Positions of conjugation of bile acids with glucose and *N*-acetylglucosamine in vitro[1]

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Abstract In order to establish the position of conjugation of bile acids with glucose or *N*-acetylglucosamine, glucosides of chenodeoxycholic and hyodeoxycholic acids and of ^{13}C-labeled cholic, lithocholic, chenodeoxycholic, hyodeoxycholic, and ursodeoxycholic acids, and *N*-acetylglucosaminides of ursodeoxycholic, isoursodeoxycholic, 3-dehydro-ursodeoxycholic, and ursodeoxycholylglycine were synthesized in vitro. The conjugates were purified by anion-exchange chromatography and reversed-phase HLPC and were analyzed by gas chromatography–mass spectrometry. The glucosides of chenodeoxycholic and hyodeoxycholic acids were also analyzed after periodate and chronic acid oxidation. All conjugates were analyzed by fast atom bombardment mass spectrometry with collision-induced dissociation. Glucose conjugation was shown to occur at C-3 in all bile acid glucosides studied. In contrast, the selective *N*-acetylglucosaminidation of 7β-hydroxy bile acids was shown to occur at the 7β-position.—**Marschall, H-U., W. J. Griffiths, J. Zhang, H. Wietholtz, H. Matern, S. Matern, and J. Sjövall.** Positions of conjugation of bile acids with glucose and *N*-acetylglucosamine in vitro. *J. Lipid Res.* 1994. **35:** 1599–1610.

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Bile acid glucosides, glucuronides, and *N*-acetylglucosaminides are excreted in similar amounts, about 0.3 μmol/24 h, in urine of healthy subjects (1). Bile acids carrying a 6α-hydroxy group, e.g., hyodeoxycholic acid, are glucuronidated at C-6 both in vivo (2) and in vitro (3–5) in contrast to bile acids lacking a hydroxyl group at this position, e.g., chenodeoxycholic acid, which are glucuronidated at C-3 (2). *N*-Acetylglucosaminidation seems to be an even more selective glycosidic conjugation reaction as only 7β-hydroxylated bile acids were found to be conjugated with *N*-acetylglucosamine in vivo (6) and in vitro (7). However, the position of conjugation has not been established. Similarly, the positional selectivity of sugar nucleotide-independent bile acid glucosidation has not been studied (8–10). The aim of this study was to establish the position of the sugar moiety in bile acid glucosides and *N*-acetylglucosaminides synthesized in vitro.

METHODS

Materials

The sources and the purity of most materials are described elsewhere (11–13). [24-^{13}C]cholic (CA) and [24-^{13}C]lithocholic (LCA) acids with a ^{13}C excess of 90% were obtained from MSD isotopes (Montreal, Canada); ^{13}C-labeled chenodeoxycholic (CDCA), hyodeoxycholic (HDCA), and ursodeoxycholic (UDCA) acids with a ^{13}C excess of 90–91% were synthesized as described (14).

Synthesis of 7β-hydroxy-3-oxo-5β-cholan-24-oic acid (3-dehydro-UDCA)

One mg of UDCA was oxidized for 2 h at 37°C with 0.3 units of 3α-hydroxysteroid dehydrogenase (dissolved in 1 ml 10 mM KH_2PO_4, pH 7.2) in a total volume of 30 ml containing 20 mM $Na_2H_2P_2O_7$, pH 9.5, and 40 mM β-NAD. The product was extracted with Sep-Pak C_{18} (Waters Associates, Milford, MA) and purified by anion-exchange chromatography on Lipidex-DEAP (Packard-Becker, Groningen, The Netherlands), from which it was eluted with 0.1 M acetic acid in 70% ethanol, and by reversed-phase HPLC (11). Using a gradient from 50–100% methanol containing 1% acetic acid, the 3-dehydro derivative of UDCA eluted at 36–40 min.

Abbreviations: FAB, fast atom bombardment; MS, mass spectrometry; CID, collision-induced dissociation; GC, gas chromatography; GLC, gas–liquid chromatography; HPLC, high performance liquid chromatography; *m/z*, mass/charge ratio; RI, retention index; TMS, trimethylsilyl; UDP, uridine diphosphate; gly, glycine; tau, taurine; C, cholyl; CA, cholic acid, 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid; CDCA, chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholan-24-oic acid; HDCA, hyodeoxycholic acid, 3α,6α-dihydroxy-5β-cholan-24-oic acid; LCA, lithocholic acid, 3α-hydroxy-5β-cholan-24-oic acid; UDC, ursodeoxycholyl; UDCA, ursodeoxycholic acid, 3α,7β-dihydroxy-5β-cholan-24-oic acid; isoUDCA, isoursodeoxycholic acid, 3β,7β-dihydroxy-5β-cholan-24-oic acid; 3-dehydro-UDCA, 7β-hydroxy-3-oxo-5β-cholan-24-oic acid; Glc, glucose; GlcNAc, *N*-acetylglucosamine; (24).

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Synthesis and derivatization of bile acid glucosides

Glucosides of unlabeled CDCA and HDCA were synthesized in microgram amounts as described (8). Briefly, 0.1 mM of CDCA and HDCA, respectively, were incubated for 5 h at 37°C with 10 mg of human liver microsomes in 31 ml of a reaction mixture containing 0.1 M sodium acetate buffer, pH 5.0, 15 mM $MgCl_2$ and 2 mM octyl-β-D-glucopyranoside. The glucosides were separated from unreacted bile acids by extraction of the latter with chloroform. They were purified by anion-exchange chromatography on Lipidex-DEAP, from which they were eluted with 0.1 M acetic acid in 70% ethanol, and by reversed-phase HPLC (11). The glucosides of CDCA and HDCA eluted at 51–55 min. Additionally, glucosides of ^{13}C-labeled CA, CDCA, HDCA, LCA and UDCA were synthesized as described (8). These conjugates were only purified by anion-exchange chromatography. The presence of ^{13}C in the side chain helped to confirm the origin of fragment ions in the mass spectrometric analyses.

Derivatization of bile acid glucosides for gas–liquid chromatography (GLC) and gas chromatography–mass spectrometry (GC/MS)

Aliquots of all bile acid glucosides were methylated with diazomethane and converted to the trimethylsilyl (TMS) ether derivative (4). In order to determine the site of conjugation, formyloxy and formyloxy-oxo derivatives were prepared from the unlabeled glucosides of CDCA and HDCA (2, 4). About 20 μg of the purified conjugates was incubated with 20 mg sodium periodate in 0.7 ml water for 1 h at 37°C. The solution was extracted with Sep-Pak C_{18} and the products were purified on Lipidex-DEAP (4). The material in the acetic acid fraction was extracted with Sep-Pak C_{18} and methylated with diazomethane. Aliquots of the periodate cleavage product were either oxidized with 10 μl of Jones' reagent in 1 ml acetone at 0°C or converted to the TMS ether derivative.

Derivatization of bile acid glucosides for negative-ion fast atom bombardment mass spectrometry (FABMS)

Negative-ion FAB spectra of bile acids show prominent pseudomolecular anions. Taurine-conjugated bile acids give more intense spectra than other natural bile acids. FAB/CID (collision-induced dissociation) spectra of their $[M-H]^-$ pseudomolecular ions show charge-remote fragmentation patterns which are of value in structural studies (12). A simple method for the derivatization of bile acids with taurine has recently been developed (13) and was applied to the bile acid glucosides and *N*-acetylglucosaminides in the present study. The bile acids listed in the tables were derivatized by reacting about 1 μg of bile acid with excess taurine and EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride) in a buffer of pyridine hydrochloride, pH 5, in water for 2 h. The taurine conjugates were separated from excess reagents on a Sep-Pak C_{18} bed (13).

Synthesis of *N*-acetylglucosaminides of UDCA, isoUDCA, 3-dehydro-UDCA and UDC-gly

N-Acetylglucosaminides of UDCA, isoUDCA, 3-dehydro-UDCA, and UDC-gly were synthesized as described (6, 7) with the following modifications: UDCA, isoUDCA, 3-dehydro-UDCA, and UDC-gly, respectively, each 0.1 mM, were reacted for 1 h at 37°C in 18 ml of a mixture containing 3 mM UDP-*N*-acetyl-D-glucosamine, 0.1 M sodium acetate, pH 6.2, 2 mM $MgCl_2$, 0.002% Brij 58, 0.05 mM ADP, and about 4 mg of human kidney or liver microsomes. Unreacted bile acids were extracted with chloroform and the *N*-acetylglucosaminides were purified by anion-exchange chromatography and reversed-phase HPLC as above. The *N*-acetylglucosaminides of UDCA and isoUDCA eluted at 35–40 min from the HPLC column, the conjugate of 3-dehydro-UDCA at 25–30 min, and the conjugate of UDC-gly at 15–20 min.

Derivatization of bile acid *N*-acetylglucosaminides for GLC, GC/MS and FABMS

Aliquots of the purified *N*-acetylglucosaminide of 3-dehydro-UDCA were converted to the methyloxime by reacting for 1 h at 60°C with methoxyammonium chloride in 0.1 ml pyridine (4) for FABMS or reduced for 1 h at 45°C with 1 mg sodium borohydride in 1 ml ethanol. The starting material and the products were converted to methyl ester TMS ether derivatives for GLC and GC/MS as were the *N*-acetylglucosaminides of UDCA and isoUDCA (6). For FAB/CID, the *N*-acetylglucosaminides of UDCA, isoUDCA, 3-dehydro-UDCA, and UDC-gly were derivatized with taurine as described above.

Reference bile acid derivatives

3α-Hydroxy-6-oxo-5β-cholanoic, 3α-hydroxy-7-oxo-5β-cholanoic, and 7α-hydroxy-3-oxo-5β-cholanoic acids were obtained from Steraloids (Wilton, NH). Methyl 6α-hydroxy-3-oxo-5β-cholanoate was synthesized as described (4). After methylation of the free acids with diazomethane, 60 μg of each oxo-bile acid was reacted with formic acid to give the formate esters (2, 4).

Gas–liquid chromatography and gas chromatography–mass spectrometry

GLC and GC/MS were performed using the equipment and conditions previously described (11). Column A was a 24 m × 0.32 mm I.D. fused silica capillary coated with cross-linked methyl silicone (film thickness 0.25 μm; Quadrex Corp., New Haven, CN). Column B was a 25 m × 0.32 mm I.D. fused silica capillary coated with a polar silicone gum containing 25% 4-phenoxyphenyl, 2% vinyl, and 73% methyl groups (Unicoat UC-1625, KSV

Chemicals, Helsinki, Finland). All derivatives were analyzed on both columns. For GLC, the samples were injected on column in 1 μl hexane at 60°C. The temperature was then taken to 280°C at 30°C/min. After elution of *n*-hexatriacontane added as standard, the temperature was taken to 300°C at 30°C/min. Retention indices were calculated in relation to the *n*-alkanes C_{30} and C_{36} at 280°C, and C_{40}, C_{44}, and C_{46} at 300°C, respectively. For GC/MS, a falling needle injection system was used and the samples were analyzed isothermally at 280°C and 300°C, respectively.

Negative-ion fast atom bombardment mass spectrometry

Negative-ion FAB spectra were recorded on a VG-AutoSpec-Q mass spectrometer (VG Analytical, Manchester, U.K.) fitted with an FAB source and a cesium ion gun. The ion gun was operated at an anode potential of 25 kV. The instrument was tuned for maximum sensitivity at a resolution of 1000 (5% valley). The spectrometer accelerating potential was 8 kV and ions were detected at the fourth field free region off-axis detector, immediately preceding the quadrupole mass filter. The quadrupole was not used in the present study. Bile acid samples were prepared in 70% aqueous methanol and dissolved in a drop of glycerol that coated the FAB target.

Collision-induced dissociation (CID) spectra were generated using helium as the collision gas in the first field free region gas cell at a pressure which gave a reading of 1.5×10^{-7} Torr on the nearby analyser 1 ion gauge. This was sufficient to cause a 50% reduction in parent $[M-H]^-$ ion beam intensity. Daughter ion linked scans (B/E is constant) were recorded on the $[M-H]^-$ ions. The scan range was 10–1000 Da, of duration 2 s/decade. Between 10 and 500 scans were collected in the continuous mode and averaged.

RESULTS

Position of the glucose moiety of the glucosides of unlabeled CDCA and HDCA by gas chromatography–mass spectrometry

The methyl ester TMS ether derivatives of the glucosides of CDCA and HDCA eluted at RI 4354 and 4238, respectively, from column A, and at RI 4395 and 4280 from column B, respectively. GC/MS analysis showed typical fragment ions of the sugar moiety at *m/z* 204 and 217, and at *m/z* 371 and 461 from the bile acid moiety (1, 9).

After periodate oxidation and conversion into methyl ester TMS ether derivatives, two products were formed from the glucoside of CDCA: the derivative of CDCA (RI 3195 on column A and 3360 on column B), identified by comparison with the derivative of the authentic compound, and the derivative of 3α-formyloxy-7α-hydroxy-5β-cholanoate (RI 3250 on column A and 3642 on column B). The latter was tentatively identified from the molecular ion at *m/z* 506 and the fragment ions at *m/z* 370 (base peak), 339, 255, and 213. Chromic acid oxidation of the product formed by periodate oxidation and methylation yielded a compound that eluted at RI 3404 on column A and at 4038 on column B (**Fig. 1**). It was identified as methyl 3α-formyloxy-7-oxo-5β-cholanoate by comparison with the reference compound. There was no peak of methyl 7α-formyloxy-3-oxo-5β-cholanoate eluting at RI 3357 on column A and at RI 4130 on column B. Therefore, the glucose moiety must be linked to the C-3 position of CDCA.

Two compounds were also found after periodate oxidation and derivatization of the glucoside of HDCA: the derivative of HDCA (RI 3206 on column A and 3383 on column B), identified by comparison with the derivative of the authentic compound, and the derivative of 3α-formyloxy-6α-hydroxy-5β-cholanoate (RI 3283 on

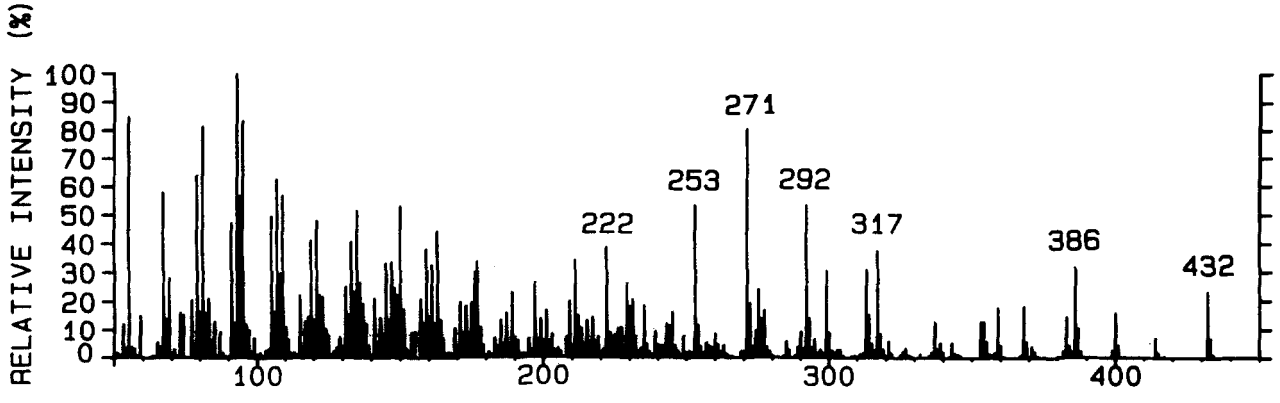

Fig. 1. Mass spectrum of methyl 3α-formyloxy-7-oxo-5β-cholanoate obtained by oxidation of chenodeoxycholic acid glucoside prepared with human liver microsomes.

column A and 3677 on column B), tentatively identified from the molecular ion at *m/z* 506 and the fragment ions at *m/z* 370 (base peak), 345, 323, and 274. Chromic acid oxidation of the product formed by periodate oxidation and methylation yielded a compound that eluted at RI 3436 on column A (**Fig. 2**). This was identified as methyl 3α-formyloxy-6-oxo-5β-cholanoate by comparison with the reference compound. There was also a peak eluting at RI 3372 on column A which was the RI of reference methyl 6α-formyloxy-3-oxo-5β-cholanoate (4). The formation of both formyloxy-oxo derivatives was also observed after periodate oxidation of the 6α-glucuronide of HDCA (4) and was explained as intramolecular rearrangement of the formyl group. However, 6α-formyloxy-3α-hydroxy-5β-cholanoate, which is the periodate oxidation product of HDCA 6α-glucuronide (4), was not found after periodate oxidation of HDCA glucoside. Therefore, the glucoside moiety must be linked to the C-3 position of HDCA.

Position of the glucose moiety of bile acid glucosides by negative-ion FABMS and FAB/CID

The calculated molecular weights of 539 of the glucoside of [24-^{13}C]LCA, of 555 of the glucosides of ^{13}C-labeled CDCA, HDCA, and UDCA, and of 571 of the glucoside of [24-^{13}C]CA were confirmed by FABMS showing pseudomolecular anions at *m/z* 538, 554, and 570, respectively (**Table 1**). The RI of the methyl ester TMS ether derivatives of these conjugates on the apolar column A were 4195 (Glc-[24-^{13}C]LCA), 4354 (Glc-[24-^{13}C]CDCA), 4238 (Glc-[24-^{13}C]HDCA), 4296 (Glc-[24-^{13}C]UDCA), and 4433 (Glc-[24-^{13}C]CA), respectively. The respective RIs of these compounds on the polar column B were 4432, 4395, 4280, 4377, and 4353, respectively.

The negative-ion FAB spectra of the glucosides listed in Table 1 were dominated by pseudomolecular anions. The FAB/CID spectra of these compounds are described in Table 1. The dominant fragmentations involve loss of the sugar from the steroid ring system. There is little cleavage through the steroid ring system and the position of glucose conjugation cannot be determined. Shown in **Fig. 3A** is the FAB/CID spectrum of the glucoside of [24-^{13}C]HDCA. To obtain information as to the position of glucose moiety, FAB/CID spectra were recorded after derivatization with taurine.

The FAB spectra of the derivatized bile acid glucosides (Table 1) are dominated by $[M-H]^-$ pseudomolecular ions, the intensities of which are approximately one order of magnitude greater than that of the underivatized precursors. The negative-ion FAB/CID spectra of the taurine derivatives are much more complex than those of the underivatized bile acid glucosides (cf. Figs. 3A, 3B). Table 1 shows the fragmentation patterns for the derivatized bile acid glucosides studied.

Shown in Fig. 3B is the FAB/CID spectrum of derivatized [24-^{13}C]HDCA glucoside. The most intense fragmentation involves loss of the glucose moiety (Z_2, Z_1, Y, X, X + 14). However, steroid ring and side chain fragmentations also occur (A_0-H). From previous studies in this laboratory (12, 13, 15) it is known that intense ring fragmentations of cholanoyltaurines occur at positions A_3, B_3, C_3, and D_2. A fragment ion is observed at 427 Da that is consistent with fragmentation A_3 of 3-Glc-[24-^{13}C]HDC-tau. Other less intense fragmentations are observed at positions A_2 and A_1 that are consistent with the glucose linkage being at C-3. Fragment ions consistent with cleavages at positions B_3 and D_2 are also observed. If glucose were linked at position C-6, fragmentation A_3 would give an ion at 589 Da. No such ion is

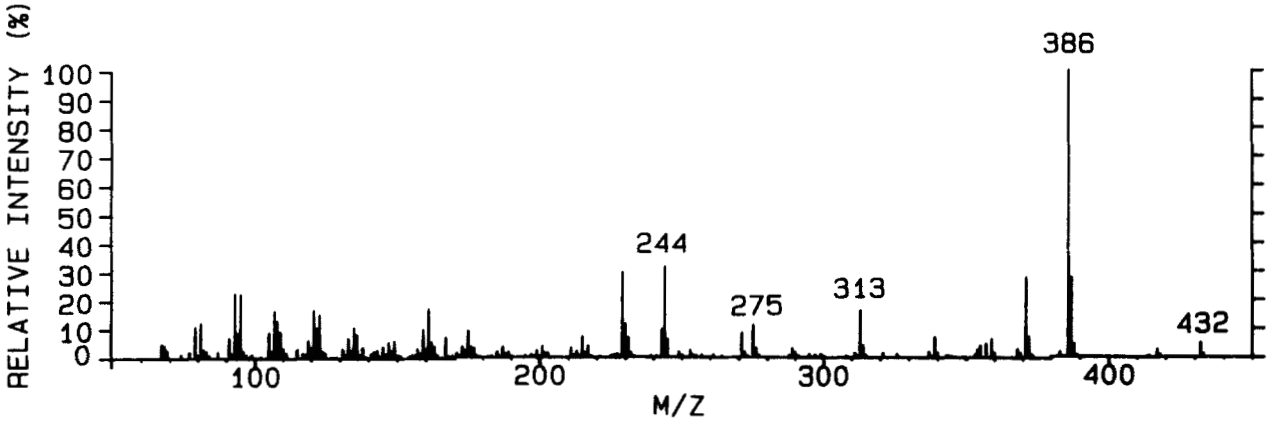

Fig. 2. Mass spectrum of methyl 3α-formyloxy-6-oxo-5β-cholanoate obtained by oxidation of hyodeoxycholic acid glucoside prepared with human liver microsomes.

TABLE 1. FAB/CID fragmentation of 24-^{13}C-labeled bile acid glucosides before and after derivatization with taurine

Fragmentation[b]	Bile Acids [M-H][-a]																	
	LCA 538		LC-tau 645		UDCA[c] 554		UDC-tau 661		CDCA[c] 554		CDC-tau 661		HDCA 554		HDC-tau 661		CA[c] 570		CA-tau 677	
	m/z	%	*m/z*	%	*m/z*	%	*m/z*	%	*m/z*	%	*m/z*	%	*m/z*	%	*m/z*	%	*m/z*	%	*m/z*	%
– 16	522	20	629	40									538	20						
– 18					536	15	643	45	536	15	643	60			643	40	552	20	659	80
– 32			613	5			629	10			629	10								
Z_2	418	5	525	10	434	5	541	5	434	10	541	10	434	10	541	10	450	10	557	15
Z_1	404	5	511	50	420	5	527	40	420	10	527	60	420	10	527	60	436	10	543	65
Z_0	388	5															420	10		
Y[d]	376	100	483	60	392	100	499	45	392	100	499	35	392	100	497	45	408	100	515	50
X	358	30	465	100	374	20	481	100	374	35	481	100	374	20	481	100	390	50	497	100
X + 14/A_0	344	20	451	75	360	15	467	35	360	25	467	60	360	15	467	35	376	40	483	90
A_1			437	5	348	5	453	5			453	5			451	10			467	10
A_2			423	10							439	10			439	10			455	10
A_3			411	25	320	5	427	20			427	15			427	20			443	30
A_3 + 18			395[e]	5			409	5			409	5			409	10			425	15
B_1			369	5							385	10			383	35				
B_2			355	5			371	5			371	5			357	5				
B_3			343	10	236	5	343	10			343	15	236	5	343	20			359	20
C_1			315	5			314	5												
C_3			299	5																
D_1			247	5			247	5												
D_2			235	5			235	5			235	5			235	10				
E			207	25			207	10			207	20			207	46			207	20
F			179	30			179	10			179	15			179	30			179	15
G			166	5											166	5				
H			152	5											152	5				
I			124	5																

[a]The table shows fragment ions from glucosides of 24-^{13}C-labeled bile acids all containing 24-^{13}C. Unlabeled glucosides of HDCA and UDCA gave ions one mass unit lower but otherwise identical fragmentation patterns as the respective labeled compounds. Abbreviations: FAB/CID, fast atom bombardment/collision-induced dissociation; LCA, lithocholic acid; LC, lithocholyl; tau, taurine; UDCA, ursodeoxycholic acid; UDC, ursodeoxycholyl; CDCA, chenodeoxycholic acid; CDC, chenodeoxycholyl; HDCA, hyodeoxycholic acid; HDC, hyodeoxycholyl; CA, cholic acid.

[b]Only fragmentations with a relative abundance of 5% and higher are considered. Percentages are given to the nearest 5%.

[c]Other fragment ions were 117 (– 437, 5%) in the case of UDCA and CDCA, and 541 (– 29, 10%), 291 (– 279, 10%), 197 (– 373, 10%), and 123 (– 447, 45%) in the case of CA, respectively.

[d]Fragmentation Y involves loss of the sugar moiety. The resultant ion may be saturated or monounsaturated, and a doublet is observed.

[e]A_3 + 16.

observed (Fig. 3B). Further fragmentations E, F, G, and H correspond to cleavages of the side chain. The fragmentations Z_2–H, as shown in Table 1, are all of the charge-remote type, i.e., occur at positions remote from the charged taurine group.

The FAB/CID spectra of all the derivatized bile acid glucosides shown in Table 1 confirm that the position of glucose conjugation is at C-3 in each case.

Position of the *N*-acetylglucosamine moiety of the *N*-acetylglucosaminides of UDCA, isoUDCA, and 3-dehydro-UDCA

The calculated molecular weights of 595 of the *N*-acetylglucosaminides of UDCA and isoUDCA (GlcNAc-UDCA and GlcNAc-isoUDCA) were confirmed by FABMS (6) showing a pseudomolecular ion at *m/z* 594. The RI of the methyl ester TMS ether derivatives of these conjugates on the apolar column A were 4411 in the case of GlcNAc-UDCA and 4436 in the case of GlcNAc-isoUDCA. The respective RIs of these compounds on the polar column B were 4708 and 4665.

The enzymatically prepared 3-dehydro-UDCA was obtained in a total yield of 88.3%. Unreacted UDCA (9.9%), and isoUDCA (1.8%), formed as a side product, eluted approximately 30 min later in the reversed-phase HPLC. After HPLC, the purity was estimated by GLC to be 98.3%. The RI of the methyl ester TMS ether derivative was 3314 on column A at 280°C and 3848 on column B at 300°C. The molecular weight of 593 of the *N*-acetylglucosaminide of 7β-hydroxy-3-oxo-5β-cholanoic acid synthesized in vitro was confirmed by FABMS showing a pseudomolecular anion at *m/z* 592. After treatment with methoxyammonium chloride the ion shifted to *m/z* 621 confirming the free oxo group of 7β-*N*-acetylglucosaminido-3-oxo-5β-cholanoic acid and thus the position of the *N*-acetylglucosamine moiety at C-7. The methyl es-

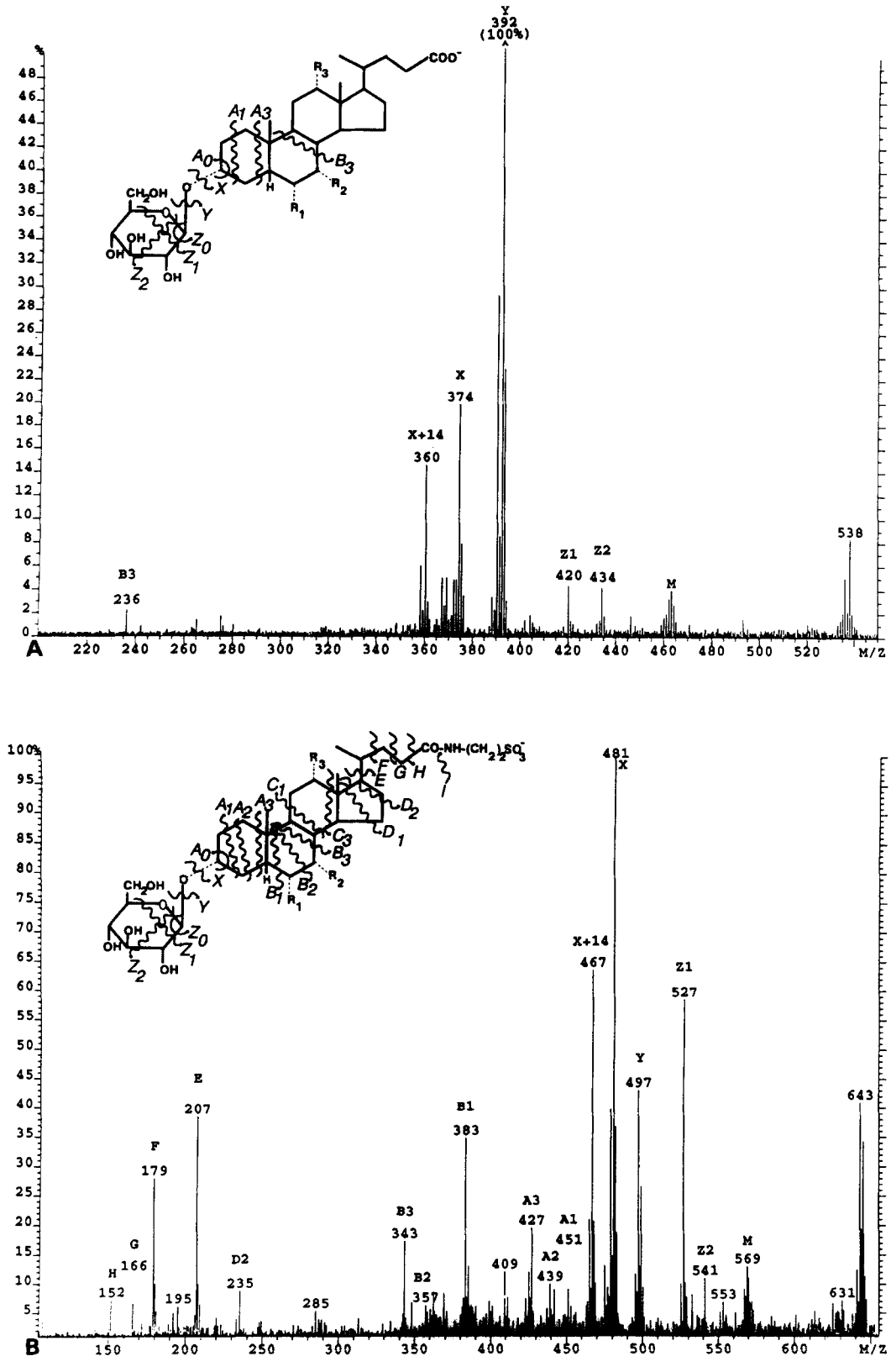

Fig. 3. A: Daughter ions produced by collision-induced dissociation (CID) of the $[M-H]^-$ ion at *m/z* 554 from ^{13}C-labeled hyodeoxycholic acid glucoside (Glc-[24-^{13}C]HDCA) prepared with human liver microsomes. B: Daughter ions produced by CID of the $[M-H]^-$ ion at *m/z* 661 from taurine derivatized Glc-[24-^{13}C]HDCA. R_1 = OH, R_2 = H, R_3 = H.

ter TMS ether derivative eluted at RI 4518 from column A and at an extrapolated RI of 5055 from column B. The differences between the RI of the derivatives of the *N*-acetylglucosaminides of isoUDCA, UDCA, and 3-dehydro-UDCA on column B at 300°C (4665–4708–5055) are approximately the same as the differences between the unconjugated derivatives of isoUDCA, UDCA, and 3-dehydro-UDCA (3405–3449–3848) at 280°C on the same column. This supports the structural relationships between the conjugates.

Sodium borohydride reduction of the 7β-*N*-acetylglucosaminido-3-oxo-5β-cholanoic acid and derivatization yielded a product with an RI of 4413 on column A, i.e., the RI of the derivative of GlcNAc-UDCA, showing that the reduced product was the *N*-acetylglucosaminide of UDCA. Only traces of the derivative of GlcNAc-isoUDCA were found confirming that the 3-oxo group of 7β-*N*-acetylglucosaminido-3-oxo-5β-cholanoic acid was preferentially reduced to a 3α-hydroxy group.

The calculated molecular weight of 652 of the *N*-acetylglucosaminide of UDC-gly was confirmed by FABMS showing a pseudomolecular anion at *m/z* 651. The methyl ester TMS ether derivative of this conjugate could not be analyzed by GLC. The FAB/CID spectra of the *N*-acetylglucosaminides of UDCA, isoUDCA, 3-dehydro-UDCA, and UDC-gly were dominated by fragmentation involving sugar loss. This is shown in **Table 2 and Fig. 4A, Fig. 5A** and **Fig. 6A.** The FAB/CID spectra

TABLE 2. FAB/CID fragmentation of bile acid *N*-acetylglucosaminides before and after derivatization with taurine

Fragmentation[b]	Bile Acids [M-H][-][a]											
	UDCA/isoUDCA 594		UDC-/isoUDC-tau 701		3-Dehydro-UDC 592		3-Dehydro-UDC-tau 699		UDC-gly[c] 651		UDC-gly-tau[d] 758	
	m/z	%	*m/z*	%	*m/z*	%	*m/z*	%	*m/z*	%	*m/z*	%
– 16					576	5	683	50	635	45	742	65
– 18	576	5	683	30								
– 32/A_0			669	5			671	5	619	5	726	10
A_2			641	5			641	5			698	10
A_3			629	10			629	10	579	10	686	10
– 120			581	5			579	5	531	5	638	5
– 133	461	5			459	5						
Z_1	419	5	526	25	417	5	524	30	476	25	583	40
Y[e]	391	100	496	35	389	100	494	45	448	90	553	30
X	373	10	480	100	371	10	478	100	430	100	537	100
X + 14							464	30			523	20
X + 16	357	5	464	20	357	5			414	15		
X + 28			452	5			450	5	402	5	509	5
X + 40							438	5			497	5
X + 42			438	5								
X + 44									386	5		
X + 56			424	5			422	5			481	5
X + 68							410	10				
X + 72			408	5					358	5	465	5
X + 94							384	5				
X + 96			384	5							441	5
X + 108			372	5			370	5	322	5	429	5
B_3			342	15			342	5	292	5	399	10
C_3			288	5			288	5			345	5
D_2			234	10			234	5	184	5	291	5
E	99	5	206	20	99	5	206	25	156	5	263	15
F			178	15			178	15	128	5	235	60
G			165	5			165	5	114	5	222	25
H			151	5			151	5	99	5	208	5
I			123	5			123	5			179	5
J			106	5			106	5			166	5
x[f]					219	5			219	5		
y[f]	202	5			202	5			202	5		

[a] Abbreviations: FAB/CID, fast atom bombardment/collision-induced dissociation; UDCA, ursodeoxycholic acid; UDC, ursodeoxycholyl; tau, taurine; gly, glycine.

[b] Only fragmentations with a relative abundance of 5% and higher are considered. Percentages are given to the nearest 5%.

[c] Other fragment ions were 609 (– 42, 5%), 594 (– 57, 5%), 575 (– 76, 10%), 548 (– 103, 5%), 518 (– 133, 5%), 464 (– 187, 10%), 279 (– 371, 5%), and 179 (– 454, 5%).

[d] Other fragment ions were 716 (– 42, 5%), 682 (– 76, 10%), 625 (– 133, 5%), 453 (– 305, 10%), 150 (– 608, K, 5%), and 123 (– 635, L, 10%).

[e] Fragmentation Y involves loss of the sugar moiety. The resultant ion may be saturated or monounsaturated, and a doublet is observed.

[f] Charge retained on the sugar moiety.

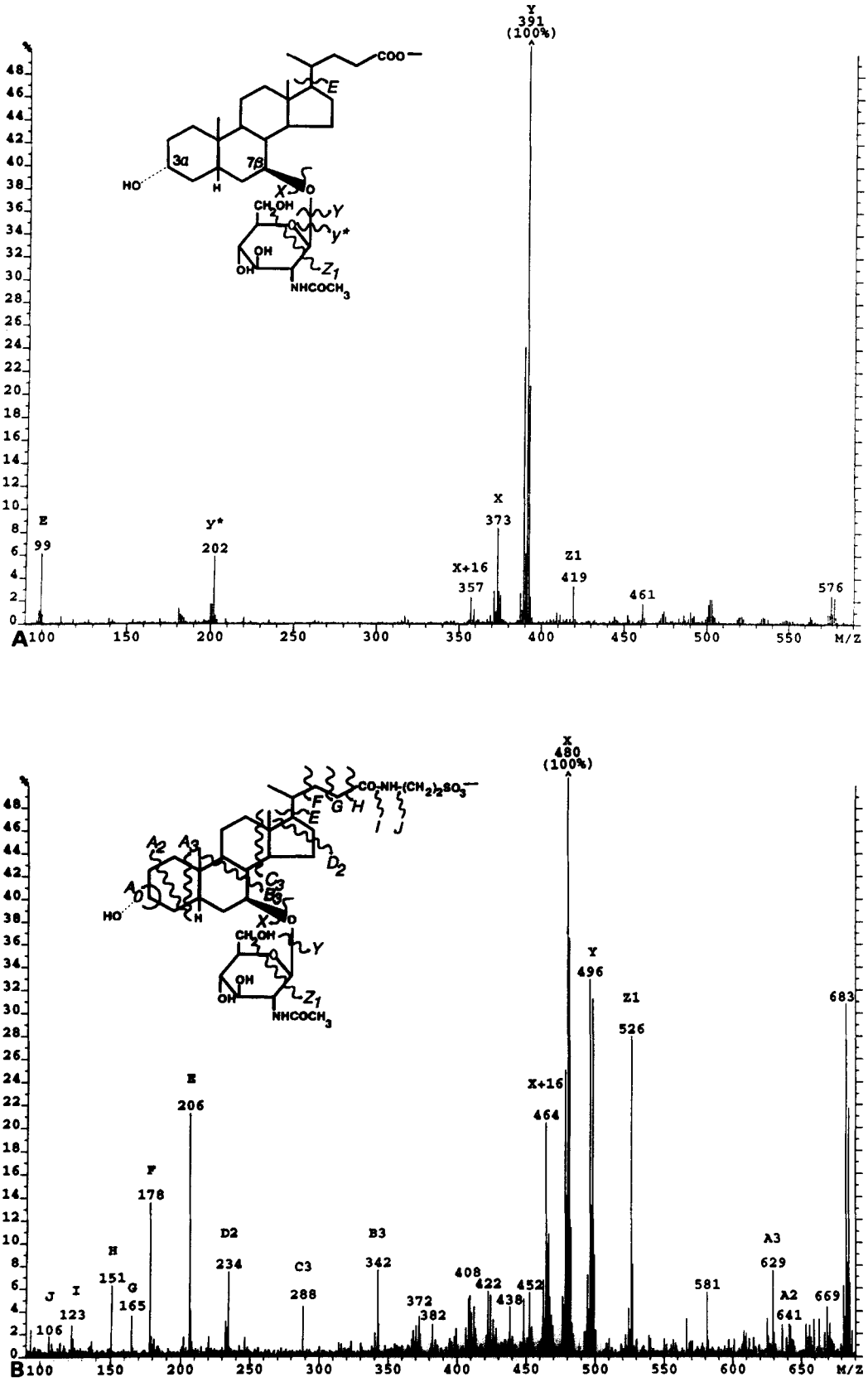

Fig. 4. A: Daughter ions produced by CID of the $[M-H]^-$ ion at *m/z* 594 from ursodeoxycholic acid *N*-acetylglucosaminide (GlcNAc-UDCA) prepared with kidney or liver microsomes. B: Daughter ions produced by CID of the $[M-H]^-$ ion at *m/z* 701 from taurine derivatized GlcNAc-UDCA.

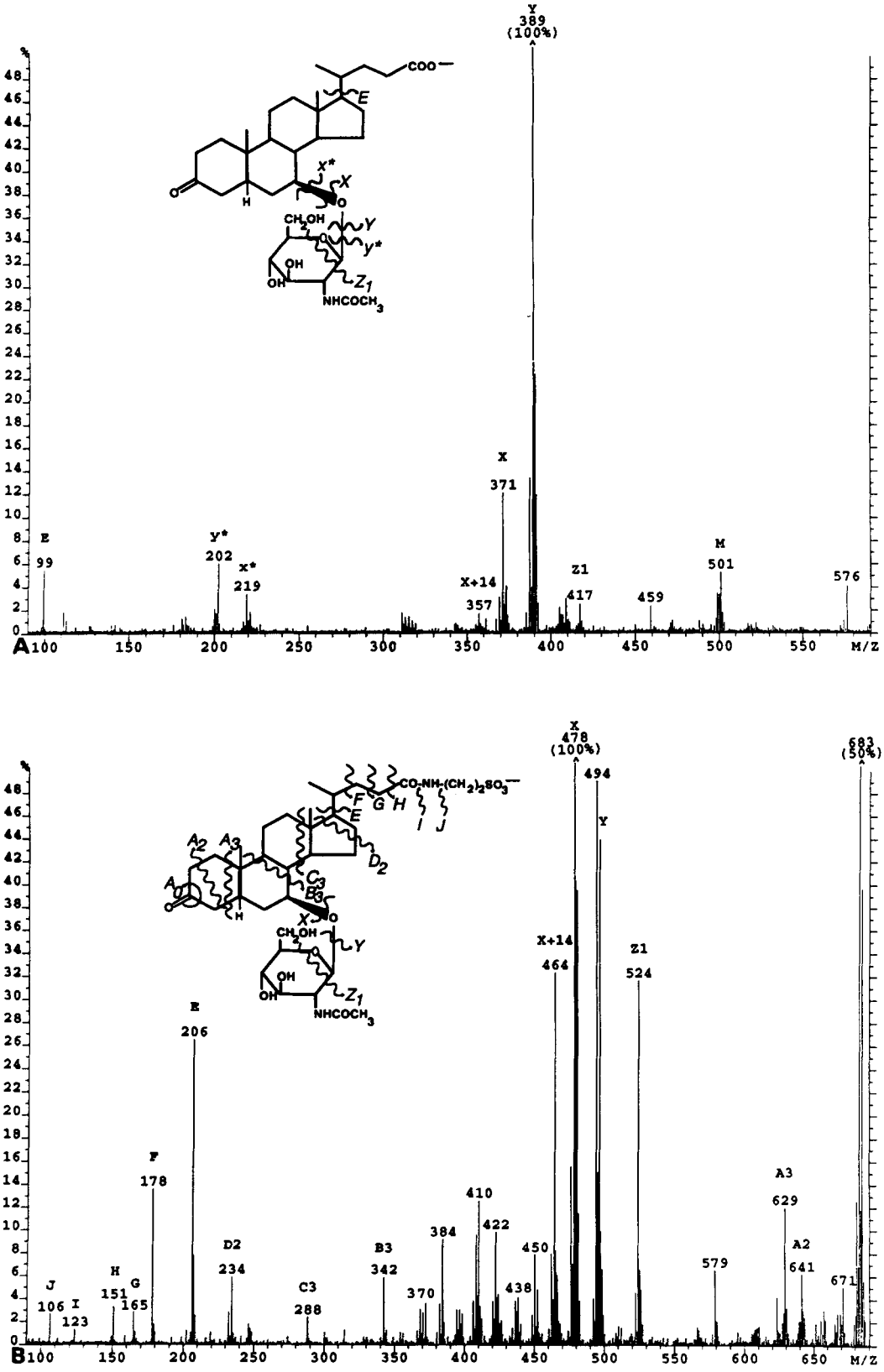

Fig. 5. A: Daughter ions produced by CID of the $[M-H]^-$ ion at *m/z* 592 from 3-dehydro-ursodeoxycholic acid *N*-acetylglucosaminide (GlcNAc-3-dehydro-UDCA) prepared with human kidney or liver microsomes. M is derived from a matrix adduct ion. B: Daughter ions produced by CID of the $[M-H]^-$ ion at *m/z* 699 from taurine derivatized GlcNAc-3-dehydro-UDCA.

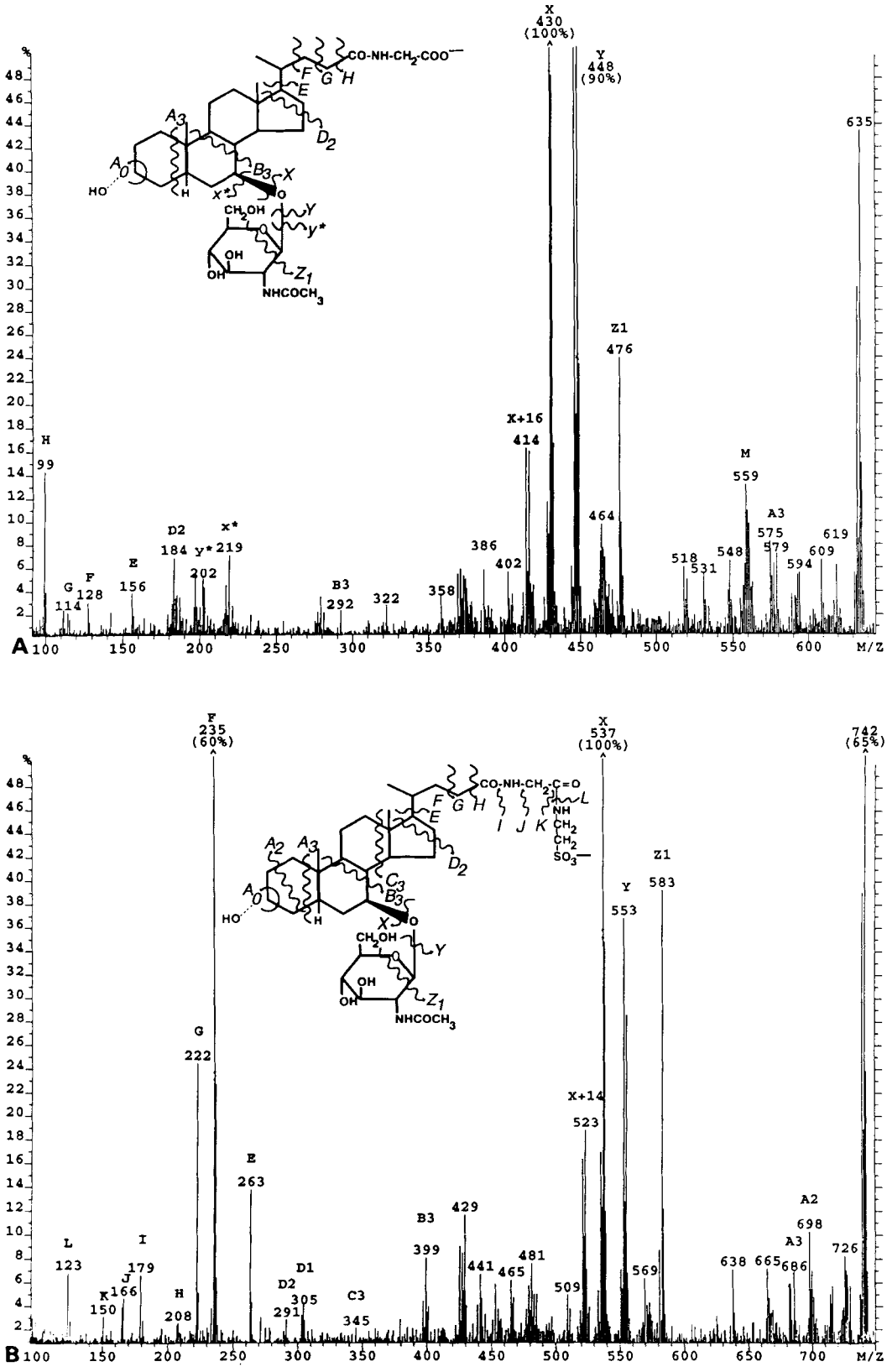

Fig. 6. A: Daughter ions produced by CID of the $[M-H]^-$ ion at *m/z* 651 from *N*-acetylglucosaminidyl-ursodeoxycholylglycine (GlcNAc-UDC-gly) prepared with human kidney or liver microsomes. B: Daughter ions produced by CID of the $[M-H]^-$ ion at *m/z* 758 from taurine derivatized GlcNAc-UDC-gly.

of the taurine derivatives were much more informative as is seen in Table 2 and Figs. 4B, 5B, and 6B. The fragmentation patterns of the four derivatized bile acids were similar and dominated by fragmentations involving the sugar (Z_1, Y, and X). Fragment ions containing the intact steroid ring structure differed by 2 Da between the 3-oxo and the 3-hydroxy forms. This is illustrated by comparing Figs. 4B and 5B. The GC/MS evidence suggested that the sugar conjugation was at the 7β-position. Therefore, fragmentation A_3 should lead to an ion of 629 Da in case of the derivatized *N*-acetylglucosaminides of UDCA, isoUDCA, and 3-dehydro-UDCA and to an ion of 686 Da in case of the derivatized *N*-acetylglucosaminide of UDCgly. The respective ions are observed for all four derivatives in addition to ions corresponding to cleavages B_3, C_3 and D_2 in the steroid ring. More complex fragmentations X + 14 - X + 108 involve loss of the sugar group accompanied by cleavage of the steroid rings.

DISCUSSION

It is well established that glucuronidation is a preferential conjugation pathway for 6α-hydroxylated bile acids like HDCA. The major part of urinary bile acid glucuronides contains a 6-hydroxy group (2) and HDCA and other 6-hydroxy bile acids are efficiently glucuronidated in vitro at the 6-position (3–5) whereas bile acids lacking a 6-hydroxy group are glucuronidated at the 3-position (2). The present study shows that sugar nucleotide-independent glucosidation of CDCA and HDCA (8) occurs at C-3 as is the case with glucuronidation of CDCA. The preferable glucuronidation of 6-hydroxy bile acids like HDCA is supported by previously measured kinetic data. Calculations of V_{max}/K_m (16) from previous studies of the in vitro glucuronidation and glucosidation of CDCA and HDCA by human microsomal UDP-glucuronosyltransferase and sugar nucleotide-independent glucosyltransferase (4, 17, 18) show that glucuronidation and glucosidation of CDCA and glucosidation of HDCA have very similar efficiencies in vitro. In contrast, HDCA is about 100 times more efficiently glucuronidated with human liver or kidney microsomes.

The glucosides of CDCA and HDCA investigated were formed in vitro by a sugar nucleotide-independent glucosyltransferase using octyl-β-D-glucopyranoside as the glucose donor (8). Quite recently, the synthesis of glucosides of 6-hydroxylated bile acids by a UDP-sugar-dependent mechanism was also observed in human liver microsomes with reaction rates similar to the 6-glucuronidation of HDCA (19). In a previous study, where the urinary excretion of bile acid glucosides and glucuronides in healthy volunteers and patients with cholestatic liver diseases were compared, only the excretion of glucuronides was increased in cholestasis. This suggests that bile acid glucosidation is not an important detoxication mechanism for bile acids in cholestasis (10).

The configuration of the glycosidic linkage in bile acid glucosides has not been determined. However, the formation with octyl-β-D-glucopyranoside as donor and the hydrolysis with β-glucosidase and not with α-glucosidase (1, 11) support a β-glucosidic linkage. This is also the configuration of a steroid glucoside isolated from rabbit urine (20).

N-Acetylglucosaminidation has recently been shown to be a selective conjugation reaction for 7β-hydroxylated bile acids like UDCA (6). Only bile acids containing a 7β-hydroxy group were identified among the bile acid *N*-acetylglucosaminides in urine from healthy humans as well as patients with cholestatic liver disease. Significant activities of hepatic or renal bile acid *N*-acetylglucosaminyltransferase were only observed towards 7β-hydroxylated bile acids (6). These findings suggested the 7β-hydroxyl group to be the site of conjugation with *N*-acetylglucosamine, which has been definitively established in the present study. The FAB/CID mass spectra of the *N*-acetylglucosaminides of UDCA, isoUDCA, and 3-dehydro-UDCA as well FABMS and GC/MS analyses of the 3-dehydro-UDCA-*N*-acetylglucosaminide gave independent confirmation of the 7-position of the *N*-acetylglucosamine moiety.

Also in this case, the configuration of the glycosidic linkage has not been determined. The hydrolysis with an enzyme specific for β-*N*-acetylglucosaminides (1, 11) and the β-configuration of a steroid *N*-acetylglucosaminide from human urine (21) make the latter configuration most likely. The recent synthesis of a number of bile acid 3- and 7-*N*-acetylglucosaminides will make a direct comparison with authentic β-anomers possible (22).

In conclusion, selective glycosidic conjugation reactions of bile acids are dependent on the position and orientation of hydroxyl groups in the steroid ring. 6-Hydroxy bile acids are preferentially glucuronidated at the 6-hydroxyl group whereas *N*-acetylglucosaminidation is selective for 7β-hydroxy bile acids and occurs at the 7β-position. Whether this latter selectivity of conjugation is related to the beneficial effects of UDCA observed in cholestatic liver diseases, e.g., in primary biliary cirrhosis (23), remains to be studied.

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