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On-line high speed lipid extraction for nanoflow liquid chromatography-tandem mass spectrometry

Ju Yong Lee, Joon Seon Yang, Se Mi Park, Seul Kee Byeon, Myeong Hee Moon*

Department of Chemistry, Yonsei University, Seoul, 120-749, South Korea

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ABSTRACT

An on-line lipid extraction method is introduced by utilizing a short capillary extraction column using HILIC and C4 particles prior to nanoflow liquid chromatography-tandem mass spectrometry (nLC–MS/MS). The on-line extraction using a urine sample spiked with PL standards showed similar or slightly higher recovery values (86%–96%) of phospholipids (PLs) compared to those obtained by the conventional off-line extraction based on the Folch method with or without using the air-exposed drying process. In this study, we demonstrated that PL oxidation can occur during the air-exposed drying process of lipid extracts in standard liquid–liquid extraction procedures, which was confirmed by the oxidized PL (OxPL) molecules that were generated from an off-line extraction using a few PL standards. Quantitative comparison of these OxPL species between on- and off-line extraction followed by nLC–MS/MS with multiple reaction monitoring (MRM) analysis showed a significant decrease (2–10 fold) in unwanted OxPL species when on-line extraction was employed. While the number of identified PLs from a urine sample was somewhat lower than those by off-line extraction, the number of OxPLs was significantly reduced (from 70 to 22) with on-line extraction. The new method offers high speed (~5 min) automated extraction of PLs with nLC–MS/MS analysis and presents the possibility of handling a biological sample with a very limited amount of lipids.

1. Introduction

Lipidomics is the study of lipid networks in biological systems and generally needs molecular characterization and quantitation of lipids to elucidate their functions and interactions with proteins, cells, and other metabolites. Lipids are the main components of cellular membranes; moreover, they are involved in important functions such as energy storage, signal transduction between cells and proteins, cell proliferation, and death [1,2]. Recently, using lipidomic analysis for developing biomarkers has attracted considerable attention because lipids are known to be directly or indirectly related to the development of various adult diseases such as diabetes, cardiovascular diseases, and some cancers [3–7]. However, since lipids are very diverse in their molecular structures and polarities, extracting and analyzing lipid species of different categories simultaneously is difficult; therefore, lipidomic analysis required comprehensive and accurate analytical methods.

Mass spectrometry (MS) plays an important role in lipid analysis because of the simultaneous detection of a number of molecular ions and determination of their molecular structures. Liquid chromatography-electrospray ionization-tandem MS (LC-ESI–MS/MS) provides separation of complicated lipid mixtures in their intact states, followed by both qualitative and quantitative lipid profiling [8–10]. Nanoflow reversed-phase LC (nRPLC) with ESI–MS/MS has been utilized for analyzing phospholipids (PLs) from human plasma and urine samples with a limit of detection (LOD) of low fmol levels [11–13]; moreover, ultrahigh performance LC (UPLC) with ESI–MS/MS with enhanced resolution and speed has been utilized for identifying more than 400 lipids from rat plasma [14]. Recently, hydrophilic interaction chromatography (HILIC) method has shown its applicability to separate various lipid species from egg yolk [15]. Supercritical fluid chromatography (SFC) coupled with MS has been utilized for lipid profiling [16,17] and ultrahigh-performance SFC has demonstrated its high-throughput capability of analyzing 436 lipids in 6 min [18]. Although the abovementioned approaches can accelerate the performance and speed of lipid analysis, sample preparation, which includes lipid extraction from biological materials, requires attention because extracting all lipid classes simultaneously with a high recovery rate is difficult. Typical lipid extraction based on liquid–liquid extraction (LLE) has been widely utilized using the Folch method [19] or the Bligh and Dyer method [20]; however, these methods generally require large amounts of samples and removal of water when handling urine or blood samples requires

* Corresponding author.
E-mail addresses: mhmoon@hotmail.com, mhmoon@yonsei.ac.kr (M.H. Moon).

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a few hours. The solid phase extraction method offers a reduced matrix effect with higher selectivity compared to LLE; however, completing the extraction/purification steps, including the use of a series of solvents, requires time [21,22]. The quick, easy, cheap, effective, rugged, and safe (QuEChERS) method was applied for extracting lipids from human plasma and urine samples. Note that it shows high speed (<15 min) preparation of lipids with levels of lipid identification comparable to those of the Folch method [23]; however, adsorbents used to remove water in the QuEChERS method are not re-usable, which makes it less suitable for on-line extraction. Although the above methods provide an efficient extraction/clean-up of lipids, special care is required when extracting lipids from biological samples because, depending on the lipid classes to be analyzed, each method requires a slight modification for selecting organic solvents. Moreover, there is a possibility of inducing unwanted oxidation of lipid molecules from exposure to air during the drying step. Lipid oxidation generally occurs in biological systems via enzymes and/or reactive oxygen species (ROS); moreover, lipid oxidation typically results in hydroxylation of unsaturated acyl chains of PLs, cleavage of acyl chain into short-chain products or lysophospholipids (LPLs), and the dissociation of head groups of PLs [24,25]. Because oxidation of lipids alters biological membrane structures and leads to an increase in oxidized low-density lipoproteins, which is a key factor for the development and progression of age-related chronic diseases such as atherosclerosis [26,27], minimizing external lipid oxidation during extraction for accurate lipid analysis is important. In a recent report on the quantitative profiling of oxidized PLs in different lipoproteins from patients with coronary artery disease, special care was required to prevent unwanted lipid oxidation during extraction [28]. External lipid oxidation can be minimized by using N_2 during the drying steps or by using an on-line lipid extraction followed by direct analysis using MS or LC–MS. On-line lipid extraction may offer high speed extraction, which reduces the entire analysis time, however it has been hardly found except a report in which laser capture microdissection was utilized to collect fluorescently labeled brain tissue patches and carry out on-column lipid extraction prior to LC–MS^n [29]. The latter method demonstrated an identification of 58 lipids, but it was limited to mostly PC and PE with few Cer, and etc.

This study introduces an on-line lipid extraction device that can be utilized between an autosampler and an LC column during LC-ESI–MS/MS. This device is designed with a capillary based extraction column with a combination of packing materials, which can be applied for high speed lipid extraction by injecting urine samples after a simple treatment to remove proteins or macromolecules. The efficiency of the on-line lipid extraction column was evaluated using various particles (C4, C8, C18, and HILIC) packed in a short capillary tube by comparing the recoveries from a urine sample spiked with standard PLs between on-line and few off-line extraction methods. Also this study reveals that PL oxidation can occur with air exposure during the drying steps in typical extraction methods by confirming the molecular structures of the produced OxPLs when a few standard PLs underwent known extraction methods. Finally, the new method was applied for the comparison of urine samples' OxPL profiles between on- and off-line extraction methods.

2. Experimental

2.1. Materials & chemicals

Nineteen PL standards were purchased from Avanti Polar Lipid, Inc. (Alabaster, AL, USA): 14:0-LPE (lysophosphatidylethanolamine), 18:0-LPE, 12:0/12:0-PE (phosphatidylethanolamine), 14:0/14:0-PE, 14:0-LPG (lysophosphatidylglycerol), 18:0-LPG, 12:0/12:0-PG, 15:0/15:0-PG, 16:0-LPC (lysophosphatidylcholine), 16:0/16:0-PC (phosphatidylcholine), 20:0/20:0-PC, 14:0/14:0-PS (phosphatidyserine), 18:0/18:0-PS, 16:0/18:2-PI (phosphatidylinositol), d18:0/12:0-SM (sphingomyeline), d18:1/16:0-SM for the extraction efficiency test, 16:0/18:1-PE and 18:0/22:6-PG for the experiments to confirm oxidation products, and 16:0/16:0-PG as an internal standard (IS). Each PL standard was dissolved in 1:1 chloroform/MeOH at a concentration of 1 nmol/μL to make a stock solution. For the case of polar PLs like lysophospholipids, use of chloroform was minimized then small quantity of water (<15%) was added to enhance dissolvation. Then each stock solution was mixed to make a mixture at a concentration of 50 pmol/μL of each lipid in 8:2 MeOH/water for spiking to urine sample. A human urine sample was obtained from a healthy male volunteer (age 27) and stored under −20 °C before usage. Ammonium hydroxide and ammonium formate were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA), HPLC grade solvents (acetonitrile, methanol, isopropanol, and water) were obtained from J. T. Baker (Phillipsburg, NJ, USA). $MgSO_4$ and CH_3COONa, which were used for QuEChERS, were obtained from Agilent Technologies (Palo Alto, CA, USA).

2.2. Off-line lipid extraction

Three extraction methods were utilized in this study. In the conventional Folch method, 200 μL of either urine or water both spiked with standard lipid mixtures was first mixed with 40 μL of methanol and 100 μL of chloroform. After 30 min, the mixture was centrifuged at 5000*g* for 10 min. The lower phase was then collected and dried in the vacuum centrifuge or under dried N_2 gas without using a vacuum centrifuge. For the Folch method with methyl-t-butylether/methanol (Folch with MTBE/MeOH), 200 μL of either urine or water both spiked with standard lipids was mixed with 40 μL of methanol and 100 μL of MTBE followed by vortexing for 30 min [30]. After centrifuging at 5000*g* for 10 min, the top layer containing lipids was collected and the bottom layer was transferred to another vial to extract remaining lipids by adding 100 μL of methanol followed by centrifugation for another 10 min. The supernatant was then collected and combined with the previously collected top layer. This mixture was dried under vacuum centrifuge or under dried N_2 gas to avoid exposure to air. Dried lipid extracts are dispersed in a solvent mixture composed of 10 μL of chloroform, 90 μL of 1:9 (v/v) acetonitrile:methanol, and 100 μL of LC mobile phase A (to be described later). The modified QuEChERS method for extraction of lipids follows the procedure described in the earlier report [23]. Briefly, the above described 200 μL of either urine or water both spiked with standard lipids is added to a prepacked extraction kit (250 mg of $MgSO_4$, 50 mg of CH_3COONa, and a glass ball) and mixed with 400 μL of 2:1 (v/v) chloroform:methanol for 1 min. The mixture is then centrifuged at 10,000*g* for 10 min, and the upper layer was transferred to 50 mg of C18 particles. The latter is centrifuged again at the same condition, and the upper layer is added to a separate vial and diluted to a final volume of 200 μL using the mobile phase A for nLC-ESI–MS/MS analysis.

2.3. On-line lipid extraction column

The on-line extraction column was prepared in the laboratory by packing various particles in a capillary (100 μm I.D. × 360 μm O.D. × 4 cm). Tested bead materials were Nucleodur® HILIC (5 μm), which was obtained from Macherey-Nagel (Duren, Germany); Magic® C4 and C8 resins (5 μm, 200 Å each), which was obtained from Bruker-Michrom (Auburn, CA, U.S.A.); and Watchers® ODS-P C18 particles (3 μm, 100 Å) from Isu Industry Corp. (Seoul, Korea). For packing the on-line extraction column, one end of the capil-