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Mass Spectrometry Advances in Lipidomica: Collision Induced Decomposition of Kdo₂-Lipid A

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Abstract

Mass spectrometry has made significant advances in the analysis of lipid substances, both simple and complex present in extracts of eukaryotic and prokaryotic cells. The development of the ionization techniques of electrospray ionization and matrix assisted laser desorption ionization have both been applied to the analysis of lipids. The example of the types of structural information that can be obtained from MALDI-TOF tandem mass spectrometry is exemplified by the analysis of Kdo₂-lipid A, a complex lipopolysaccharide known to activate Toll-like 4-receptors on mammalian cells. Analysis of Kdo₂-lipid A obtained from an *E. coli* WBB06 was found to generate an abundant [M-H]⁻ ion at m/z 2236.4 and a more abundant carbon-13 isotope at m/z 2237.4. Furthermore, collisional activation of the lipid A portion of the molecule at m/z 1796.3 resulted in a series of ions corresponding to the loss of all four fatty acyl groups as neutral carboxylic acids. An altogether different challenge of mass spectrometry applied to the area of lipid analysis is that of quantitative analysis. Two rather different requirements have emerged. One with high precision and accuracy for the measurement of relatively few lipid species that are produced at very low concentrations and typically interact with specific receptor proteins. A rather different challenge is that for the analysis of abundant lipid classes, which are composed of multiple molecular species that can approach several hundred under certain circumstances.

INTRODUCTION

Over the past two decades mass spectrometry has emerged as the premier method by which to structurally characterize and quantitate complex lipids found in living cells. Such efforts are one of the major goals of investigators as they begin to address problems in the area of lipidomica. Much of the current applications of mass spectrometry have built upon advances that were made over 20 years ago with the development of desorption ionization techniques in largely chemical research investigations that were found to generate intact ions from nonvolatile biomolecules including lipids such as glycerophospholipids. Gross and co-workers (1,2) demonstrated that fast atom bombardment mass spectrometry could generate molecular ion species, both [M+H]⁺ or [M-H]⁻ for all glycerophospholipids constituents of

cellular membranes. Furthermore, collisional activation of these ion species was found to generate decomposition product ions characteristic of both the polar head group (positive ions) and the fatty acyl substituent (negative ions). When electrospray ionization was commercialized, it therefore became possible to use these fundamental observations of tandem mass spectrometry to analyze lipids (3-5), even as they emerged from the HPLC, thus engaging online mass spectrometric analysis of chromatographic effluents to deal with the problem of complex mixtures of lipids typically present within cells. Substantial progress has continued to take place and now the very powerful tools of chromatographic separation, electrospray ionization, and tandem mass spectrometry are available to analyze glycerophospholipids as well as other complex lipids. Nonetheless, significant challenges remain in the analysis of lipids both in terms of the qualitative and quantitative analytical regimes.

Another technique that has emerged in the past two decades has been the use of matrix assisted laser desorption ionization (MALDI) to generate abundant ions of nonvolatile biologically relevant molecules (6). While much of the emphasis of the use of this technique has been in the analysis of proteins (7), nonetheless, there has been some demonstration that this technique can be used for the analysis of lipid substances (8-10). Much of the fundamental behavior of collisional activation of electrospray generated $[M+H]^+$ ions of glycerophospholipids were also observed for MALDI generated $[M+H]^+$ ions. This has led to a general understanding that the collision induced decomposition behavior of many molecular ion species (both positive and negative ions) are fundamentally the same and independent of the mode by which these nonvolatile ions are generated, either by electrospray ionization, fast atom bombardment, or MALDI.

METHODS AND MATERIALS

Escherichia coli mutant WBB06 was grown on LB broth with added calcium chloride (11). The Kdo₂-lipid A and its phosphoethanolamine-modified derivative were extracted with organic solvents and purified by chromatography on DEAE cellulose, as described previously (11). Kdo₂-lipid A was separated from the phosphoethanolamine modified derivative by chromatography on a 0.5 × 10 cm column of silica acid in the solvent chloroform/methanol/water/acetic acid (25:15:4:2, v/v/v). Phosphoethanolamine modification of Kdo₂-lipid A was induced by the presence of 5 – 50 mM calcium chloride in the growth medium (11). Only Kdo₂-lipid A was subjected to mass spectrometry. The analysis of the phosphoethanolamine derivative will be the subject of a future communication. Positive and negative ion mass spectra were obtained using a Qstar XL tandem hybrid quadrupole time-of-flight mass spectrometer (Applied Biosystems) equipped with both electrospray ionization (ESI) and orthogonal matrix-assisted laser-desorption ionization (MALDI) ion sources. For ESI, preparations of lipid A were diluted to 2.5 μM in a mixture of chloroform, methanol, and water (2:3:1) and admitted to the ion source at a flow rate of 5 μL/min. MALDI spectra were obtained using a matrix of 6-aza-2-thiothymine (saturated solution) and 10% ammonium citrate deposited from a 1:1 mixture of acetonitrile and water (11) with irradiation from a nitrogen laser at 337 nm. Samples were prepared by first depositing and air-drying 0.33 μL of the matrix solution followed by an equal volume of the chloroform-methanol-water solution containing approximately 1 nmol of the lipid A

preparation. Negative ion collision induced dissociation of precursor ions was performed using Ar at energies from –60 to –80 V. Spectra were recorded as the average obtained from 30 to 80 laser shots.

RESULTS AND DISCUSSION

Bacterial endotoxin or lipopolysaccharide (LPS) is a complex glycopospholipid substance known to activate immune cells through the Toll-like 4 receptor. The active component of LPS is the so-called lipid-A portion of the molecule (12). MALDI has been employed previously to determine the molecular weight of lipid A (11); however, the emergence of tandem mass spectrometry coupled with MALDI has permitted detailed studies of the decomposition of the molecular ion species generated from lipid A, specifically from a truncated form of LPS termed Kdo₂-lipid A (Scheme 1), which can be purified without hydrolysis from heptose-deficient mutants of *E. coli* (11).

MALDI of a preparation of Kdo₂-lipid A (Figure 1) revealed a monoisotopic molecular weight of 2237.4 Da, although the most abundant negative molecular ion species $[M-H]^-$ was observed at m/z 2237.4. Since the elemental composition of Kdo₂-lipid A is C₁₁₀H₂₀₁N₂O₄₀P₂, the probability of a molecular ion containing all carbon-12, nitrogen-14, oxygen-16, and hydrogen-1 is less probable than that having at least one heavy isotope, most probably carbon-13. It was also clear from the MALDI of this lipid A preparation that multiple molecular species of lipid A were present, in particular ± 14 daltons from the expected molecular weight of the molecule. Clearly, odd chain fatty acyl substituents were present in the lipid A molecule and were biochemically incorporated during the biosynthesis of this molecule by *E. coli*. There were also abundant ions observed at m/z 2017, 1797, and 1586 which were generated during MALDI, and may be the loss of 1 Kdo (m/z 2017) or 2 Kdo (m/z 1799) units but may also be separate lipid A-related species (m/z 1586). Interestingly, electrospray ionization of this same species generated predominantly $[M-2H]^{2-}$ at m/z 1117.5 with substantially less decomposition, suggesting that there were prompt fragmentations of the Kdo-lipid A during MALDI ionization. The origins of these MALDI fragment ions are indicated in the structure inset in Figure 1.

Collision induced decomposition of the $[M-H]^-$ ion from Kdo-lipid A (m/z 2237) revealed quite abundant decomposition ions, again corresponding to the loss of the Kdo sugars. These were observed at m/z 2017.4 and 1797.3 (Figure 1). Since the loss of the Kdo sugar (Scheme 2) was in fact a MALDI generated ion (m/z 1797.3), it was also possible to perform collision induced decomposition of this secondary ion (Figure 2). When this was carried out, a series of quite abundant ions were observed, corresponding to the loss of various ester moieties from lipid A as neutral carboxylic acids. Such losses have been a commonly observed process of fatty acyl substituents of complex lipids (13). This loss of each of the ester side chains made it possible to examine quite precisely each esterified fatty acyl group.

The six fatty acyl chains are linked as two amides and four esters, with the esters distributed so that each of the four acyl groups linked to Kdo₂-lipid A are structurally unique (Scheme 1). One tetradecanoyl ester (A) is linked to a β -hydroxy tetradecanoyl ester, forming a diester complex (G). Collision activation of the lipid A fragment ion (LA) resulted in the ion

at m/z 1568.1 for the loss of ester A and m/z 1341.9 for the loss of the diester complex. A shorter chain docosanoate ester (C) is linked with a β -hydroxyl fatty acyl group, but this β -hydroxy-tetradecanoyl group is linked to the amino nitrogen atom at carbon-2 of one hexose. Loss of this fatty acyl group (C) as a neutral carboxylic acid resulted in the ion at m/z 1596.2. The final ester group (E) is a β -hydroxy tetradecanoyl group esterified to carbon-3 of the second hexose of lipid-A fragment and is not further esterified. Loss of this group as a free acid resulted in the ion observed at m/z 1552.1. Each of these ions corresponding to the loss of a neutral carboxylic acid was accompanied by loss of a further phosphate (H_3PO_4) from one of the two phosphate ester moieties (Figure 2), most likely the anomeric phosphate group.

Since the minor molecular species likely corresponded to esterification of odd chain fatty acids, it was possible to explore whether or not there was a specific site where the odd chain fatty acid was esterified at each of the four ester bonds of lipid A. However, it was clear that this was a random esterification process since many of the losses of the odd chain fatty acids were reflected by minor ions for each of the loss of the free carboxylic acids.

Quantitative analysis

The challenges of quantitative analysis by mass spectrometry are many in that it is difficult to control all of the parameters affecting ion abundance and relate that measurement to the absolute quantity of a particular analyte. Nonetheless, a number of investigators have made the assumption that abundance of an ion, even in a complex mixture is equal to the mole ratio of each component present. There have been numerous examples where this has been shown to be a very dangerous assumption because of the various factors affecting ionization of molecules and subsequent measurement by mass spectrometry. This is certainly the case for electrospray ionization since it is a surface phenomenon where small droplets eventually reach a point in size at which ions are either directly ejected in the ion evaporation mechanism (14) or the residual solvent evaporates leaving the bare ion, which can be analyzed by mass spectrometry (15). Irrespective of the exact mechanism by which ions are formed, it is clear that surface events on and within these droplets can affect the appearance of ions. This is particularly true for species which are surface active and many lipids indeed are surface active agents. Investigators have clearly shown that there is a dependency of ion yield in complex mixtures of lipids based upon structural feature such as the number of double bonds, exact fatty acyl substituents, and the presence of other ionized species such as alkali metals (16,17). However, a major determinant of ion yield can be the type of lipid molecule being analyzed; for example, the class of phospholipid whether it is a glycerophosphocholine or glycerophosphoinositol will greatly influence the formation of both positive and negative ions relative to each other. Furthermore, the concentration of other components present in a complex mixture can greatly affect ion yield for components in that mixture (17).

These concerns have resulted in alternative strategies to quantitate lipids based upon analytical demands of precision and accuracy. Two very different approaches are needed - one approach involves stable isotope dilution quantitation where high precision and accuracy is required, and typically the lipid of interest is a unique species, likely a lipid

mediator that works through a G-protein coupled receptor. A second approach involves a very different class of lipids which are normally quite abundant and exist within cellular membranes as a complex mixture of very closely related species, also termed molecular species. This is certainly the case for glycerolipids, including glycerophospholipids and the neutral lipids triacylglycerols and diacylglycerols. The demand of this latter situation is that there are so many constituents in these mixtures that an internal standard for each component is impossible to implement. Furthermore, since these are typically abundant at relatively high concentrations within a cell, the more important question is whether or not such species change relative to each other. In one sense, any one of the components can serve as the standard since relative changes to one arbitrary component can be observed. Thus, one should be able to assess total quantitation by using a single internal standard. Here the demands of precision and accuracy are substantially less demanding than those for the lipid mediator case. One important strategy which has emerged has been the use of chromatographic separation to substantially decrease the complexity of the mixture, at least at the level of separating individual classes of lipids such as glycerophospholipid classes and neutral lipid classes. For example, when quantitation of a class of phospholipids is a desired target, then class separation could be achieved by normal phase chromatography. Then individual, but class specific internal standards would be co-separated with each of the individual classes.

Another confounding feature that still remains, even within a single lipid class, is that there are often components which differ from each other by the addition of one or more double bonds. This renders the stable isotopic contribution of two daltons $[M+2]$ equal in mass to another, otherwise identical component with one less double bond in a fatty acyl group. This two dalton difference in mass, can confound the analysis especially when the lower mass component is significantly more abundant than the higher mass component (one less double bond) and thus the stable isotope contribution from the lower mass component $[M+2]$ is of the same general order of magnitude or in fact sometimes greater than the abundance of the more saturated analog. This means that it is necessary to correct for stable isotope contribution from the lower mass and correct the abundance of the higher mass entity if one is to obtain a true representation of the components present in the complex mixture. The generally accepted means by which one can correct for this situation is to calculate the naturally occurring stable isotope contributions based upon the abundance of the lower mass ion and subtract that abundance from the observed abundance of the $[M+2]$ ion with the difference assumed to be the true abundance of the more saturated component. Yet there are two concerns with this approach. The first complication is that the total amount of time spent measuring any of the ions in a complex mixture is typically quite short, ranging from typically 5-100 msec, and thus one has limited ion statistics in terms of estimating the true ion signal. This inherent noise in the signal can amplify the error in the isotope calculation and subsequent subtraction. Another confounding feature is that the exact values for naturally occurring isotopes may not truly reflect carbon isotope effects within the biological system. The newly published accepted values for the major stable isotopes of common elements found in biology are listed in Table 1. These values are typically used to calculate isotope abundances using the general strategy based on probability theory and as outlined by Beynon (18). In order to avoid the need to correct for isotope abundances, it is also possible

to separate species that differ by one or more double bonds by chromatography because they have different lipophilicity. Thus, reversed phase HPLC is a powerful technique to significantly reduce this potential problem.

Stable isotope dilution quantitation

Another form of quantitation is to use a stable isotope dilution strategy, which has emerged as one of the most powerful mass spectrometric techniques for quantitative analysis in terms of high precision and accuracy. The general method employed in this approach is to have available a synthetic variant of the lipid of interest which has multiple stable isotopes present so that it has a molecular weight greater than three or more daltons from that of the target lipid. This stable isotope labeled analog is then mixed with the crude biological matrix for which the analyte of interest is to be measured prior to any extraction technique. The tacit assumption, which is in general valid for virtually all isolation and chromatographic techniques, is that during isolation and purification of the endogenous target or [M-H] analyte it is impossible to separate the stable isotope labeled variant. The mass spectrometric measurement involves measuring just two species, the stable isotope labeled variant and the target molecule, either as a single ion such as electrospray or MALDI generated $[M+H]^{+-}$ or ion currents in a tandem mass spectrometric measurement, typically termed multiple reaction monitoring (MRM). This latter approach is more specific for the analyte molecule, but the stable isotope variant must have a different set of ions which are monitored as the quantitative measure. The ratio of the ion abundances observed for the target molecule and the internal standard are then used as a mole ratio measurement of the quantity of analyte. This ratio is then employed to calculate the exact abundance of the analyte molecule after an appropriate calibration curve has been established. In order to generate this standard curve, the primary standard becomes the critical determinant of accuracy of the method and the ability to mix precisely the same amount of stable isotope labeled internal standard to each sample, as well as the standard curve samples, becomes the major determinant of precision. When carried out correctly there is a linear relationship between the ratio of the ion abundances and the quantity of analyte.

There are limits to this linearity of the stable isotope dilution curve if one exceeds the isotopic purity of the internal standard. The ratio of abundances asymptotically approaches the value of the internal standard when it is analyzed at very high dilutions to the primary standard. Alternatively, when there is a very high dilution of the internal standard relative to the primary standard, the ratio of ion abundances asymptotically reaches the naturally occurring stable isotope abundance at the same mass of the internal standard. Of course this latter situation can be minimized by having more stable isotopes in the internal standard in order to increase the difference in mass between the internal standard and the analyte molecule. Since this is not always possible and one must work with one or two dalton difference between the internal standard and the analyte molecule, the linear range of the working curve must be carefully analyzed so that one does not inappropriately dilute the internal standard with too high of a level of analyte molecule.

CONCLUSION

Mass spectrometry will continue to remain as the primary technique to characterize lipids structures and also to carry out quantitative analysis of highly abundant lipids in complex mixtures as well as certain target lipids present in very low concentrations generated by cells. A development of newer techniques of ionization will undoubtedly impact on specific protocols and possibly even improve the throughput by which one can analyze complex mixtures of lipids present within cells or generated following cellular stimulation. An understanding of the lipid components within the cell will greatly enhance our understanding of cellular biology and the integrated nature of proteins, lipids, and genes in biochemical processes.

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Figure 1.

Matrix assisted laser desorption time-of-flight mass spectrometry of Kdo₂-lipid A (negative ions) using 6-aza-2-thiothymine in 10% ammonium citrate as matrix. Mass spectra were obtained in a tandem quadrupole time-of-flight mass spectrometer. Inset figures correspond to an expansion of the mass scales from m/z 2230 – 2550 and m/z 2200 – 2280, respectively.

Figure 2.

Collision induced decomposition of the lipid A fragment ion m/z 1796.3 in a tandem quadrupole time-of-flight mass spectrometer using matrix assisted laser desorption ionization. The product ions correspond to the lipid A portion of the molecule (LA) and the corresponding loss of the fatty acids as free carboxylic acids as indicated in Scheme 2 as the simple fatty acids A, C, and D, and the complex fatty acid G. Other significant ions correspond to the loss of phosphoric acid moieties from the lipid A ion (LA) and individual fatty acyl groups.

Scheme 1.
Kdo₂-Lipid A

m/z 1796.3 (LA)

Scheme 2.

Table 1Current Accepted Exact Mass and Isotopic Abundance Commission on Atomic Weights and Abundances¹

	Exact Mass	Percent Natural Abundance	IUPAC Approval Date
Carbon	12(definition)	98.930%	
	13.003354	1.070%	1995
Hydrogen	1.007825	99.989%	
	2.014101	0.012%	1981
Nitrogen	14.00307	99.636%	
	15.000108	0.364%	1999
Oxygen	15.9949	99.757%	
	16.99913	0.038%	
	17.99916	0.205%	1969
Sodium	22.98976	100.000%	1995
Phosphorous	30.97376	100.000%	1995

¹ De Laeter *et al.* Pure Appl. Chem. 75:683-800 (2003).