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A chemical derivatization based UHPLC-LTQ-Orbitrap mass spectrometry method for accurate quantification of short-chain fatty acids in bronchoalveolar lavage fluid of asthma mice

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ABSTRACT

Recent studies have demonstrated the important role of short-chain fatty acids (SCFAs) in the maintenance of homeostasis of respiratory immunity. However, there is still no report focus on the determination of SCFAs level in bronchoalveolar lavage fluid (BALF), the most common sample used for screening biomarkers of the pulmonary diseases. Herein, an ultra-high-performance liquid chromatography with LTQ-Orbitrap mass spectrometer (UHPLC-LTQ-Orbitrap) oriented 3-nitrophenylhydrazine (3-NPH)-based derivatization method was developed for the quantification of SCFAs in BALF. To achieve accurate quantitation, d4-acetate was used as internal standard to compensate for the matrix effects. Method validation showed a good linearity (R^2 > 0.9992) with wide concentration range, and the intra-day and inter-day precision for determination of eight SCFAs in BALF samples was ≤ 14.79%. The quantitation accuracy, assessed by relative recoveries, ranged from 90% to 110% for target SCFAs at three concentration levels. Matrix effects ranged from 85% to 115%, and the lower limits of quantification of these targeted SCFAs were varied from 3 to 24 nmol/L. The SCFAs-targeted method was then applied to determine the changed levels in BALF samples from OVA-induced asthma mice and normal mice. In addition, the universality of our developed method was also demonstrated by determining the SCFAs concentrations in feces, serum and lung tissue samples from asthma and normal mice. These results indicate that 3-NPH derivatization based UHPLC-LTQ-Orbitrap provides accurate view of global SCFAs alternation in different samples, giving a support to deduce the origin of SCFAs in lung. The present study is of great importance for understanding the role of SCFAs in modulation of host metabolism and immunity.

1. Introduction

Short-chain fatty acids (SCFAs) are a subset of fatty acids produced by the gut microbiota during the fermentation of fibers and dietary carbohydrates [1]. There is now a growing evidence to show that SCFAs play an important role in the maintenance of health and the development of disease [2]. Some of these studies even suggested a role for the gut microbiota-derived SCFAs in modulating respiratory diseases, such as asthma and chronic obstructive pulmonary disease (COPD) [3–5]. Interestingly, independent investigations have recently demonstrated that the human respiratory tract (spans from the nostrils to the lung alveoli) is also inhabited by niche-specific communities of bacteria [6]. Therefore, it is intriguing that the respiratory microbiota-derived SCFAs might also be involved in the modulation of host metabolism and immunity.

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However, rare research has focused on the determination of respiratory microbiota-derived SCFAs, which limits the understanding of their possible functions in maintenance of homeostasis of respiratory immunity.

Bronchoalveolar lavage (BAL) is often used in immunological researches as a mean of sampling cells or pathogen levels in the lung [7]. And bronchoalveolar lavage fluid (BALF) is the most common sample used to determine inflammatory cells, cytokines, and protein composition of the pulmonary airways [8–11]. Recently, BALF samples are being investigated to screen metabolite biomarkers of lung disease. Analytical platforms for metabolite profiling of BALF include high performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (HPLC/QTOF-MS)-based lipidomics and metabolomics, and gas chromatography-mass spectrometry (GC/MS)-based metabolomics [12–15]. The monitors of metabolome in BALF could reveal comprehensive pulmonary metabolic signatures in lung disease through specific alterations in carbohydrates, lipids, sterols, and energy metabolic pathways [14,16]. However, up to now, no method has been reported for the determination of SCFAs in BALF samples. Its utility as a biofluid for SCFAs quantitation may be limited because of its high protein and salt content but low concentrations of SCFAs [17].

Fortunately, several analytical techniques which mainly included chemical derivatization procedure coupled with GC/MS or LC–MS have been used to analyze the SCFAs in other biological samples, such as blood, urine and feces samples [18–22]. Based on the previous studies, we intend to use 3-nitrophenylhydrazine (3-NPH) to derivatize SCFAs in BALF for ultra-high-performance liquid chromatography with LTQ-Orbitrap mass spectrometer (UHPLC-LTQ-Orbitrap) quantitative analysis. Because of the high speed of MS scans and the high efficiency of high energy collision-induced dissociation on the LTQ-Orbitrap instrument, the MS/MS spectra can be readily acquired for the derivatived SCFAs [23,24]. Its high-ion-transmission results in short accumulation times, which distinguishes it for the purpose of LC–MS analysis [24–26]. This merit appears attractive for overcoming the main problems encountered with quadrupole instruments, especially for their low mass resolution resulted significant overlapping problems in the analysis of complex metabolite mixtures [24,27]. Herein, 3-NPH was used for pre-analytical chemical derivatization by quantitatively converting BALF-derived SCFAs to their 3-nitrophenylhydrazones under a mild reaction condition for subsequent UHPLC-LTQ-Orbitrap analysis. To the best of our knowledge, this is the first report about quantitative analysis of SCFAs in BALF. The profile of SCFAs in BALF has been successfully applied to discriminate between asthmatic mice and normal controls.

2. Materials and methods

2.1. Chemicals and reagents

Standards: acetate (C_2, 99.8%), propionate (C_3, 99.5%), isobutyrate (C_4, 99.5%), butyrate (C_4, 99.5%) and valerate (C_5, 99.5%) were purchased from Shanghai Macklin Biochemical Co. Ltd (Shanghai, China). Isovalerate (C_5, 99.5%), 2-methylbutyrate (C_5, 98%), caproate (C_6, 99.5%) and d4-acetate (99.5%) were purchased from Aladdin (Shanghai, China). Derivatization reagents: 3-nitrophenylhydrazine hydrochloride (3-NPH) and 1-ethyl (3-dimethyllaminopropyl) carbodiie hydrochlide (EDC·HCl) were purchased from Aladdin (Shanghai, China); pyridine was purchased from Sinopharm Chemical Reagent Co. Ltd, R.P.C. (Shanghai, China). LC–MS grade acetonitrile (ACN) and formic acid were purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained by filtration of distilled water using a Milli-Q system (Millipore, USA).

2.2. Sampling bronchoalveolar lavage fluid from asthmatic mice

Female BALB/c mice (18–20 g, 6–8 weeks old) were purchased from the Laboratory Animal Center of Yangzhou University (Yangzhou, China). All animals were maintained in isolation under controlled light and temperature conditions. BALB/c mice were subcutaneously (s.c.) sensitised to ovalbumin (OVA, 50 μg; Grade BR, yuanye Bio-Techology Co.,l-td, Shanghai, China) with 50 μL of Incomplete Freund's Adjuvant (IFA; Sigma, U.S.A.) in 50 μL of 0.9% saline on days 0, 7 and 14. On day 22–28, mice were challenged with an aerosolised solution of 5% OVA (w/v) in 0.9% saline for 30 min every day. The mice of control group were treated with saline instead of the OVA/IFA solution during immunization and challenge. Bronchoalveolar lavage was performed by using a cannula surgically inserted into the trachea. Ice-cold PBS (1.0 mL × 3) was instilled into the airways and a consistent volume of BALF (2.4–3.0 mL) was recovered from each mouse. BALF samples were kept at −80 °C prior further analysis.

2.3. The preparation of BALF samples and 3-NPH-based derivatization

After lyophilization [28], each BALF sample was redissolved in 90 μL acetonitrile. The solution was then vortexed for 2 min followed by centrifugation at 12,000 rpm for 10 min. And then, 20 μL of the supernatant was transferred to an eppendorf tube for further derivatization.

The derivative reagent (2 mL) was constituted of 1 mL of 200 mmol/L 3-NPH in 50% acetonitrile, 200 μL of 600 mmol/L EDC in 50% acetonitrile, and 800 μL 7.5% pyridine in 50% acetonitrile. The samples were mixed with derivative reagent at the ratio of 1:1 (v/v; 40 μL in total). The whole process was performed in an ice bath. Afterwards, the mixture was permitted to react in a water bath at 40 °C for 30 min. After reaction, the mixture was cooled down at 4 °C and finally diluted to 1 mL with 10% acetonitrile. Before injection, the mixed system was centrifuged at 12,000 rpm for 10 min, and 720 μL of the supernatant was added with 30 μL of 3-NPH-derivatived d4-acetate (internal standard, IS) at a final concentration of 800 nmol/L.

2.4. Preparation of standard solutions, calibration and quality control (QC) samples

The mixture of stock standard solution containing acetate (10 mmol/L), propionate (5 mmol/L), isobutyrate (2.5 mmol/L), butyrate (5 mmol/L), 2-methylbutyrate (1.25 mmol/L), isovalerate (1.25 mmol/L), valerate (2.5 mmol/L) and caproate (10 mmol/L) was prepared in 50% acetonitrile. Before use, the mixture was diluted with 50% acetonitrile to give a series of mixed standards working solutions. The d4-acetate stock solution (1 mmol/L) was also prepared in 50% acetonitrile, which was reacted as above process to prepare 3-NPH-derivatived d4-acetate (IS, 20 μmol/L). Quality control (QC) samples were prepared from each biological sample to ensure broad metabolite coverage. All solutions were stored at 4 °C before use.

2.5. The solvent-only and matrix-matched calibration curves

To prepare the solvent-only curve, 90 μL of acetonitrile was spiked with 10 μL mixed standards working solution series, respectively. After being homogenized, 20 μL of the mixed solution was transferred for derivatization, and was diluted to 1.0 mL with 10% acetonitrile followed by centrifugation. Then, 30 μL of IS working solution (1 mmol/L) was added to 720 μL of sample supernatant to prepare working solution series (96-15360 nmol/L for acetate, 9.6-7680 nmol/L for propionate, 96-3840 nmol/L for