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Simultaneous metabolomics and lipidomics analysis based on novel heart-cutting two-dimensional liquid chromatography-mass spectrometry

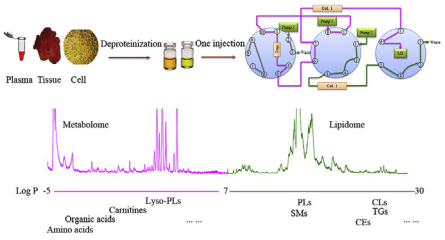
Shuangyuan Wang [a, b], Lina Zhou [a], Zhichao Wang [a, b], Xianzhe Shi [a, **], Guowang Xu [a, *]

[a] CAS Key Laboratory of Separation Science for Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China
[b] University of Chinese Academy of Sciences, Beijing 100049, China

HIGHLIGHTS

- A 2D-LC-MS method based on a heart-cutting and column switching strategy was established.
- Comprehensive information of metabolome and lipidome was simultaneously covered within one injection for the first time.
- The method was suitable to large-scale metabolomics studies with small amount of samples.

GRAPHICAL ABSTRACT



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ABSTRACT

Increasing metabolite coverage by combining data from different platforms or methods can improve understanding of related metabolic mechanisms and the identification of biomarkers. However, no one method can obtain metabolomic and lipidomic information in a single analysis. In this work, aiming at collecting comprehensive information on metabolome and lipidome in a single analytical run, we developed an on-line heart-cutting two-dimensional liquid chromatography-mass spectrometry (2D-LC-MS) method. Complex metabolites from biological samples are divided into two fractions by using a precolumn. The first fraction is directly transferred and subjected to metabolomics analysis. Most lipids are retained on the precolumn until the mobile phases for lipidomics flow through; then they are subjected to lipidomics analysis. Up to 447 and 289 metabolites in plasma, including amino acids, carnitines, bile acids, free fatty acids, lyso-phospholipids, phospholipids, sphingomyelins etc. were identified within 30 min in the positive mode and negative mode, respectively. A comparison of the newly developed method with the conventional metabolomic and lipidomic approaches showed that approximately 99% features obtained by the two conventional methods can be covered with this 2D-LC method. Analytical characteristics evaluation showed the method had a wide linearity range, high sensitivity, satisfactory recovery and repeatability. These results demonstrate that this method is reliable, stable and well qualified in metabolomics analysis, particularly for large-scale metabolomics studies with small amount of samples.

1. Introduction

Metabolites in biological samples are extremely complex. An analytical method that can identify as many metabolites as

* Corresponding author.
** Corresponding author.
E-mail addresses: shixianzhe@dicp.ac.cn (X. Shi), xugw@dicp.ac.cn (G. Xu).

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possible is extremely important for full metabolic characterization and biomarker discovery. Reversed-phase liquid chromatography-mass spectrometry (RPLC-MS) has become the most widely used platform in metabolomics studies because of its excellent ability to separate analytes with good repeatability [1–4], and has been successfully applied in many areas of metabolomics studies [5–7]. But these metabolomics methods with water and acetonitrile or methanol mobile phases mainly identify metabolites such as organic acids, carnitines, lysophospholipids (lyso-PLs) etc. Most lipids such as PLs, sphingomyelins (SMs), triglycerides (TGs), cholesterol esters (CEs), and cardiolipins (CLs), are nearly impossible to be eluted. Accordingly, lipids are often analyzed separately as a special metabolite species, and thus lipidomics methods have emerged to complement the conventional metabolomics methods [8–10]. The mobile phases of lipidomics are usually composed of iso-propanol, acetonitrile and water, which are greatly different from those of the conventional metabolomics methods [11,12].

More recently, as the improvement of the performance of hydrophilic interaction liquid chromatography (HILIC) columns, HILIC-MS methods have been widely applied in metabolomics studies [13,14]. The metabolites in central carbon pathways that could not be well retained in RPLC methods present good retention and separation. However, serious co-elution of lipids is the main disadvantage of HILIC-MS methods [15,16]. Thus, two analyses are necessary to cover comprehensive metabolomics and lipidomics information, owing to the significant difference in polarity [17,18]. Ivanisevic et al. established a dual LC-MS approach for simultaneous coverage of metabolome and lipidome [19], where HILIC and RPLC methods were used for central carbon metabolites and lipids, respectively.

An inherent advantage of two-dimensional (2D) LC approaches is the improved resolution achieved by combining different separation mechanisms, for example, orthogonal normal-phase (NP) LC or HILIC with RPLC [20–23]. Stoll et al. developed a high-temperature 2D-LC method for the separation of tryptic peptides [24]. A maximum peak capacity of 1350 in 20 min was achieved through this technique. The advantages of 2D-LC are attracting increasing attention in the development of high-throughput “omics” methods [21]. Wei et al. established a high-throughput multiplexed LC-MS system for targeted metabolomics analysis. This system enabled well to separate amino acids, sugars, nucleic acids, and organic acids, but 3 injectors were needed [25]. Klavins et al. established a parallel LC separation method to quantitatively analyze target metabolites involved in the central carbon metabolism [26]. Although these methods have improved resolution, they have not significantly extended the metabolite coverage.

Here, by combining on-line pre-fractionation and column-switching 2D-LC-MS, we developed a new method with comprehensive metabolite coverage to achieve simultaneous metabolomics and lipidomics analysis in a single analytical run. Briefly, complex metabolites from biological samples are firstly divided into two fractions by using a precolumn. Lyso-PC 19:0, which has an intermediate logP value of 7.2, serves as the boundary of the two fractions. The first fraction, containing amino acids, organic acids, carnitines, fatty amides, most lyso-PLs *etc.* is directly transferred and subjected to a metabolomics analysis with a C18 column and an acetonitrile/water mobile phase. Metabolites with the greater hydrophobicity are retained on the precolumn until mobile phases for lipidomics flow through. After being transferred, the second fraction, including PLs, SMs *etc.* is subjected to lipidomics analysis on a T3 column with an iso-propanol/acetonitrile/water mobile phase. Through this 2D-LC method, metabolome and lipidome can be covered simultaneously in a single analysis.

2. Experimental

2.1. Chemicals and reagents

Descriptions of all standards are listed in Table S1 (Supplementary materials). Acetyl carnitine − d3, decanoylcarnitine − d3, phenyl alanine − d5, cholic acid − d4 (CA − d4), chenodeoxycholic acid − d4 (CDCA − d4), phosphatidylcholine (PC) (19:0/19:0), phosphatidylethanolamine (PE) (17:0/17:0), ceramide (Cer) (d18:1/17:0), and sphingomyelin (SM) (d18:1/12:0) were used as internal standards (IS). Specific information is listed in Table S2 (Supplementary materials). Acetonitrile (ACN), iso-propanol (IPA) and methanol (MeOH) of HPLC grade were purchased from Merck (Darmstadt, Germany). Formic acid and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, USA). Water used in this research was purified using Milli-Q system (Millipore, Bedford, USA).

2.2. Sample preparation

A plasma sample pooled from equal aliquots of plasma from ten healthy humans was used to establish and evaluate the 2D-LC-MS method in the present study. Fifty μL pooled sample was pipetted into a 1.5 mL eppendorf tube and mixed with 200 μL MeOH (containing IS) to precipitate proteins. Then, 50 μL IPA was added and vortexed for 60 s. The resulting solution was centrifuged for 10 min at 14,000 rpm, 10 °C and 250 μL supernatant was transferred and freeze-dried at vacuum condition. The dried residue was stored at −80 °C before use and reconstituted in 50 μL IPA/water (1:1, v/v) for injection. The plasma sample and standard mixture were prepared with this protocol unless special statement.

For extraction of 10 mg wet liver tissue of mice or prostate cancer cells (48 h culture of a prostate cancer cell line PC-3, 2.5×10^6), 500 μL 80% MeOH (containing IS) was firstly added and homogenized for 60 s at the frequency of 25 Hz using a mixer mill instrument (MM400, Retsch, Haan, Germany). Then 100 μL IPA was added to the mixture and vortexed for 60 s. After the mixture was centrifuged for 10 min at 14,000 rpm, 500 μL supernatant was freeze-dried and reconstituted in 50 μL IPA/H_2O (1:1, v/v) for injection.

2.3. Comprehensive analysis of metabolome and lipidome using the novel 2D-LC method

LC separation was performed by using Shimadzu Prominence (for metabolomics analysis) and Nexera (for lipidomics analysis) systems (Shimadzu, Kyoto, Japan), respectively. A LC-20AD pump was used as the make-up pump (pump 3). A short Acquity BEH C8 column (2.1 × 5 mm, 1.7 μm, Waters, Milford, USA) was optimized as the precolumn to separate metabolome from lipidome. An Acquity BEH C18 column (column 1, 2.1 × 50 mm, 1.7 μm, Waters) was used for metabolic profiling analysis, the mobile phases A1 and B1 were water with 0.1% formic acid and ACN with 0.1% formic acid, respectively. For lipidomics analysis, an Acquity HSS T3 column (column 2, 2.1 × 50 mm, 1.7 μm, Waters) was used, the mobile phase A2 was 60% ACN in water and B2 was 90% IPA in ACN. Both A2 and B2 contained 10 mM ammonium acetate. The mobile phase in the make-up flow was water. The gradient conditions for column 1 and column 2 are listed in Table 1. The flow rates of the columns 1 and 2 were 0.35 and 0.3 mL min^{-1}, respectively. The temperature of column oven was 55 °C and the injection volume was 5 μL.

Specific schematic illustration of this method is shown in Fig. 1. Three valves were involved in this newly developed 2D-LC method. A 2-way 8-port valve and a 2-way 10-port valve, which were bought from Valco (Houston, USA), were used for analytical