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Development and validation of a UHPLC-ESI-MS/MS method for the simultaneous quantification of mammal lysophosphatidylcholines and lysophosphatidylethanolamines in serum

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ABSTRACT

Recent investigations based on non-targeted metabolomics have proposed lysophospholipids (Lyso-PLs) as biomarkers of different diseases. In particular, lysophosphatidylcholines (Lyso-PCs) and lysophosphatidylethanolamines (Lyso-PEs) have been associated with serious lipid pathologies. Methods to determine the different molecular species in a biological sample and to quantify even less abundant species are required for the evaluation of the Lyso-PL pattern as a novel comprehensive biomarker of dyslipidemia. This study describes the development and validation of an ultra-high-performance liquid chromatography coupled to tandem mass spectrometry assay for the determination of a large number of Lyso-PCs and Lyso-PEs in biological samples. The method was validated in rat serum using two simple methanol-based extractions with low sample volumes (5–50 μL) that covered the wide concentration range of these metabolites. In total, thirty-one Lyso-PLs were separated and quantified with low method limits of detection and quantification, reaching values of 0.2 and 0.8 nM, respectively. The method was subsequently applied in the identification of Lyso-PL-related changes produced by the chronic intake of a cafeteria diet. The results showed alterations in the majority of Lyso-PCs and Lyso-PEs in rat serum. Furthermore, multivariate analysis indicated that the comprehensive evaluation of serum Lyso-PLs could be an excellent indicator of the nutritional phenotype associated with an increased risk of lipid disorders.

1. Introduction

Glycerol-based lysophospholipids (Lyso-PLs) are structural components of cellular membranes that can also act as signaling molecules in a wide range of physiological and pathological events including inflammation, reproduction, nervous and vascular system development and carcinogenesis [1]. The molecular structure of Lyso-PLs is very simple and consists of a hydrophobic acyl chain attached to the *sn1*- or *sn2*-position of the glycerol backbone and a hydrophilic phosphate head group in the *sn3*-position whose nature determine the diverse classes of Lyso-PLs.

Among circulating lyso forms, those containing a choline group, namely lysophosphatidylcholines (Lyso-PCs), are the most abundant, with serum levels of several hundred micromolar [2,3]. Lyso-PCs have been mainly related to inflammatory diseases and atherosclerosis [4–7]. Conversely, lysophosphatidylethanolamines (Lyso-PEs) exhibit a smaller head group than Lyso-PCs and are present at lower circulating levels, reaching a maximum concentration of several hundred nanomolar [8,9]. Information regarding the biological significance of Lyso-PE in serum is scarce compared to Lyso-PCs, although both classes of Lyso-PLs have been postulated as biomarkers of the progression of different pathologies. Lipid profiling of patients with different development statuses of atherosclerotic plaques showed that certain modifications in the circulating levels of Lyso-PCs and Lyso-PEs were associated with disease severity [10]. Continuing with a holistic view, a recent study noted the importance of both Lyso-PLs as serum indicators of hepatocarcinogenesis progression in humans [11]. *In vivo* investigations demonstrated that dysregulation of serum and hepatic

Abbreviations: Lyso-PL, lysoglycerophospholipid; Lyso-PC, lysophosphatidylcholine; Lyso-PE, lysophosphatidylethanolamine; ST, standard chow; CAF, cafeteria diet; LSV and HSV, low and high sample volume extractions; PCA, principal component analysis; ROC, receiver operating characteristic

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levels of several Lyso-PCs and Lyso-PEs occurs in rodents fed high-calorie diets [12–14], while differential patterns of secreted Lyso-PLs have been observed in a cellular model of steatosis [9]. Therefore, the examination of circulating levels of Lyso-PLs holds remarkable potential in the diagnosis of lipid disorders and could be used to assess the therapeutic effects of drugs. Since the pathways leading to the synthesis of Lyso-PCs and Lyso-PEs are related [15], it would be of interest to quantify them in a high-throughput analysis with sufficient specificity to differentiate Lyso-PLs containing different side chains.

A variety of methodologies, including coupled techniques based on chromatography, have been developed for the evaluation of Lyso-PCs and Lyso-PEs in biological samples. One of the most common techniques involves separation by thin-layer chromatography (TLC) on silica gel plates [16]. However, once Lyso-PLs are isolated, they must be extracted for further analysis of the fatty acid composition, and thus this methodology is laborious and time-consuming [17,18]. Moreover, the quantification of molecular species present in low abundance using this approach is very difficult. In recent years, chromatographic techniques have become more robust and specific. The use of reverse phase high-performance liquid chromatography (HPLC) coupled to fluorescence [19], ultraviolet (UV) [20], radioactive [21], phosphorus [22], and evaporative light-scattering [23] detection permits enhanced separation of Lyso-PLs containing different acyl chains from complex mixtures with high resolution. However, these quantitative techniques have low sensitivity and poor selectivity for Lyso-PLs. By contrast, electrospray ionization mass spectrometry (ESI–MS) stands out as suitable technique due to the easily ionizable nature of the polar head group of Lyso-PLs [24]. The soft ionization achieved using ESI–MS offers a series of advantages that make it an ideal technique for the analysis of biological samples. ESI–MS detects intact molecules with very high sensitivity, and ESI-tandem MS (ESI–MS/MS) permits the determination of the structural composition of the relevant ions. Thus, HPLC-ESI–MS/MS can lead to real and simultaneous identification and quantification of the many compounds present in a biological sample. As a result, this approach is currently used in studies evaluating the Lyso-PL content of experimental rodent and human samples [2,25,26]. The recent emergence of ultra-HPLC (UHPLC) with sub-2-μm column particles has enabled much greater operating pressures compared with HPLC, thus reducing analysis times without sacrificing efficiency [27,28]. Such properties are essential for clinical research and the evaluation of biomarkers since these studies require the evaluation of a large number of samples. Because Lyso-PEs are the Lyso-PLs more recently postulated as biomarkers in mammals, most of the present quantitative methods which are focused on Lyso-PLs have considered few or none molecular species within the lipid subclass.

Therefore, the main objective of the present study was to develop a rapid, sensitive and reproducible methodology based on UHPLC-ESI–MS/MS for the exhaustive characterization of Lyso-PCs and Lyso-PEs as part of the evaluation of the comprehensive biomarker in serum. First, circulating members belonging to the two lipid subclasses were identified in a pooled serum sample using a UHPLC coupled to a quadrupole time-of-flight mass spectrometer (UHPLC-QTOF). Second, a quantitative method was applied using two simple methanol-based procedures (to cover the entire concentration range of the metabolites of interest) and coupling of the UHPLC to a triple quadrupole mass spectrometer (UHPLC-QqQ). To validate the developed method, several quality parameters were determined by spiking the rat serum with different Lyso-PL standards. The suitability of the quantitative methodology was demonstrated by comparison of the circulating levels of Lyso-PLs found in two groups of rats with different diets. Subsequent multivariate data analysis of all identified Lyso-PLs was also conducted in both animal groups to evaluate the efficiency of the biomarker in serum.

2. Material and methods

2.1. Chemicals and reagents

Methanol (Scharlab, Barcelona, Spain), acetonitrile (Millipore, Darmstadt, Germany), isopropanol, glacial acetic acid (Panreac Applichem, Barcelona, Spain), chloroform and 7.5 M ammonium acetate solution (Sigma-Aldrich, St. Louis, MO, USA) were of the highest grade commercially available. Ultrapure water was obtained from a Milli-Q advantage A10 system (Madrid, Spain). Lyso-PL standards were all of > 99% purity and were purchased from Avanti Polar Lipids (Birmingham, AL, USA). Lyso-PC standards included 1-tridecanoyl-*sn-glycero*-3-phosphocoline, Lyso-PC (13:0); 1-palmitoyl-*sn-glycero*-3-phosphocoline, Lyso-PC (16:0); 1-stearoyl-*sn-glycero*-3-phosphocoline, Lyso-PC (18:0); and 1-arachidoyl-*sn-glycero*-3-phosphocoline, Lyso-PC (20:0). Lyso-PE calibrators were 1-palmitoyl-*sn-glycero*-3-phosphoethanolamine, Lyso-PE (16:0); 1-stearoyl-*sn-glycero*-3-phosphoethanolamine, Lyso-PE (18:0); and 1-oleoyl-*sn-glycero*-3-phosphoethanolamine, Lyso-PE (18:1). The standards contained a small proportion of the corresponding *sn2*-Lyso-PL isomer. The standards were individually dissolved in methanol/chloroform/water (65:35:8 v/v/v) at 2 mg/mL and stored in dark-glass vials at −20 °C prior to use. On the day of LC–MS/MS analysis, a mixed standard solution with a concentration of 100 mg/L was prepared using methanol and diluted with water/isopropanol/acetonitrile (4:3:3 v/v/v) to the desired concentrations. Lyso-PC (13:0) was separately handled similarly to the standards for use as internal standard (IS). Butylated hydroxytoluene (BHT; Sigma-Aldrich, St. Louis, MO, USA) was used as an antioxidant.

2.2. Animal procedure

Serum samples were obtained from female Sprague-Dawley rats (3-month-old). The animals were maintained under standard conditions of temperature (22 ± 1 °C) and relative humidity (50 ± 10%) with a light/dark period of 12 h. The rats were randomly divided into two groups (n = 5 per group) with free access to food and water. For 8 weeks, the animals were fed *ad libitum* either standard chow (ST) or a highly palatable diet rich in fat and carbohydrates known as the cafeteria diet (CAF) [14]. The CAF was renewed daily and consisted of the followings components (quantity per rat/day): bacon (8–12 g), biscuits with pâté (12–15 g) or cream cheese (10–12 g), sweet roll (8–10 g), carrot (6–8 g), milk with sugar (220 g/L; 50 mL) and ST chow. After overnight fasting, ST- and CAF-fed rats were alternately sacrificed by beheading to avoid interference due to chemical drugs, and total blood was collected in falcon tubes. Serum samples were obtained by allowing blood to clot at room temperature for 30 min. The samples were then centrifuged at 2000 g and 4 °C for 15 min, aliquoted and stored at −80 °C until metabolite extraction and LC–MS/MS quantification. All procedures were performed in accordance with the European Communities Council Directive regarding the protection of experimental animals (86/609/EEC).

2.3. Identification of Lyso-PCs and Lyso-PEs in rat serum

Serum volumes of 10 μL from each animal were pooled (n = 10) for determination of the specific members of Lyso-PCs and Lyso-PEs in rat serum. Metabolites were extracted from serum using a hydroalcoholic solution. Briefly, 900 μL of methanol/water (8:1 v/v) was added to 100 μL of pooled sample, vortexed (30 s) and ultrasonicated (30 s). The mixture was incubated on ice for 10 min and centrifuged (20,000*g*, 10 min, 4 °C). The supernatants were dried under nitrogen flow and redissolved in 200 μL of water/methanol (1:1 v/v) prior to injection.

The exhaustive characterization of the two circulating subclasses of lipids was performed using a UHPLC 1290 coupled to a Q-TOF 6550 Series mass spectrometer equipped with a Dual ESI source using Agilent Jet Stream Technology (AJS ESI) (Agilent Technologies, Palo Alto, CA,

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