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A rapid and adaptable lipidomics method for quantitative UPLC-mass spectrometric analysis of phosphatidylethanolamine and phosphatidylcholine *in vitro*, and in cells

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Abstract

Phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are highly prevalent phospholipids in mammalian membranes. There are currently no methods for detection of minute levels of these phospholipids or simultaneously with products of the utilization of these phospholipid substrates by phospholipase A₂ (PLA₂) enzymes. To examine the substrate utilization of PE and PC by PLA₂, we developed a method to accurately detect and measure specific forms of PE and PC as low as 50 femtomoles. Validation of this method consisted of an enzymatic assay to monitor docosahexaenoic acid and arachidonic acid release from the hydrolysis of PE and PC by group IV phospholipase A₂ (cPLA₂α) coupled to the generation of lyso-PE (LPE) and lyso-PC (LPC). In addition, the PE and PC profiles of RAW 264.7 macrophages were monitored with zymosan/lipopolysaccharide-treatment. Finally, genetic validation for the specificity of the method consisted of the downregulation of two biosynthetic enzymes responsible for the production of PE and PC, choline kinase A (CHKA) and ethanolamine kinase 1 (ETNK1). This new UPLC ESI-MS/MS method provides accurate and highly sensitive detection of PE and PC species containing AA and DHA allowing for the specific examination of the substrate utilization of these phospholipids by PLA₂ *in vitro* and in cells.

Keywords

phosphatidylethanolamine; phosphatidylcholine; mass spectrometry; QTRAP 5500 LC-MS/MS; Group IVA cytosolic phospholipase A₂

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Introduction

Phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are membrane glycerophospholipids that acts as a storage lipid for various fatty acids, such as arachidonic acid (AA) and docosahexaenoic acid (DHA). (1, 2) PE is structurally comprised of a glycerol backbone linked to two fatty acids chains, at the sn₁ and sn₂ positions, and a phosphoethanolamine group at the sn₃ position. PC is also structurally comprised of a glycerol backbone linked to two fatty acid chains, at the sn₁ and sn₂ positions, and a phosphocholine group at the sn₃ position. (3, 4) Typically, a saturated fatty acid is bound in the sn₁ position, whereas an unsaturated fatty acid is linked at the sn₂ locus. PE and PC can interact with specific phospholipases (e.g., cPLA₂α) at cell membranes to cleave the sn₂ fatty acid chain resulting in the downstream production of either pro-inflammatory or pro-resolution eicosanoids based on whether or not AA or DHA, respectively, are being released from these phospholipid reservoirs. (4)

Ultra-performance liquid chromatography electrospray ionization tandem mass spectrometry (UPLC ESI-MS/MS) is an extremely useful tool in the endeavor to quantitatively and qualitatively create a complete lipid profile of cells when studying various disease states, such as sepsis and inflammation. A major difficulty of many UPLC ESI-MS/MS methods is that the full separation of different lipid species/classes can be extremely difficult because of the need for different solvent conditions as discussed in previous reports from our laboratory. (4–7)

Prior to the development of triple quad mass spectrometers with higher sensitivity, PC and PE were both difficult to examine *in vitro* and in cells. Furthermore, fatty acids in the sn₂ position of phospholipids were studied via radiolabeling, an expensive and cumbersome process (2). To combat these issues many recent strides have been made in the field, including the ability to quantify polyunsaturated fatty acids (PUFA) by mass spectrometry, and quantification of phospholipids via both targeted and untargeted LCMS. (8–11) Although these methods remain relevant for some studies, we have further improved and optimized detection of PC and PE for studies that require higher sensitivity. Herein we detail a new method that utilizes reverse-phase UPLC ESI-MS/MS coupled with a simple modified Bligh-Dyer extraction that provides a much lower limit of detection for currently published PE and PC methods. The separation and solvent used in this method is also applicable to the detection of fatty acids that are generally found in the sn₂ position of PC and PE, such as eicosapentaenoic acid (EPA), AA and DHA. Thus, we demonstrate that this new UPLC ESI-MS/MS method is extremely sensitive and versatile allowing for the analysis of the utilization of phospholipid substrates (e.g., PE and PC) by PLA₂ species, both *in vitro* and in cells as well as adaptable to other biomatrices.

Material and Methods

Chemicals and Reagents

RAW 264.7 Macrophage cell lines were bought from ATTC and cultured in Dulbecco's Modified Eagle Medium (DMEM (1X)) with 10% Fetal Bovine Serum (FBS) and 1% Pen/Strep. Cells were cultured and passaged in T75 Falcon cell culture flasks every 3–4 days, as

needed. For collection, cells were scrapped in DMEM with 10% FBS and counted using a Millipore sceptor. A549 cells were purchased from ATTC and cultured in 50:50 DMEM and Roswell Park Memorial Institute (RPMI) with 10% FBS and 10% Pen Strep. Dharmafect 1, ONTARGETplus non-targeting pool (D-001810–10-05), ON-TARGETplus Human ETNK1 (55500) siRNA, and ON-TARGETplus Human CHKA siRNA (1119) was purchased from Dharmacon for the siRNA knockdown of ETNK1 and CHKA. Anti-ethanolamine kinase antibody (ab38284), and Anti-Choline kinase alpha antibody (ab88053) were purchased from abcam for the western blotting. PE and PC standards (18:0 Lyso PE, 16:0 Lyso PE, 16:0/20:4 PE, 16:0/22:6 PE, 18:0/20:4 PE, 18:0/22:6 PE, 17:0/17:0 PE, 17:0 Lyso PC, 18:0 Lyso PC, 16:0 Lyso PC, 16:0/20:4 PC, 16:0/22:6 PC, 18:0/20:4 PC, 18:0/22:6 PC) and Ceramide-1-Phosphate (C18:1 C1P) were purchased from Avanti Polar Lipids and stored at -80°C . Methanol, Acetonitrile, Isopropanol, and HPLC grade water used in extractions and solvents were purchased from Thermo Fisher Scientific.

UPLC ESI-MS/MS conditions

PE species are separated using an Acquity UPLC CSH C18 $1.7\ \mu\text{m}$ $2.1 \times 100\ \text{mm}$ column via UPLC using a Shimadzu 2-D UPLC Nexera System in conjunction with a QTRAP 5500 LC-MS/MS (SCIEX). Mass spectrometry parameters are: Polarity-Negative, Ion Source: Electrospray, Q1 Resolution: Low, Q3 Resolution: Unit, Collision Activated Dissociation: Medium, MCA: No, Curtain Gas: 30 psi, IonSpray Voltage: $-4500\ \text{V}$, Temperature: 500°C , Ion Source Gas 1: 40 psi, Ion Source Gas 2: 60 psi, Entrance Potential: $-10\ \text{V}$. MRM transitions with corresponding declustering potentials, collision energies, and collision cell exit potentials are shown in Table 1.

PC species are separated using an Acquity UPLC CSH C18 $1.7\ \mu\text{m}$ $2.1 \times 100\ \text{mm}$ column via UPLC using a Shimadzu 2-D UPLC Nexera System in conjunction with a QTRAP 5500 LC-MS/MS (SCIEX). Mass spectrometry parameters are: Polarity-Positive, Ion Source: Electrospray, Q1 Resolution: Low, Q3 Resolution: Unit, Collision Activated Dissociation: Medium, MCA: No, Curtain Gas: 30 psi, IonSpray Voltage: $4500\ \text{V}$, Temperature: 500°C , Ion Source Gas 1: 40 psi, Ion Source Gas 2: 60 psi, Entrance Potential: $12\ \text{V}$. MRM transitions with corresponding declustering potentials, collision energies, and collision cell exit potentials are shown in Table 2.

UPLC conditions for both PC and PE are Pumping Mode: Binary Flow, Total Flow: $0.7000\ \text{mL/min}$, Injection Volume: $5\ \mu\text{L}$, Column Oven: 60°C . The solvents used for reverse phase UPLC separation across a 10-min run are: Solvent A, 60:40 acetonitrile/water with 0.1% formic acid and 10mM ammonium formate and Solvent B, 10:90 acetonitrile/isopropanol with 0.1% formic acid and 10mM ammonium formate. Solvent conditions for UPLC separation were 10% Solvent B from 0–1 min, linear increase from 10–60% Solvent B from 1–4min, constant 60% Solvent B from 4–5min, linear increase from 60–100% Solvent B from 5–7min, constant 100% Solvent B from 7–8min, drop from 100–10% solvent B from 8–8.1min, and constant 10% Solvent B from 8.1–10.1min.

Mixed Micelle/Kinetics Assays

A mixed micelle, as an *in vitro* form of validation, was prepared as previously reported by us (A 4mL assay buffer was made using 320 mM HEPES, 600 mM NaCl, 19.42 μ M CaCl_2 (10 μ M free Ca^{2+}), 10 μ M EGTA, 4 mM DTT, and 2.39% H_2O .(2) For kinetics analysis micelles were created through drying down six separate concentrations of 16:0–20:4 PC and 16:0–20:4 PE by nitrogen and reconstituting in 8mM TritonX. Micelle concentrations are as follows: 100 μ M PC/PE (4.7% mol PC/PE), 300 μ M PC/PE (13% mol PC/PE), 500 μ M PC/PE (20% mol PC/PE), 800 μ M PC/PE (28.6% mol PC/PE), and 1200 μ M PC/PE (37.5% mol PC/PE).

To prepare the enzyme, the appropriate amount of wild type cPLA₂ α was calculated to equal 250 ng of protein per 100 μ L reaction. The enzyme was then mixed with 30% glycerol and 80mM HEPES. 25 μ L of the assay buffer was then combined with 25 μ L of micelle, followed by adding 50 μ L of the protein mix, totaling a final reaction volume of 100 μ L. A total of 5 reactions (500 μ L) were prepared for each of the six concentrations. Immediately following addition of the enzyme to the micelle/assay buffer mixture a timer was started and mixtures were placed into a 37 °C bead bath. At 15, 30, and 45 min, a 100 μ L aliquot was pipetted from each concentration into 500 μ L of MeOH containing a mixture of 250 pmol of 17:0 Lyso PC and 250 pmol of 17:0/17:0 PE to quench the reaction. After quenching, samples were run via UPLC-LC/MS. Statistical and kinetic analysis were performed using GraphPad Prism 6 software from GraphPad Software Inc.

For single point micelle analysis, a micelle composed of 18:0/20:4 PE with and without C18:1 C1P was prepared by mixing 15% mol of substrate with 4% mol of C18:1 C1P. This mixture was dried down in nitrogen and reconstituted in 8 mM Triton-X100 to form micelles. The protein and assay buffer and made the same way as described in kinetics analysis. The micelle reaction mixed with protein and assay buffer was then incubated at 37 °C for 45 min followed by a modified Bligh-Dyer extract to extract the PE as stated in the “PE Extraction Procedures” section.

Macrophage Validation

RAW 264.7 Macrophages were scraped from T75 falcon flasks and 1.5e6 cells were plated per 60 mm dish. Cells were incubated for 22 hours. Upon completion of the 22-hour incubation, cells were serum starved for 2 hours and subsequently treated with 100 ng/mL of LPS for 2 more hours. Following LPS treatment, cells were treated with 480 μ g zymosan/60 mm dish and taken at 5 time points of 0, 30, 60, 90, and 120 min of zymosan treatment. All samples were centrifuged at 800 G, and cell pellets were re-suspended in 200 μ L PBS followed by a modified Bligh-Dyer extract to extract PE and PC as stated in the “Lipid Extraction” section.

Genetic Validation

A549 lung adenocarcinoma cell line was utilized for siRNA transfection of CHKA and ETNK1. Cells were seeded on 6-well plates for western blotting and lipidomic analysis. Media was removed from cells and washed with PBS. Trypsin-EDTA was added and allowed to incubate at 48°C and 5% CO_2 . Complete media containing FBS was added and

transferred to 15ml conical tube. The cells were then centrifuged at 800 G for 5 min. Supernatant was removed and resuspended in complete media. A hemocytometer was used to determine cell concentration. 100,000 cells were then plated in each well and allowed to incubate overnight. The following day the cells were transfected with either non-coding siRNA control or CHKA and ETNK1 siRNA using Dharmafect 1 in reduced serum media at a 200 nM final concentration. Cells were incubated for 24 hours before removing reduced serum media. Fresh complete media was added and allowed to incubate for 48 hours.

Immunoblotting

Cells were harvested 72 hours following siRNA transfection. Media was removed and washed with PBS. Cells were scraped and collected in PBS solution. Samples were centrifuged at 2000 G for 7 min. Supernatant was removed, followed by addition of detergent and protease and phosphatase inhibitor. Cells were then pulse sonicated for approximately 10 seconds. Protein concentration was then quantified using NanoDrop (ThermoFisher Scientific, Waltham, MA). Amount of Laemmli buffer to add was calculated, added, and boiled for 5 min. Samples were run on a 10% polyacrylamide gel with precision plus kaleidoscope ladder (Bio-Rad Laboratories, Hercules, CA) at 50 volts for 30 min, then 80 volts for the remaining time. Once completed, gel was transferred onto methanol-activated polyvinylidene difluoride (PVDF) membrane using semi-dry transfer. Membrane was washed in phosphate buffered saline with tween 20 (PBST), followed by blocking in 5% blocking grade milk for 1 hour on shaker. Primary antibody was added directly to milk and allowed to incubate at room temperature for 1 hour. The membrane was left to incubate in primary antibody at 4 °C overnight. The following morning membrane was washed in PBST for 1 hour. Following PBST wash, secondary antibody in 5% milk was added and set to incubate at room temperature for 1 hour. The membrane was again washed in PBST for 2 hours, followed by a 30-min wash in PBS. Blots were imaged using horseradish peroxidase enzyme (HRP) substrate solution.

Lipid Extraction/Quantification

A modified Bligh-Dyer extraction method was used to extract PE from the RAW 264.7 macrophage cell pellets, the A549 cell pellets, and the *in vitro* mixed micelle.(6) For the RAW 264.7 macrophages, cell pellets were brought up in 200 µL of PBS followed by the addition of 10 µL of 0.5 ng/µL IS. For the mixed micelle assay, 10 µL of 0.5 ng/µL IS was added to the 100 µL of micelle mix. Following IS addition for both micelles and cell pellets, 1 mL of methanol and 0.5 mL of chloroform was added. Samples were then sonicated and transferred to the 48 °C water bath for 8 hours. Samples were then sonicated and centrifuged at 2500 G for 10 min. The supernatant was transferred to a new tube, dried down, and re-suspended in 500 µL of methanol. Samples were again centrifuged at 2500 G for 10 min, transferred to injection vials, and were submitted for PE mass spectrometry analysis on the QTRAP 5500 LC-MS/MS. Data analysis was performed using Analyst Software. Quantification of unknown peaks was done using the following formula: $[(\text{Analyte Concentration}/\text{Analyte Signal}) = (F)(\text{Internal Standard Concentration}/\text{Internal Standard Signal})]$ where F is signal response factor (determined using calibration curves) used to account for ionization differences between the internal standard and analytes.

Results

Mass spectrometry source settings and linear signal vs concentration curves show high UPLC ESI-MS/MS sensitivity for PE and PC species.

Previously published PE and PC methods have either shown HPLC separation without coupling to mass spectrometric analysis or showed only the mass spectrometry ionization profiles of PE and PC without coupling to HPLC.(12,13) Other groups have also published HPLC ESI-MS/MS phospholipid methods that were performed on older mass spec systems with lower sensitivity, lacked AA- and DHA- containing PE and PC species in the analysis or were focused on several different classes of glycerophospholipids rather than optimizing for PE and PC.(14)

To circumvent these issues as well as provide a versatile method for examining PC and PE levels in many biological assays, a method for both PC and PE was developed using UPLC ESI-MS/MS coupled to a SCIEX QTRAP 5500 LC-MS/MS to accurately quantify PE and PC. The PE method is able to accurately quantify 17:0/17:0 PE (used as internal standard), 18:0 Lyso PE, 16:0 Lyso PE, 16:0/20:4 PE, 16:0/22:6 PE, 18:0/20:4 PE, and 18:0/22:6 PE standards as low as 5 fmol. The PC method is able to accurately quantify 17:0 Lyso PC (used as internal standard), 18:0 Lyso PC, 16:0 Lyso PC, 16:0/20:4 PC, 16:0/22:6 PC, 18:0/20:4 PC, and 18:0/22:6 PC standards as low as 5 fmol. Negative mode ionization was chosen for the PE standards based on previous work showing that PE readily ionizes in negative mode.(14) A basic structure of PE and chromatograms of the aforementioned PE standards are shown in Figure 1A and Figure 1B displaying the ability for this method to detect PE by using specific retention times, MRM transitions, and mass spectrometric parameters (Table 1) both at 5 fmols and 50 fmols. Table 1 shows the retention times of these analytes, respective precursor and product ion masses, declustering potentials, collision energy, and entrance potentials as determined by direct infusion optimization on the SCIEX QTRAP 5500 mass spectrometer. Positive mode ionization was chosen for the PC standards based on the ability for the PC head group to readily ionize in the positive mode. A basic structure of PC and chromatograms of the aforementioned PC standards are shown in Figure 1C and Figure 1D displaying the ability for this method to detect PC by using specific retention times, MRM transitions, and mass spectrometric parameters (Table 2), both at 5 fmols and 50 fmols. Table 2 shows the retention times of these analytes, respective precursor and product ion masses, declustering potentials, collision energy, and entrance potentials as determined by direct infusion optimization on the SCIEX QTRAP 5500 mass spectrometer. The C18 column and UPLC solvents were chosen based on previously known solvents that are used in lipidomic and metabolomic LCMS.(15) The gradient settings were modulated and optimized several times until acceptable separation between peaks was found.

To investigate the sensitivity limits of the PE and PC UPLC ESI-MS/MS methods, a mixture of 17:0/17:0 PE, 18:0 Lyso PE, 16:0 Lyso PE, 16:0/20:4 PE, 16:0/22:6 PE, 18:0/20:4 PE, 18:0/22:6 PE, 17:0 Lyso PC, 18:0 Lyso PC, 16:0 Lyso PC, 16:0/20:4 PC, 16:0/22:6 PC, 18:0/20:4 PC, and 18:0/22:6 PC was prepared and analyzed from concentrations of 5 fmol to 400 fmol. The signal response (cps) was measured by taking the area under the peak. A

linear relationship was able to be formed by plotting signal response vs concentration of PE and PC species with correlation coefficients above 0.99 (Figure 2A and B). These results were then plotted as a log-log plot with log of signal response vs log of PE and PC concentrations, also generating a linear relationship with correlation coefficients above 0.99 (Figure 2C and D). The intra-day and inter-day QC samples were also within an acceptable range (Table 3 & 4). This method shows the standards to have at least a 5x signal to noise ratio at 5 fmol, which exceeds U.S. F.D.A. guidelines for Bioanalytical Method Validation of 5x signal to noise for 10 fmols per injection for each chain length.(16)

In vitro validation using a mixed micelle assay for assessing cPLA₂ α activity

In the past to assess cPLA₂ α activity *in vitro*, the utilization of sn₂-AA of PC labeled with ³H or ¹⁴C was required. This assay was both expensive and labor intensive, and the radiolabeled forms of PC have become difficult to obtain. As DHA and EPA-derived lipid mediators have been shown to play important roles in many biological functions, the assessment of the utilization of substrate lipids with sn₂-DHA and -EPA by PLA₂ enzymes has become important. Unfortunately, radiolabeled substrate phospholipids are not available. Thus, one of the goals of the development of this method was to provide an *in vitro* assay to assess the activity of PLA₂ enzymes toward multiple substrates using a mixed-micelle based assay.(2,3) Indeed, other studies have used a mixed micelle-based assay to quantify the cPLA₂ α catalyzed release of AA from the membrane bound glycerophospholipid, PC.(17) In accordance with this information, a mixed micelle assay was used as a form of both *in vitro* method validation for PE and PC utilization as well as show mitigation of Triton-X100 contamination and demonstrate the versatility of the assay for both *in vitro* and cellular studies. For single point analysis, an altered Bligh-Dyer extraction was performed on the mixed micelle assay samples followed by analysis via UPLC ESI-MS/MS. The resulting data from this assay are presented in Figure 3A, which shows the utilization of substrate and increase in 18:0 Lyso PE and free AA, resulting from the C1P-mediated activation of cPLA₂ α using 18:0/20:4 PE as a substrate.

Along with the data in Figure 3A, our laboratory and others have previously shown that C1P interacts with cPLA₂ α to increase the release of AA and the respective lyso-species from membrane phospholipids.(2,17–20) To further validate the assay, PE and PC utilization of cPLA₂ α in the presence and absence of C1P was examined kinetically. In line with our previous reports using the radiolabeled mixed-micelle assay, C1P enhanced cPLA₂ α activity with 16:0/20:4 PC (Figure 3B). Kinetic activity was measured by release of 16:0 lyso-PC and 16:0 lyso-PE, resulting from the C1P mediated cPLA₂ α interaction with both 16:0/20:4 PC and 16:0/20:4 PE (Figures 3B and 3C). An allosteric fit was used, as it has been shown that membranes can serve as allosteric activators of PLA₂.(21) K_{0.5}, the concentration of substrate that allows the enzyme to operate at half V_{max}, and V_{max}, and the maximum enzyme velocity, are shown in Tables 5 and 6 for PC and PE respectively. Both micelle assays demonstrate that this is a reliable UPLC ESI-MS/MS method to quantify PE and PC levels as well as PLA₂ products *in vitro* for both single point and enzymatic kinetic studies.

Biological validation of the UPLC-ESI MS/MS method using RAW 264.7 macrophages

To demonstrate that this method can also be utilized to examine PC and PE levels, and utilization of these substrate phospholipids by PLA₂ in cells, RAW 264.7 macrophages were chosen as a cellular model. Indeed, previous papers have examined the effect LPS and zymosan treatment on phospholipid and eicosanoid levels in this cell model.(2,3,22) Balsinde and coworkers reported that when murine macrophages were exposed to LPS treatment, followed by zymosan, an activator of cPLA₂ α , AA-containing PC species acted as net donors of AA from the cPLA₂ α mediated release of free fatty acids, whereas the AA-containing PE species showed no significant decreases following treatment. In accord with these reported findings, when RAW 264.7 macrophages were treated with LPS, followed by zymosan there was significant decrease in AA-PC at 90 and 120 minutes. This suggests PC acts as net donor of arachidonic acid (most likely to produce pro-inflammatory eicosanoids) during LPS+zymosan stimulation as shown in murine macrophages by Balsinde et al. (Figure 4A). Although it is not statistically significant, it is interesting to note that DHA-PC decreased between 30 and 120 minutes, possibly pointing to PC being more widely used as an overall donor of sn-2 fatty acids during LPS+zymosan stimulation when compared to PE (Figure 4A). Using the same cellular system, our UPLC-ESI MS/MS method was utilized to assay PE, and the PE data correlated with published reports (Figure 4B). Indeed, Rouzer et al and Balgoma et al showed no significant decrease of AA-containing PE species between LPS treatment and a combined LPS and zymosan-treatment of the RAW 264.7 macrophages. We discovered no significant decrease in AA or DHA containing PE species, but we did however find a significant increase in 16:0 Lyso PE at the 90-min LPS+zymosan treatment, but not at 120-min LPS+zymosan treatment, possibly due to release of different sn-2 chain fatty acid not being analyzed in our method, but still suggesting our findings are indeed similar to Rouzer et al. as PE does not act as net donor of either AA or DHA in RAW 264.7 macrophages. Overall, these data show that this new method can be utilized to quantitate the utilization of PC and PE in cells by PLA₂ species.

Genetic alteration of Choline Kinase A and Ethanolamine Kinase 1 validate the specificity of the UPLC-ESI MS/MS method.

To further validate our method using a molecular approach, siRNA was utilized to specifically downregulate the biosynthetic enzymes for both PE and PC production in mammalian cells. Specifically, we targeted ethanolamine Kinase 1 (ETNK1), as this enzyme controls the initial rate limiting step of the biosynthesis of PE in the Kennedy Pathway.(23) Choline kinase alpha (CHKA) was also downregulated as this enzyme controls the initial rate limiting step of the biosynthesis of PC in the Kennedy Pathway. Downregulation of ETNK1 in A549 cells showed significantly reduced levels of all 16:0 and 18:0 PE species (Figure 5A). Downregulation of CHKA in A549 cells showed significantly reduced levels of all 16:0 and 18:0 PC species (Figure 5B). PE findings were also cross-validated using qualitative, untargeted lipidomics, which also demonstrated a significant reduction in 16:0/20:4, 18:0/20:4, and 16:0/22:6 PE. (Figure 5C). Validation of siRNA downregulation was accomplished via western immunoblotting for ETNK1 and CHKA (Figure 5D). These data show that our UPLC-ESI MS/MS method is specific and quantitative for examining the levels of PE and PC in cells.

Discussion

The few reported methods for measuring endogenous PC and PE *in vitro* have suffered from a multitude of problems. Indeed, measurement of PE and PC *in vitro* and *in vivo* had proven to be incomplete and lack sensitivity.(11–15,24) For example, there are no current methods for the detection of low quantities of these phospholipids. Further, these methods did not allow for the simultaneous detection of PLA₂ products for adaptability to *in vitro* enzymatic assays or examination of substrate use in cells. This new UPLC ESI-MS/MS method will offer researchers a valuable tool to examine biological levels PE and PC in various biomatrices as well as determine substrate utilization when free fatty acids are cleaved from membrane phospholipids (e.g., PE and PC). Through a very simple extraction, this new method is able to efficiently separate and analyze various PE and PC species without having to modify solvent conditions or the column, allowing for very accurate, low-level analysis of PE and PC, both *in vitro* and *in vivo*.

It is extremely important to analyze the substrate utilization of PLA₂ species *in vitro* and in cells. Indeed, the interaction of cPLA₂α with PE and PC, and subsequent release of lyso-PE, lyso-PC, and free fatty acids from PE and PC, which plays key roles in the inflammatory response.(4,6,7) To date, little is known about the differential utilization of these PLA₂ substrates in cells in various contexts due to the lack of accurate analytical methods to discern these mechanism. Thus, it is important to be able to accurately quantify assorted PE and PC species in the cell membrane. Prior to LCMS, the primary method for detecting cleavage products from the interaction of PLA₂ and membrane phospholipids was done using radiolabeling of the sn₂ fatty acid.(18) Radiolabeling is slower and more cumbersome compared to LCMS analysis as well as being a potential risk/hazard. Furthermore, appropriate radiolabeled substrates for examining the release of DHA and EPA from phospholipid reservoirs are not commercially available. Those available for sn-2 AA are very expensive and somewhat cost limiting versus a LCMS protocol. Previous published methods have also suffered from older, less sensitive instruments as well as a lack of focus on PE and PC, resulting in poor detection or a much more general change in phospholipid classes rather than specific species.(13,24,25) This new UPLC ESI-MS/MS method will offer researchers a valuable tool to look at biological levels of PE and PC, and determine substrate use when free fatty acids, AA and DHA, are cleaved from these membrane phospholipids. This is important for the purpose of determining pre-clinical treatment for patients with sepsis or severe inflammation due to the downstream effects AA and DHA have on eicosanoid synthesis.

Lastly, the methods described provide highly sensitive and targeted UPLC ESI-MS/MS assays for the quantification of both PC and PE. These new methods hold several advantages over similar currently published methods for not only high sensitivity, an LLOQ of 5 femtomoles on-column, but also their adaptability for use *in vivo*, *in vitro*, and in cells. Furthermore, other currently published methods studied only lyso-PC and -PE species, or performed nontargeted analysis of multiple phospholipids.(11,25) These methods are also novel and significant because they are beneficial for studying immune response. Both methods focus on precursor phospholipids known to be utilized for their sn₂ fatty acids species, which when converted into eicosanoids play a major role in both the pro-

inflammatory and anti-inflammatory response in wound healing and sepsis.(4–7,19,20,26–28) Unfortunately, PE is negatively ionized, and PC is positively ionized causing the minor drawback in that they are unable to be run simultaneously in a single method on the SCIEX QTRAP 5500 as this system is unable to rapidly switch polarities. To combat this problem, the same solvents and column are used so no physical changes to the LCMS system must be made between running samples for both PC and PE. Overall, the methods described in this study were the first PC and PE methods to be validated by multiple traditional biological assays combined with *in vitro* micelle assays.

In conclusion, measurement of phospholipid substrates *in vitro* and in cells has proven to be insensitive and unfocused until the development of the UPLC ESI-MS/MS method described herein. These new UPLC ESI-MS/MS methods will offer researchers a valuable tool for determination of the activities of PLA₂ species toward specific phospholipid substrates in cells and *in vitro* for the purpose of delineating biological mechanisms and pre-clinical drug development for diseases associated with inflammation such as diabetes, atherosclerosis, and cancer. The increased sensitivity offered by these new methods will provide more accurate results when trying to determine the cause of these disease states. Overall, the UPLC ESI-MS/MS method presented in this study supplies a new tool for researchers to further explore the biological functions of PE and PC as well as the role of differential substrate utilization by PLA₂ species in various biological mechanisms and disease states.

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Figure 1a **Figure 1b**

Figure 1c **Figure 1d** **Figure 1.**

To accurately identify the retention times of the different PE and PC species, internal standards of PE and PC are used to find the elution times of the PE and PC species during reverse phase UPLC ESI-MS/MS. These same standards are also used to create distinct MRM chromatograms at 50 fmol/injection for (A) 16:0/20:4 PE, 16:0/22:6 PE, 18:0/20:4 PE, 18:0/22:6 PE, 16:0 Lyso PE, 18:0 Lyso PE, and 17:0/17:0 PE. (B) Basic structure of phosphatidylethanolamine. (C) MRM chromatograms at 50 fmol/injection for 16:0/20:4 PC, 16:0/22:6 PC, 18:0/20:4 PC, 18:0/22:6 PC, 16:0 Lyso PC, 18:0 Lyso PC, and 17:0 Lyso PC.

(D) Basic structure of phosphatidylcholine. Inset: LLOQ chromatograms at 5 fmol for respective PE and PC species

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Figure 2a

Figure 2b

Figure 2c

Figure 2d

Figure 2.

The detection method showed a linear response for PE and PC species from 5 fmol to 400 fmol. Separate internal standard concentrations were made with all 7 PE and PC species (16:0/20:4 PE, 16:0/22:6 PE, 18:0/20:4 PE, 18:0/22:6 PE, 16:0 Lyso PE, 18:0 Lyso PE, and 17:0/17:0 PE for the PE calibration curves and 16:0/20:4 PC, 16:0/22:6 PC, 18:0/20:4 PC, 18:0/22:6 PC, 16:0 Lyso PC, 18:0 Lyso PC, and 17:0 Lyso PC for the PC calibration curve. Each curve ranged from 5 mol/injection to 400 fmol/injection. **(A)** Shows a linear response of the signal response (cps) vs the fmol PE/injection. This data is an average of 3 sample injections $\pm$ SEM. **(B)** Shows the $\text{Log}_{10}[\text{Signal Response, cps}]$ vs $\text{Log}_{10}[\text{fmol PE/injection}]$. The data is an average of 3 sample injections $\pm$ SEM. **(C)** Shows a linear response of the signal response (cps) vs the fmol PC/injection. This data is an average of 3 sample injections $\pm$ SEM. **(D)** Shows the $\text{Log}_{10}[\text{Signal Response, cps}]$ vs $\text{Log}_{10}[\text{fmol PC/injection}]$. The data is an average of 3 sample injections $\pm$ SEM.

Figure 3a **Figure 3b** **Figure 3c** **Figure 3.**

Using UPLC ESI-MS/MS an in vitro PE quantification of a single point mixed-micelle assay showed the (A) C1P and cPLA₂ α mediated release of 18:0/20:4 PE sn-2 bound arachidonic acid along with 18:0 Lyso PE. The modified Bligh-Dyer extraction is detailed in Experimental and the data presented represents an $n=3 \pm \text{SD}$. The results have been blanked from a sample with no enzyme or C1P and normalized to ng/mL. (B) Mixed-micelle kinetics assay of the C1P and cPLA₂ α mediated release of 16:0/20:4 PC sn-2 bound arachidonic acid along with 16:0 Lyso PC. 16:0 Lyso PC nmoles/min/mg has been used as a measure of kinetic activity. Data presented represents an $n=3 \pm \text{SD}$. (C) Mixed-micelle kinetics assay of the C1P and cPLA₂ α mediated release of 16:0/20:4 PE sn-2 bound arachidonic acid along with 16:0 Lyso PE. 16:0 Lyso PE nmoles/min/mg has been used as a measure of kinetic activity. All data presented represents an $n=3 \pm \text{SD}$. An allosteric sigmoidal non-linear regression analysis has been used as a best fit for both kinetics experiments.

Figure 4.

Comparison of control RAW 264.7 macrophages with LPS and zymosan treated RAW 264.7 macrophages. Non-treated control cells (blue and brown) were plated and scraped 7 hours following a 2-hour serum starve with no treatment. LPS treated macrophages (red) were treated with 100 ng/mL of LPS for 7 hours following a 2-hour serum starve. Zymosan treated (green, purple, orange, yellow) macrophages were treated with 480 μ g/60mm dish of zymosan for 2 hours following 5 hours of 100 ng/mL of LPS treatment and a 2-hour serum starve. **(A)** There is no significant decrease of AA-containing or DHA-containing PE species, between LPS (red) and LPS + zymosan (green, purple, orange, yellow) treatment, although at 90 min of LPS + zymosan treatment there is significance in 16:0 Lyso PE. **(B)** There is a significant decrease of 16:0/20:4 PC at both 90 and 120 min of LPS + zymosan treatment, although no significance of 16:0/22:6 PC between LPS (red) and LPS + zymosan (green, purple, orange, yellow) treatment. (The modified Bligh-Dyer extraction is detailed in Experimental. The data presented represents an $n=3 \pm$ SD. Statistical analysis was performed using a MANOVA with a Tukey HSD Post-hoc Test. Significance of a p value <0.05 is shown as a * above each point.

Figure 5a

Figure 5b

Figure 5c

Figure 5d

Figure 5.

siRNA knockdown of ETNK1 and CHKA in A549 cells. **(A)** UPLC ESI-MS/MS results revealed significant decreases 16:0 Lyso PE, 16:0/20:4 PE, 16:0/22:6 PE, 18:0 Lyso PE, 18:0/20:4 PE, and 18:0/22:6 PE in A549 cells treated with ETNK1 compared to cells treated with scrambled siRNA. **(B)** UPLC ESI-MS/MS results revealed significant decreases 16:0 Lyso PC, 16:0/20:4 PC, 16:0/22:6 PC, 18:0 Lyso PC, 18:0/20:4 PC, and 18:0/22:6 PC in A549 cells treated with CHKA compared to cells treated with scrambled siRNA. **(C)** A549 cells treated with ETNK1 were also sent to the VCU Lipidomics Core to be run for untargeted lipidomics. Results showed a decrease of 16:0/20:4 PE, 16:0/22:6 PE, and 18:0/20:4 PE when treated with ETNK1 when compared to the scrambled siRNA A549s. **(D)** Western blotting of ETNK1 and CHKA when treated with siETNK1 and siCHKA respectively. Non-specific band in CHKA marked by *.⁽²⁹⁾ The modified Bligh-Dyer extraction used for PE and PC extraction is detailed in the Experimental. The UPLC ESI-MS/MS data presented in **(A)** and **(B)** represents an n=4. Untargeted lipidomics presented in **(C)** represent n=3. Statistical analysis was performed using a MANOVA with a Tukey HSD Post-hoc Test. Significance of a p value <0.05 is shown as a * above each point.

Table 1

SCIEX 5500 QTRAP mass spectrometer settings and retention times for reverse phase chromatographic separation of PE species DP: declustering potential, CE: collision energy, EP: entrance potential. Transitions and settings were determined manually via direct infusion of each analyte separately. Settings and transitions were chosen based on the best signal obtained during infusion using manual tuning in Sciex Analyst software.

PE Species	Precursor Ion (m/z) (-ve ionization) Q1 Mass	Product Ion (m/z) (-ve ionization) Q3 Mass	DP	CE	EP	Retention Time (min)
16:0 Lyso PE	452.0	255.1	-110	-32	-10	1.71
18:0 Lyso PE	480.1	283.1	-110	-34	-10	3.06
16:0/22:6 PE	762.3	327.1	-100	-38	-10	5.70
16:0/20:4 PE	738.3	303.1	-140	-38	-10	5.82
18:0/22:6 PE	790.4	327.1	-140	-38	-10	6.18
18:0/20:4 PE	766.3	303.1	-140	-38	-10	6.31
17:0/17:0 PE	718.4	269.1	-110	-44	-10	6.64

Table 2

SCIEX 5500 QTRAP mass spectrometer settings and retention times for reverse phase chromatographic separation of PC species DP: declustering potential, CE: collision energy, EP: entrance potential. Transitions and settings were determined manually via direct infusion of each analyte separately. Settings and transitions were chosen based on the best signal obtained during infusion using manual tuning in Sciex Analyst software.

PC Species	Precursor Ion (m/z) (-ve ionization) Q1 Mass	Product Ion (m/z) (-ve ionization) Q3 Mass	DP	CE	EP	Retention Time (min)
16:0 Lyso PC	496.3	184.0	103	31	9	1.06
18:0 Lyso PC	524.4	184.0	109	34	8	1.94
16:0/22:6 PC	806.6	184.0	100	36	12	4.67
16:0/20:4 PC	782.5	184.0	92	38	12	4.76
18:0/22:6 PC	834.6	184.0	104	37	10	5.06
18:0/20:4 PC	810.6	184.0	114	37	10	5.18
17:0 Lyso PC	510.3	184.0	99	33	8	1.44

Table 3

Intra-day and inter-day precision and accuracy for PE

PE Species	Nominal Concentration (nM)	Intra-day precision and accuracy Mean (n=4) (nM)	Mean accuracy (%)	CV (%)	Inter-day precision Mean (n=4 days) (nM)	CV (%)
16:0 Lyso PE	20	19.78	98.91	3.27	21.42	3.79
	40	39.20	98.00	2.13	41.37	3.91
	80	77.89	97.36	1.65	81.01	4.35
	100	96.02	96.02	2.90	105.99	3.83
18:0 Lyso PE	20	20.00	100.02	3.83	21.99	2.68
	40	38.79	96.97	2.60	42.84	1.09
	80	82.59	103.24	2.25	111.08	26.59
	100	92.66	92.66	2.62	116.50	16.01
16:0/22:6 PE	20	19.64	98.19	3.45	22.82	5.81
	40	39.95	99.87	3.02	44.98	5.78
	80	79.28	99.10	2.29	90.64	7.13
	100	98.30	98.30	0.59	113.31	5.51
16:0/20:4 PE	20	19.19	95.94	4.78	21.97	3.99
	40	38.78	96.95	1.25	43.44	3.58
	80	79.28	99.10	3.60	87.76	4.93
	100	97.95	97.95	1.40	110.69	3.67
18:0/22:6 PE	20	18.32	91.58	4.44	21.63	5.63
	40	39.35	98.38	1.13	43.96	7.99
	80	80.85	101.07	1.99	89.09	7.44
	100	99.53	99.53	1.15	111.97	7.10
18:0/20:4 PE	20	19.34	96.68	4.04	22.07	5.57
	40	38.90	97.25	1.25	43.53	4.83
	80	79.59	99.49	1.82	86.98	6.63
	100	98.67	98.67	2.50	109.77	4.29
17:0/17:0 PE	20	17.96	89.81	2.45	22.00	10.34
	40	39.16	97.90	2.54	47.18	8.93
	80	80.54	100.67	1.89	90.43	10.83
	100	99.16	99.16	0.85	114.09	7.92

Table 4

Intra-day and inter-day precision and accuracy for PC

PC Species	Nominal Concentration (nM)	Intra-day precision and accuracy Mean (n=4) (nM)	Mean accuracy (%)	CV (%)	Inter-day precision Mean (n=4 days) (nM)	CV (%)
16:0/20:4 PC	20	20.19	100.93	3.63	23.21	3.37
	40	38.60	96.51	3.73	41.60	2.78
	80	74.05	92.56	3.38	88.11	2.95
	100	97.44	97.44	3.96	102.31	2.39
16:0/22:6 PC	20	20.18	100.89	4.56	23.54	3.23
	40	38.72	96.81	3.69	42.82	2.74
	80	74.88	93.60	3.16	91.66	2.58
	100	98.71	98.71	3.60	106.72	2.58
16:0 Lyso PC	20	20.28	101.42	3.13	18.05	8.13
	40	39.23	98.08	2.52	31.38	5.28
	80	76.01	95.01	3.72	67.11	4.39
	100	99.07	99.07	0.97	76.41	4.06
18:0/20:4 PC	20	20.44	102.20	7.36	24.29	0.43
	40	38.73	96.83	4.43	43.81	1.83
	80	74.17	92.71	4.84	93.03	0.58
	100	97.00	97.00	6.17	107.92	0.93
18:0/22:6 PC	20	20.48	102.39	4.49	23.97	1.58
	40	39.51	98.77	3.18	43.29	1.26
	80	75.61	94.51	3.45	92.68	0.51
	100	100.18	100.18	3.05	107.53	0.88
18 Lyso PC	20	20.35	101.74	1.94	19.69	7.74
	40	39.66	99.15	2.21	34.98	6.37
	80	75.92	94.90	2.31	74.85	5.07
	100	99.83	99.83	0.99	86.24	4.48
17 Lyso PC	20	19.76	98.81	2.21	19.72	6.36
	40	39.21	98.03	1.96	35.93	5.80
	80	76.07	95.09	1.77	77.47	6.06
	100	99.45	99.45	2.07	88.85	4.28

Table 5

$K_{0.5}$ and $V_{\max}$ values of PC kinetics. $K_{0.5}$ is the concentration of substrate at which maximal enzyme velocity is half. $V_{\max}$ is the maximum enzyme velocity in “nmoles/min/mg” of 16:0 Lyso PC.

CIP	Approximate $V_{\max}$ (nmoles/min/mg)	Approximate $K_{0.5}$ (mol fraction PC)
0 mol %	1.745 ± 0.1269	0.1145 ± 0.01001
4 mol %	6.852 ± 0.5644	0.07633 ± 0.01021

Table 6

$K_{0.5}$ and $V_{\max}$ values of PE kinetics. $K_{0.5}$ is the concentration of substrate at which maximal enzyme velocity is half. $V_{\max}$ is the maximum enzyme velocity in “nmoles/min/mg” of 16:0 Lyso PE.

CIP	Approximate $V_{\max}$ (nmoles/min/mg)	Approximate $K_{0.5}$ (mol fraction PE)
0 mol %	3.289 ± 0.2236	0.06021 ± 0.008366
4 mol %	6.676 ± 0.3448	0.04304 ± 0.006522