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Towards complete structure elucidation of glycerophospholipids in the gas phase through charge inversion ion/ion chemistry

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Abstract

Shotgun lipidomics has recently gained popularity for lipid analysis. Conventionally, shotgun analysis of glycerophospholipids via direct electrospray ionization tandem mass spectrometry (ESI-MS/MS) provides glycerophospholipid (GPL) class (*i.e.*, head group composition) and fatty acyl composition. Reliant on low-energy collision induced dissociation (CID), traditional ESI-MS/MS fails to define fatty acyl regiochemistry along the glycerol backbone or carbon-carbon double bond position(s) in unsaturated fatty acyl substituents. Therefore, isomeric GPLs are often unresolved, representing a significant challenge for shotgun-MS approaches. We developed a top-down shotgun-MS method utilizing gas-phase ion/ion charge inversion chemistry that provides near-complete GPL structural identification. First, in negative ion mode, CID of mass-selected GPL anions generates fatty acyl carboxylate anions via fragmentation of ester bonds linking the fatty acyl substituents at the *sn*-1 and *sn*-2 positions of the glycerol backbone. Product anions, including fatty acyl carboxylate ions, were then derivatized in the mass spectrometer via an ion/ion charge inversion reaction with tris-phenanthroline magnesium dications. Subsequent CID of charge inverted fatty acyl complex cations yielded isomer-specific product ion spectra that permit (i) unambiguous assignment of carbon-carbon double bond position(s) and (ii) relative quantitation of isomeric fatty acyl substituents. The outlined strategy was applied to the analysis of targeted GPL extracted from human plasma, including several proposed plasma biomarkers. A single experiment thus facilitates assignment of the GPL head group, fatty acyl composition, carbon-carbon double bond position(s) in unsaturated fatty acyl chains, and, in some cases, fatty acyl *sn*-position and relative abundances for isomeric fatty acyl substituents. Ultimately, this MS^{*n*} platform paired with ion/ion chemistry permitted identification of major, and some minor, isomeric contributors that are unresolved using conventional ESI-MS/MS.

Graphical Abstract

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Associated Content

Supporting Information

Additional information discussed in the text that support the presentation of the work (PDF)

Introduction

Glycerophospholipids (GPLs) are universal cellular components, accounting for nearly 60 *mol*% of lipid mass in eukaryotic cells.¹ The primary role of GPLs is to define the lipid bilayer (cellular membrane), which encompasses not only individual cells but also intracellular organelles.² While most cellular GPL serve as architectural components of the lipid bilayer, GPLs also function as energy storage and signaling molecules.² The general GPL structure includes three main components: a glycerol backbone, a functionalized phosphate ester group, and fatty acyl (or alkyl ether) chains. The phosphate moiety is esterified at the *sn*-3 position of the glycerol backbone and is also coupled to a polar functional group (*e.g.*, choline, ethanolamine, serine, inositol, or glycerol), commonly referred to as the head group. The most commonly observed GPL structure in eukaryotes is the diacyl (phosphatidyl) subclass wherein fatty acids (FAs) are esterified at the *sn*-1 and *sn*-2 positions of the glycerol backbone. Thus, extensive molecular diversity results from variations both in the head group and the fatty acyl substituents where composition can vary based on chain length, degree of unsaturation, site(s) of unsaturation, and stereochemistry. Considering just the commonly occurring headgroups and a pool of just 40 fatty acids, thousands of theoretical GPL structures can be constructed, including a multitude of isomeric or isobaric molecular structures.³

Mass spectrometry (MS) has proven to be a useful tool for both quantitative and qualitative analysis of lipids, including GPLs. Early MS studies of GPL used soft ionization methods such as fast-atom bombardment (FAB)^{4, 5}, chemical ionization⁶, and field desorption.^{7, 8} However, these ionization methods were largely supplanted by electrospray ionization (ESI).¹ ESI readily generates both the $[M - H]^-$ and $[M + H]^+$ ions for GPL in the negative ion and positive ion modes, respectively. The preference for a GPL to form protonated or deprotonated molecules is dictated by acid-base chemistry (or, in the case of choline, the existing fixed positive charge) of the polar head group, and thus there exists a strong propensity of an individual GPL class to be detected in positive or negative ion mode ESI.^{1, 2, 9} Specifically, anionic GPL including phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatic acid (PA), are readily observed in the negative ion mode as $[M - H]^-$ species.

Conversely, phosphatidylcholines (PC) lipids are neutral polar molecules, possessing a quaternary ammonium moiety that permits efficient ionization of PC in positive ion mode, generating $[M + H]^+$ ions. PC ionization in negative ion mode can also be achieved and relies on the addition of a dopant to the ESI solution to facilitate formation of an adduct ion (*e.g.*, $[M + X]^-$ where $X = Cl$ or CH_3CO_2).

While liquid chromatography (LC) coupled to MS has been a mainstay in lipid analysis, shotgun electrospray ionization tandem mass spectrometry (ESI-MS/MS) has gained popularity due to ease of use, reduced sample volumes, and shorter analysis times.¹ Utilizing ESI-MS/MS, collision-induced dissociation (CID) of GPL ions provides information regarding GPL head group and fatty acyl composition. GPL classes can be individually detected via exploitation of neutral loss and precursor ion scans.¹⁰ Additionally, in negative ion mode, CID of the GPL anion cleaves ester bonds at the *sn*-1 and *sn*-2 positions, liberating fatty acyl chains as abundant carboxylate product ions. The information extracted from such ESI-MS/MS experiments can facilitate assignment of the lipid subclass and acyl chain composition of a given GPL resulting in a so-called molecular lipid assignment (*e.g.*, PC 16:0_18:1).¹¹ While informative, the molecular lipid assignment fails to define both (i) the relative position of acyl chains on the glycerol backbone (*i.e.*, *sn*-position) or (ii) the site(s) of carbon-carbon double bonds in unsaturated acyl chain. The absence of this structural information is problematic for a number of reasons. Firstly, failing to define *sn*- and double bond location masks the possible presence of multiple isomeric contributors and thus fails to represent the natural diversity of given GPL. Secondly, alterations in chemical structure influence both biophysical and biochemical properties of lipids. For example, differences in double bond position(s) in fatty acyl substituents can have profound effects on GPL physical properties such as solubility, fluidity, and thermodynamic stability that have consequences for their biological function(s).^{12–14} Furthermore, a growing body of research suggests that alterations in the relative abundances of lipid isomers – indistinguishable by conventional ESI-MS/MS – could be sensitive markers for the onset and progression of numerous chronic diseases such as type-2 diabetes¹⁵, cardiovascular disease¹⁶, obesity¹⁷, neurodegenerative disease¹⁸, and several types of cancer^{19–23}. Thus, analytical methods capable of providing more complete structural assignments of GPL are integral to furthering current understanding of lipid biochemistry and the roles of lipids in health and disease.²⁴

As carbon-carbon double bond localization in unsaturated GPL cannot be readily achieved with ESI-MS/MS methods relying on traditional, low-energy CID, a number of solution-based derivatization strategies and gas-phase ion-chemistries have been explored to assign sites of unsaturation in GPLs. Pioneered by the Xia group, solution phase Paternò-Büchi (PB) reaction involves derivatization of unsaturated lipids via selective addition of acetone to carbon-carbon double bond(s) (C=C) upon exposure to 254-nm ultraviolet radiation.^{25–28} Low-energy CID of PB-modified lipids generates a pair of diagnostic product ions revealing the location of each double bond. Ultimately, while effective, the PB reaction is reliant on wet-chemical modification of unsaturated lipids and can suffer from low reaction yields. In turn, low derivatization efficiency can lead to partially modified or unreacted lipid species. As an alternate approach, recent work suggests solution-based epoxidation of unsaturated lipids can be achieved with high efficiency.²⁹ CID of the epoxidated lipid ion provides C=C

location(s), yet this approach is largely unexplored compared to its PB counterpart and still reliant on solution-based lipid derivatization.

Based on gas-phase chemistry, ozone-induced dissociation (OzID) has received considerable attention for the structural elucidation of unsaturated lipids.^{30–32} Specifically, OzID is a unique ion-activation method that exploits ion/molecule reactions between mass-selected unsaturated lipid ions and ozone vapor. Often in tandem with low-energy CID, OzID permits the identification of not only sites of unsaturation but also assignment of the *sn*-position of fatty acyl chains in GPLs, and in some instances, double bond geometry (*i.e.*, *E/Z*) has been assigned.³³ Despite a wealth of structural information obtained using OzID, ion-molecule reactions can be slow, due to low reagent molecule densities and/or small rate constants, leading to low product ion abundances and thus longer integration times can be required, impacting on sensitivity. While recent implementations of OzID have exploited higher pressure regions within the mass spectrometer to improve reaction efficiency, ion-ion reactions have potential advantages over ion-molecule chemistries for efficient structural characterization in the gas-phase.^{34, 35}

Recently, we reported the use of gas-phase ion/ion charge inversion reactions for the structural elucidation of fatty acids.^{36, 37} In comparison to solution-based strategies, use of gas-phase ion/ion reactions for lipid analysis presents several advantages. For example, electrospray and solution conditions can be optimized for each reagent, including lipid analytes. Furthermore, gas-phase ion/ion reactions provide the ability to switch between charge states on-demand and undertake facile, highly efficient and structure-selective derivatization. For instance, we have previously reported that singly deprotonated FA anions (*i.e.*, $[FA - H]^-$) react in the gas phase with tris-phenanthroline magnesium dications, $[MgPhen_3]^{2+}$, to form the long-lived electrostatic complex ion composed of the magnesium dication, phenanthroline ligand, and deprotonated fatty acid (*i.e.*, $[FA - H + MgPhen]^+$). Low-energy CID of $[FA - H + MgPhen]^+$ permits unambiguous FA identification and consequent isomer discrimination and relative quantitation.³⁷ Herein, we describe a top-down shotgun lipidomics approach toward in-depth GPL structural elucidation utilizing gas-phase ion/ion charge inversion and derivatization chemistries. To do this experiment, the GPL was first ionized, mass-selected, and collisionally activated in negative ion mode to liberate fatty acyl carboxylate anions (*i.e.*, $[FA - H]^-$) carried by the glycerophospholipid precursor. Subsequent ion/ion reaction between these product anions and the charge inversion reagent dications (*i.e.*, $[MgPhen_3]^{2+}$) resulted in the generation $[FA - H + MgPhen]^+$ ions. Re-isolation and CID of charge inverted FA complex cations yield diagnostic spectra permitting confident assignment of FA double bond position(s) and calculation of relative composition of isomeric FA mixtures. In total, this experiment provides near-complete structural information on the initial mass-selected GPL including information head group assignment, fatty acyl composition, site(s) of unsaturation, and, in some cases, relative abundances of isomeric fatty acyl substituents. Application of the developed ion/ion method to the analysis of targeted GPLs in human plasma extracts reveals the presence of isomeric GPLs that would be unresolved by conventional ESI-MS/MS platforms.

Experimental

Materials

HPLC-grade methanol, water, and chloroform were purchased from Fisher Scientific (Pittsburgh, PA). Magnesium chloride, ammonium acetate, 1,10-phenanthroline (Phen), and citrated human blood plasma were purchased from Sigma-Aldrich (St. Louis, MO). All lipids standards were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL).

Lipid Extraction and Preparation of nESI Solutions

Solutions of lipid standards were prepared in methanol to a final concentration of 5 μM . Magnesium chloride and 1,10-phenanthroline were combined in methanolic solution to a final concentration of 20 μM .

Lipids were extracted from human plasma according to the Folch method.³⁸ Briefly, 50 μL of human plasma was added to 375 μL of methanol and vortexed for 30 s. Then, 750 μL of chloroform was added to the mixture. The resulting mixture was incubated for 1 h at room temperature in a shaker. Next, 312.5 μL of water was added induce phase separation. After sitting for 10 min at room temperature, the mixture was centrifuged at 1000 g for 10 min. Following centrifugation, the chloroform (lower) layer was collected and subsequently dried in a vacuum centrifuge. The dried organic phase was reconstituted in 1000 μL of methanol with 10 mM ammonium acetate prior to MS analysis.

Nomenclature

When possible, we adopt the shorthand notation recommended by Liebisch *et al.*¹¹ Briefly, GPL classes are abbreviated as follows: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidic acid (PA). Fatty acyl substituents are described by the total number of carbons, as indicated before the colon, the total number of double bonds, as indicated after the colon, and identified double bond position(s), as indicated within parentheses (*i.e.*, 18:1(9) represents 18-carbon chain with 1 double bond between carbon-9 and -10 as numbered from the carboxylate moiety). If geometry is known, the double bond positional number is followed by *Z* for *cis* or *E* for *trans*, *e.g.*, 18:1(9*Z*). Where regiochemistry of fatty acyl chains is identified, the fatty acyl substituents reported before and after a forward-slash (*i.e.*, PC 16:0/18:1) represent the fatty acids esterified to the *sn*-1 and *sn*-2 position of the glycerol backbone respectively. However, if regiochemistry is unknown, the fatty acyl substituents are separated by an underscore (*i.e.*, PC 16:0_18:1).

Mass Spectrometry

All data were collected on a Sciex QTRAP 4000 hybrid triple quadrupole/linear ion trap mass spectrometer (SCIEX, Concord, ON, Canada) with modifications analogous to those previously described.³⁹ Alternately pulsed nano-electrospray ionization (nESI) allows for sequential injection of lipid anions and tris-phenanthroline magnesium dications, $[\text{Mg}(\text{Phen})_3]^{2+}$.⁴⁰ First, the lipid anion was generated in negative ion mode via nESI. The lipid anion was then isolated during transit through Q1 and sent to the high-pressure collision cell, q2, for storage. In q2, ion-trap CID of the lipid anion generated the fatty acyl

anions, denoted $[FA - H]^-$. Next, direct positive nESI was used to generate $[Mg(Phen)_3]^{2+}$. Following ionization, $[Mg(Phen)_3]^{2+}$ was mass-selected in Q1 and transferred to q2 for storage. In q2, product ions resulting from collisional activation of the lipid anion, including $[FA - H]^-$ anions, and $[Mg(Phen)_3]^{2+}$ dications were simultaneously stored for 500 ms. The ion/ion reaction produced charge-inverted product ions. Next, beam-type CID was used to simultaneously collisionally activate product ions and transfer them to the low-pressure linear ion trap (LIT), Q3. Charge inverted FA complex cations (*i.e.*, $[FA - H + MgPhen]^+$) were then mass-selected using a 1 Th window and subjected to collisional activation via single frequency resonance excitation ($q = 0.383$). Product ions generated from CID were analyzed via mass-selective axial ejection (MSAE).⁴¹

Results

Generation of $[FA - H + MgPhen]^+$ complex cations from GPL anions

Scheme 1 illustrates the generation of $[FA - H + MgPhen]^+$ complex cations from the gas-phase ion/ion reaction between reagent dications and fatty acyl carboxylate anions liberated from a GPL precursor anion. In negative ion mode, GPLs are first ionized via direct negative nESI, generating abundant $[M - H]^-$ for the classes PE, PS, PI, PG, and PA. Due to the quaternary ammonium moiety of PC, ionization of PC lipids in the negative ion mode required generation of the chloride or acetate adduct ion (*i.e.*, $[PC + X]^-$ where $X = Cl$ or CH_3CO_2). Next, the GPL anion was mass-selected during transit through Q1 and then transferred to the high-pressure collision cell q2. Once in q2, single frequency resonance excitation was used to collisionally activate the lipid precursor anion. Ion-trap CID of the mass-selected $[M - H]^-$ ion for unsaturated PE, PI, PG, and PA synthetic standards directly generated $[FA - H]^-$ anions via cleavage of the ester bonds at the *sn*-1 and *sn*-2 positions. Consistent with previous reports,^{42–47} additional fragment ions reflecting neutral losses of the *sn*-1 and *sn*-2 substituents as acids and ketenes were also observed. To release FA anions from PC and PS lipid precursor anions, two q2 CID steps were required. Specifically, CID of $[PC + X]^-$ ($X = Cl$ or CH_3CO_2) generated the demethylated PC anion (*i.e.*, $[PC - CH_3]^-$), while activation of $[PS - H]^-$ produced a fragment ion derived from neutral loss of the serine head group (loss of $C_4H_5NO_2$, 87 Da). Subsequent ion-trap CID of the $[PC - CH_3]^-$ and $[PS - H - C_4H_5NO_2]^-$ ions resulted in cleavage of the *sn*-1 and *sn*-2 ester bonds and generation of $[FA - H]^-$ anions.

To generate charge inverted FA complex cations, all fragment ions, including $[FA - H]^-$ ions, were allowed to react with the tris-phenanthroline magnesium dications. The $[FA - H]^-$ anions are transformed in the gas-phase to $[FA - H + MgPhen_2]^+$ by ion/ion reaction, as previously described.^{36, 37} All ion/ion product ions were then activated via beam-type CID (BT CID). BT CID resulted mostly in the formation of $[FA - H + MgPhen]^+$ ions and, ultimately, fatty acyl double bond positions can be pinpointed from $[FA - H + MgPhen]^+$ CID spectral matching to a previously constructed FA library.³⁷

Identification of double bond position(s) in synthetic GPL

We first demonstrate the overall approach using synthetic PE 16:0/18:1(9*Z*). Direct negative ion nESI of PE 16:0/18:1(9*Z*) generated abundant $[PE - H]^-$ anions at *m/z* 716.5 (Figure

1a). To probe fatty acyl composition of this reference compound, ion-trap CID of $[\text{PE} - \text{H}]^-$ was employed. The MS/MS spectrum of PE 16:0/18:1(9Z), as shown in Figure 1c, displays structurally informative product ions relating to the fatty acyl substituents. Consistent with previous reports⁴², product ions arising from losses of both fatty acyl substituents were observed. For example, the product ions observed at m/z 478.3 and m/z 452.3 reflect losses of the *sn*-1 (*i.e.*, $[\text{PE} - \text{H} - \text{R}_1'\text{CH}=\text{C}=\text{O}]^-$) and *sn*-2 (*i.e.*, $[\text{PE} - \text{H} - \text{R}_2'\text{CH}=\text{C}=\text{O}]^-$) acyl chains as ketenes, whereas those observed at m/z 460.3 and m/z 434.3 represent losses of the *sn*-1 and *sn*-2 acyl chains as fatty acids, denoted $[\text{PE} - \text{H} - \text{R}_1\text{COOH}]^-$ and $[\text{PE} - \text{H} - \text{R}_2\text{COOH}]^-$, respectively (Figure 1c). Dominating the CID spectrum are the $[\text{16:0} - \text{H}]^-$ (m/z 255.2) and $[\text{18:1} - \text{H}]^-$ (m/z 281.2) carboxylate fragment anions reflective of the fatty acyl substituents carried by the PE precursor ion at the *sn*-1 and *sn*-2 positions, respectively. The greater abundance of the $[\text{18:1} - \text{H}]^-$ fragment ion relative to the $[\text{16:0} - \text{H}]^-$ ion is in good agreement with previous observations,⁴² suggesting the formation of the carboxylate anion from the *sn*-2 acyl substituent. It is important to note that for unknown lipids the relative abundances of the carboxylate anions alone (or related ketene and fatty acid loss fragments) are insufficient to quantitatively assign fatty acyl regiochemistry (*i.e.*, *sn*-1 or *sn*-2) without calibration to standards. It can, however, provide a useful guide as to the dominant regiochemistry (*i.e.*, the most abundant regioisomer).⁴⁸

The charge inversion process begins with the product ions generated from CID of $[\text{PE} - \text{H}]^-$ (shown in Figure 1c). All fragment ions generated via activation of the deprotonated PE anion (Figure 1c) were subjected to ion/ion reaction with the $[\text{MgPhen}_3]^{2+}$ dications. Charge inversion proceeds via the formation of long-lived electrostatic complexes comprised of the anion, two phenanthroline ligands, and the magnesium metal dication. The product ion spectrum resulting from the ion/ion reaction between the fragment ions of $[\text{PE} - \text{H}]^-$ and $[\text{MgPhen}_3]^{2+}$ is shown in Figure 1d. The dominant mutual storage product ions observed are the charge-inverted FA cations $[\text{16:0} - \text{H} + \text{MgPhen}_2]^+$ (m/z 639.3) and $[\text{18:1(9Z)} - \text{H} + \text{MgPhen}_2]^+$ (m/z 665.4). Notably, the relative abundances of the FA anions (*i.e.*, $[\text{FA} - \text{H}]^-$ from Figure 1c) and the charge-inverted FA cations (*i.e.*, $[\text{FA} - \text{H} + \text{MgPhen}_2]^+$ from Figure 1d) were conserved. More so, FA anion charge inversion appears to be nearly unit efficient, as there was no evidence for FA anion neutralization via proton-transfer reaction with tris-phenanthroline magnesium complex cations.³⁶ Charge inverted product ions reflecting the loss of the *sn*-1 fatty acyl as a fatty acid (*i.e.*, $[\text{PE} - \text{H} - \text{R}_1\text{COOH} + \text{MgPhen}_2]^+$) and a ketene (*i.e.*, $[\text{PE} - \text{H} - \text{R}_1'\text{CH}=\text{C}=\text{O} + \text{MgPhen}_2]^+$) were also observed at m/z 844.4 and m/z 862.4, respectively. Similarly, the product ions representing *sn*-2 fatty acyl loss as both a fatty acid, $[\text{PE} - \text{H} - \text{R}_2\text{COOH} + \text{MgPhen}_2]^+$ (m/z 818.4), and ketene, $[\text{PE} - \text{H} - \text{R}_2'\text{CH}=\text{C}=\text{O} + \text{MgPhen}_2]^+$ (m/z 836.4), were also charge-inverted via ion/ion reaction with $[\text{MgPhen}_3]^{2+}$ dications. Following the ion/ion reaction, BT CID was then employed yielding the spectrum shown in Figure 1e. The dominant product ions observed in Figure 1e are $[\text{18:1(9Z)} - \text{H} + \text{MgPhen}]^+$ (m/z 485.3) and $[\text{16:0} - \text{H} + \text{MgPhen}]^+$ (m/z 459.3), generated via the neutral loss of a phenanthroline ligand from each of the analogous charge-inverted FA cations observed in Figure 1d. Also depicted in Figure 1e are a variety of low-abundance product ions reflecting neutral losses of the ethanolamine head group and a phenanthroline ligand from their respective charge-inverted mutual storage product ions. Specifically, these fragment ions were assigned as $[\text{PE} - \text{H} - \text{R}_1'\text{CH}=\text{C}=\text{O} - \text{CH}_2\text{CH}_2\text{NH} +$

MgPhen]⁺ (*m/z* 639.3), [PE – H – R₁COOH – CH₂CH₂NH + MgPhen]⁺ (*m/z* 621.3), [PE – H – R₂'CH=C=O CH₂CH₂NH + MgPhen]⁺ (*m/z* 613.3), and [PE – H – R₂COOH – CH₂CH₂NH + MgPhen]⁺ (*m/z* 595.3). Lastly, to assign double bond position unambiguously, the desired [FA – H + MgPhen]⁺ ion was mass-selected with unit resolution and activated via ion-trap CID. In the case of PE 16:0/18:1(9Z), the C9=C10 double bond position was confirmed via observation of a the spectral gap (highlighted in green shading) in the resulting CID product ion spectrum of [18:1(9Z) – H + MgPhen]⁺ (Figure 1f).³⁶ Briefly, the spectral gap shown in Figure 1f results from dramatic suppression in the abundance of product ions arising from carbon-carbon cleavages vinylic to the double bond (*i.e.*, *m/z* 345.2 and *m/z* 371.2) and fragmentation of the C9=C10 double bond (*i.e.*, *m/z* 357.2). More so, perturbation of the 14 Da spacing, further confirms the C9=C10 double bond position, as the differential between *m/z* 345.2 and *m/z* 357.2 is 12 Da.

In total, the workflow first relies on ionization and collisional activation of the GPL precursor ion in negative ion mode to release fatty acyl substituents as carboxylate anions. As all GPL classes can be ionized in negative ion mode, fatty acyl anions can be liberated from any GPL precursor anion regardless of head group composition using low-energy CID. Thus, gas-phase ion/ion chemistry can be applied to assign GPL structure, including double bond position(s), independent of the polar head group present. To demonstrate, this method was applied to analyze the following synthetic GPL standards of varying head group and fatty acyl composition: PE 18:0/20:4(5Z,8Z,11Z,14Z), PC 16:0/18:1(9Z), PS 16:0/18:1(9Z), PI 16:0/18:1(9Z), PG 16:0/18:1(9Z), and PA 16:0/18:1(9Z). Data for these experiments are shown in Supporting Information Figures S1 – S8. As previously noted^{42–47}, each GPL class (*i.e.*, PE, PS, PC, *etc.*) exhibits some class-specific fragmentation behavior upon collisional activation, and charge inversion behavior is consequently influenced. That said, all GPL classes yield abundant fatty acyl anions, and thus other variations in CID behavior do not interfere with the ability to confidently pinpoint carbon-carbon double bond position(s) across all species. Indeed, the final CID mass spectra of [18:1(9Z) – H + MgPhen]⁺ derived by ion/ion chemistries from PC, PS, PI, PG, and PA complex lipids are identical and can further be matched explicitly to reference spectra previously generated from unesterified oleic acid.

Identification of human plasma lipids

Human blood plasma is an exceedingly complex biological fluid rich in lipids, as more than 1500 lipids have been putatively identified.^{49, 50} Recently, characterization of the human blood plasma lipidome has received much attention, as changes in the plasma lipidome have been widely studied with hopes of identifying potential disease biomarkers. Specifically, plasma lipid biomarkers have been proposed for a variety of disease pathologies including prostate cancer, Alzheimer's disease, cardiovascular disease, and many others.⁵¹ However, many of these potential plasma lipid biomarkers have only been identified at the sum compositional level; neglecting to clearly differentiate amongst potential isomeric structures that likely exist. To demonstrate the utility of gas-phase ion/ion chemistry for the structural characterization of GPL, we applied this approach to examine GPL extracted from human plasma. Results obtained from the analysis of human plasma extract via direct negative ionization are shown in Figure 2. As mentioned above, precursor ion or neutral loss scanning

can be used to detect individual GPL classes at the sum compositional level exploiting class-specific fragmentations.¹ For example, in positive ion mode, PCs were identified using precursor ion scanning of m/z 184 (Figure S9), while neutral loss scanning of 141 Da detected PEs (Figure S10). Information from these scan modalities can be compiled to generate targeted lists of GPL, which in turn, can be used to guide subsequent structural interrogation via ion/ion chemistry. Herein, we report the detailed structural identification of three GPL extracted from human plasma. Explicitly, PC 34:2, PI 38:4, and PE 36:2 were investigated using the gas-phase charge inversion MSⁿ process. Table 1 summarizes the structural identities of targeted GPL confirmed with ion/ion chemistry.

Upon direct negative mode ionization of the human plasma extract, the most abundant GPL-related anions were found to be derived from PC 34:2. Using the gas-phase workflow described here, PC 34:2 was found to be composed of at least two isomers. Following mass-selection of the [PC 34:2 + OAc][−] anion (m/z 816.6), collisional activation via ion-trap CID generated [PC 34:2 − CH₃][−] (m/z 742.5) (Figure S11). Subsequent CID of the demethylated PC 34:2 anion is illustrated in Figure 3a and reveals the presence of at least two isomeric species. From Figure 3a, the dominant product ions observed at m/z 255.2 and m/z 279.2 reflect the 16:0 and 18:2 fatty acyl carboxylate anions, respectively. As a result, PC 16:0_18:2 was found to be the major contributor to the PC 34:2 sum composition. The product ion observed at m/z 480.3 was generated by the neutral loss of the 18:2 fatty acyl as a ketene from the demethylated PC 34:2 anion (*i.e.*, [PC 34:2 − CH₃ − C₁₆H₂₉CH=C=O][−]). Importantly, this product ion is significantly more abundant than the corresponding [PC 34:2 − CH₃ − C₁₄H₂₇CH=C=O][−] ion at m/z 504.3 (see inset Figure S12) indicating that the dominant regiochemistry can be assigned as PC 16:0/18:2. This assignment is consistent with a recent study of human plasma that found the ratio of PC 16:0/18:2 to PC 18:2/16:0 to be 89 : 11.⁵² The [16:1 − H][−] (m/z 253.2) and [18:1 − H][−] (m/z 281.2) anions were also observed at very low abundances, signifying the presence of PC 16:1_18:1 as the minor isomeric constituent (Figure S12).

To pinpoint double bond position(s) in unsaturated fatty acyl substituents, all product ions generated via CID of the [PC 34:2 − CH₃][−] anion (Figure 3a) were subjected to reaction with [MgPhen₃]²⁺ dications. The dominant product ions observed were the charge-inverted fatty acyl anions, denoted [18:2 − H + MgPhen₂]⁺ (m/z 663.3) and [16:0 − H + MgPhen₂]⁺ (m/z 639.3), as shown in Figure 3b. Additional product ions observed at m/z 864.4 and m/z 1126.8 represent [PC 34:2 − CH₃ − C₁₆H₂₉CH=C=O + MgPhen₂]⁺ and [PC 34:2 − CH₃ + MgPhen₂]⁺, respectively. Beam-type CID was then employed to generate the [18:2 − H + MgPhen]⁺ (m/z 483.3) and [16:0 − H + MgPhen]⁺ (m/z 459.3) complex cations (Figure 3c). Subsequent unit mass-selection and ion-trap CID of the [18:2 − H + MgPhen]⁺ ion at m/z 483.3 provided identification of double bond positions for the 18:2 fatty acyl, as highlighted in Figure 3d. The 18:2 fatty acyl was identified based on *de novo* spectral analysis and comparison to a reference spectrum derived from linoleic acid as 18:2(9,12). In turn, the major isomeric component of PC 34:2 was identified as PC 16:0/18:2(9,12). Note that the [18:1 − H + MgPhen]⁺ (m/z 485.3) and [16:1 − H + MgPhen]⁺ (m/z 457.3) complex cations were also generated, but the relative abundances of these product ions were extremely small, prohibiting further interrogation via CID and identification of unsaturation sites. In sum, our

assignment of the dominant isomeric of PC 34:2 in plasma is in good agreement with previous reports of both *sn*-positional and carbon-carbon double bond assignments.^{35, 52}

As mentioned above, the human plasma lipidome has been widely investigated with hopes of identifying lipid biomarkers for a multitude of disease pathologies.⁵¹ Thus, as additional examples, we chose to investigate two GPL proposed as plasma biomarkers. First, serum-based PIs have been reported to have a significant association with bipolar disorder (BD).⁵³ Specifically, alterations in PI levels have been observed in patients exhibiting BD when compared to non-diseased individuals. In our study, we identified the double bond positions in plasma PI 38:4 as PI 18:0_20:4(5,8,11,14) using the gas-phase ion/ion procedure described above (Figure S13). Furthermore, the relative abundance of the $[\text{PI } 38:4 - \text{H} - 20:4]^-$ ion at m/z 581.3 in the negative ion CID (Figure S13a) indicates the dominant regioisomer to be the canonical PI 18:0/20:4(5,8,11,14), noting that contributions from the alternate *sn*-isomer are not excluded based upon these data. Based on this single experiment, PI 18:0/20:4(5,8,11,14) appears to be the only major contributor to PI 38:4.

In an additional example, we interrogated plasma-based PE 36:2. Recently, PE 36:2 has been recognized as a potential biomarker for lung cancer.⁵⁴ Using the ion/ion reaction approach, we identified numerous isomeric contributors for PE 36:2. CID of the deprotonated PE 36:2 anion (*i.e.*, $[\text{PE } 36:2 - \text{H}]^-$) at m/z 742.6) exposed the presence of at least three isomeric lipids (Figure 4a). PE 18:0_18:2 comprises as the major fatty acyl contributor to PE 36:2, as indicated by the dominant 18:0 and 18:2 fatty acyl carboxylate anions observed at m/z 283.2 and m/z 279.2. Furthermore, the fragment ion observed at m/z 480.3 was generated via the neutral loss of the 18:2 fatty acyl as a ketene from the deprotonated PE 36:2 anion. Two additional minor isomeric contributors were also identified. Explicitly, the $[\text{18:1} - \text{H}]^-$ (m/z 281.2) fatty acyl anion can be attributed to PE 18:1_18:1, while the $[\text{16:0} - \text{H}]^-$ (m/z 255.2) and $[\text{20:2} - \text{H}]^-$ (m/z 307.3) carboxylate anions indicate PE 16:0_20:2. Sites of unsaturation were identified using gas-phase ion/ion chemistry, and results of the ion/ion reaction and subsequent BT CID are displayed in Figure 4b. Using mass-selection and CID of the $[\text{18:2} - \text{H} + \text{MgPhen}]^+$ cation (m/z 483.3), as detailed in Figure 4c, we confidently determine the structure of the major isomeric contributor to be PE 18:0_18:2(9,12). Upon interrogation of the $[\text{18:1} - \text{H} + \text{MgPhen}]^+$ complex cation via ion-trap CID, the 18:1 fatty acyl was found to be an isomeric mixture of 18:1(11) and 18:1(9) (Figure 4d). From Figure 4d, the product ions denoted in red indicate the 18:1(9) isomer, while those depicted in blue signify the 18:1(11) isomer. Exploiting the previously developed multiple linear regressions approach in conjunction with library spectra generated from *cis*-vaccenic (18:1(11*Z*)) and oleic (18:1(9*Z*)) acids³⁷, relative abundances of the 18:1(9) and 18:1(11) fatty acyls were calculated to be 92 ± 2 and 8 ± 2 , respectively. It is important to note that we cannot distinguish amongst isomeric contributors such as PE 18:1(9)_18:1(9), PE 18:1(9)_18:1(11), or PE 18:1(11)_18:1(11) using the ion/ion method presented here. Furthermore, while we can identify the presence of PE 16:0_20:2 in human plasma, we were unable to localize double bond positions in the 20:2 fatty acyl substituent, as the charge-inverted 20:2 fatty acyl complex cation was too low in abundance for subsequent interrogation. Based on these results, it is clear that plasma biomarkers identified only at the sum compositional level can exist as isomeric mixtures, suggesting a need for isomeric discrimination biomarker discovery research.

Conclusions

In this work, we demonstrated a top-down shotgun lipidomics approach employing gas-phase charge inversion ion/ion chemistry that provides detailed structural characterization of glycerophospholipids. The glycerophospholipid, originating either from an authentic standard or lipid extract, is first ionized and mass-selected in the negative ion mode. Subsequent collisional activation of the isolated lipid anion liberates fatty acyl carboxylate anions carried by the GPL precursor at the *sn*-1 and *sn*-2 positions. Following generation of $[FA - H]^-$ anions from the GPL precursor, fatty acyl anions are transformed in the gas-phase to $[FA - H + MgPhen]^+$ complex cations via ion/ion reaction with $[MgPhen_3]^{2+}$ dications. These charge-inverted FA complex cations can be individually mass-selected and collisionally activated to produce product ion spectra that enable confident identification of double bond positions(s) and calculation of relative compositions of isomeric contributors. In total, charge inversion ion/ion chemistry offers a sensitive, rapid, and entirely gas-phase MSⁿ process for detailed structural elucidation of GPL. Key advantages of this strategy include (i) the ability to use spectral libraries based upon readily-available fatty acid standards to characterize and quantitate the fatty acyl chains present in GPLs where standards are rare and expensive; and (ii) the ability to assign - in some instances - *sn*-position and double bond position. The latter is particularly significant as it represents a key step toward complete structure elucidation of GPLs in complex mixtures. As demonstrated with the analysis of three GPLs found in human plasma, the incorporation of ion/ion chemistry enables the identification of major, and some minor, isomeric contributors that are unresolved using conventional ESI-MS/MS experiments. Furthermore, these results indicate that a previously suggested plasma biomarker for lung cancer exists as a mixture of isomeric components, indicating the possible need for isomer differentiation during biomarker discovery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Sequence of MS events used to structurally interrogate synthetic PE 16:0/18:1(9Z). (a) Structure of ionized PE 16:0/18:1(9Z) (*i.e.*, [PE – H][–]). The blue and red font indicate the *sn*-1 and *sn*-2 fatty acyls respectively, (b) Reaction scheme detailing identification procedure for the PE 16:0/18:1(9Z) anion, (c) Ion-trap CID spectrum of [PE – H][–] (*m/z* 716.5). (d) Charge inversion spectrum generation via the gas-phase ion/ion reaction between product ions generated from CID of the [PE – H][–] precursor ion as shown in (c) and [MgPhen₃]²⁺ dications, (e) Subsequent beam-type (BT) CID spectrum generated post-ion/ion reaction, (f) Ion-trap CID product ion spectrum following monoisotopic mass-selection and collisional activation of [18:1(9Z) – H + MgPhen]⁺ (*m/z* 485.3). Note that circles (●/○) indicate positive ion mode analysis, while squares (■/□) indicate negative ion mode analysis.

Figure 2.
Direct infusion negative ion mode nESI mass spectrum of human plasma extract.

Figure 3.

Demonstration of gas-phase charge inversion ion/ion chemistry for the analysis of PC 34:2 in human plasma extract. (a) Ion-trap CID spectrum resulting from activation of $[PC\ 34:2 - CH_3]^-$. (b) Product ion spectrum following ion/ion reaction of fragment ions generated via activation of $[PC\ 34:2 - CH_3]^-$ and $[MgPhen_3]^{2+}$ dications. (c) Beam type CID product ion spectrum post-ion/ion reaction. (d) CID spectrum of $[18:2 - H + MgPhen]^+$.

Figure 4.

Demonstration of gas-phase charge inversion ion/ion chemistry for the analysis of PE 36:2 in human plasma extract, (a) Ion-trap CID spectrum resulting from activation of [PE 36:2 - H]⁻. (b) Product ion spectrum following ion/ion reaction of fragment ions generated via activation of [PE 36:2 - H]⁻ and [MgPhen₃]²⁺ dications and subsequent beam type CID. (c) CID spectrum of [18:2 - H + MgPhen]⁺. (d) CID spectrum of [18:1 - H + MgPhen]⁺.

Scheme 1.

Generation of charge inverted FA complex cations, $[FA - H + MgPhen]^+$, derived directly from GPL anion in the gas-phase via ion/ion reaction between $[FA - H]^-$ anions released from a GPL precursor ion and $[MgPhen_3]^{2+}$ reagent dications.

Table 1.

Summary of lipids identified in human plasma extract using gas-phase charge inversion ion/ion chemistry.

GPL Sum Composition	Precursor Ion (<i>m/z</i>)	Fragment Ion (<i>m/z</i>)	GPL Structure
PC 36:2	816.6	255.2, 279.2	PC 16:0_18:2(9,12) **
		253.2 *, 281.2 *	PC 16:1_18:1
PI 38:4	885.6	283.2, 303.2	PI 18:0_20:4(5,8,11,14)
PE 36:2	742.6	283.2, 279.2	PE 18:0_18:2(9,12)
		281.2	PE 18:1_18:1
			18:1(9) = 92 ± 2
			18:1(11) = 8 ± 2
		255.2 *, 307.3 *	PE 16:0_20:2

* Unable to identify double bond position(s) via charge inversion ion/ion chemistry due to low ion abundance

** Major isomer PC 16:0/18:2(9,12) – see text for discussion