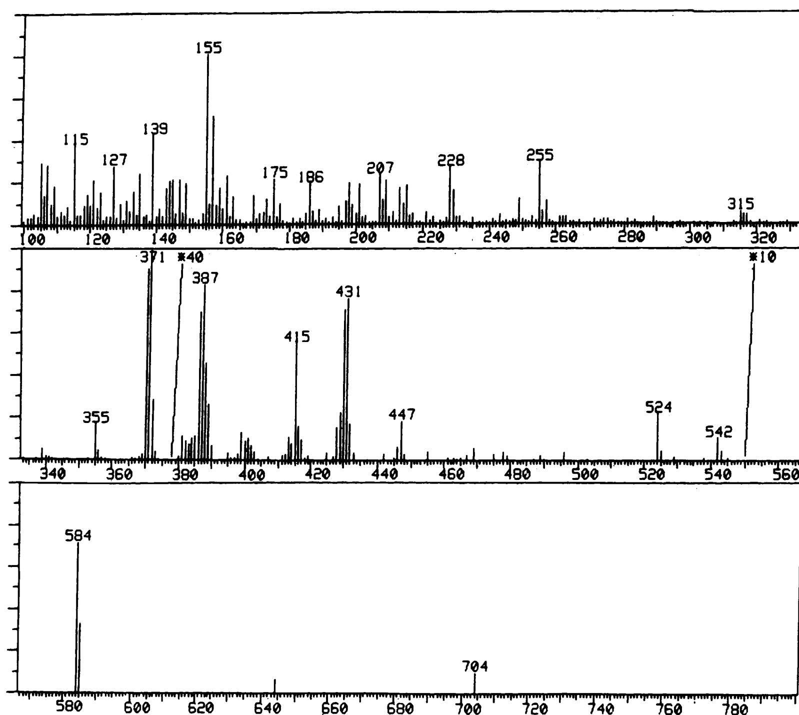
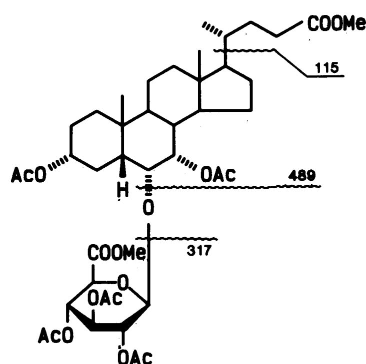


Figure 5. Mass spectrum and fragmentation pattern of the methyl ester/peracetate of hyodeoxycholic acid 6-O-glucuronide. The electron impact ionization (20 eV) spectrum was obtained using solid probe insertion.

group (2). On the other hand, in the case of in vitro glucuronidation of a 6-hydroxy bile acid, hyodeoxycholate, the reaction product was identified as the 3-*O*-glucuronide (7, 16). In view of this discrepancy we decided to perform a rigorous structure determination on the enzymatic reaction products of two selected 6-hydroxy bile acids, namely the glucuronides of hyocholic and hyodeoxycholic acids. If a sufficient amount of the glucuronide ($> 50\ \mu g$) is available, high-field proton NMR spectrometry of methyl ester/acetate derivatives provides a full structural identification of the metabolites. In addition to the identification of the glucuronidation site, NMR spectra contain sufficient structural information to confirm that the reaction product is in fact a β-D-glucuronide (this is usually assumed but not explicitly proven by mass spectrometry—compare reference 2), and that no additional modifications of the steroidal nucleus took place. Mass spectra of methyl ester/acetate derivatives of glucuronides yield complementary information.

The 6-*O*-glucuronides were the sole products of the enzymatic reaction with hyocholic and hyodeoxycholic acids. Within the detection limit of our methods (few percent of the main compound), no 3-*O*-glucuronides or acyl-linked glucuronides were found. This is in agreement with the indirect conclusion of Almé and Sjövall on urinary metabolites (2) but, as noted above, at variance with the results of Sacquet et al. (7). On the basis of lack of activity of 3α-hydroxysteroid dehydrogenase toward a glucuronide of hyodeoxycholic acid isolated from bile and urine of patients who ingested the bile acid, the latter authors concluded that the metabolite is the 3-*O*-glucuronide. The discrepancy between this and our result could be explained by steric hindrance in binding of the glucuronide to the steroid dehydrogenase. This interpretation is supported by the examination of a molecular model of the 6-*O*-glucuronide of hyodeoxycholic acid which reveals that the bulky glucuronic acid moiety remains in the vicinity of the 3α-hydroxyl group. In fact, our sample of authentic 6-*O*-glucuronide of hyodeoxycholic acid was not oxidized by 3α-hydroxysteroid dehydrogenase (from *Pseudomonas testosteroni*), whereas hyodeoxycholic acid reacted as expected (Little, J. M., unpublished result). The present unambiguous structure determination of metabolites obtained in vitro provides strong evidence for 6-*O*-glucuronidation of 6α-hydroxylated bile acids in the human.

Thus, the bile acid substrates used in this study fall into two distinct categories. All bile acids that possess a free 6α-hydroxyl group are glucuronidated at high rates; the 6α-OH is the target group for the reaction. Blocking of this group abolishes the reaction completely; no glucuronidation of the available 3α-hy-



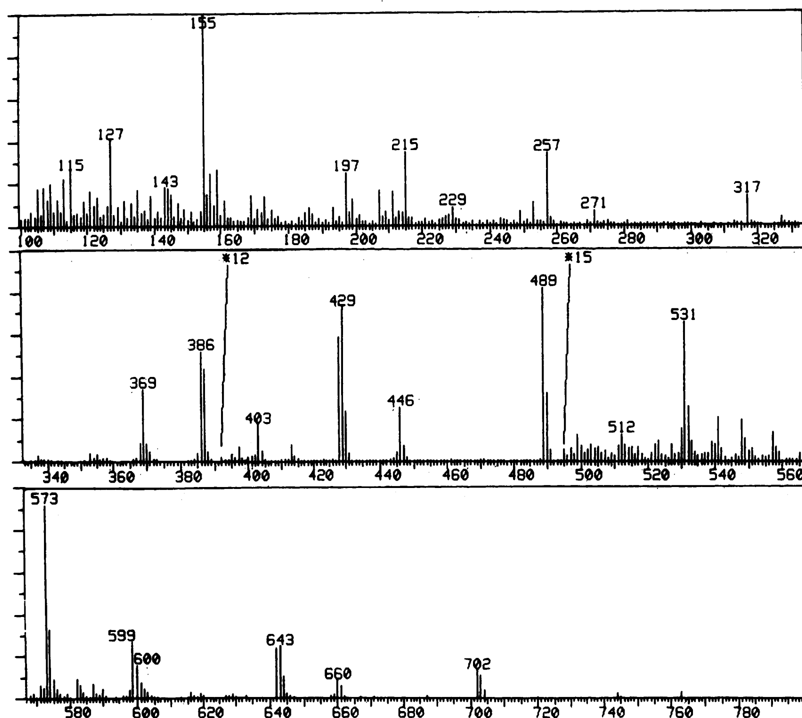


Figure 6. Mass spectrum and fragmentation pattern of the methyl ester/peracetate of hyocholic acid 6-*O*-glucuronide. See legend to Fig. 5 for conditions.

droxyl group takes place. Inversion of the 6α-OH to 6β-OH causes a dramatic decrease in the glucuronidation rate. The second category of substrates consists of bile acids that are glucuronidated on the 3α-hydroxyl group (the position of the glucuronyl moiety was determined previously by Almé and Sjövall (2) and was not reinvestigated here except for ruling out the possibility of a carboxyl-linked glucuronide, data not shown). These bile acids are glucuronidated at significantly lower rates than the 6α-hydroxylated substrates. This raises the obvious question concerning the enzymology of the process. Inasmuch as the enzyme or enzymes responsible for bile acid glucuronidation have not been purified from human material, any answers have to remain speculative. Essentially, the two possibilities formulated previously (2), namely that of separate UDP-glucuronosyltransferases specific for the two positions and that of one enzyme preferring 6α-OH to 3α-OH but accepting both, remain viable.

The broader question concerns the physiological function of 6-*O*-glucuronidation. We subscribe to the idea, whose elements have been expressed earlier by others (2, 3), that it constitutes a part of a metabolic response to toxic hydrophobic bile acids, especially lithocholate but also some dihydroxy bile acids. Even though additional hydroxylation of the steroidal nucleus and/or the formation of the 3-*O*-sulfate appear to be the major detoxification process for lithocholate (see reference 36 for a review), other options also exist. Conjugation of lithocholate with taurine causes, counterintuitively, a decrease in water solubility (37, 38). Direct glucuronidation of lithocholate results in a compound that is still poorly soluble and even more cholestatic than the parent bile acid, at least in the rat (37). 6α-hydroxylation, which leads to the formation of hyodeoxycholate from lithocholate and of hyocholate from the less toxic but nevertheless still cholestatic chenodeoxycholate, avoids this difficulty. The 6α-hydroxy bile acids formed in this process are efficiently glucuronidated and excreted (7, 8). Thus, in the proposed scheme, a bile acid would be committed to excretion by 6α-hydroxylation, which in turn would trigger 6-*O*-glucuronidation and clearance.

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