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Quantitation of phosphatidic acid and lysophosphatidic acid molecular species using hydrophilic interaction liquid chromatography coupled to electrospray ionization high resolution mass spectrometry

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ABSTRACT

A method for a highly selective and sensitive identification and quantitation of lysophosphatidic acid (LPA) and phosphatidic acid (PA) molecular species was developed using hydrophilic interaction liquid chromatography (HILIC) followed by negative-ion electrospray ionization high resolution mass spectrometry.

Different extraction methods for the polar LPA and PA species were compared and a modified Bligh & Dyer extraction by addition of 0.1 M hydrochloric acid resulted in a ≈1.2-fold increase of recovery for the 7 PA and a more than 15-fold increase for the 6 LPA molecular species of a commercially available natural mix compared to conventional Bligh & Dyer extraction. This modified Bligh & Dyer extraction did not show any artifacts resulting from hydrolysis of natural abundant phospholipids.

The developed HILIC method is able to separate all PA and LPA species from major polar membrane lipid classes which might have suppressive effects on the minor abundant lipid classes of interest. The elemental compositions of intact lipid species are provided by the high mass resolution of 100,000 and high mass accuracy below 3 ppm of the Orbitrap instrument. Additionally, tandem mass spectra were generated in a parallel data dependent acquisition mode in the linear ion trap to provide structural information at molecular level. Limits of quantitation were identified at 45 fmol on column and the dynamic range reaches 20 pmol on column, covering the range of natural abundance well.

By applying the developed method to mouse brain it can be shown that phosphatidic acid contains less unsaturated fatty acids with PA 34:1 and PA 36:1 as the major species. In contrast, for LPA species a high content of polyunsaturated fatty acids (LPA 20:4 and LPA 22:6) was quantified.

1. Introduction

Phosphatidic acid (PA, Supplemental Fig. S1A) is the simplest membrane phospholipid class and has a key function as an intermediate in phospholipid and glycerolipid metabolism. Due to its intermediate function, the steady state concentration of PA is very low in cellular systems [1]. Beside these functions, phosphatidic acid is involved in multiple regulatory cascades such as signal transduction, membrane trafficking, secretion and cytoskeletal rearrangement [2]. Lysophosphatidic acid (LPA, Supplemental Fig. S1B), a degradation product of phosphatidic acid, is a bioactive lipid which is important in a multitude of cellular processes, like bone remodeling, inflammation and migration [3–6]. The action of LPA is transduced by G protein-coupled receptors at the cells surface [7]. These receptors are expressed in cells of the nervous, immune and cardiovascular system [8,9] where they cause atherosclerosis, inflammation and cancer. Hence, LPA can be regarded as an important biomarker for several diseases such as acute coronary syndrome or ovarian cancer [10,11].

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Due to its high selectivity and sensitivity, mass spectrometry is frequently used for analysis of lipids [12–14]. Nevertheless, PA and LPA are often not detected by standard lipidomic platforms [15,16] for several reasons. In contrast to most other phospholipid classes, PA and LPA do not have any lipid class specific fragment ions, which could be used in class specific precursor ion scans at sufficient sensitivity, because the fragment ion at m/z 153 can also be observed for phosphatidylglycerol (PG) or phosphatidylinositol (PI). Furthermore, the phosphate group of PA and LPA needs to be protonated to avoid smearing high pressure liquid chromatography (HPLC) peaks (unpublished observation), which is incompatible with the pH of many solvents typically used for negative-ion electrospray ionization (ESI). Finally, the most demanding challenge is the very low concentration of PA and even more so of LPA compared to other phospholipids in most biological systems [17].

The majority of published methods for analysis of PA and LPA relies on triple quadrupole mass spectrometry coupled to reversed phase HPLC [18–22]. Other examples for chromatographic approaches coupled to triple quadrupole or ion trap mass spectrometry would be normal phase [23,24], hydrophilic interaction liquid chromatography (HILIC) [25] or supercritical fluid chromatography after derivatization [26,27]. Furthermore, matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry without previous chromatographic pre-separation was successfully used for analysis of LPA [28,29].

In contrast to these low resolution methods, our approach takes advantage of the ultrahigh mass resolution (100,000) and high mass accuracy ($\leq$3 ppm) available from an Orbitrap instrument, which greatly enhances identification certainty. Additionally, tandem mass spectra acquired at a linear ion trap deliver valuable fragments corroborating structure proposals for molecular species. HILIC separation of individual phospholipid classes for as little ion suppression effects as possible completes the setup.

2. Material and methods

2.1. Chemicals

Methanol, acetonitrile, 1-butanol, methyl-tert-butyl ether (MTBE) and disodium hydrogen phosphate were purchased from Sigma–Aldrich (St. Louis, MO, USA), 2-propanol, ammonium formate and citric acid monohydrate from Fluka Analytical (Buchs, Switzerland) and chloroform, formic acid and hydrochloric acid 37% from Merck (Darmstadt, Germany). LIPID MAPS quantitative lipid standards (1,2-dilauroyl-sn-glycero-3-phosphate, 1-dodecanoyl-2-tridecanoyl-sn-glycero-3-phosphate, 1-heptadecanoyl-2-(5Z,8Z,11Z,14Z-eicosatetraenoyl)-sn-glycero-3-phosphate, 1-heneicosanoyl-2-(4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl)-sn-glycero-3-phosphate, 1-heptadecanoyl-2-(9Z-tetradecenoyl)-sn-glycero-3-phosphate (ammonium salt), 1-(10Z-heptadecenoyl)-sn-glycero-3-phosphate, 1-dodecanoyl-2-tridecanoyl-sn-glycero-3-phosphocholine, 1-heptadecanoyl-2-(5Z,8Z,11Z,14Z-eicosatetraenoyl)-sn-glycero-3-phosphocholine, 1-heneicosanoyl-2-(4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl)-sn-glycero-3-phosphocholine, 1-heptadecanoyl-2-(9Z-tetradecenoyl)-sn-glycero-3-phosphocholine, 1-(10Z-heptadecenoyl)-sn-glycero-3-phosphocholine, 1-dodecanoyl-2-tridecanoyl-sn-glycero-3-phosphoethanolamine, 1-heptadecanoyl-2-(5Z,8Z,11Z,14Z-eicosatetraenoyl)-sn-glycero-3-phosphoethanolamine, 1-heneicosanoyl-2-(4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl)-sn-glycero-3-phosphoethanolamine, 1-heptadecanoyl-2-(9Z-tetradecenoyl)-sn-glycero-3-phosphoethanolamine, 1-dodecanoyl-2-tridecanoyl-sn-glycero-3-phospho-L-serine (ammonium salt), 1-heptadecanoyl-2-(5Z,8Z,11Z,14Z-eicosatetraenoyl)-sn-glycero-3-phospho-L-serine (ammonium salt), 1-heneicosanoyl-2-(4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl)-sn-glycero-3-phospho-L-serine (ammonium salt), 1-heptadecanoyl-2-(9Z-tetradecenoyl)-sn-glycero-3-phospho-L-serine (ammonium salt), 1-dodecanoyl-2-tridecanoyl-sn-glycero-3-phospho-(1′-myo-inositol) (ammonium salt), 1-heptade-canoyl-2-(5Z,8Z,11Z,14Z-eicosatetraenoyl)-sn-glycero-3-phospho-(1′-myo-inositol) (ammonium salt), 1-heneicosanoyl-2-(4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl)-sn-glycero-3-phospho-(1′-myo-inositol) (ammonium salt) and 1-heptadecanoyl-2-(9Z-tetradecenoyl)-sn-glycero-3-phospho-(1′-myo-inositol) (ammonium salt)) were purchased from Avanti Polar Lipids (Alabaster, AL, USA); natural phospholipids (1-oleoyl-2-hydroxy-sn-glycero-3-phosphate, L-α-phosphatidic acid (egg PC), L-α-phosphatidylinositol (bovine heart), L-α-phosphatidylcholine (bovine liver), L-α-lysophosphatidylcholine (bovine liver), L-α-phosphatidylethanolamine (corn germ), L-α-lysophosphatidylethanolamine (egg yolk), L-α-phosphatidylglycerol (egg PC), L-α-phosphatidylserine (porcine brain) and cardiolipin (ammonium salt) (heart, bovine)) were purchased from Larodan Fine Chemicals AB (Malmö, Sweden). Deionized water was obtained from a MilliQ Gradient A10 system (Millipore, Billerica, MA, USA).

Lipid shorthand nomenclature is used according to Liebisch et al. [30].

2.2. Mouse information and tissue homogenization

A male C57BL/6JRj mouse was euthanized and the brain was dissected and flash frozen in liquid nitrogen. The brain was homogenized in a BioPulverizer (BioSpec Products, Bartlesville, OK) and triplicates of approximately 25 mg of tissue homogenate were weighed and extracted using the modified Bligh & Dyer HCl method described below.

2.3. Lipid extraction

Evaluation of extraction efficiency was performed on standard mixtures of naturally occurring phospholipid standards by comparing the extract to the unextracted standard mixture. Prior to extraction of tissue samples, 20 pmol of PA 24:0 were added to the sample to account for analyte losses during extraction. After extraction, the organic phase was dried in a vacuum centrifuge (Thermo Fisher Scientific Inc., Waltham, MA).

2.3.1. Bligh & Dyer (B&D, modified after [31])

To 10 μL of phospholipid standard mixture were added 2.5 mL of methanol and 2.5 mL of chloroform and the mixture was then incubated for 60 min in an overhead shaker at room temperature. 2.25 mL of deionized water was added to induce phase separation and after additional 10 min of shaking the mixture was centrifuged for 3 min at 2000 × g and the lower phase was collected.

2.3.2. Bligh & Dyer HCl (B&D HCl) [32]

To phospholipid standard mixture or tissue homogenate were added 800 μL of a 1:1 (v/v) mixture of 0.1 M HCl and methanol and the mixture was vortexed for 1 min. After addition of 400 μL of chloroform and vortexing for 1 min, the mixture was centrifuged for 3 min at 2000 × g and the lower phase was collected.

2.3.3. MTBE [33]

To phospholipid standard mixture were added 1.5 mL of methanol and 5 mL of MTBE and the mixture was incubated for 60 min in an overhead shaker at room temperature. After addition of 1.25 mL of deionized water and 10 min of additional incubation the mixture was centrifuged for 3 min at 2000 × g and the upper phase was collected.