papers and notes on methodology

Ammonia gas: an improved reagent for chemical ionization mass spectrometry of bile acid methyl ester acetates

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Summary The ammonia chemical ionization mass spectra of 28 methyl ester acetate derivatives of bile acids and related compounds have been determined by gas–liquid chromatography–mass spectrometry. Advantages of ammonia ionization over the previously studied isobutane ionization include a 130–270% enhancement in the sensitivity of base peak monitoring, and direct determination of molecular weight from the base peak ($M + NH_4^+$) in the mass spectrum of any of the derivatives. Minor ions in the ammonia spectra also allow selective detection of 3-keto compounds and can indicate unsaturation or double bond conjugation in the molecule. The significance of these studies for the detection and quantitation of bile acids is discussed. — **DeMark, B. R., and P. D. Klein.** Ammonia gas: an improved reagent for chemical ionization mass spectrometry of bile acid methyl ester acetates. *J. Lipid Res.* 1981. **22:** 166–171.

Supplementary key words gas–liquid chromatography–mass spectrometry

Our laboratory has reported the gas–liquid chromatographic properties of a number of bile acids as their methyl ester acetates (1) and we have investigated the electron impact (EI) and isobutane chemical ionization (CI) mass spectra of these compounds obtained by gas–liquid chromatography–mass spectrometry (GLC–MS) (2). The EI spectra provide valuable structural information because of the extensive fragmentation occurring in this mode of ionization, while isobutane CI provides highly simplified spectra. These CI spectra usually display a base peak resulting from the loss of acetate groups from the protonated molecular ion (MH^+) leading to major peaks at $MH^+ - (n \times 60)$, where n is the number of acetate groups lost. Isobutane chemical ionization of bile acids as their methyl ester acetates thus avoids scission of the carbon-carbon bonds and aids in the screening of complex mixtures of monohydroxy, dihydroxy, trihydroxy, and tetrahydroxy bile acids as well as their ketone analogues (2–6).

The methane CI characteristics of unacetylated bile acid methyl esters have been recently reported (7). This nonselective mode of ionization is useful in identification work, as it produces more fragments than isobutane. However, when quantitative measurements are of paramount importance, such as in stable isotope ratio measurements of ^{13}C- and ^{18}O-labeled bile acids in kinetic or biosynthetic studies (8–10), three further attributes of the ionization process become critical. The first is the degree to which formation of alternative ion species can be avoided, i.e., loss of acetate groups from the molecular ion, as these fragmentation pathways reduce the ion yield available for quantitation. The second is the molecular weight of the base peak; the higher this value is, the less susceptible it will be to interference from matrix contaminants. The third is the selectivity of the ionization process, or the degree to which ionization is limited to the compounds of interest.

The ammonia CI mass spectrum of underivatized cholic acid obtained by direct probe has been recently reported (11). The major ion peak in the mass spectrum is the molecular ion ammonium adduct ($M + NH_4^+$). Ammonia CI mass spectra have also been reported for cholesterol esters (12) and a variety of C_{27}-steroids (13, 14). The base peaks in these spectra are largely dependent upon the degree of conjugation and unsaturation in the steroid ring nucleus as well as the ion source temperature. In this report we have investigated the use of ammonia as a reagent gas in the gas–liquid chromatography–chemical ionization mass spectrometry (GLC–CIMS) analysis of 28 methyl ester acetate derivatives of bile acids and related compounds at an ion source temperature of 160°C. We have found that ammonia offers several important advantages over isobutane when used as a reagent gas in the GLC–CIMS analysis of bile acid mixtures.

Abbreviations: EI, electron impact; CI, chemical ionization; GLC–MS, gas–liquid chromatography–mass spectrometry; amu, atomic mass unit; OAc, acetate; m/z, mass to charge ratio.

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MATERIALS AND METHODS

Chemicals

All bile acids and related compounds were obtained from sources noted previously (2). Free acids were converted to methyl esters (15) and hydroxyl groups were acetylated (16). Up to five bile acid derivatives were combined to form eight different standard mixtures in which each derivative was present at a concentration of 1–3 μg/μl in acetone. Mixtures were formulated on the basis of the gas–liquid chromatographic retention time of the individual components to give well-resolved chromatographic peaks. One μl of each mixture was injected for GLC-CIMS analysis.

Mass spectra generation

The bile acid standard mixtures were analyzed by GLC–CIMS. The gas chromatograph employed was a Varian Series 1400 (Varian Associates, Palo Alto, CA) using a glass column (1 mm i.d. × 183 cm) packed with 1% Poly S-179 on 100/120 mesh Gas Chrom Q (1) (Applied Science Labs, Inc., State College, PA). Column oven temperature was 260°C; the temperature at the injector and at the GLC outlet–MS inlet was 285°C. Helium was the carrier gas at a flow rate of 9.0 ml/min. A Biospect quadrupole mass spectrometer (Chemetron Medical Products, St. Louis, MO) was operated with ammonia or isobutane as reagent gas. The ion source temperature was 160°C and the source pressure was 1.3 torr ammonia or 1.0 torr isobutane. Mass spectra were recorded between 350 and 625 amu at a rate of 22.5 amu/sec using a PDP 12 computer (Digital Equipment Corp., Maynard, MA). For each derivative, an integrated mass spectrum was generated by summing the ion intensities of the stored mass spectra corresponding to the mass scans for the chromatographic peak of the compound. Depending on peak width, 7–27 scans were summed for each of the 28 compounds studied.

Sensitivity of ammonia versus isobutane

For relative sensitivity measurements, a mixture of methyl ester acetates of lithocholic, deoxycholic, chenodeoxycholic, and cholic acids was prepared. On the same day, an aliquot containing 1–2 μg of each component was analyzed by GLC–CIMS for each of the reagent gases studied (ammonia or isobutane). The source pressure of reagent gas was adjusted to give maximum intensity of the base peak ion under each reagent condition. The ion intensity of the base peak in the mass spectrum of each derivative was integrated during the elution time of its respective chromatographic peak, using a stable isotope ratiometer multiple ion detector (17), and identical integration times were used with both reagent gases.

RESULTS

Table 1 lists the relative abundance of all ions produced in the ammonia CI mass spectrum of each of the bile acid derivatives studied. Isotopic satellite ions have been omitted. Since all spectra were obtained by GLC–CIMS analysis, the relative chromatographic retention time coordinates are also shown.

In every case studied, the base peak in the ammonia CI spectrum corresponds to the molecular weight of the compound plus 18, the molecular ion ammonium adduct ($M + NH_4^+$). In the vast majority of cases, ions corresponding to loss of acetate groups account for less than 5% of the base peak; in two cases (Nos. 1 and 10) no ions other than the M + 18 ion are detected. There are only two compounds in which loss of acetate groups accounts for ions in the mass spectrum that are greater than 10% of the base peak. These compounds, cholesterol acetate (No. 27) and 3β OAc Δ^5-methyl cholenic acid (No. 15), have in common the 3β OAc Δ^5 configuration.

The table includes spectra of several compounds that might be present as artifacts of the derivatization process. These include partially acetylated compounds (Nos. 3, 5, and 7) as well as 5β 3α methoxy, 7α, 12α, diOAc methyl cholic acid (No. 8). In each of these spectra, the minor peaks result only from the loss of an acetate group ($M + NH_4^+ - 60$) rather than a hydroxy or methoxy group.

Minor peaks that do not relate to loss of acetate groups are observed in the mass spectra of the 3-keto bile acid derivatives (Nos. 19–22). These are at m/z equal to the molecular weight of the compounds and can be attributed to the loss of a molecule of water from the ammonium adduct of the molecular ion (see Discussion).

Table 2 shows the comparison between isobutane and ammonia as reagent gases for the GLC–CIMS analysis of a standard mixture of bile acid derivatives. The average ion intensity of the base peak monitored under ammonia CI conditions was 130–270% greater, depending on the compound, than when the base peak was monitored under isobutane ionization conditions for the same integration time.

TABLE 1. Ammonia chemical ionization mass spectra of bile acid methyl ester acetates

No.	Functional Group	R.R.T.[a,b]	M.W.[a]	Mass Spectrum: Base Peak Rel. Int.[a] = 100 m/z ($M + NH_4^+$)	Loss of HOAc: m/z	Loss of HOAc: Rel.[a] Int.	Other Ions: m/z	Other Ions: Rel.[a] Int.
	C_{24} Methyl cholanic acids							
1	5β 3αOAc (Lithocholic acid)	0.70	432	450	390	0		
2	5β 3α,12α diOAc (Deoxycholic acid)	1.00	490	508	448	3		
3	5β 3αOAc, 12αOH	1.96	448	466	406	3		
4	5β 3α,7α, diOAc (Chenodeoxycholic acid)	1.35	490	508	448	5		
5	5β 3αOAc, 7αOH	2.35	448	466	406	4		
6	5β 3α,7α,12α, triOAc (Cholic acid)	1.78	548	566	506	9	446[c]	1
7	5β 3α,7α, diOAc, 12αOH	3.58	506	524	464	9		
8	5β 3αOMe, 7α,12α, diOAc	1.00	520	538	478	6		
9	5β 3αOAc, 7βOAc (Ursodeoxycholate)	1.93	490	508	448	3		
10	5β 3α,6α, diOAc (Hyodeoxycholic acid)	1.78	490	508	448	0		
11	5β 3α,6α,7α, triOAc (Hyocholic acid)	2.24	548	566	506	1		
12	5β 3α, 6β, 7β, triOAc (β-Muricholic acid)	3.42	548	566	506	4		
13	5β 3α, 6β, 7α, triOAc (α-Muricholic acid)	2.46	548	566	506	2		
14	5β 3α, 12α, diOAc $\Delta^{8(14)}$	1.06	488	506	446	2		
15	3β OAc Δ^5	0.90	430	448	388	36	371[d]	2
16	5α 3α, 12α, diOAc (Allodeoxycholic acid)	1.21	490	508	448	3		
17	5α 3α, 7α, 12α, triOAc (Allocholic acid)	2.05	548	566	506	6	446[c]	1
18	5α 3β, 12α, diOAc	1.54	490	508	448	1		
19	5β 3-keto	1.04	388	406			388[e]	3
20	5β 3-keto, 7αOAc	2.30	446	464	404	2	446[e]	1
21	5β 3-keto, 12αOAc	1.59	446	464	404	2	446[e]	1
22	5β 3-keto, 7α, 12α, diOAc	2.83	504	522	462	3	504[e]	1
23	5β 3αOAc, 7-keto	2.66	446	464	404	1		
24	5β 3αOAc, 12-keto	2.31	446	464	404	2		
25	5β 3α, 12α, diOAc, 7-keto	3.33	504	522	462	2		
26	5β 3α, 7α, diOAc, 12-keto	3.96	504	522	462	3	402[c]	1
	C_{27} Compounds							
27	3β, OAc Δ^5 (Cholesterol)	0.32	428	446	386	30	369[d]	4
28	5β 3α, 7α, 12α, triOAc Me Coprostanic acid (trihydroxycoprostanic acid)	2.35	590	608	548	7		

[a] Abbreviations used: R.T.T., relative retention time; M.W., molecular weight; Rel. Int., relative intensity.
[b] Retention time relative to the methyl ester acetate of deoxycholic acid (11.0 min).
[c] Ion corresponds to ($M + NH_4^+ - 2$ HOAc).
[d] Ion corresponds to ($M + NH_4^+ - NH_4OAc$).
[e] Ion corresponds to ($M + NH_4^+ - H_2O$).

DISCUSSION

Ammonia mass spectra of bile acid derivatives

It is apparent from the ammonia CI mass spectra of bile acid methyl ester acetates that ammonia ionization is a lower energy process producing less fragmentation than ionization with isobutane. This is due to the relative proton affinities of the conjugate bases of the two reagent ions. The proton affinity of NH_3 (207 kcal/mol) (18) is greater than that of isobutylene (195 kcal/mol) (19, 20). Therefore, protonation of a sample from the reagent ion in isobutane CI [$(CH_3)_3C^+$] will be more exothermic than either proton transfer from the reagent ion in ammonia CI (NH_4^+) or electrophilic attachment of NH_4^+ to the sample (21, 22). Because of the high proton affinity of NH_3, proton transfer from NH_4^+ has only been observed with amines, amides (23–25), conjugated ketones (13, 14, 26), and some unsaturated steroids (13). In compounds which are not sufficiently basic to remove a proton from NH_4^+, collision-stabilized complexation with NH_4^+ has been observed (27, 28).

The ammonia mass spectra of the twenty-eight bile acid derivatives (Table 1) show an absence of protonated molecular ions (MH^+) and of ions that result from MH^+ fragmentation, indicating that the low basicity of the derivatives prohibits proton transfer from NH_4^+. The base peak of each of these derivatives is the ammonium molecular ion adduct ($M + NH_4^+$), and all minor ion peaks in the mass spectra can be derived from this base peak.

Electron-rich conjugated keto bile acid derivatives as well as certain unsaturated derivatives might be expected to yield MH^+ peaks in their ammonium

TABLE 2. Sensitivity of base peak monitoring with isobutane or ammonia[a]

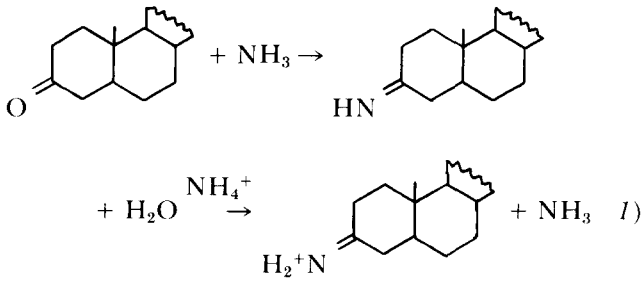
Bile Acid Methyl Ester Acetate	Time (sec) Base Peak Monitored	Isobutane		Ammonia		
		Base Peak m/z	Integrated Ion Intensity	Base Peak m/z	Integrated Ion Intensity	% Increase over Isobutane (average)
Lithocholic acid	75	373	115,640	450	376,100	210%
			135,074		347,980	
			92,453		356,717	
			114,389 avg.		360,266 avg.	
Deoxycholic acid	100	371	135,942	508	528,250	270%
			151,261		484,260	
			124,663		504,283	
			137,289 avg.		505,598 avg.	
Chenodeoxycholic acid	110	371	145,240	508	343,770	130%
			162,175		318,800	
			126,448		322,849	
			144,621 avg.		328,473 avg.	
Cholic acid	120	429	120,857	566	379,950	200%
			138,446		341,614	
			101,387		355,825	
			120,230 avg.		359,130 avg.	

[a] Data were from three successive GLC–MS analyses with each reagent gas. Source pressure was 1.0 torr isobutane or 1.3 torr ammonia. One μl containing 1–2 μg of each component was injected and the base peak was integrated for the time indicated for each derivative at its retention time.

CI mass spectra. Related C_{27}-steroids of this type were found to be sufficiently basic to be protonated by NH_4^+ and often produced MH^+ as their base peaks, especially at high source temperatures (190–200°C) (13, 14). We did not study conjugated keto-bile acid derivatives, but we did obtain ammonia CI spectra of three derivatives containing a single carbon-carbon double bond (Nos. 14, 15, and 27), and these compounds did not produce MH^+ ions in their mass spectra. However, loss of the 3β OAc group from the base peak ammonium complex (as either HOAc or NH_4OAc) in the 5-ene compounds (Nos. 15 and 27) accounted for a much larger fraction of their mass spectra than was observed for any of the other derivatives, indicating that electrophilic attachment of NH_4^+ to these more basic unsaturated 5-ene compounds is a more exothermic reaction.

It is likely that certain conjugated keto bile acids and unsaturated dienes will possess a degree of basicity that is high enough to permit proton transfer from NH_4^+ and produce MH^+ ions as either base peaks or minor peaks in their CI mass spectra. Base peaks that relate strictly to MH^+ can be easily distinguished from those that correspond to $(M + NH_4^+)$. Protonated molecular ions will yield only odd mass ion base peaks for bile acid derivatives, or any other compound containing carbon, hydrogen, and/or oxygen, while all mono-ammonium adduct ions of these compounds will have only even mass ion base peaks in their mass spectra. Thus, the molecular weight of any bile acid-like derivative can be obtained from the base peak in its ammonia CI mass spectrum. Furthermore, a degree of conjugation or unsaturation may be inferred from the presence of MH^+ peaks or from a significant increase in the relative intensity of ions corresponding to the loss of acetate groups from the base peak complex (i.e., the 5-ene compounds, Nos. 15 and 27).

The ammonia CI mass spectral characteristics can also be used to detect and distinguish 3-keto compounds from other mono-keto isomers. The 3-keto bile acid methyl ester acetates (Nos. 19–22) contain minor ions in their mass spectra that do not relate to the loss of acetate groups at m/z equal to their molecular weights. Apparent molecular ions have also been observed in the mass spectra of steroid alcohols (13) and these ions were assigned to substitution of the hydroxyl group by NH_3. However, this cannot be the case in the ammonia spectra of the 3-keto bile acids, where the free hydroxyl groups have been acetylated. The molecular weight ion observed in these compounds very likely corresponds to a protonated Schiff base of the 3-keto group, formed in a neutral reaction between ammonia and the ketone, followed by protonation by the NH_4^+ reagent ion (equation *1*).



Schiff base formation has been observed in the ammonia CI mass spectra of aldehydes (27) and probably also accounts for the minor molecular ion peak in the ammonia CI spectrum of 3-keto-Δ^5-cholestene (13). Similar protonated Schiff base ions were not observed with the 7-keto or 12-keto bile acid derivatives (Nos. 23–26) or with the C_{27} 6-keto or 7-keto steroids (13), indicating the selectivity of ammonia for the 3-keto function.

Applications of ammonia CI to bile acid metabolism studies

Our laboratory has utilized isobutane chemical ionization mass spectrometry in conjunction with gas–liquid chromatography in the characterization of bile acid mixtures in a variety of collaborative clinical studies (3–6, 8–10). When characterizing a complex mixture of the bile acid fraction by isobutane GLC–CIMS in liver disease patients, identification of atypical compounds or artifacts is particularly important (3). Knowledge of the molecular weight can be a great help in bile acid identification, but with the exception of the keto-bile acids, molecular weight information cannot be obtained from the base peaks in the isobutane (2) or methane (7) CI mass spectra. The present work, however, shows that ammonia CI yields higher mass base peaks $(M + NH_4)^+$ from which molecular weight information can be directly determined for any of the bile acid derivatives.

The presence of intact ammonium molecular ion adducts as the base peak in the mass spectrum would also be a great advantage in ^{18}O inhalation studies of bile acid and cholesterol biosynthesis. Recently, Björkhem and Lewenhaupt (10) reported such a study in rats but they were unable to determine ^{18}O incorporation directly in chenodeoxycholic acid; no molecular ion was obtained in its EI or CI mass spectrum. Our work shows that ammonia CI could be used for direct measurement of ^{18}O incorporation into the primary bile acids by isotope ratio measurements of their methyl ester acetate derivatives.

The standard mixture of bile acid derivatives studied shows that the sensitivity of selected base peak monitoring can be increased by a factor of 2–3 when ammonia is substituted for isobutane as the CI reagent gas. This will be an advantage in both quantitation and screening studies, particularly for quantitation of the low levels of bile acids in normal serum or for detection of trace levels of atypical compounds in the bile, serum, or urine of the diseased patient.

Ammonia CI is also a more selective process than isobutane CI (25, 27). Neither proton transfer nor attachment of NH_4^+ is observed in the ammonia CI spectra of simple ethers, alcohols, phenols, nitro compounds, hydrocarbons, or aromatics (27). This increased selectivity should facilitate isotope ratio measurements of serum bile acids for the purpose of kinetic studies using stable isotope-labeled bile acids.

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