Formation of three types of glucuronides of 6-hydroxy bile acids by rat liver microsomes[1]

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Abstract The glucuronidation of 6-hydroxylated bile acids by rat liver microsomes was studied in vitro; for comparison, several major bile acids lacking a hydroxyl group in position 6 were also investigated. The highest reaction rates were found for lithocholic and deoxycholic acid (10.2 ± 0.2 and 7.3 ± 1.4 nmol/mg • min, respectively); our results for these substrates agree well with published values. Glucuronidation rates for the 6β-hydroxylated bile acids 3α,6β-dihydroxy-5β-cholanoate (murideoxycholate) and 3α,6β,7β-trihydroxy-5β-cholanoate (β-muricholate) were only slightly lower (3.7 ± 0.3 and 3.6 ± 0.3 nmol/mg · min). 6α-Hydroxylated bile acids were glucuronidated at rates that were lower than those for their 6β-hydroxy counterparts. Rigorous product identification by high-field proton NMR of methyl/acetyl derivatives revealed that while bile acids lacking a 6-hydroxyl group gave rise exclusively to the typical 3-O-glucuronide, the presence of a hydroxyl group in position 6 led to the formation, in ratios depending on the substrate, of three types of conjugate: the 3-O-, the 6-O-, and the carboxyl-linked (acyl-) glucuronide. The latter is the first example of an acyl glucuronide of a bile acid of conventional (C_{24}) size.—**Zimniak, P., A. Radominska, M. Zimniak, and R. Lester.** Formation of three types of glucuronides of 6-hydroxy bile acids by rat liver microsomes. *J. Lipid Res.* 1988. **29:** 183–190.

Supplementary key words 6-hydroxy bile acid glucuronides • hydroxyl-linked glucuronides • carboxyl-linked glucuronides • acyl glucuronides • nuclear magnetic resonance

The bile of some rodents contains significant amounts of bile acids hydroxylated in position 6β of the steroidal nucleus (1–3). The presence of these compounds is a consequence of a bile acid-specific 6β-hydroxylase activity in the liver (4–11). Rodents differ in this respect from many other mammals that are capable of hydroxylating bile acids in position 6α rather than 6β. In the pig, for instance, hyodeoxycholic and hyocholic acids constitute the predominant biliary bile acids (12, 13). The presence of a bile acid 6α-hydroxylase in human liver is almost certain (14), and 6α-hydroxy bile acids become detectable in disease states (15). Our previous study of the glucuronidation of 6-hydroxylated bile acids by human liver microsomes (16) revealed that the presence of the 6-OH group constitutes a signal that redirects the UDP-glucuronosyltransferase from the 3α-OH of the bile acid to the 6-OH. The process can be viewed as a defense mechanism against the presence of an excess of bile acids (15–17): in cholestatic liver disease, elevated levels of bile acids activate the 6α-hydroxylase, whose reaction products are rapidly glucuronidated on the 6-OH group (16) and efficiently excreted (18). The present study was initiated to ascertain whether the 6-OH group has a similar effect in the rat where 6-hydroxylation is the rule rather than an exception. Mechanistically, the problem is of additional interest because of stereochemical considerations. In the biologically predominant 5β-H series of bile acids, the two hydroxyl groups that are known to be potential targets for the UDP-glucuronosyltransferase reaction, namely 3α-OH (19) and 6α-OH (15, 16), are equatorial. Their axial counterparts are glucuronidated at a lower rate, as shown for 3β-OH groups with rat liver microsomes (20) and for 6β-OH groups with human liver microsomes (16). It was not clear whether axial hydroxy groups are generally less active in the UDP-glucuronosyltransferase reaction, or whether the specificity of the enzyme is a simple reflection of the type of substrate that is more abundant in a given species. A comparison of glucuronidation rates of 6α- and 6β-hydroxylated bile acids by the rat, in which substrates bearing the axial 6β-OH predominate, would help to answer this question.

Abbreviations: hyocholic acid, 3α,6α,7α-trihydroxy-5β-cholanoic acid; hyodeoxycholic acid, 3α,6α-dihydroxy-5β-cholanoic acid; allohyodeoxycholic acid, 3α,6α-dihydroxy-5α-cholanoic acid; murideoxycholic acid, 3α,6β-dihydroxy-5β-cholanoic acid; β-muricholic acid, 3α,6β,7β-trihydroxy-5β-cholanoic acid; cholic acid, 3α,7α,12α-trihydroxy-5β-cholanoic acid; deoxycholic acid, 3α,12α-dihydroxy-5β-cholanoic acid; ursodeoxycholic acid, 3α,7β-dihydroxy-5β-cholanoic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid; lithocholic acid, 3α-hydroxy-5β-cholanoic acid; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance.

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MATERIALS AND METHODS

Chemicals

The sources of bile acids were as given in reference 16. β-Muricholic acid was a generous gift from Dr. D. Alvaro, Universita Degli Studi De Roma. All compounds were checked for chemical purity by TLC and by gas–liquid chromatography.

The acetonide of hyocholic acid (3α-hydroxy-6α,7α-isopropylidenedioxy-5β-cholanoic acid) was synthesized by a modification of a method described previously (21): 0.5 g (1.22 mmol) of hyocholic acid was suspended in 10 ml of dimethoxypropane and approximately 1 mg of *p*-toluenesulfonic acid was added. The mixture was sonicated in a bath-type sonicator until dissolution was complete (approximately 30 sec). After 10 min at room temperature, the reaction mixture was diluted with 50 ml of aqueous 1% sodium carbonate and was adjusted to pH 7–8 with 6 N HCl; care was used not to acidify the solution below this pH at any time to prevent hydrolysis of the product. The reaction mixture was extracted twice with ethyl acetate, the combined extracts were backwashed with water, dried with Na_2SO_4, and evaporated. TLC (silica gel; benzene–acetone 2:1) revealed the major desired product (R_f = 0.31) and a minor product, probably a mixed acetal (R_f = 0.52). The crude yield was 95%. The acetonide was purified on a 2.2 ×12 cm silica gel column eluted with benzene–acetone 2:1. ^{1}H-NMR: 0.69s, Me-18; 0.92s, Me-19; 0.97d (J = 5.8), Me-21; 1.36s and 1.55s, acetonide Me (2×); 3.81bs, H-3β; 4.23m, overlapped, H-6β and H-7β. Methyl ester (methyl 3α-hydroxy-6α,7α-isopropylidenedioxy-5β-cholanoate): ^{1}H-NMR: 0.68s, Me-18; 0.91s, Me-19; 0.94d (J = 6.0), Me-21; 1.35s and 1.56s, acetonide Me (2×); 3.67s, OMe; 3.79bs, H-3β; 4.23 overlapped, H-6β and H-7β. Methyl ester/acetate (methyl 3α-acetoxy-6α,7α-isopropylidenedioxy-5β-cholanoate): ^{1}H-NMR: 0.71s, Me-18; 0.87s, Me-19; 0.96d (J = 5.8), Me-21; 1.34s and 1.52s, acetonide Me (2×); 2.05s, OAc; 3.70s, OMe; 4.15m and 4.33m, H-6β and H-7β; 4.62m, H-3β.

D-Saccharic acid-1,4-lactone (saccharolactone), uridine diphosphate glucuronic acid, ammonium salt, and Brij 58 were from Sigma Chemical Co, St. Louis, MO. Uridine diphosphate [U-^{14}C]glucuronic acid (225–325 mCi/mmol) was from New England Nuclear, Boston, MA, or from ICN, Irvine, CA. Bond Elut cartridges (C_{18}, size: 6 cc) were from Analytichem International, Harbor City, CA.

Animals and preparation of microsomes

Rats (Sprague-Dawley, male, 180–230 g) were used in all experiments. Microsomes were prepared as previously described (20), and could be stored at −70°C for at least 6 months without loss of activity.

Bile acid:UDP-glucuronosyltransferase assay

Bile acid:UDP-glucuronosyltransferase was assayed using the TLC separation method (20) and/or the Bond Elut cartridges (C_{18}) elution method (16, 22). The incubation conditions were as previously described (16, 20) except that the reactions were run in 100 mM Na-HEPES buffer, pH 6.5. The bile acid concentration was 100 μM. In preliminary experiments, this concentration was determined to be saturating but not inhibitory for all substrates used (data not shown). Approximately 50 μg of microsomal protein was incubated for 10 min in a total volume of 60 μl. The glucuronosyltransferase reaction was linear with time for 20 min and linear with protein concentration up to 1.5 mg/ml. Specific activities of enzymes are expressed as nmol of bile acid glucuronide formed per mg protein and per min, and are reported as mean ± SD. Protein was measured using the Bio-Rad protein assay originally described by Bradford (23), using bovine albumin as standard.

Preparative reactions and separation of bile acid glucuronides

Preparative enzymatic reactions contained approximately 15 mg of microsomal protein in a total volume of 15 ml. The microsomes were activated (permeabilized) with Brij 58, and the bile acid substrates were prepared in the form of mixed micelles with the same detergent as previously described (20). Bile acids were used at a final concentration of 100 μM. After 150 min at 37°C, the reaction was stopped with a three- to five-fold excess of ice-cold 0.1 M glycine-trichloracetic acid buffer, pH 2.8, and applied to Bond Elut cartridges (C_{18}) as previously described (16). Up to 90–95% of the bile acid substrate was glucuronidated under these conditions. The methanol eluate containing unreacted bile acid and bile acid glucuronide products was evaporated and methyl ester–acetates were prepared as described previously (20). The resulting derivatives were separated by HPLC (see below).

High performance liquid chromatography

Preparative high performance liquid chromatography was carried out using a single piston mini-pump (LDC/Milton Roy Co., Riviera Beach, FL) and a refractive index detector Model 771 (Micromeritics, Norcross, GA). Methyl ester-acetates of bile acid glucuronides were separated isocratically on a 600-RP C_{18} reversed phase column (6.4 × 150 mm, Alltech, Deerfield, IL) with methanol–water (80:20) as the eluent at a flow rate of 1.13 ml/min.

NMR spectrometry

Fourier transformed ^{1}H-NMR spectra were measured at 300 MHz in $CDCl_3$ using a General Electric QE-300 instrument. Chemical shifts are reported relative to the

$CHCl_3$ signal which was set to 7.260. Spectra were taken at ambient temperature (approximately 25°C). The pulse width was experimentally set before each series of measurements to yield a flip angle of 90°; for the spectra shown, it was 7 μsec. The acquisition time was 6.06 sec, and the acquisition data size was 32786 points for a sweep width of 2659 Hz (approx. from 9 to 0 ppm). Typically, 256 scans were accumulated. Exponential multiplication apodization (with a line broadening of 0.2 Hz) was applied to the data before Fourier transformation.

RESULTS

Substrate specificity

The specific activities of rat liver microsomes for glucuronidation of various bile acid substrates are shown in **Fig. 1.** The reaction rates with the common bile acids, all of which lack a hydroxyl group in position 6 (see panel B) are similar to literature values. Even though some differences in absolute activities exist between data from different laboratories (22, 24–26), they can be explained by variations in pretreatment of animals, isolation methods for microsomes, or assay conditions, and do not change the pattern of specificity, which usually is lithocholate ≥ deoxycholate > ursodeoxycholate > chenodeoxycholate > cholate. We have observed the same order (Fig. 1B). The glucuronidation activity of rat microsomes toward 6-hydroxylated bile acids has not, to our knowledge, been previously measured. Our results are shown in Fig. 1A.

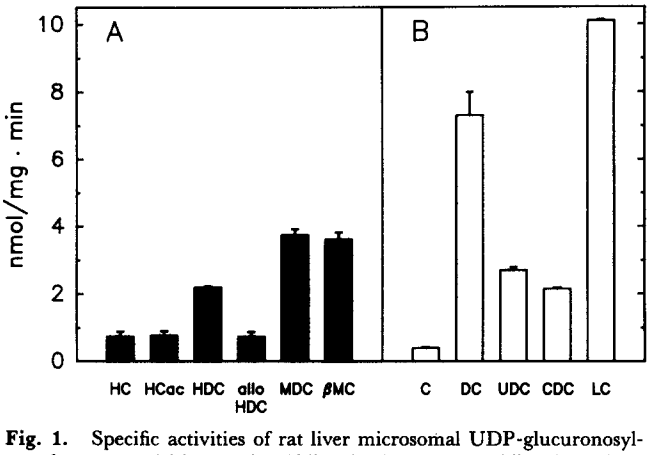

Fig. 1. Specific activities of rat liver microsomal UDP-glucuronosyltransferase toward 6-hydroxylated bile acids (panel A) and bile acids lacking a hydroxyl group in position 6 (panel B). The values plotted are means ± SD (n = 5–8, with "n" denoting the number of separate experiments, each done in triplicate). The products shown in panel B (open bars) are 3-O-glucuronides; the closed bars in panel A represent total activities, i.e., the sums of all positional isomers of glucuronide formed. All reactions were carried out at 100 μM of the bile acid substrate. Abbreviations: HC, hyocholic acid; HCac, hyocholic acid 6,7-acetonide; MDC, murideoxycholic acid; βMC, β-muricholic acid; C, cholic acid; DC, deoxycholic acid; UDC, ursodeoxycholic acid; CDC, chenodeoxycholic acid; LC, lithocholic acid.

The two bile acids possessing a 6β-hydroxyl group, murideoxycholate and β-muricholate, were better substrates than the 6α-hydroxylated bile acids hyodeoxycholate and hyocholate. A change of configuration at C-5, from hyodeoxycholate to allohyodeoxycholate, resulted in a significant reduction of the glucuronidation rate, while blocking of 6-OH and 7-OH in the acetonide of hyocholic acid did not cause a significant change in activity.

Product identification

A preliminary characterization of enzymatic reaction products was carried out by TLC. A group separation of bile acid glucuronides into hydroxyl-linked and carboxyl-linked (acyl-) species is possible in an alkaline solvent system (20). This method was used in the present study, with a minor modification of the solvent to obtain a better separation of glucuronides of di- and trihydroxylated bile acids. The substrates shown in Fig. 1B gave rise to hydroxyl-linked glucuronides only. In the case of lithocholic acid, we have previously identified the product of the enzymatic reaction obtained under conditions identical to those used in this study as the 3-O-β-glucuronide by rigorous spectral techniques (20). Except for the glucuronide of cholic acid, of which insufficient amounts were available, the glucuronides of the other bile acids listed in Fig. 1B have been assigned the same structure on the basis of their NMR spectra (**Table 1**). This is in agreement with the structure of the human metabolites of the same bile acids (15, 19, 27).

The composition of reaction products obtained with 6-hydroxylated bile acids (Fig. 1A) was more complex. A group separation by TLC revealed that all substrates gave rise to both hydroxyl- and carboxyl-linked glucuronides. The separation of glucuronides of hyodeoxycholic acid is shown in **Fig. 2** as an example. The proportions of the two metabolites varied depending on the substrate and are listed in **Table 2**. The assigned structures were verified by NMR (see below).

Typically, the glucuronic acid moiety is attached to the 3-OH of bile acids (15, 19, 27). Almé and Sjövall (15) have found, however, that the glucuronide of hyodeoxycholic acid isolated from human urine is the 6-O-glucuronide. The later assignment of the 3-O-glucuronide structure to a human biliary and urinary metabolite of the same bile acid (28) was probably incorrect (16), and we established recently that the 6-O-glucuronide is the only in vitro reaction product obtained with human liver microsomes from various 6-hydroxylated bile acids (16). Because hyodeoxycholic acid was extensively studied in the systems mentioned above, we have chosen it to determine which positional isomer of the hydroxyl-linked glucuronide is formed by rat liver microsomes. A preparative enzymatic synthesis was carried out and a fraction containing the glucuronides as well as the unreacted substrate was obtained by solid phase extraction. This fraction, after methylation and acetylation,

TABLE 1. 300 MHz ^{1}H-NMR spectra of methyl/peracetyl derivatives of bile acid glucuronides

Proton	Glucuronide of: Deoxycholic Acid	Ursodeoxycholic Acid	Chenodeoxycholic Acid
H-1′	4.63d (J = 7.9)	4.64d (J = 7.6)	4.62d (J = 7.8)
H-2′	4.96t (J = 8.4)	4.95t (J = 8.4)	4.97t (J = 8.5)
H-3′, H-4′	5.17-5.29m	5.21-5.26m	5.19-5.26m
H-5′	4.02d (J = 9.1)	4.01d (J = 9.7)	4.01d (J = 9.7)
H-3	3.57br (H-3β)	3.56br (H-3β)	3.44br (H-3β)
H-7		4.75m (H-7α)	4.84dd ($J_{7\beta6\beta}$ = $J_{7\beta8\beta}$ = 2.6; $J_{7\beta6\alpha}$ = 0.7) (H-7β)
H-12	5.05bs (H-12β)		
Me-18	0.71s	0.69s	0.64s
Me-19	0.88s	0.95s	0.90s
Me-21	0.80d (J = 6.1)	0.91d (J = 5.8)	0.92d (J = 7.9)
MeO	3.66s(one) 3.75s(one)	3.66s(one) 3.77s(one)	3.66s(one) 3.74s(one)
AcO	2.02s(three) 2.07s(one)	1.97s(one) 2.02s(two) 2.05s(one)	2.02s(two) 2.05s(two)

The glucuronides were obtained from preparative incubations of the bile acids with rat liver microsomes as the source of UDP-glucuronosyltransferase. Protons of the sugar moiety carry a "prime" index, whereas steroidal protons are not indexed.

was further separated by HPLC. Four peaks, two of them poorly resolved, were obtained (**Fig. 3**). Material eluting in the region of peaks A and B was combined and rechromatographed in a more polar solvent system (CH_3OH-H_2O 75:25 (v/v) rather than 80:20 used previously). A peak broadening but no better separation was the result (not shown). It was, however, possible to take cuts from the ascending and descending parts of the peak that contained material of sufficient purity for structure determination. The NMR spectrum of fraction D (not shown) unequivocally identified it as the methyl/acetyl derivative of hyodeoxycholic acid. This fraction, constituting the excess of unreacted substrate from the enzymatic incubation, was not further investigated. The NMR spectra of peaks A, B, and C are shown in **Fig. 4**. The interpretation of the spectra was similar to that discussed previously (16, 20, 29) and was straightforward. On the basis of the characteristic signals of the sugar moiety, the number of methoxyl groups, as well as the downfield acyl shift of protons H-3β and H-6β (broken arrows in Fig. 4), fraction A was identified as the carboxyl-linked glucuronide of hyodeoxycholic acid, thus validating the conclusion drawn from the TLC group separation. Fraction B is a hydroxyl-linked glucuronide as evidenced by the resonances of the sugar protons and the presence of two methoxyl groups. In this compound, proton H-3β shows no downfield shift, whereas H-6β is shifted from 4.0 ppm to 5.1 ppm, indicating acetylation of 6α-OH. This proves that the glucuronyl moiety is attached in position 3. In fraction C, also a hydroxyl-linked glucuronide, the situation is reversed: H-3β but not H-6β is shifted downfield. This identifies fraction C as the 6α-O-β-D-glucuronide of hyodeoxycholic acid. The ratio of radioactivity in HPLC peaks A + B and C was measured by liquid scintillation counting, while the ratio of hydroxyl- to carboxyl-linked glucuronides (corresponding to B + C and A, respectively) was obtained by quantitative scanning of the radiochromatogram (TLC plate, Fig. 2). The contri-

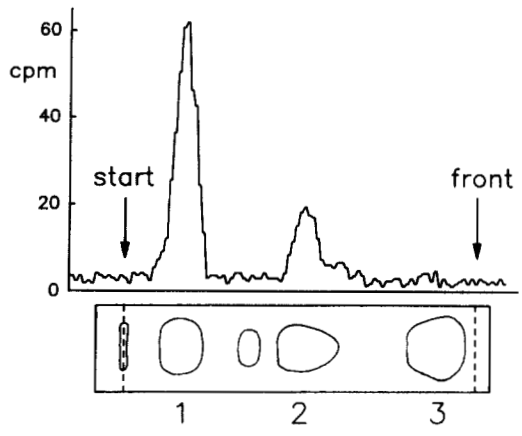

Fig. 2. Radiochromatogram of glucuronides of hyodeoxycholic acid formed in vitro by rat liver microsomes. Incubation conditions (with unlabeled bile acid and UDP-[^{14}C]glucuronic acid) were as described in the Methods section. The products (and unreacted bile acid) were isolated by solid phase extraction and applied to a silica gel TLC plate. The plate was developed in ethanol-ethyl acetate-conc. NH_3 40:40:20 (v/v/v) and radioactivity was localized using a Berthold linear analyzer. The spots were subsequently visualized using Krowicki's reagent (38). Spot 1, hydroxyl-linked glucuronides (sum of 3-O- and 6-O-glucuronide); spot 2, carboxyl-linked glucuronide; spot 3, unreacted hyodeoxycholic acid.

TABLE 2. Distribution of hydroxyl- and carboxyl-linked glucuronides formed in incubation of rat liver microsomes with various 6-hydroxylated bile acids

Substrate	Type of Glucuronide	
	Hydroxyl-Linked	Carboxyl-Linked
	%	
Hyocholic acid	36 ± 10	64 ± 10
Hyocholic acid 6,7-acetonide	37 ± 3	63 ± 3
Hyodeoxycholic acid	68 ± 5[a]	32 ± 5
Allohyodeoxycholic acid	26 ± 9	74 ± 9
Murideoxycholic acid	60 ± 16	40 ± 16
β-Muricholic acid	56 ± 11	44 ± 11

Group separation of glucuronides was carried out by TLC as described (20) except that the solvent system was ethanol–ethyl acetate–conc. NH_3 40:40:20 (v/v/v). The further separation of subclasses of hydroxyl-linked glucuronides of hyodeoxycholate was achieved by HPLC (see text). Means ± SD (n = 4–12) are listed.
[a] 3-O-Glucuronide: 16%, 6-O-glucuronide: 52% of total.

bution of each positional isomer of glucuronide can be calculated from the above data. The 6-O-glucuronide was the predominant metabolite of hyodeoxycholic acid; compared to it, there was only one-third as much 3-O-glucuronide and half as much carboxyl-linked glucuronide (Table 2).

DISCUSSION

All bile acids that have been tested in the course of the present investigation gave rise to glucuronides when incubated with UDP-glucuronic acid and rat liver microsomes; for those substrates that have been studied previously, the rates of glucuronidation found by us agreed well with literature values. Nevertheless, the bile acids can be sharply divided into two classes according to their behavior in the glucuronidation reaction: the first class consists of those substrates that possess a hydroxyl group in position 6, the second of those that lack such group. The latter class of bile acids gives rise exclusively to 3-O-glucuronides, as shown by proton NMR spectra of their methyl/peracetyl derivatives. This is not unexpected since this structure has been assigned previously to glucuronides of many different bile acids, especially those isolated from human urine, plasma, and bile (15, 19, 27), even though a direct and rigorous structure determination of the rat metabolites has not been previously reported.

Bile acids carrying a hydroxyl group in position 6 are common in rodents. In rat bile, the major compound of this class appears to be β-muricholate. Its contribution, 10–20% of the total bile acid pool (1–3), makes it second only to cholate and, according to some sources, chenodeoxycholate. In contrast to other species, especially the pig, in which 6α hydroxylation predominates, the 6-hydroxy bile acids of the rat are of the 6β configuration. Their presence is due to an active hepatic microsomal 6β-hydroxylase that is capable of converting lithocholate and chenodeoxycholate to murideoxycholate and β-muricholate, respectively (4, 5, 7–11). In the group of 6-hydroxylated compounds, the two latter bile acids were the best substrates for the rat microsomal UDP-glucuronosyltransferase; the reaction rate was higher than with any of the 6α-hydroxylated bile acids. However, all 6-hydroxy bile acids shared a characteristic that set them apart from compounds lacking this functional group: they gave rise to more than one positional isomer of glucuronide, namely the two hydroxyl-linked glucuronides identified in the case of hyodeoxycholic acid as 3-O-glucuronide and 6-O-glucuronide, and the carboxyl-linked (acyl) glucuronide. Whereas the 6-O-glucuronide of hyodeoxycholic acid has been previously isolated from human urine (15), and 6-O-glucuronides have been found to be the sole in vitro reaction products of various 6-hydroxylated bile acids with human liver microsomes (16), the carboxyl-linked glucuronides of 6-hydroxy bile acids constitute a new class of metabolites of C_{24} bile acids. This type of conjugate has been recently identified, both in vivo and in vitro (20, 29), as a metabolite of short-chain bile acids, a heterogeneous group of acidic steroids (30) that are quantitatively minor in the adult organism but abundant in meconium (31, 32). Short-chain bile acids differ from each other in their metabolic origin. While some (the C_{23} and C_{22} compounds) are probably derived from C_{24} bile acids and/or from cholesterol, the C_{21} and C_{20} compounds are catabolites of steroid hormones (33, 34). Even though the biological significance of short-chain bile acids remains largely unknown, their minute concentrations in adults appear to preclude a physiological function similar to that of the major C_{24} bile acids. This functional as well as metabolic separation of short-chain bile acids might have led to the conclusion that the

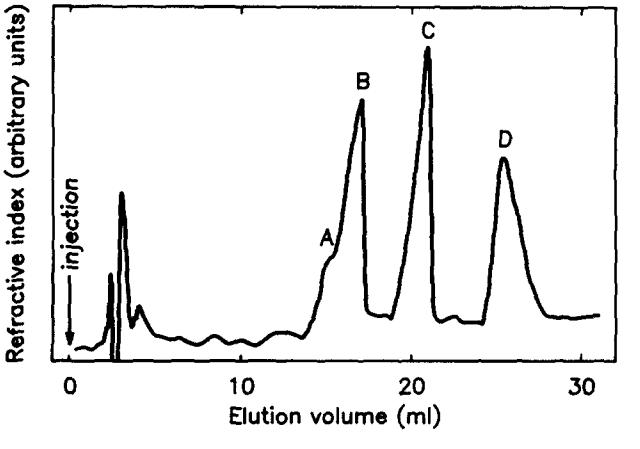

Fig. 3. Separation of methyl/peracetyl derivatives of hyodeoxycholic acid glucuronides by HPLC. A reversed phase column with methanol–water 80:20 (v/v) was used; see Methods for further details. A: carboxyl-linked glucuronide; B: 3-O-glucuronide; C: 6-O-glucuronide; D: hyodeoxycholic acid (all as methyl/peracetyl derivatives). Structure assignment was by ^{1}H-NMR (see text).

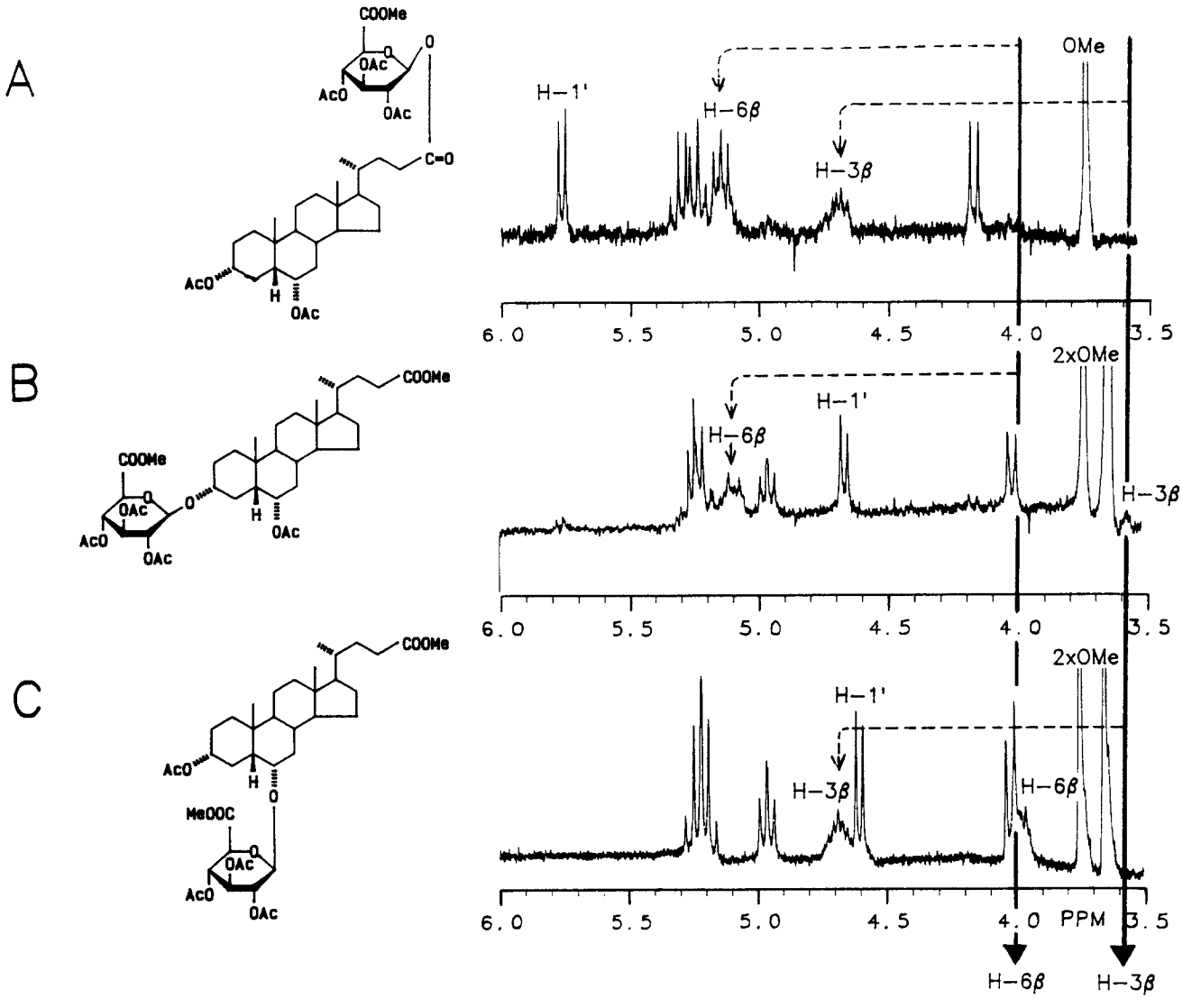

Fig. 4. 300 MHz ^{1}H-NMR spectra and formulas of methyl/peracetyl derivatives of the three positional isomers of hyodeoxycholic acid glucuronide. A: carboxyl-linked glucuronide; B: 3-O-glucuronide; C: 6-O-glucuronide. The small amounts of A and B that were available (only a small fraction of the partially separated peaks could be used, see text) are reflected in the quality of their spectra. The vertical lines denote the chemical shifts of protons H-3β and H-6β in methyl hyodeoxycholate, i.e., in the parent bile acid in which groups 3α-OH and 6α-OH are free (spectrum not shown); these chemical shifts do not change significantly upon glucuronidation of 3α-OH and 6α-OH. Acetylation of these hydroxyl groups leads to a downfield shift of the vicinal protons H-3β and H-6β that is shown by arrows where applicable.

formation of carboxyl-linked glucuronides, while important for short-chain bile acids, is insignificant or absent for the predominant C_{24} bile acids. The demonstration of the formation of carboxyl-linked glucuronides of major and physiologically important bile acids necessitates a revision of this point of view, even though the metabolic significance of the process, especially if compared to the hydroxyl-directed glucuronidation, remains unclear.

A comparison of the glucuronidation of bile acids by rat (this communication) and by human (16) liver microsomes is interesting from the enzymological point of view. In both species, bile acids lacking a hydroxyl group in position 6 give rise to 3-O-glucuronides only, while the presence of 6-OH leads to a change in the type of biotransformation. In the human it is a complete redirection of glucuronidation to the 6-OH, while in the rat 6-O-glucuronidation, residual 3-O-glucuronidation, and the formation of the acyl glucuronide coexist. Mechanistically, it therefore appears that the region of the bile acid molecule that encompasses carbon C-6 constitutes a recognition site for UDP-glucuronosyltransferases and is involved in determining which substrate is recognized by which isoenzyme. Chemical modifications of this part of the molecule, including the synthesis of photoaffinity labels bearing a reactive group at C-6, might therefore become useful in distinguishing between isoenzymes of bile acid-specific UDP-glucuronosyltransferases, as well as help in the elucidation of the structure of the bile acid binding site.

It should be pointed out that, in rat liver microsomes, 6β-hydroxylated bile acids were superior substrates for glucuronidation as compared to 6α-hydroxylated compounds (this study), while the situation was reversed in human liver microsomes (16). This parallels the presence of 6β-hydroxylases in the rat and 6α-hydroxylases in the human liver (14). This correlation is consistent with the notion that P-450-dependent hydroxylases and UDP-glucuronosyltransferases for a given substrate may be, at least functionally, tightly coupled (35).

The physiological significance of glucuronidation of bile acids in positions different from the 3α-hydroxyl group is at present not clear. A possible advantage for the organism could lie in the fact that a 6β-hydroxylation of lithocholic acid and glucuronidation of the resulting murideoxycholic acid, either on the 6β-hydroxyl or on the carboxyl group, would tend to reduce the formation of lithocholate 3-O-glucuronide; the latter is strongly cholestatic and, in fact, it appears to more toxic in the rat than the parent bile acid (36). Whether the process has a functional significance beyond the diversion of the glucuronidation reaction from the 3α-hydroxyl group remains to be elucidated. There are some indications that this might be the case. The glucuronide of hyodeoxycholic acid is very efficiently cleared by the kidneys, at least in humans (18). Thus, the 6-O-glucuronide moiety could serve as a tag that commits the bile acid molecule to rapid excretion. A confirmation of this possibility, and the elucidation of a possible role of the acyl-linked glucuronyl moiety, will require a comparative study of the metabolic and physiologic behavior of the three positional glucuronide isomers.

In summary, we have shown that the presence of a hydroxyl group in position 6 of a bile acid molecule causes a redirection of the rat liver microsomal UDP-glucuronosyltransferase activity: in addition to the expected 3-O-glucuronide, two other positional isomers of glucuronide are formed. One is the 6-O-glucuronide, a product that is also synthesized in the human liver, and the other is the acyl glucuronide. The latter metabolite has not been previously described for C_{24} bile acids.

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