Identification of Ethanolamine Plasmalogens from Complex Lipid Mixtures by MS/MS and Ag Adduction

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A neutral loss scan of 141, corresponding to a phosphoethanolamine head group, has been commonly used for the determination of various glycerophosphoethanolamine species in complex lipid mixtures. However, the neutral loss of 141 Da is not a major fragmentation pathway in the collision-induced dissociation (CID) of ethanolamine plasmalogens in the positive-ion mode. Thus, the use of the neutral loss scan of 141 can be problematic to observe all possible ethanolamine phospholipids in biological samples. Ethanolamine plasmalogens could easily form adducts with Ag(I) ions, and the CID of Ag(I)-adducted ethanolamine plasmalogens provided abundant head group loss of 141 with higher collision energy. Thus, all ethanolamine plasmalogens could be identified from a neutral loss scan of 141 after Ag(I) adduction. This novel approach could be a useful diagnostic tool to observe most of the possible glycerophosphoethanolamine species in complex lipid mixtures.

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Introduction

Glycerophosphoethanolamines are a class of phospholipids that have an ethanolamine head group. The neutral loss of 141 Da has been used as a diagnostic tool for the determination of glycerophosphoethanolamine species in complex lipid mixtures. However, ethanolamine plasmalogens do not provide abundant neutral loss of phosphoethanolamine (141 Da), so a new approach is necessary for the determination of all possible glycerophosphoethanolamines from complex biological samples. Plasmalogen is a type of ether phospholipid that contains an O-alk-1'-enyl residue attached to the glycerol moiety at sn-1 position. In mammals, the sn-1 position is typically derived from C16:0, C18:0 or C18:1 vinyl ether moieties.¹ Plasmalogens are commonly found in animal tissues, and they contain mostly ethanolamine or choline as their head groups. The vinyl ether double bond of plasmalogens is targeted by various oxidants, such as peroxy radicals and metal ions.² Thus, the oxidation of the vinyl ether may prevent the oxidation of highly unsaturated fatty acids, and was suggested to protect against the oxidation of other lipids, including cholesterol.³⁻⁵ The peroxidation of lipids has been known to play a key role in the pathogenesis of Alzheimer's disease, diabetes, atherosclerosis, and many other diseases. In Alzheimer's disease, a decreased amount of plasmalogens is associated with increased lipid peroxidation.⁶⁻⁸ Therefore, determining the ethanolamine plasmalogen levels would be interesting to explore beneficial effects of plasmalogens, and should be monitored along with other glycerophosphoethanolamine species.

The analysis of plasmalogens follows the general method for phospholipid analysis. Plasmalogen analysis conventionally

relies on thin-layer chromatography (TLC) to separate and identify the various phospholipid classes, with relatively nonspecific detection techniques, such as UV detection.⁹ The vinyl ether bond at the sn-1 position of plasmalogens is labile to acid-catalyzed hydrolysis, which results in a lysophospholipid and a fatty aldehyde. The analysis of the fatty aldehyde typically involves scraping of the TLC spots, and derivatization for gas chromatography (GC) or gas chromatography-mass spectrometry (GC-MS) to identify specific fatty aldehydes in each phospholipid class. Another method is to use enzymes, such as phospholipase C, to remove the polar moiety of plasmalogens and to identify alkenyl-acyl glycerols by high-performance liquid chromatography (HPLC) or by GC after derivatization.¹⁰

Mass spectrometry (MS) becomes a powerful tool for the identification and structural characterization of phospholipids, and is often combined with HPLC or GC to enhance the selectivity and sensitivity of the analysis.¹¹⁻¹⁴ Liquid chromatography-mass spectrometry (LC-MS) can directly analyze various lipids as intact molecules, and preserve the information inherent in their chemical structures.^{4,15} Liquid chromatography-tandem mass spectrometry (LC-MS/MS) provides structural information of phospholipids from their fragmentation patterns.^{16,17} Collision-induced dissociation (CID) is the most commonly used tandem mass spectrometric technique. The CID of the $[M+H]^+$ or $[M-H]^-$ of phospholipids results in fragment ions related to a polar head group, a fatty acyl and vinyl ether group, which enables structural analysis of phospholipids including plasmalogens.¹⁸⁻²⁰ One of the principal fragmentation pathways during CID in the positive-ion mode is cleavage of the phosphate-glycerol bond, which leads to the elimination of the polar head group as a neutral or charged species. These characteristic fragmentation patterns allow us to determine the phospholipid class using a precursor ion or a neutral loss scan.

Ethanolamine plasmalogens could constitute more than half of

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the ethanolamine phospholipid pool depending on organs.²¹ Neutral loss scan of 141, corresponding to the phosphoethanolamine head group, has been commonly used for the determination of various glycerophosphoethanolamine species (such as diacyl or 1-O-alkyl-2-acyl) in complex lipid mixtures.²² However, the neutral loss of 141 Da is not a major fragmentation pathway in the CID of ethanolamine plasmalogens.^{3,23} Thus, most of the ethanolamine plasmalogens could not be observed with the neutral loss scan of 141. The two major fragment ions in the CID of $[M+H]^+$ of ethanolamine plasmalogens are ions involving both of the head group loss (141 Da) and cleavage at the sn-1 position, so their m/z provides information about the vinyl ether residue attached to the sn-1 position. For example, each CID spectrum of ethanolamine plasmalogens, containing C16:0, C18:0, and C18:1 at the sn-1 position, provides a major fragment ion of m/z 364, 392, or 390, respectively; another major peak is the complementary ion to each of these. On the other hand, the ion abundance corresponding to the neutral loss of 141 Da is rather small, and the neutral loss scan of 141 could not observe all possible ethanolamine plasmalogens from biological samples.^{3,23} Thus, several precursor ion scans (*e.g.* precursor ion of 364, 392 or 390) may be needed to find all ethanolamine plasmalogens in biological samples, which consume more time, labor, and materials.

Metal ions, such as Li(I) or Ag(I), have been used to form metal-phospholipid adducts for the structural determination of glycerophosphoethanolamine species.^{2,16} CID of alkali metal (*e.g.* Li(I))-adducted ethanolamine plasmalogens resulted in abundant product ions from cleavage at the sn-1 position and the sn-2 position in the positive-ion mode, along with the small neutral loss peak (–141 Da).^{1,2} Ag(I) has been used to reveal structural information of several phospholipids, but not plasmalogens. Ag(I) adduction enabled a complete structural characterization of phosphatidylethanolamine; thus, information about the phospholipid class and fatty acyl chains could be obtained from the CID of Ag(I)-adducted phospholipid in the positive-ion mode.²⁴

The neutral loss scan of 141 from protonated or Li(I)-adducted plasmalogens cannot observe ethanolamine plasmalogens in complex lipid mixtures, due to its low abundances in the positive-ion mode.^{1–3,23} Thus, the neutral loss scan of 141 could not be a useful tool to discover ethanolamine plasmalogens from complex biological samples, and a new diagnostic strategy is needed. To the best of our knowledge, this is the first application for Ag(I) adduction to be used for the identification of ethanolamine plasmalogens. The CID of Ag(I)-adducted ethanolamine plasmalogens provided the predominant head-group loss of 141 Da. Two major glycerophosphoethanolamine species (ethanolamine plasmalogens and phosphatidylethanolamines) were determined using the neutral loss scan of 141 after Ag(I)-adduction with a lipid mixture. While the neutral loss scan of 141 enabled the determination of phosphatidylethanolamine with/without Ag(I) adduction, ethanolamine plasmalogens were observed only after Ag(I) adduction.

Experimental

Sample preparation

Ethanolamine plasmalogens (PE(P-18:0/22:6), PE(P-18:0/20:4) and PE(P-18:0/18:1)) were purchased from Avanti Polar Lipids (Alabaster, AL). Phosphatidylethanolamine (soybean) was purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions of 100 mM ethanolamine plasmalogen and 1 mg/ml

phosphatidylethanolamine were made in methanol. Silver acetate was purchased from Sigma-Aldrich. A 1:1 mixture of 1 µg/ml of phosphatidylethanolamine and 1 µM of ethanolamine plasmalogen solutions was prepared and used as a standard lipid mixture. For Ag(I)-adduction with the standard lipid mixture, 20 µL of the standard lipid mixture was vacuum centrifuged to remove the solvent; then 10 µL of a 0.1 mM silver acetate solution was added to the dried matter. A solution of Ag(I)-adduction with lipid mixtures was prepared at least 30 min prior to MS/MS analysis.

Mass spectrometry

Mass-spectrometric detection was carried out on an AB Sciex QTRAP 5500 tandem mass spectrometer (AB Sciex, Framingham, MA) equipped with a pneumatically assisted “turbo V” electrospray ionization (ESI)-source. The instrument was operated in the positive-ion mode with an ion-spray voltage of 4500 V using 25 psi curtain gas, 40 psi nebulizer gas and 71 psi drying gas at a temperature of 400°C. The mass spectra of CID and neutral loss scan were collected after direct injection of a sample solution with a flow of 7 µL/min, and summed over 100–150 scans. The mass spectral data were analyzed with Analyst 1.5.1 software.

Results and Discussion

CID of ethanolamine plasmalogens

CID has been used to explore structural information of ethanolamine plasmalogens from various samples.^{1,2,23,25} Fragmentations of ethanolamine plasmalogens in CID showed characteristic ions corresponding to the combined cleavage of the head group and vinyl ether group attached to the sn-1 position in the positive-ion mode. The CID of PE(P-18:0/20:4) provided m/z 361 and 392, as shown in Fig. 1. The ions at m/z 361 and 392 are characteristic ions of the sn-2 and sn-1 position, respectively, and these two ions are complementary to each other. The ion at m/z 392 involves combined cleavage at the phosphoethanolamine head group and the sn-1 position, and thus could be used to characterize ethanolamine plasmalogens with C18:0 at the sn-1 position. Likewise, fragment ions of m/z 364 represent ethanolamine plasmalogens with C16:0 at the sn-1 position. Each precursor ion scan of 392, 390, and 364 could be performed to detect ethanolamine plasmalogens which have C18:0, C18:1, C16:0 at the sn-1 position, individually.²³ However, the head-group loss, $[M+H-141]^+$, was not a major fragment ion of ethanolamine plasmalogens at the various collision energies shown in Fig. 1, while it has been well known that head-group loss is the most dominant fragment ion of phosphatidylethanolamines, regardless of the collision energies, as shown in Fig. 2.

Phosphatidylethanolamine always provides very abundant head-group loss, $[M+H-141]^+$, during the CID process, thus neutral loss scan (141 Da) should observe all possible phosphatidylethanolamines in various biological samples.²² However, ethanolamine plasmalogens give abundant characteristic fragment ions corresponding to the combined cleavage of the head group and the vinyl ether residue attached to the sn-1 position during the CID process, but head-group loss, $[M+H-141]^+$, is a minor fragmentation pathway, and is hard to be observed. Berry *et al.* investigated the fragmentation mechanism of ethanolamine plasmalogens using deuterium exchange and acid hydrolysis, and proposed that the vinyl ether double bond of plasmalogens would withdraw the electron density away from the C–O bond that attached the sn-1

Fig. 1 CID of protonated ethanolamine plasmalogen, [PE(P-18:0/20:4)+H]⁺. Several collision energies were used to compare the fragmentation patterns: (A) 16, (B) 26, and (C) 46 V.

substituent to the glycerol backbone.²³ Such characteristics of plasmalogens might attribute less abundant neutral loss of 141 Da. Thus, the neutral loss scan of 141 cannot be used to detect ethanolamine plasmalogens from lipid mixtures. Instead, several precursor ion scans corresponding to certain plasmalogen classes, such as C18:0, C18:1 or C16:0 at the sn-1 position, has been performed to observe the potential ethanolamine plasmalogen species from biological samples.

CID of Ag(I)-adducted ethanolamine plasmalogens

Ag(I) adduction provided abundant head-group loss and fatty acyl chain cleavages during the CID process, which enabled the complete structural characterization of phosphatidylethanolamines.²⁴ Thus, the enhanced signal intensity of the head-group loss from Ag(I) adduction would be useful to observe all possible

Fig. 2 CID of protonated phosphatidylethanolamine, [PE(33:1)+H]⁺. Several collision energies were used to compare the fragmentation patterns: (A) 16, (B) 26, and (C) 46 V.

ethanolamine plasmalogens, which are easily missed by a neutral loss scan of 141 of protonated forms.

Figure 3 shows fragmentation patterns of Ag(I)-adducted PE(P-18:0/20:4) depending on the collision energies. The signal abundance of the neutral loss of the head group, [M+Ag-141]⁺, increased with higher collision energy. On the contrary, the neutral loss from the protonated form, [M+H-141]⁺, did not show much difference along with different collision energies, and rather decreased with a higher collision energy, as shown in Fig. 1. PE(P-18:0/18:1) and PE(P-18:0/22:6) also showed similar fragmentation tendencies (data not shown). In addition, Fig. 3 provides other structural information, such as *m/z* 393, [R₂COOH+Ag-H₂O]⁺, and *m/z* 411, [R₂COOH+Ag]⁺, where R₂COOH is a fatty acyl group attached at the sn-2 position. The fatty acyl group at the sn-2 position has one double bond, which has a greater Lewis acid-base interaction with the Ag(I) ion than a saturated fatty acid at the sn-1 position, and was observed as [R₂COOH+Ag]⁺ and [R₂COOH+Ag-H₂O]⁺, while [R₁COOH+Ag]⁺ and [R₁COOH+Ag-H₂O]⁺ were not detected. A similar

Fig. 3 CID of Ag(I)-adducted ethanolamine plasmalogen [PE(P-18:0/20:4)+Ag]⁺. Several collision energies were used to compare the fragmentation patterns: (A) 16, (B) 26, and (C) 46 V.

observation was also made with various classes of phospholipids.²⁴ Although Ag(I) interacts with unsaturated fatty acyl groups, the major interaction of Ag(I) with phospholipids would be the ionic interaction due to the negatively charged phosphate groups. Thus, the most dominant fragmentation often involves cleavages occurring at head groups, $[M-141+H]^+$.²⁴ Berry *et al.* carefully explored the collisional activation of protonated ethanolamine plasmalogens.²³ They also discussed that vinyl ether double bond present at the sn-1 position withdrew the electron density away from the C-O bond, which hindered the neutral loss of 141 Da.²³ We also observed a similar phenomenon for protonated ethanolamine plasmalogens, as shown in Fig. 1. However, it has been known that Ag ions interact with the lone-pair electrons of the N and O atoms of the polar amide group of polyvinyl pyrrolidone, and these interactions are used for stabilizing the Ag nanospheres.²⁶ Thus, the existence of the Ag(I) ion would prevent the withdrawal of the electron density away from phosphoethanolamine caused by the vinyl ether residue, due to the interaction between the Ag ion

and many O atoms of the phosphoethanolamine headgroup, which resulted in and enhanced the signal abundance of the neutral loss of 141 Da during the CID process.

Metal ions including monovalent (*e.g.* Li(I)) and divalent metals (*e.g.* Mn(II)) have been investigated for phosphatidylethanolamine and ethanolamine plasmalogens, and have provided the predominant neutral loss of the vinyl amine (43 Da).^{2,16} However, the neutral loss of 43 was not detected in the CID of protonated phosphatidylethanolamine and ethanolamine plasmalogens. Thus, a comparison of neutral loss scans of 43 before and after such metal adduction should not distinguish between phosphatidylethanolamine and ethanolamine plasmalogens. On the other hand, the neutral loss of 141 is a predominant fragmentation pathway for protonated phosphatidylethanolamine, but not for protonated ethanolamine plasmalogens. Thus, phosphatidylethanolamine and ethanolamine plasmalogens could be distinguished from the comparison of neutral loss scan of 141 before and after Ag(I) adduction in the positive-ion mode.

We also explored transition metal adduction (*e.g.* Zn(II) or Mn(II)) with ethanolamine plasmalogens. Their CID spectra provided several fragment ions useful for structural characterization, depending on the fatty acyl groups attached at the sn-2 position shown in Fig. S1 (Supporting Information). However, the neutral loss of 141 could not be obtained from the CID of other metal adduction with ethanolamine plasmalogens. Ho *et al.* investigated the fragmentation mechanism of adducts of ethanolamine glycerophospholipid and transition metals.¹⁶ They suggested that two hydrogen abstractions from the phospholipids were required to produce the neutral loss of 141 Da, and the pathway as not feasible comparing to other fragmentation pathways.¹⁶ Thus, it is assumed that transition-metal adduction with ethanolamine plasmalogens undergoes a similar fragmentation pathway, and unlikely produces the neutral loss of 141 Da.

Neutral loss scan of Ag adducts with phosphatidylethanolamine and ethanolamine plasmalogens

Based on the result described above, a collision energy of ~46 V (instrumental parameter value) could be used for the neutral loss scan of 141 for Ag(I)-adducted lipid mixtures, which possibly identify all glycerophosphoethanolamine species from biological samples. To test this hypothesis, two major glycerophosphoethanolamines (phosphatidylethanolamine and ethanolamine plasmalogens) were mixed and either protonated or adducted with Ag(I). The neutral loss scan of 141 with protonated forms could observe only phosphatidylethanolamine, as shown in Fig. 4(A); however, neutral loss scan after Ag(I) adduction provided both phosphatidylethanolamine and all three ethanolamine plasmalogens shown in Fig. 4(B). As shown in the figure, phosphatidylethanolamine did not go to complete adduct formation, and both of the protonated and Ag(I)-adducted phosphatidylethanolamines were observed. However, ethanolamine plasmalogens could be observed only after Ag(I)-adduction, as expected.

Although Ag(I) has equally abundant 1st and 3rd isotopes, and seems to make neutral loss scan mass spectra complicated, this typical isotopic pattern of Ag(I) would help in the identification of Ag(I) adducts. The insets in Fig. 4(B) show detailed views of the indicated peaks. Ions at *m/z* 704.6 and 718.5 are protonated phospholipids based on the isotopic pattern of the phospholipids that did not undergo adduct formation completely. On the other hand, the ions at 858.5 and 882.5 are Ag(I) adducts, because they have the typical isotopic pattern of Ag ions. Most of the newly appeared peaks after adduction had this typical isotopic

Fig. 4 (A) Neutral loss scan of 141 of protonated standard lipid mixture. A collision energy of 36 V was used. (B) Neutral loss scan of 141 of the mixture of Ag(I)-adducted standard lipid mixture. A collision energy of 46 V was used. Possible compositions of phosphatidylethanolamine are shown with the corresponding m/z values. Exact compositions are shown for ethanolamine plasmalogens. The identification of each m/z in the above mass spectra is: 690.6, PE(32:1); 704.6, PE(33:1); 718.6, PE(34:1); 732.6, PE(35:1); 744.6, PE(36:2); 758.6, PE(37:2); 796.4, 690.6+Ag; 810.4, 704.6+Ag; 824.4, 718.6+Ag; 838.4, 732.6+Ag; 850.4, 744.6+Ag; 864.4, 758.6+Ag; 836.5, PE(P-18:0/18:1)+Ag; 858.5, PE(P-18:0/20:4)+Ag; 882.5, PE(P-18:0/22:6)+Ag.

pattern. The most abundant peak before Ag(I) adduction was PE(33:1), but PE(34:1) was the most abundant one after Ag(I) adduction, as shown in Fig. 4(B). The relative signal abundances among phospholipids changed slightly after Ag(I) adduction, which might be due to different adduction and fragmentation efficiencies. This strategy was also applied to a real biological sample (Figs. S2, S3, and S4), and identified ethanolamine plasmalogens present in the sample. PE(P-16:0/20:4), PE(P-18:0/20:3), and PE(P-18:0/22:6) were identified by comparing the neutral loss scan of 141 Da before and after Ag(I) adduction. The challenge for this strategy would be to apply this method with the LC/MS/MS system for quantitation, because the adduction between Ag(I) and ethanolamine plasmalogens was not stable during the LC separation process. However, Ag(I) adduction could be valuable to find unknown ethanolamine plasmalogens from complex lipid mixtures using MS only, especially when the biomolecules play an important role in a biological system.

Conclusions

Ethanolamine plasmalogens could easily form adducts with Ag(I) ions, and the CID of Ag(I)-adducted plasmalogens provided an abundant head group loss of 141 at a collision

energy of 46 – 56 V. On the other hand, phosphatidylethanolamine provided the predominant head group loss of 141 with or without Ag(I) adduction in the positive-ion mode. Thus, ethanolamine plasmalogens could be identified by comparing the mass spectra of the neutral loss scan of 141 before and after Ag(I) adduction; or ethanolamine plasmalogens could be identified from the neutral loss scan spectrum after Ag(I) adduction, where phosphatidylethanolamines show both protonated and Ag adducted forms, while ethanolamine plasmalogens appear only as Ag adducted forms. Two major glycerophosphoethanolamine species (ethanolamine plasmalogens and phosphatidylethanolamines) were determined using the neutral loss scan of 141 after Ag(I)-adduction with a lipid mixture. This novel approach could be a useful diagnostic tool to observe all possible ethanolamine plasmalogens in complex lipid mixtures with a single neutral loss scan.

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Supporting Information

The CID of Mn(II) or Zn(II)-adducted ethanolamine plasmalogen, the full and zoomed-in spectra of neutral-loss scan of 141 from mouse liver extract before and after Ag(I) adduction, the CID of m/z 856 from Ag(I) adducted mouse liver extract. This material is available free of charge on the Web at <http://www.jsac.or.jp/analsci/>.

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