Journal of Chromatography A, xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Rapid and simple extraction of lipids from blood plasma and urine for liquid chromatography-tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 31 October 2013
Received in revised form 7 January 2014
Accepted 10 January 2014
Available online xxx

Keywords:
Lipids
Dispersive solid-phase extraction
QuEChERS
Blood plasma
nLC-ESI-MS/MS

ABSTRACT

A simple and fast lipid extraction method from human blood plasma and urine is introduced in this study. The effective lipid extraction from biological systems with a minimization of the matrix effect is important for the successful qualitative and quantitative analysis of lipids in liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). The method described here is based on the modification of the quick, easy, cheap, effective, rugged and safe (QuEChERS) extraction method, which was originally developed for pesticide residue analysis in food, for the purpose of isolating lipids from biological fluids. Applicability of QuEChERS method for lipids was evaluated by varying organic solvents for the extraction/partitioning of lipids in $MgSO_4$/CH_3COONa for the removal of water and by varying sorbents (primary secondary amines, graphitized carbon black, silica, strong anion exchange resins and C18 particles) for the dispersive solid-phase extraction (dSPE) step. This study shows that 2:1 (v/v) $CHCl_3$/CH_3OH is effective in the extraction/partitioning step and that 50 mg of C18 particles (for 0.1 mL plasma and 1 mL of urine) are more suitable for sample cleanup for the dSPE step of the QuEChERS method. Matrix effects were calculated by comparing the recovery values of lipid standards spiked to both plasma and urine samples after extraction with those of the same standards in a neat solution using nanoflow LC-ESI-MS/MS, resulting in improved MS signals due to the decrease of the ion suppression compared to the conventional Folch method. The modified QuEChERS method was applied to lipid extracts from both human urine and plasma samples, demonstrating that it can be powerfully utilized for high-speed (<15 min) preparation of lipids compared to the Folch method, with equivalent or slightly improved results in lipid identification using nLC-ESI-MS/MS.

1. Introduction

Lipids are major components of biological membranes and are involved in a number of metabolic processes, such as intercellular interactions, secretion, and energy storage [1]. Due to the important roles of lipids, a comprehensive lipid profile is necessary to study the mechanisms of lipid-related diseases, such as diabetes, Alzheimer's disease, atherosclerosis, and breast cancer [2,3]. Since lipids are so diverse their molecular structures, which are classified into eight categories based on their polar nature and common molecular backbone structures [4,5], it is difficult to simultaneously analyze all lipid classes.

Analytical methods that are frequently employed for lipid analysis are liquid chromatography-mass spectrometry (LC-MS) and direct analysis with MS via electrospray ionization (ESI). LC provides highly sensitive separation of lipid classes, depending on their polarity, in normal-phase LC (NPLC), or high-resolution separation of individual lipid molecules based on hydrophobicity of alkyl chains in lipids in reversed-phase LC (RPLC) [6–9]. With the coupling of LC with ESI-MS or ESI-MS/MS, lipid profiling can be conducted both qualitatively and quantitatively, along with the structural determination [10,11]. ESI-MS provides a fast analysis of lipids along with structural determination using high-resolution MS or from tandem MS experiments; however, ion suppression of highly abundant species can be of concern when complex lipid mixtures are to be analyzed [12–14].

Despite the high performances of these sophisticated analytical methods, the important pre-requisite for the successful analysis is the efficient extraction of highly complicated lipid mixtures of biological origin, such as cells, tissues, plasma, and urine, along with the removal of interfering substances, such as proteins, sugars, and other small molecules. Since lipids are so diverse in their hydrophobic and hydrophilic properties, it is often complicated to simultaneously extract all lipid classes with a high recovery

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http://dx.doi.org/10.1016/j.chroma.2014.01.024

rate. Traditionally, lipid extraction has been carried out with the Folch method [15] or the Bligh and Dyer method [16] based on liquid-liquid extraction. The liquid-liquid extraction (LLE) method requires a large sample, but generally yields low recovery as well as showing a matrix effect in LC-MS analysis [17,18]. Particularly for plasma or urine samples, the removal of water without losing recovery is an essential step. When the typical freeze-drying method is added, it often takes a long time (~12 h for urine) [19–21]. While a solid-phase extraction (SPE) method is effective in reducing the matrix effect and more selective than the LLE method, it requires a long period of time to carry out a series of purification/extraction processes, such as the preconditioning of sorbent materials, use of multiple solvents, and solvent waste fractionation steps [22–24]. Recently, a rapid extraction/cleanup approach, known as the QuEChERS (quick, easy, cheap, effective, rugged, and safe) method, was introduced by Anastassiades et al. in 2003 [25] for the multi-residue analysis of pesticides contained in food, and it was rapidly applied to various foods and environmental samples [26–28]. The method was further developed to extract polycyclic aromatic hydrocarbons in fish [29], veterinary drug residues in animal tissue [30], mycotoxins in cereals [31], and pharmaceuticals from whole blood [32,33]. It is known that the QuEChERS method reduces the preparation time (< 30 min) required to complete the extraction and clean-up processes. The extraction/partitioning step of QuEChERS involves extraction with CH_3CN partitioned from aqueous matrix using anhydrous magnesium sulfate to absorb water and sodium acetate to enhance phase separation. The second step of QuEChERS is the dispersive solid-phase extraction (dSPE) to cleanup impurities by using sorbents such as primary secondary amine (PSA), graphitized carbon black (GCB), and C18 particles.

This study focuses on the applicability of a QuEChERS-based approach toward the extraction and cleanup of various lipids from human blood plasma and urine samples for nanoflow LC-ESI-MS/MS analysis. The QuEChERS method, to our knowledge, has not been applied for lipid extraction. An initial evaluation was conducted by the recovery test of the extraction/partitioning step by varying organic solvents (CH_3CN, CH_3OH, $CHCl_3/CH_3OH$, and MTBE/CH_3OH) with nineteen lipid standards, followed by a recovery test of the dSPE step using different sorbents such as PSA, GCB, silica, strong anion exchange (SAX) resins, and C18 particles. To evaluate the influence of the matrix, recovery values of lipid standards spiked to both plasma and urine samples before and after extraction using the modified QuEChERS method selected from the initial evaluation were compared to calculate "matrix effects" using the equation proposed by Matuszewki et al. [34].

2. Experimental

2.1. Reagents

A total of 19 standard lipid molecules used for the evaluation of the proposed extraction method were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA): 12:0-lysophosphatidylcholine (LPC), 12:0/12:0-phosphatidylcholine (PC), 12:0- lysophosphatidylethanolamine (LPE), 12:0/12:0-phosphatidylethanolamine (PE), 14:0-lysophosphatidylglycerol (LPG), 12:0/12:0-phosphatidylglycerol (PG), 16:0/18:2- phosphatidylinositol (PI), 14:0/14:0-phosphatidylserine (PS), 18:0-lysophosphatidic acid (LPA), 12:0/12:0-phosphatidic acid (PA), $(14:0)_4$-cardiolipin (CL), d18:0/12:0-sphingomyeline (SM), d18:1/12:0-monohexosylceramide (MHC), d18:1/12:0-lactosylceramide (LacCer), d18:1/12:0-Ceramide (Cer), d18:0-sphinganine (Sa), 18:2- cholesterol ester (CE), 18:1/18:1-diacylglycerol (DG), and 16:0/18:1/18:2-triacylglycerol (TG). Ammonium formate, ammonium hydroxide, and chloroform were all of MS grade from Sigma (St. Louis, MO, USA). All solvents used for nLC-MS/MS and for extraction were all HPLC grade from Avantor Performance Materials (Phillipsburg, NJ, USA): water, CH_3OH, CH_3CN, and isopropanol. Lipid standards were dissolved in 1:1 (v/v) $CHCl_3$:CH_3OH solution except for the lysophospholipids (LPC, LPE, LPA, and LPG), which were dissolved in 1:1:3 (v/v/v) H_2O:$CHCl_3$:CH_3OH. Each standard was diluted to 9:1 (v/v) CH_3OH:CH_3CN for nLC-ESI-MS/MS analysis. For the preparation of the standard lipid mixture sample, the concentration of each standard was adjusted to 50 μmol/L. Internal standards (13:0/13:0-PC and 15:0/15:0-PG for positive and negative ion mode, respectively) were added to extracted sample at a concentration of 1 μmol/L after all sample preparation procedures.

For the removal of water and extraction/partitioning of lipids in the first step of the QuEChERS method, $MgSO_4$ and CH_3COONa from Agilent Technologies (Palo Alto, CA, USA) were utilized. To reduce the matrix effect through the second step, sorbent materials used were PSA (primary secondary amine), GCB (graphitized carbon black), and end-capped C18 particles (grafted silica) from Agilent and SAX (strong anion exchange) and silica from Macherey-Nagel Co. (Düren, Germany).

Human urine and plasma samples were obtained from a healthy male volunteer (age 27).

2.2. Lipid extraction

2.2.1. Folch method for plasma and urine samples

Extraction of lipids from standard mixtures, plasma, and urine samples by the Folch method followed the methods utilized in earlier studies [35,39]. For plasma samples, 100 μL of a plasma mixture containing standards was dried in a vacuum centrifuge, a model Bondiro MCFD 8508 freeze dryer with a concentrator from Ilshin Lab Co. (Yangju, Korea), for 6 h. To the dried powder, 300 μL of CH_3OH and 600 μL of chloroform were added in sequence. After vortexing for 1 h, 180 μL of MS-grade water was added for phase separation followed by centrifugation at 5000 rpm for 5 min. Then, the lower organic layer was transferred to another centrifuge tube and dried in the vacuum centrifuge. The resulting dried lipids were reconstituted in 200 μL of 1:1 (v/v) $CHCl_3$:CH_3OH and diluted in 200 μL of CH_3OH. The final solution was kept at 4 °C.

For the extraction of urine samples, 1 mL of urine in a 15-mL centrifuge tube was first frozen in liquid nitrogen and then dried in the vacuum centrifuge for 12 h. Before freeze-drying the urine sample, the centrifuge tube was covered with MilliWrap, a PTFE membrane filter with 0.45 μm pores, from Millipore (Billerica, MA, USA), without the screw cap to prevent lipids from evaporating, a measure applied to all drying steps. The resulting powder was dissolved in 0.90 mL of 2:1 (v/v) $CHCl_3$:CH_3OH and left for 1 h at room temperature. After adding 180 μL of MS-grade water, it was centrifuged at 5000 rpm for 5 min. The rest of the procedures are the same as those applied for plasma samples.

2.2.2. Modified QuEChERS method

In the first step of the QuEChERS method for the extraction/partitioning of lipids, 100 μL of plasma was placed in a 2-mL Eppendorf tube containing the 0.150-g pre-packed extraction preparation (125 mg of $MgSO_4$, 25 mg of CH_3COONa, and a glass ball). Then, 200 μL of an extraction solvent and 25 μL of water were added in sequence. To select an efficient extraction solvent, four different organic solvents (CH_3CN, CH_3OH, 2:1 (v/v) $CHCl_3$:CH_3OH, and 2:1 (v/v) methyl-*tert*-butyl ether (MTBE):CH_3OH) were utilized to test the recovery rate of each lipid. Since MTBE has been reported as an efficient organic solvent for the modification of the Folch method [38,39], it was utilized for comparison with the Folch method. For urine samples, 1 mL of urine was placed in a 15-mL Falcon tube containing 1.50 g of the same pre-packed extract