Mass Spectral Analysis of Glucuronides†

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Evaluation of the mass spectra of a number of ether-linked glucuronides indicates that a considerable amount of structural information may be deduced. A number of ions derived from the sugar acid moiety serve to identify an unknown as a glucuronide. The molecular weight of the conjugate, and by subtraction that of the aglycon, may be determined. Fragmentation around the acetal linkage varies according to whether the hydroxyl group is aromatic or aliphatic. Further information about the aglycon structure and also about the point of conjugation can be obtained from appropriate fragmentation in the drug moiety itself.

The structural elucidation of urinary glucuronides has presented a challenge to toxicologists and pharmacologists for many years. The glucuronides or mixtures of conjugates are commonly hydrolyzed and the free aglycons are identified. A more elegant and more tedious approach has been to identify the intact glucuronides by comparison of their chromatographic or electrophoretic‡ behavior with that of known standards. Recently several laboratories[1,2] have demonstrated that trimethylsilyl derivatives of urinary glucuronides can be separated by gas chromatography. The potential of mass spectrometry combined with gas chromatography as a facile method of separation and direct identification in clinical research underway in our laboratories prompted us to examine the mass spectral fragmentation patterns of a number of ether-linked glucuronides. With the aid of high-resolution mass measurements, deuterium and halogen labeling, and defocused metastable measurements, we have attempted to derive some general principles by which metabolites may be identified as glucuronides and by which structures or partial structures may be deduced from mass spectra, without synthetic standards.

Results and Discussion

The glucuronides used in this study were conjugates of 6-bromo-2-naphthol (Figure 1), chloramphenicol (Figure 2), 4-(*N,N*-dimethylsulfamoyl)phenol (Figure 3), 8-hydroxyquinoline, 4-nitrophenol, phenolphthalein, androsterone, and testosterone. These were analyzed by mass spectrometry and by gas chromatography–mass spectrometry as the trimethylsilyl derivatives of methyl esters. 8-Hydroxyquinoline and testosterone were also analyzed as the per(trimethylsilyl) derivatives without prior conversion to methyl esters. In addition, spectra were obtained of the tris(trimethyl-d_9-silyl) derivative of the methyl ester of 6-bromo-2-naphthylglucuronide and of the tetrakis(trimethyl-d_9-silyl) derivative of 8-hydroxyquinoline glucuronide. Labeling studies with the 6-bromo-2-naphthylglucuronide derivative were particularly attractive since the deuterium atoms labeled the sugar acid portion of the molecule, and the isotopes of bromine with their 1:1 abundance ratio allowed ions to be distinguished which contained substantial portions of the aglycon moiety. High-resolution spectra were obtained of tris(trimethylsilyl)-6-bromo-2-naphthylglucuronide methyl ester, of tetrakis(trimethylsilyl)-8-hydroxyquinoline glucuronide, and of tetrakis(trimethylsilyl)-phenolphthalein glucuronide methyl ester. For purposes of comparison, spectra were also obtained of trimethylsilyl derivatives of the corresponding aglycons.

These compounds contain a variety of functional groups all of which may influence fragmentation. The extent to which a functional group localizes charge density and directs fragmentation in a polyfunctional molecule has been empirically correlated with its ionization potential.[3,4] The ionization potentials[5] of simple compounds containing the three functional groups of the derivatized cyclic sugar acid are listed in Table I. As these values would suggest, considerable fragmentation occurs in the glucuronide derivatives in and adjacent to the trimethylsilyloxy groups (enhanced by their statistical abundance as well) while little or no primary fragmentation can be detected in the carbomethoxy group. Important bond cleavage also occurs in and around the acetal linkage, consistent with the relatively low ionization potential of its simple homolog. The chemical nature of the aglycon will determine how well it competes with the sugar acid moiety for charge and how much information its fragments contribute to the spectrum.

Molecular Weights. Molecular ions were observed in all spectra obtained by inserting the sample directly into the source of the mass spectrometer. Molecular ions were not always observed using the gas-chromatographic inlet system, perhaps reflecting enhanced thermal excitation. In all the compounds studied loss of a methyl group led to M −.15 ions of abundance greater than that of the molecular ion. Peaks at M − 73 [M − $(CH_3)_3Si$], M − 90 [M − $(CH_3)_3SiOH$], and M − 105 (M − 15 − 90) also occurred to varying extents in all the spectra. If molecular ions are scarce or missing, the molecular weight of the glucuronide may be deduced from this set of ions formed by fragmentation in the trimethylsilyl ether groups. Used this way, these ions may be considered as the "molecular ion set."

Identification as a Glucuronide. A number of ions are formed in the fragmentation of all the glucuronides which are derived from portions of the sugar acid ring.

Cleavage of the exocyclic acetal bond leads to loss of the aglycon and formation of ions of mass 407 as shown (see Tables II and III). When the carboxylic acid is esterified

Table I. Ionization Potentials[5] of Model Compounds

$CH_3\overset{\overset{O}{\|}}{C}OCH_3$	10.27 ± 0.02 eV
$CH_3OSi(CH_3)_3$	9.79 ± 0.04 eV
$CH_3\overset{\overset{H}{\|}}{C}OCH_3$ $\quad\quad\|$ $\quad OCH_3$	9.65 ± 0.03 eV

†This material was presented in part at the Twentieth Annual Conference on Mass Spectrometry and Allied Topics, Dallas, Texas, June 5–9, 1972.

‡L. Chung and D. S. Coffey, unpublished results.

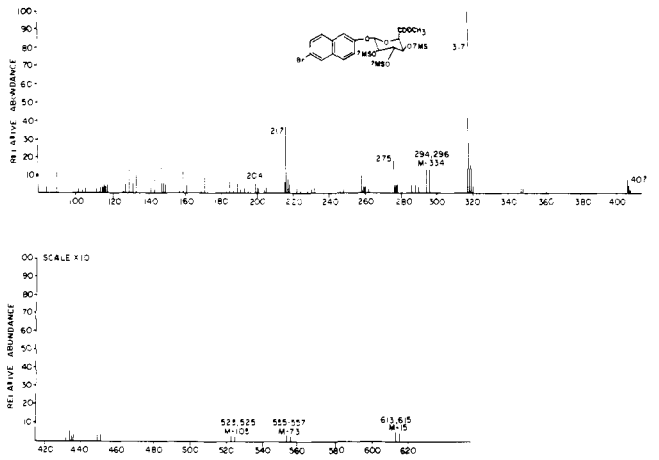


Figure 1. Mass spectrum of tris(trimethylsilyl)-6-bromo-2-naphthyl-glucuronide methyl ester.

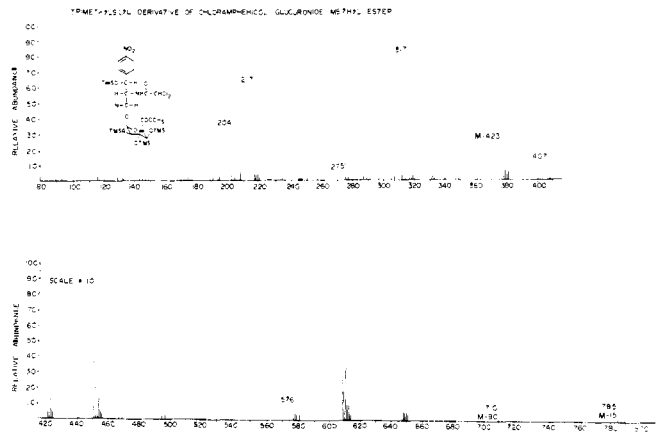


Figure 2. Mass spectrum of tetrakis(trimethylsilyl)chloramphenicol glucuronide methyl ester.

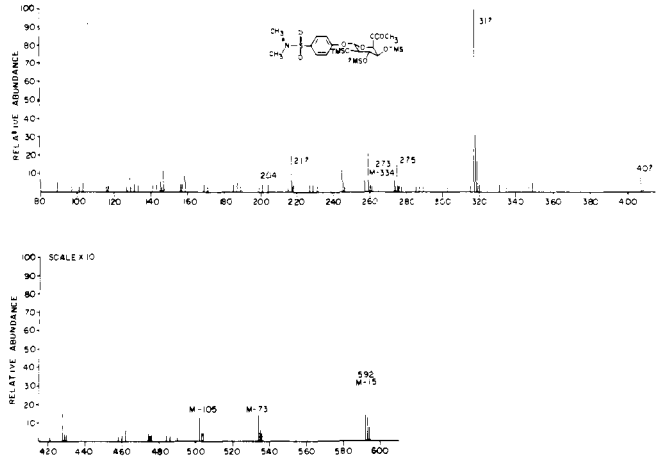
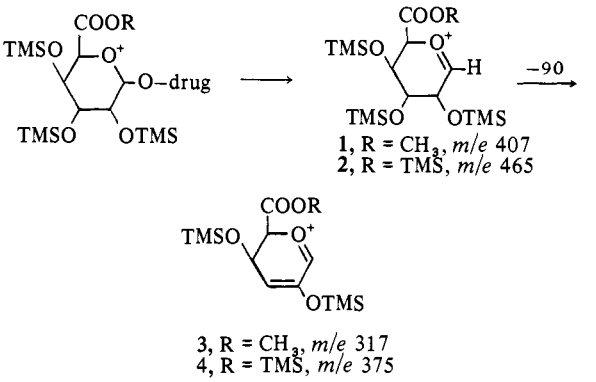
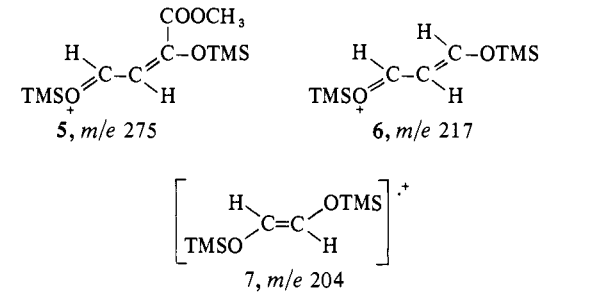


Figure 3. Mass spectrum of tris(trimethylsilyl)-4-(*N,N*-dimethyl-sulfamoyl)phenol glucuronide methyl ester.

with a trimethylsilyl group, ions are produced of mass 465. In most spectra ions **1** and **2** are accompanied by ions one mass unit lower, 406 and 464, formed by loss of an additional hydrogen atom. Elimination of $(CH_3)_3SiOH$, 90 atomic mass units, generates ions of mass 317 or 375, **3** or **4**, usually represented by the base peak in the spectrum. This transition was confirmed in metastable defocusing studies (Table IV). These ions are not commonly found in



the fragmentation of silylated carbohydrates[6] and can be used to identify an unknown sample (eluted from a gas chromatograph, for example) as a glucuronide or related aldohexuronic acid derivative. Ions of mass 275 [333 in per(trimethylsilylated) derivatives] also contain the carboxyl ester. Some of the fragment ions common to all silylated carbohydrates[6] appear in glucuronide spectra. These include ions **6** and 7 of masses 204 and 217. The intensity of the peak at *m/e* 204 is, in our experience, a good indication of the extent of pyrolysis of the sample in the interface or inlet system. A number of other peaks are observed below *m/e* 200 which are also common to the spectra of most silylated carbohydrates.



The elemental compositions of ions **1**, **3**, **5**, **6**, and 7 established in the high-resolution spectrum of tris(trimethylsilyl)-6-bromo-2-naphthylglucuronide methyl ester are presented in Table II. The masses of ions **1**–7 observed in the spectra of trimethyl-d_9-silyl analogs (Table III) are consistent with the compositions suggested.

Aglycon Attachment and Structure. The molecular weight of the aglycon may be established by subtraction

Table II. Accurate Mass Values from the Spectrum of Tris(trimethylsilyl)-6-bromo-2-naphthylglucuronide Methyl Ester

Obsd mass	Formula	Theor mass
630.1310	$C_{26}H_{41}O_7Si_3^{81}Br$	630.1323
628.1322	$C_{26}H_{41}O_7Si_3^{79}Br$	628.1343
615.1077	$C_{25}H_{38}O_7Si_3^{81}Br$	615.1089
613.1136	$C_{25}H_{38}O_7Si_3^{79}Br$	613.1109
572.1119	$C_{24}H_{35}O_7Si_2^{81}Br$	572.1084
570.1111	$C_{24}H_{35}O_7Si_2^{79}Br$	570.1105
557.0874	$C_{23}H_{32}O_7Si_2^{81}Br$	557.0850
555.0913	$C_{23}H_{32}O_7Si_2^{79}Br$	555.0869
525.0567	$C_{22}H_{28}O_6Si_2^{81}Br$	525.0588
523.0579	$C_{22}H_{28}O_6Si_2^{79}Br$	523.0608
407.1710	$C_{16}H_{35}O_6Si_3$	407.1741
406.1635	$C_{16}H_{34}O_6Si_3$	406.1663
317.1267	$C_{13}H_{25}Si_2O_5$	317.1240
296.0033	$C_{13}H_{15}SiO^{81}Br$	296.0056
294.0057	$C_{13}H_{15}SiO^{79}Br$	294.0076
275.1144	$C_{11}H_{23}Si_2O_4$	275.1148
217.1072	$C_9H_{21}Si_2O_2$	217.1080
204.0993	$C_8H_{20}Si_2O_2$	204.1001
202.0795	$C_8H_{18}Si_2O_2$	202.0845

Table III. Peak Shifts in Spectra of Deuterium-Labeled Analogs[a]

A	B	C	D
628 (M)	655	609 (M)	645
613 (M – 15)	637	594 (M – 15)	627
555 (M – 73)		536 (M – 73)	
(M – 90)	556	519 (M – 90)	546
523 (M – 15 – 90)	538	504 (M – 15 – 90)	528
407	434	465	501
406	433	464	500
317	335	375	402
294 (M – 334)	303	333	360
275	293	217 (M – 392)	226, 235
217	235, 236		(1:1)
	(1:1)	204	222
204	222	202 (217 – 15)	208

[a] A = tris(trimethylsilyl)-6-bromo-2-naphthylglucuronide methyl ester; B = the trimethyl-d_9 analog of A; C = tetrakis(trimethylsilyl)-8-hydroxyquinoline glucuronide; D = the trimethyl-d_9 analog of C.

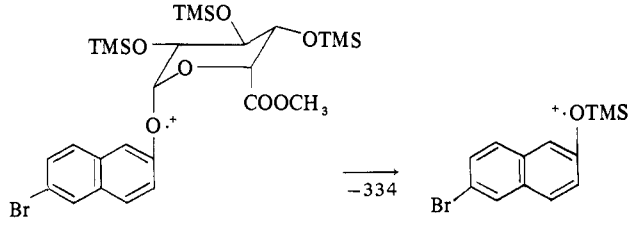
Table IV. Parent–Daughter Relationships Confirmed in the Spectrum of Tris(trimethylsilyl)-6-bromo-2-naphthylglucuronide Methyl Ester

Daughter ions	294 (M – 334)	317
Parent ions	628	630
	570	628
	555	468
	325	425
		407
		362
		347

of 406 atomic mass units from the molecular weight of the glucuronide derivative. This weight will, of course, include any trimethylsilyl groups attached to the aglycon. High-resolution measurements of the glucuronide molecular ion set should, in theory, provide information about the elemental composition of the aglycon.

In interpreting the spectrum of an unknown glucuronide, ions containing all or part of the aglycon must be identified. This may be facilitated by high-resolution mass measurements, since few drugs or endogenous metabolites will have the high ratio of oxygen to carbon which characterizes ions comprising the sugar moiety. A simpler means of distinguishing aglycon ions is provided by the multiplet of peaks generated by isotopes of chlorine or bromine present in so many drugs. A third approach is simply to subtract out the sugar acid ions common to spectra of all glucuronides.

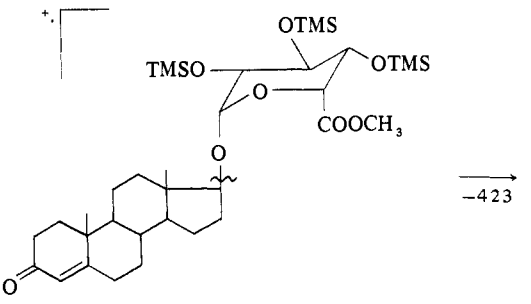
In the spectrum (Figure 1) of the 6-bromo-2-naphthylglucuronide derivative peaks are observed at *m/e* 294 and 296, which correspond to an ion containing the bromine



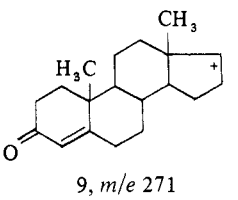
m/e 628, 630 8, *m/e* 294, 296

isotopes and the aglycon. This M – 334 ion **8** is most easily visualized as being formed by cleavage of the exocyclic acetal bond with transfer of a trimethylsilyl group as shown. The doublet appears at *m/e* 303 and 305 in the spectrum of the tris(trimethyl-d_9-silyl) analog (Table III). The precursors observed by defocused metastable measurements are listed in Table IV. Analogous loss of 334 atomic mass units leads to peaks in the middle intensity range in the spectra of 4-(*N,N*-dimethylsulfamoyl)phenol glucuronide (*m/e* 273 in Figure 3) and of all other glucuronides examined which are conjugated through an aromatic hydroxyl group. In the spectrum of the per(trimethylsilyl) derivative of 8-hydroxyquinoline glucuronide this peak is present at M – 392.

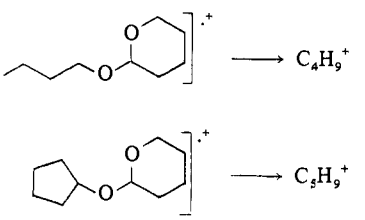
On the other hand, M – 334 peaks are small or absent in the spectra of chloramphenicol and testosterone glucuronides which possess aliphatic acetal linkages. Rather, prominent peaks occur 423 mass units below the molecular ions of these compounds, at *m/e* 377 in the spectrum (Figure 2) of the derivative of chloramphenicol glucuronide. Those M – 423 ions appear to be formed by cleavage in the exocyclic acetal bond as indicated for the testosterone derivative, with charge retention on the aglycon moiety. This fragmentation seemed unlikely, initially, since it leads to a primary carbonium ion in the spectrum of the derivative of chloramphenicol glucuronide. However, an analogous bond cleavage generates ions of moderate abundance in the electron impact induced fragmentation of alkyl tetrahydropyranyl ethers,[7,§] the simplest model compounds for the glucuronide acetal linkage.



m/e 694



9, *m/e* 271

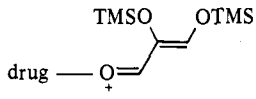


In our study M – 334 peaks were consistently observed in spectra of glucuronides containing an aromatic acetal linkage while M – 423 ions occurred in the spectra of those conjugated through an aliphatic hydroxyl group. In some cases both ions were present, but the ratio of intensities was always 6 to 1 or greater (Table V).

The spectra available also suggest that ions 191 mass units lighter than the molecular ions are formed in the fragmentation of glucuronide methyl esters linked through an aliphatic hydroxyl group, while the loss of 179 mass units from the molecular ion leads to more abundant ions from silylated methyl esters of glucuronides conjugated with an aromatic hydroxyl group.

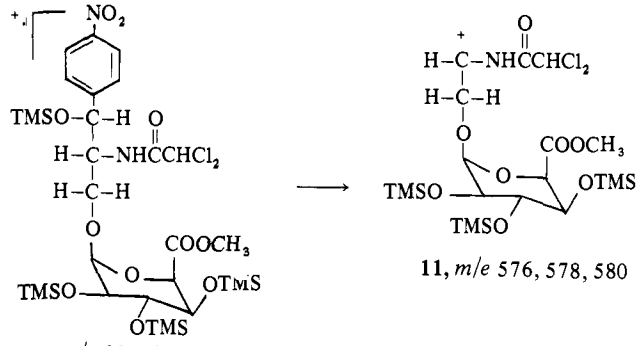
Fragmentation within the aglycon moiety should also provide information about the structure of the conjugate. For example, bond cleavage in the derivative of chloram-

§ R. Foltz, private communication.



10, M − 191

phenicol glucuronide is illustrated, which permits us to distinguish between the two hydroxyl groups in that drug as sites of conjugation. Observation of ions of mass 576 which contain two chlorine atoms confirms the conclusion[8] of an earlier investigation employing chemical degradation that

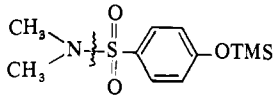


m/e 800, 802, 804

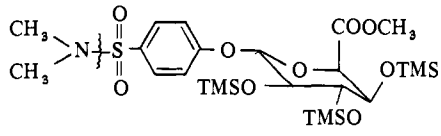
11, *m/e* 576, 578, 580

conjugation occurs on the primary hydroxyl group, rather than on the benzylic hydroxyl group.

To some extent fragmentation in the drug moiety of a glucuronide may be predicted by examining the fragmentation of trimethylsilyl derivatives of a number of related unconjugated aglycons. Thus, cleavage of the S–N bond is a major process in the fragmentation of the trimethylsilyl ether of 4-(*N,N*-dimethylsulfamoyl)phenol. The analogous loss of 44 mass units is detected from M − 15, M − 73, and M − 105 ions in the spectrum of the conjugate derivative. This cleavage would permit identification of conjugates of N-demethylated metabolites in this family of aglycons.



M − 44



M − 15 − 44
M − 73 − 44
M − 105 − 44

Table V. Ratio of M − 334 to M − 423 Peaks in Electron-Impact Spectra of the Trimethylsilyl Derivatives of Glucuronide Methyl Esters and Related Compounds

Aglycon	[M − 334]/[M − 423]
Androsterone	0.03
6-Bromonaphthol	8.6
Chloramphenicol	0.003
4-(*N,N*-Dimethylsulfamoyl)phenol	82.0
8-Hydroxyquinoline[a]	19.9
4-Nitrophenol	26.2
Phenolphthalein	180.0
Testosterone	0.17
Butyltetrahydropyran[b]	0.02

[a] [M − 392]/[M − 481] for this per(trimethylsilyl) derivative. [b] [M − 84]/[M − 101].

Conclusion

Mass spectra obtained of per(trimethylsilyl) derivatives of glucuronides or of trimethylsilyl derivatives of glucuronide methyl esters can provide confirmation that the compound is a glucuronide, evaluation of the molecular weight of the aglycon and the conjugate, and information about the aromatic or aliphatic nature of the conjugated hydroxyl group. Depending on the nature of fragmentation in the aglycon, the point of conjugation may be distinguished and structural features may be deduced.

Experimental Section

Mass spectra were obtained on an LKB 9000 gas chromatograph–mass spectrometer with the source at 250°, separator at 220°, and gas chromatograph programmed between 140 and 300° at 6°/min. A 3% OV-1 column was used. Low- and high-resolution spectra were obtained on a CEC 21-110 double focusing instrument using the direct inlet system with the source at 160°. High-resolution spectra were recorded on silver bromide photoplates purchased from Ionomet Co. Defocused metastable measurements[9] were also made with this instrument.

Glucuronides were passed through an acid-washed Dowex-50 column and subsequently treated with diazomethane and *N,O*-bis(trimethylsilyl)acetamide. We chose to work primarily with methyl esters rather than silyl esters, because methyl esters of glucuronides can be satisfactorily purified by thin-layer chromatography[8] and because silylated methyl esters are reported[10] to be more stable than per(trimethylsilyl) derivatives. Deuterium-labeled trimethylsilyl derivatives were obtained by treating the glucuronide with *N,O*-bis(trimethyl-d_9-silyl)acetamide purchased from Merck Sharp and Dohme of Canada.

Chloramphenicol glucuronide was isolated by Mr. Marc Mann following the procedure of Glasko, *et al.*[8] 4-(*N,N*-Dimethylsulfamoyl)phenol glucuronide was a gift from G. L. Sutherland of the American Cyanamid Co.[11] Other glucuronides were purchased from Sigma Chemical Co.

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