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Short communication

An integrated strategy for the rapid extraction and screening of phosphatidylcholines and lysophosphatidylcholines using semi-automatic solid phase extraction and data processing technology

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ABSTRACT

This study attempts to establish a comprehensive strategy for the rapid extraction and screening of phosphatidylcholines (PCs) and lysophosphatidylcholines (LysoPCs) in biological samples using semi-automatic solid phase extraction (SPE) and data processing technology based on ultra-performance liquid chromatography-quadrupole-time of flight-mass spectrometry (UPLC-Q-TOF-MS). First, the Ostro sample preparation method (*i.e.,* semi-automatic SPE) was compared with the Bligh-Dyer method in terms of substance coverage, reproducibility and sample preparation time. Meanwhile, the screening method for PCs and LysoPCs was built through mass range screening, mass defect filtering and diagnostic fragments filtering. Then, the Ostro sample preparation method and the aforementioned screening method were combined under optimal conditions to establish a rapid extraction and screening platform. Finally, this developed method was validated and applied to the preparation and data analysis of tissue samples. Through a systematic evaluation, this developed method was shown to provide reliable and high-throughput experimental results and was suitable for the preparation and analysis of tissue samples. Our method provides a novel strategy for the rapid extraction and analysis of functional phospholipids. In addition, this study will promote further study of phospholipids in disease research.

1. Introduction

Phospholipids are important components of biological membranes, especially phosphatidylcholines (PCs) and lysophosphatidylcholines (LysoPCs). These lipids can directly or indirectly reflect the interactions between phospholipids and pathology in biological systems through their metabolism dynamic changes [1]. Considering their biological significance, phospholipid analysis has gradually become a hot topic in the life science field. Because of the similarity in molecular structures between various phospholipids and the complexity of biological samples, it is difficult to comprehensively study specific phospholipids, or the overall phospholipid composition, using only one separation and analysis technique. Therefore, it is necessary to establish a systematic platform for efficient separation and analysis of phospholipids; such a system will aid in further studies of the relationship between phospholipids and diseases.

Currently, liquid-liquid extraction (LLE) and solid phase extraction (SPE) are commonly used in phospholipid preparations [2]. Compared with LLE, SPE can effectively simplify the preparation step, reduce the sample volume, avoid emulsification and operate automatically; thus, SPE is more conducive to phospholipid preparations in biological samples [3,4]. Automatic or semi-automatic preparation methods have gained popularity based on their ease of use. In our previous study, we found that the Ostro sample preparation method (*i.e.,* a semi-automatic preparation method, OSPM) could effectively separate small molecule metabolites and phospholipids in biological samples. We also found that this method could rapidly enrich and extract phospholipids because the unique fillers had a strong adsorption capacity for phospholipids [5].

Recently, liquid chromatography-mass spectrometry (LC–MS) has provided technical support for phospholipid analysis in complex samples because of its high sensitivity and wide dynamic range; moreover, LC–MS can also effectively eliminate the interference from isomers and reduce ionization suppression effects [6]. However, the chromatograms are complex and contained a large amount of information. Data processing technology has been used to extract and integrate information from original datasets for fur-

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ther characterization of functional phospholipids [7]. Due to the similarity and specificity in phospholipids structures, we found that mass defect filtering (MDF), diagnostic fragments filtering (DFF) and neutral loss filtering (NLF) had unique advantages in screening and identifying substances [8]. Together, the aforementioned analysis methods contribute to the rapid screening of functional phospholipids, and these methods are beneficial in distinguishing unknown substances.

Many studies have reported that PCs and LysoPCs are involved in the occurrence and development of many diseases, such as atherosclerosis, diabetes and hepatitis [9–11]. Considering the importance of these phospholipids in disease, as well as the series of technical challenges involved in the preparation, classification and identification of these phospholipids, this study utilized OSPM and data processing technology to establish an efficient method for rapidly extracting and analyzing PCs and LysoPCs in biological samples based on ultra-performance liquid chromatography-quadrupole-time of flight-mass spectrometry (UPLC-Q-TOF-MS). This novel method achieves fast, stable and high-throughput extraction and screening of phospholipids and provides a new approach for the preparation and screening of lipids potentially associated with disease.

2. Experimental

2.1. Reagents and chemicals

The information about the reagents and chemicals related to this study was described in the Supplementary section.

2.2. Biological samples

Blood and liver tissue samples were obtained from Wistar rats. The collection of blank plasma samples and animal experiment process were described in the Supplementary section.

2.3. Sample preparation

2.3.1. Bligh-Dyer method

The modified Bligh-Dyer method (BDM) was used to extract lipids. The preparation process was described in the Supplementary section.

2.3.2. Ostro sample preparation method

Lipids were extracted via vacuum using Ostro 96-well plates (Waters, Milford, USA). Plasma samples were prepared as follows: 100 μL of blank plasma was pipetted into the Ostro 96-well plate, then 900 μL of chloroform/methanol/triethylamine (4.5/4.5/1, v/v/v) was added. The mixtures were quickly and intensely mixed several times with a pipette and maintained at 4 °C for 5 mins to complete the protein precipitation. A vacuum (2–8 in Hg) was then applied to the plate for 5–8 min, and the flow-through fraction was filtered and collected in a collecting plate. The collected lipids were dried under N_2. Next, the dried fraction was dissolved in 200 μL of chloroform/methanol (1/1, v/v). Finally, the supernatant was injected into the UPLC–MS system.

The preparation process of tissue homogenates was described in the Supplementary section. Additionally, 100 μL of homogenates was loaded into the Ostro plate, and the subsequent operations were the same as the plasma samples.

2.4. Chromatography and mass spectrometry conditions

A UPLC-Q-TOF-MS system (Waters, USA) was used to separate and detect lipids. An ACQUITY UPLC HSS T3 (2.1 × 100 mm, 1.8 μm, Waters) was employed for chromatographic separation. The column temperature was kept at 55 °C, the injection volume was set at 1 μL and the flow rate was set to 0.4 mL min^{-1}. The UPLC system employed a binary solvent system, mobile phase A included 10 mM ammonium formate in acetonitrile/water (4:6, v/v), and mobile phase B included 10 mM ammonium formate in isopropanol/acetonitrile (9:1, v/v). The gradient elution program was designed as follows: 40–100% B for 0–10 min; 100–100% B for 10–12 min; 100–40% B for 12–15 min; 40–40% B for 15–17 min.

MS was performed using a quadrupole/time of flight mass spectrometer (Xevo G2 Waters, USA) equipped with electrospray ionization (ESI) in positive ion mode. The MS instrument parameters were as follows: the drying gas used high-purity nitrogen at a flow rate of 10 L/h, and the desolvation gas flow rate was 600 L/h. The capillary voltage was set at 3.0 kV, the fragmentor voltage was 6 kV, and the collision energy was 10–20 eV, 20–30 eV and 30–40 eV. The source temperature and desolvation gas temperature was set at 120 °C and 350 °C, respectively. The data acquisition range was 100 Da–1000 Da. A reference mass ($[M+H]^+$ = 556.2771) was applied to ensure the accuracy of the experimental results.

2.5. Data processing

MassLynx software (version 4.1, waters, USA) was used for aligning peaks, matching peaks and correcting peak intensities after original data acquisition. The involved functional parameters were described in the Supplementary section. Then, the processed data were imported into SIMCA-P12.0 (Umetrics, Sweden) for principal component analysis (PCA). Student's *t*-test was performed by Excel, which showed significant differences when the *p-value* was less than 0.05.

The screening process of the target compounds was as follows: The original data extracted from the MassLynx software were imported into Excel to obtain a compound Table Subsequently, according to the cracking process under different collision energies, the response value of the parent ions in the MS gradually decreased with the rise in collision energy. In contrast, the fragment ions did not have the same characteristic, as the information of the parent and fragment ions can be extracted simultaneously from the aforementioned table. Next, the theoretical mass range (MR) and mass defect range (MDR) of the PCs and LysoPCs were used to screen the parent ions. According to the principle that the peak time of the parent ions was similar to the fragment ions, we used DFF to finally screen the target compounds from large quantities of data. In positive ion mode, parent ions were required to meet the conditions of $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$. Different fragment ions may be derived from the same compound, so we needed to merge the fragment ions. Finally, the structure information was further confirmed through querying the HMDB (http://www.hmdb.ca/) and KEGG (http://www.genome.jp/kegg/).

3. Results and discussion

3.1. Evaluation of the developed method

This study utilized SPE for the rapid, comprehensive and non-targeted extraction of phospholipids through UPLC-Q-TOF/MS, which could detect qualitative or semi-quantitative information for phospholipids in maximum extent, rather than quantitative information for all levels of substances. Therefore, the sample preparation method was mainly investigated from the following four aspects.

3.1.1. Instrument precision

A mixed standard solution was parallelly injected six times to evaluate the instrument precision in positive ion mode. As shown in Table S2, the relative standard deviations (RSDs) of the retention

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