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Simultaneous quantification of straight-chain and branched-chain short chain fatty acids by gas chromatography mass spectrometry

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ABSTRACT

Biomedical research in areas such as metabolic disorders, neuromodulatory, and immunomodulatory conditions involves lipid metabolism and demands a reliable and inexpensive method for quantification of short chain fatty acids (SCFAs). We report a GC–MS method for analysis of all straight-chain and branched-chain SCFAs using pentafluorobenzyl bromide (PFBBr) as derivatization reagent. We optimized the derivatization and GC–MS conditions using a mixture containing all eight SCFA standards, i.e., five straight-chain and three branched-chain SCFAs. The optimal derivatization conditions were derivatization time 90 min, temperature 60 °C, pH 7, and $(CH_3)_2CO$:H_2O ratio 2:1 (*v*:*v*). Comparing the performance of different GC column configurations, a 30 m DB-225ms hyphenated with a 30 m DB-5ms column in tandem showed the best separation of SCFAs. Using the optimized experiment conditions, we simultaneously detected all SCFAs with much improved detection limit, 0.244–0.977 μM. We further applied the developed method to measure the SCFAs in mouse feces and all SCFAs were successfully quantified. The recovery rates of the eight SCFAs ranged from 55.7% to 97.9%.

1. Introduction

Short chain fatty acids (SCFAs) are saturated aliphatic fatty acids with less than six carbon atoms. While five straight-chain SCFAs (formic acid, acetic acid, propionic acid, butyric acid, and valeric acid) are predominantly the end products of fermentation of dietary fibers by the anaerobic intestinal microbiota [1], the three branched-chain SCFAs (isobutyric acid, 2-methylbutyric acid, and isovaleric acid) are mainly derived from the catabolism of branched-chain amino acids such as valine, leucine, and isoleucine [2]. SCFAs play an important role in homeostasis due to their metabolic, neuromodulatory, and immunomodulatory actions. They can influence the growth and composition of gut microbiota, and thereby further affect the health of the host. While SCFAs are the main source of energy for the cells in colon, excess SCFAs can have other functions such as providing daily calorie needs and being involved in the metabolism of important nutrients such as carbohydrates and fats.

Emerging evidence indicates that SCFAs are associated with multiple metabolic diseases such as obesity, hypertension, and diabetes [3–5]. In fact, SCFAs stimulate leptin expression and inhibit lipolysis in adipocytes through G-coupled protein receptors. They also activate 5′ adenosine monophosphate-activated protein kinase (AMPK) that acts as a major cellular fuel switch and a master regulator of metabolic homeostasis [6]. SCFAs also function in the synthesis of other metabolites. For instance, propionic acid may inhibit the synthesis of cholesterol in the liver [7]. Gastrointestinal disease could result in increased proteinous material in the colon and may increase the products of branched-chain amino acids [8]. Branched-chain amino acids are associated with the development of diabetes [9]. The oxidation of branched-chain amino acids provides energy for muscles, kidney, and other organs. As the derivatives of branched-chain amino acids, branched-chain fatty acids may become a signal for metabolic diseases.

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Other than metabolic diseases, a reduction in SCFAs might induce alterations in the enteric nervous system and can contribute to gastrointestinal dysmotility in Parkinson's disease [10]. Butyric acid has a regulatory role in the skin immune system via increasing the gene expression of Treg-specific transcription factor foxp3 and IL-10 and expansion of Treg cells [11]. Butyric acid, as a gut product of dietary fiber by microflora, is also believed to play a critical role in cellular epigenetic function that promote the immune system by increasing Treg cells in the gut and extraenteric organs [12].

The measurement of SCFAs in biological samples receives considerable attention because of their important roles in physiological and pathological processes [13–15]. Different chemical derivatization reagents and extraction solvents were developed for analysis of SCFAs by gas chromatography (GC) coupled with a flame ionization detector (FID) or a mass spectrometer [16–21]. SCFAs were also analyzed by HPLC equipped with an electrochemical detector (ECD), a UV detector, or a mass spectrometer [22–24]. Some of the analytical methods for analysis SCFAs were summarized in a review paper [25].

While multiple analytical methods have been developed for analysis of SCFAs as described above, these methods are not able to simultaneously detect all SCFAs, especially formic acid and branched-chain SCFAs. However, formic acid and branched-chain SCFAs are pivotal in biological studies. For instance, formic acid has important regulatory role, and a low level of urinary formic acid correlates with increased blood pressure [26]. To analyze all these SCFAs, pentafluorobenzyl bromide (PFBBr) reagent was used as a derivatization reagent for GC–MS quantification of SCFAs in whole blood and urine from humans and mice [27, 28]. PFBBr was also used to derivatize the five straight-chain SCFAs in an isotopomer enrichment assay [29]. To this point, there has not been any report to simultaneously quantify all eight straight-chain and branched-chain SCFAs from biologic samples.

The objective of this work was to develop a method for simultaneous identification and quantification of all straight-chain and branched-chain SCFAs in a biological sample. We used PFBBr reagent to derivatize SCFAs and a GC-Ion Trap MS instrument to measure the derivatized SCFAs. To achieve high sensitivity in detecting the low abundance and branched-chain SCFAs, we optimized the experiment conditions of PFBBr derivatization and the selection of GC columns. The optimized experiment conditions were then used to simultaneously identify and quantify straight-chain and branched-chain SCFAs in mouse feces.

2. Methods

2.1. Chemicals and reagents

Eight SCFA standards (sodium formate, sodium acetate, sodium propionate, sodium butyrate, isobutyric acid, sodium pentanoate, 2-methylbutyric acid, and isovaleric acid) and 2, 3, 4, 5, 6-pentafluorobenzyl bromide (PFBBr) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA).

2.2. Preparation of SCFA standards

Thirteen solutions were prepared for each of the eight SCFA standards in following concentrations: 0.977 μM, 1.95 μM, 3.91 μM, 7.81 μM, 15.63 μM, 31.3 μM, 62.5 μM, 125 μM, 250 μM, 500 μM, 1 mM, 2.5 mM, and 5 mM. These solutions were used for PFBBr derivatization. The derivatized standards were then used to optimize the experiment conditions and to construct calibration curves for SCFA quantification.

2.3. Animal and biological sample preparation

Eight-week-old male C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in a pathogen-free barrier facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Food and tap water were allowed ad libitum. The procedures of animal care were approved by the University of Louisville Institutional Animal Care and Use Committee. Two groups of C57BL/6J mice fed different diets. One group fed an isocaloric maltose dextrin solution (Group 1, n = 5) while the other group fed with Lieber DeCarli liquid diet containing 5% alcohol (Group 2, n = 9). After 24-day dietary intervention, mice were anesthetized with ketamine/xylazine (100/15 mg/kg i.m.) and feces were collected directly from the mouse colon. All fecal samples were frozen immediately in liquid nitrogen and stored in 1.5 mL Eppendorf tube at −80 °C freezer.

All fecal sample processing were performed at 4 °C to minimize the loss of volatile SCFAs, unless stated otherwise. For each sample, about 30 mg of mouse feces was weighed and ground in a 1.5 mL Eppendorf tube. After adding water at a ratio of 100 mg feces/mL water, the mixture was sonicated for 20 min and then centrifuged at 4 °C and 12,000 rpm for 20 min. The supernatant was collected for SCFAs detection and recovery determination.

2.4. PFBBr derivatization

A 50 μL of standard solution or 150 μL of supernatant collected from a biological sample was used for derivatization. 100 mM PFBBr in acetone, 0.5 M phosphate buffer (PBS, pH 7), and a sample were mixed at a ratio of 14:2:5 (*v*:*v*:*v*) to make the acetone:water 14:7 (*v*:*v*) in a 2 mL glass tube. After 1 min of vigorous vortex mixing, the mixture was incubated in a water bath at 60 °C for 1.5 h. 200 μL or 150 μL of hexane was added after the mixture of standard or sample cooled down to room temperature. The sample was then vortexed for 3 min followed by centrifugation for 5 min in a speedvac. 100 μL of supernatant (hexane phase) was then transferred to a 200 μL GC vial for GC–MS analysis. A blank sample prepared using distilled Milli-Q water was also derivatized as a reference for quality control purposes.

2.5. GC–MS analysis

A Thermo Scientific ITQ™ 1100 GC-Ion Trap MS instrument was coupled with a TRACE™ 1310 gas chromatography system and a 1310 autosampler (Thermo Fisher Scientific, Waltham, MS, USA). Two GC columns, DB-225ms (30 m × 0.25 mm 1 dc × 0.25 μm 1 dp, (50%-cyanopropylphenyl)-methylpolysiloxane) and DB-5ms (30 m × 0.25 mm 1 dc × 0.25 μm 1 dp, (5%-phenyl)-methylpolysiloxane), were used for SCFA separation. These columns were obtained from Agilent Technologies J&W (Santa Clara, CA, USA). The helium carrier gas (99.999% purity) flow rate was set to 1.5 mL/min for DB-225ms and DB-5ms columns, respectively. The flow rate was reduced to 1.0 mL/min when DB-225ms column and DB-5ms column were hyphenated together by a column connector purchased from Restek Corporation (Bellefonte, PA, USA), where DB-225ms was the first column and DB-5ms was the second column. The temperatures of inlet, ion source and transfer line were all set to 220 °C. The column temperature was programmed with an initial temperature of 80 °C for 0.5 min, then ramped to 158 °C at a rate of 10 °C/min, to 160 °C at 3 °C/min, to 220 °C at 20 °C/min, and then maintained at 220 °C for 8 min. The hyphenated column was ramped as follows: initial temperature, 80 °C for 0.5 min; 10 °C/min to 170 °C for 0.5 min; 5 °C/min to 220 °C, hold for 5 min. The energy of electron ionization (EI) was set to 70 eV.

One microliter of PFBBr derivatives was injected into GC–MS in splitless mode with a splitless time of 1.0 min. Solvent delay time was set to 6.1 min for the DB-225ms column, 5.38 min for the DB-5ms column, and 8.86 min for hyphenated columns, respectively. The mass spectral data were collected in a SIM mode (Table 1). All biological samples were analyzed on hyphenated columns. In addition, an aliquot of *n*-alkane series was also injected under each ramp condition for retention index calculation and quality control.

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