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Short communication

Measurement of endogenous lysophosphatidic acid by ESI-MS/MS in plasma samples requires pre-separation of lysophosphatidylcholine

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ABSTRACT

The levels of lysophosphatidic acid (LPA) or lysophosphatidylcholine (LPC) in plasma have been shown to be markers for several human diseases, including cancers. Here we show that the presence of LPC or other lysophospholipids (LPLs) in lipids extracted from biological samples affects accurate measurement of endogenous LPA in biological samples. We report for the first time the artificial conversion of LPC and lysophosphatidylserine (LPS) to LPA at the ion source of electrospray ionization tandem mass spectrometry (ESI-MS/MS). To avoid the interference of LPC with the quantification of LPA, a method based on high-performance liquid chromatography (HPLC) separation of LPA from LPC has been developed.

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1. Introduction

Lysophosphatidic acid (LPA) and lysophosphatidylcholine (LPC) are bioactive signaling molecules involved in many biochemical, physiological, and pathological processes. LPA and/or LPC levels in plasma or serum have been identified as potential biomarkers for certain human diseases, such as ovarian cancer [1–6], colorectal cancer [7], myeloma [8], sepsis, and other pathophysiological conditions [9–12]. Thus, for both biological functional assays and marker development, it is extremely important to develop methods which accurately and reproducibly analyze them.

Although many analytical methods have been developed for the analysis of phospholipids, including NMR method [13–17], mass spectrometry (MS)-based method is the best in terms of accurate quantification [18]. Many different laboratories now use liquid chromatography–tandem mass spectrometry (LC–MS/MS) for detection and quantitative analyses of lysophospholipids (LPLs). However, a number of different lipid preparation and LC–MS conditions have been utilized. For example, in our lab, thin layer chromatography (TLC) was used to purify LPA and then a flow injection in MS was used to quantify LPA [19], while Yoon et al. developed a direct flow injection LC–ESI-MS/MS method to analyze LPA in human plasma samples [6,20]. Recently, Shan et al. have reported that some unknown compounds in plasma produce the same parent-to-daughter ion transitions as LPA and interfere with the quantification of LPA in a direct flow injection LC–ESI-MS/MS method [21]. We have observed the same phenomenon in our studies (unpublished). Since LPA has been shown to be involved in numerous biological activities, accurate measurement of endogenous LPAs becomes critically important.

In the current work, we have investigated the "unknown compounds" in plasma that could give rise to the "LPA" signal in MS detection and found that LPCs were the major source. In addition, lysophosphatidylserine (LPS) could also generate "LPA" signal during mass spectrometric analysis. Thus, separation of LPC and/or LPS from LPA is essential for accurate detection of endogenous LPA in biological samples. The HPLC conditions used for separating LPAs from LPCs have been established.

2. Experimental

2.1. Materials

Lipid standards were purchased from Avanti Polar lipids (Birmingham, AL). Organic solvents were purchased from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). Mouse blood samples were obtained from the facial vein of mice in EDTA-containing tubes and centrifuged at 1750 × *g* for 15 min at room temperature. Plasma samples were aliquoted into siliconized eppendorf tubes (PGC Scientifics, Frederick, MD) and frozen at −80 °C until utilized.

2.2. Lipids extraction and HPLC–ESI-MS/MS

LPLs extraction was performed essentially the same as we described previously [7], except 10 μL of plasma samples (instead

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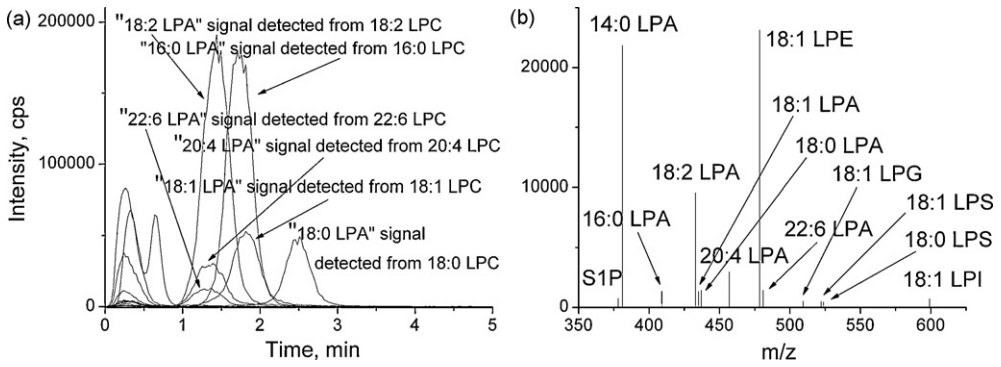

Fig. 1. Negative ion MRM chromatogram (a) of LPLs with HPLC separation, and mass spectrum (b) eluted from the HPLC column in 0–1 min. The LPLs were extracted from a mouse plasma sample.

of 100 μL) were used. MS analyses were performed using API-4000 (Applied Biosystems, Forster City, CA). Typical operating parameters were as follows: collision gas (CAD) 8 units, curtain gas (CUR) 10 psi, ion source gas 1 (GS1) 15 psi, ion source gas 2 (GS2) 35 psi, electrospray voltage 5000 V with positive ion MRM mode or −4200 V with negative ion MRM mode, and a temperature of heater at 500 °C. Multiple reaction monitoring (MRM) mode was used for measurement of LPLs. Negative and positive monitoring ions were described previously [7,19].

Samples (10 μL) were loaded through a LC system (Agilent 1100) with an auto sampler. A TARGA C18 5 μM, 2.1 mm i.d. × 10 mm TR-0121-C185 (Higgins Analytical, Southborough, MA, USA) HPLC column was used for the separation of LPC from other LPLs. The mobile phase A was MeOH/water/NH_4OH (90:10:0.1, v/v/v) and the mobile phase B was 5 mM ammonium acetate in MeOH/water (90:10, v/v). The HPLC separations were 12 min/sample using the following scheme: (1) 100% A for 3 min with a flow rate at 0.2 mL/min; (2) the mobile phase was changed from 100% A to 100% B over 2 min with the flow rate increased from 0.2 to 0.8 mL/min; (3) a constant flow rate of 0.8 mL/min for 5 min; (4) the mobile phase was changed from 100% B to 100% A in 1 min with the flow rate decreased from 0.8 to 0.2 mL/min; and (5) constant flow rate of 0.2 mL/min for 1 min. For LPCs detection, samples (10 μL) were directly injected into the MS ion source; the flow rate was 0.2 mL/min and the duration was 1.5 min/sample.

3. Results and discussions

3.1. LPAs in plasma

We have found that acidic condition was required for effective extraction of LPAs, and short-time incubation during lipid extraction was important to prevent acid-induced hydrolysis [19,22]. To determine whether a separation of other lipids from LPAs was necessary for LPA detection, we developed a HPLC method (see Section 2). A short C18 HPLC column (10 mm) was used and the inclusion of ammonium acetate (5 mM) in the mobile phase reduced the elusion times for phospholipids, such as phosphatidylethanolamine (PE) and phosphatidylcholine (PC) to 12 min. While lysophosphatidylglycerol (18:1 LPG), lysophosphatidylethanolamine (18:1 LPE), 18:1 LPS, and lysophosphatidylinositol (18:1 LPI) were not well separated from LPAs (eluted in 0.5–1 min), other lipids, including LPCs, phosphatidic acid (PA), and phosphatidylethanolamine (PE) were well separated from LPAs (Figs. 1–3). The chromatogram (Fig. 1a) and mass spectrum (Fig. 1b) of LPA species extracted from a mouse plasma sample using negative ion MRM detection mode are shown. Two sets of "LPA" MS signals were detected in fractions eluted at 0.5–1 and 1.5–4 min, respectively (Fig. 1a). Under the same HPLC–MS/MS conditions, the authentic LPAs (LPA standard compounds) were eluted between 0.5 and 1 min (Fig. 2a), indicating that the lipids eluted at 0.5–1 min should be endogenous LPA in the plasma. On the other hand, the LPA signals detected from the lipids eluted between 1.5 and 4 min could be derived from an unknown source.

3.2. The conversions of LPC and LPS to LPA

In any of the published methods for lipids extraction [7,19,22–25], LPCs were co-extracted with LPAs, albeit the efficiencies of extractions varied from method to method. Since LPC concentrations in biological samples are generally 10–100 times higher than those of LPA [7,19,23], we tested whether the second set of "LPA" signals which eluted between 1.5 and 4 min were derived from conversion of LPC. Under the same HPLC conditions, different LPC species, sphingosylphosphorylcholine (SPC) and lyso-platelet factor (lyso-PAF) (100 nM) were separated from corresponding LPAs and were eluted between 0.5 and 6 min and were detected in the *positive ion* MRM mode (Fig. 2b). When 16:0, 18:1 and 18:0 LPC standards (100 nM) were injected without other lipids and MS signals were detected in the *negative ion* detection mode, two sets of "LPA" MS signals were detected in fractions eluted at 0.5–1 and 1–4 min, respectively (Fig. 2c). The signals detected from the lipid fractions eluted between 0.5 and 1 min were very weak, which were from a very low level of contamination of LPAs in standard LPCs. However, strong "LPA" MS signals were detected in the fractions eluted from 1 to 4 min, corresponding to the elution times for

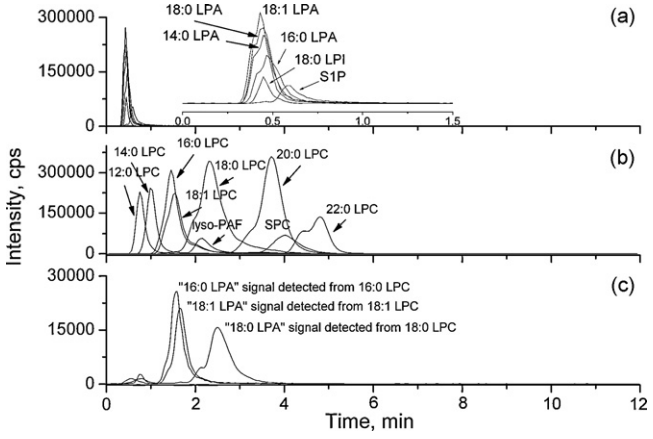

Fig. 2. The chromatogram of LPL standards detected by HPLC–ESI-MS/MS. (a) Negative ion MRM mode analysis of LPAs, S1P and LPI standards (100 nM each); the insert is a time expansion of the chromatogram; (b) positive ion MRM mode analysis of LPCs, SPC and lyso-PAF standards (100 nM each); (c) negative ion MRM mode analysis of 16:0, 18:1 and 18:0 LPC standards (100 nM each).