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An improved pseudotargeted metabolomics approach using multiple ion monitoring with time-staggered ion lists based on ultra-high performance liquid chromatography/quadrupole time-of-flight mass spectrometry

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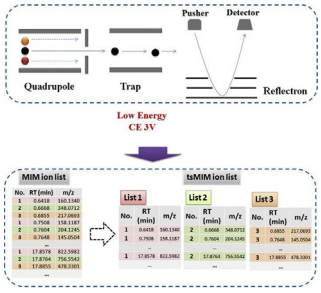
HIGHLIGHTS

- An UHPLC/Q-TOF tsMIM MS-based pseudotargeted metabolomics was proposed.
- Compared to full scan, the improved method exhibits better repeatability and a wider linear range.
- The proposed method could achieve pseudotargeted analysis on one UHPLC/Q-TOF/MS instrument.
- The developed method was successfully used to discover biomarkers for alcohol-induced liver injury.

GRAPHICAL ABSTRACT



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ABSTRACT

Pseudotargeted metabolomics is a novel strategy integrating the advantages of both untargeted and targeted methods. The conventional pseudotargeted metabolomics required two MS instruments, i.e., ultra-high performance liquid chromatography/quadrupole-time- of-flight mass spectrometry (UHPLC/Q-TOF MS) and UHPLC/triple quadrupole mass spectrometry (UHPLC/QQQ-MS), which makes method transformation inevitable. Furthermore, the picking of ion pairs from thousands of candidates and the swapping of the data between two instruments are the most labor-intensive steps, which greatly limit its application in metabolomic analysis. In the present study, we proposed an improved pseudotargeted metabolomics method that could be achieved on an UHPLC/Q-TOF/MS instrument operated in the multiple ion monitoring (MIM) mode with time-staggered ion lists (tsMIM). Full scan-based untargeted analysis was applied to extract the target ions. After peak alignment and ion fusion, a stepwise ion picking procedure was used to generate the ion lists for subsequent single MIM and tsMIM. The UHPLC/Q-TOF tsMIM MS-based pseudotargeted approach exhibited better repeatability and a wider linear range than the UHPLC/Q-TOF MS-based untargeted metabolomics method. Compared to the single MIM mode, the tsMIM significantly increased the coverage of the metabolites detected. The newly developed method was successfully applied to discover plasma biomarkers for alcohol-induced liver injury in mice, which indicated its practicability and great potential in future metabolomics studies.

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1. Introduction

Metabolomics aims at the holistic analysis of endogenous metabolites with molecular masses lower than 1000 Da and attempts to quantify the attractive metabolites in a given biological sample [1]. Liquid chromatography coupled with mass spectrometry (LC-MS) has become an indispensable analytical platform for studying metabolomics due to its high throughput, high sensitivity and selectivity, fast and reproducible analysis, and wide coverage of detected metabolites [1,2].

LC-MS-based metabolomics offers untargeted and targeted strategies with their own inherent advantages and disadvantages. Untargeted metabolomics aims to analyze all metabolites present in the biological system without prior knowledge of the components using high-resolution mass spectrometers [3]. Full-scan results provide accurate masses to facilitate metabolite identification and allow for the detection of as many of the peaks as possible [4]. However, the main limitation of full-scan MS is the narrow linear range resulting from matrix effects and detector saturation, which makes the quantification of metabolites with a wide concentration range a difficult challenge [4–7]. Additionally, the number of metabolites extracted from mass spectra and their peak areas are greatly affected by the peak alignment parameters, which will influence the results of further statistical analysis and the reproducibility of batch analyses [8,9]. Another strategy is targeted metabolomics, which only measures defined groups of chemically characterized and biochemically annotated metabolites [10]. Due to its wide linear dynamic range, high sensitivity and high repeatability, multiple reaction monitoring (MRM) performed on a triple quadrupole (QQQ) MS instrument is recognized as the golden standard for metabolite quantification, and it has been widely applied in targeted metabolomics analysis. However, by means of this targeted approach, only a limited number of known metabolites could be quantified [11,12].

Recently, a novel metabolomics strategy, referred to as the pseudotargeted approach, has been developed to integrate the advantages of both untargeted and targeted methods [13]. Metabolites and their product ions (ion pairs) were collected through data-dependent acquisition (DDA) by UHPLC/Q-TOF/MS in an untargeted manner; subsequently, the targeted ion pairs were picked and monitored using UHPLC/QQQ/MS by dynamic MRM. Compared with the conventional untargeted metabolomics, this pseudotargeted strategy displayed better repeatability and a wider linear range. Additionally, no complicated data preprocessing was required [13]. The pseudotargeted metabolomics strategy has been applied in the discovery of biomarkers for hepatocellular carcinoma [14] and gastric cancer [15]. However, the reported pseudotargeted metabolomics method required two MS instruments, i.e., Q-TOF/MS and QQQ/MS instruments, which makes method transformation inevitable. Furthermore, the picking of ion pairs from thousands of candidates and the swapping of the data between two instruments are the most labor-intensive steps of the pseudotargeted method, which greatly limit its application in metabolomic analysis [16].

To resolve the issue, we proposed an improved pseudotargeted approach to perform metabolomics using multiple ion monitoring (MIM) with time-staggered ion lists (tsMIM), which could be performed on one UHPLC/Q-TOF/MS instrument to achieve pseudotargeted analysis. The target ion list was acquired from TOF full scan-based untargeted metabolomics. To minimize the time consumption in the steps of the targeted ion picking and data swapping between instruments, which was used in the pseudotargeted approach previously reported, the MIM mode employing precursor-to-precursor ion transitions, rather than the MRM mode, was used for pseudotargeted analysis. Furthermore, to ensure data quality, MIM with time-staggered ion lists was introduced to guarantee the adequate scan point of ion peaks. As a proof of concept, the developed UHPLC/Q-TOF tsMIM MS-based pseudotargeted approach was employed to discover plasma biomarkers for alcohol-induced liver injury in mice.

2. Material and method

2.1. Chemicals

HPLC-grade acetonitrile, methanol and formic acid were purchased from Merck (Darmstadt, Germany). Ultra-pure water was prepared by a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Animals and treatments

A total of sixteen C57BL/6 mice (16–18 weeks) were housed in the individually ventilated cage (IVC) system in a standard 12 h dark/light cycle room (temperature 20 ± 2 °C, relative humidity 70± 5%) with free access to food and water. After one week of acclimation with the liquid diet, the mice were randomly divided into a control group (n = 6) and an ethanol group (n = 10). In the ethanol group, mice were fed with a modified Lieber-DeCarli alcohol liquid diet consisting of 18% protein, 19% carbohydrate, 35% fat and 28% ethanol (TROPHIC Animal Feed High-tech Co., Ltd. Nantong, China), whereas in the control group, mice were fed with a Lieber-DeCarli control diet, in which ethanol was substituted with isocaloric maltose-dextrin. After two weeks, all mice were euthanized, and blood samples were collected in heparin tubes. Plasma was separated by centrifugation at 3000*g* at 4 °C for 10 min and frozen immediately at −80 °C until metabolomics analysis. Animal treatments were approved by the Animal Ethics Committee, Institute of Chinese Medical Sciences, University of Macau (Approval No. AEC-13-002).

2.3. Sample preparation

Plasma samples were thawed at 4 °C and adequately mixed before use. A pooled "quality control" (QC) sample was prepared by mixing equal aliquots (10 μL) from all of the plasma samples for the method validation and linearity test. For method validation, 100 μL of pooled plasma was deproteinized with 300 μL of methanol. After 30 s of vortex mixing, the samples were centrifuged at 15,800 *g* for 15 min at 4 °C, the supernatant was lyophilized, and it was reconstituted in 100 μL of ultra-pure water. A 5 μL aliquot of supernatant was subjected to UHPLC/Q-TOF/MS analysis. To test the linearity of the method, 20, 40, 80, 160, and 320 μL of the QC sample were deproteinized with three volumes of methanol; 60, 120, 240, 480, and 960 μL of the supernatant were lyophilized; and the supernatant samples were reconstituted in 100 μL water. For metabolomics analysis of a real sample, 100 μL of a plasma sample was deproteinized with 300 μL of methanol, and the supernatant was lyophilized, followed by reconstitution in 100 μL of water.

2.4. UHPLC/Q-TOF MS for untargeted metabolomics analysis

Chromatographic separation was conducted on a Waters ACQUITY™ UHPLC system (Waters Corp., Manchester, UK) with an ACQUITY BEH C_{18} column (100 mm × 2.1 mm i.d., 1.7 μm) at 50 °C. The mobile phase consisted of 0.1% aqueous formic acid (phase A) and acetonitrile (phase B) under the following gradient program: isocratic 3% B (0–1 min), linear gradient from 3% to 70% B (1–8 min), isocratic 70% B (8–10 min), 70%–90% B (10–17 min), 90%–100% B (17–18 min), isocratic 100% B for 3 min and then back to 3% A in 5 min. The whole duration was 26 min at a flow-rate of