Note

Determination of Short-Chain Fatty Acids in Mouse Feces by High-Performance Liquid Chromatography Using 2-Nitrophenylhydrazine as a Labeling Reagent

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Received December 25, 2018; accepted January 22, 2019

It has been suggested that imbalances in gut microbiota are related to diseases associated with metabolism, the central nervous system, etc. Therefore, analysis of short-chain fatty acids (SCFAs) produced by gut microbiota is very important as an indicator of causation, demonstrating the effects on the host due to changes in the gut microbiota. We developed a HPLC method for the determination of SCFAs in mouse feces. After homogenization, the SCFAs in mouse feces and 2-ethylbutyric acid (internal standard) were derivatized with 2-nitrophenylhydrazine (2-NPH) in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide. The 2-NPH derivatives of SCFAs and the internal standard were separated on a reversed-phase column (octadecyl silyl column) by gradient elution using phosphoric acid (pH 2.5)–acetonitrile at 50°C and detected by absorbance measurement at 400 nm. The recovery of the method was 90–115%, with a precision (relative standard deviation) of 1.3–7.7%. The determination of SCFAs by the present method can provide useful information for biological and clinical research.

Key words short-chain fatty acid; feces; HPLC; 2-nitrophenylhydrazine

INTRODUCTION

Gut microbiota is composed of approximately 500–1000 bacterial species that inhabit the human intestine and 1011–12 microorganisms, which exceeds the total number of somatic cells in the human body. Dr. Joshua Lederberg, who received the Nobel Prize in Medicine and Physiology in 2000, proposed that we should think of each host and its parasites as a superorganism with the respective genomes yoked into a chimera of sorts. The role of gut microbiota in the development of next-generation sequencing technology is now recognized. Many reports have suggested relationships between gut microbiota and diseases, such as allergic inflammation,2–5 metabolic diseases,6,7 obesity,8,9 and central nervous system diseases.10–12 From these recent studies, the metabolites produced by intestinal bacteria are recognized as markers for elucidating how intestinal bacteria act on the host. Notably, short-chain fatty acids (SCFAs) such as acetic acid, propionic acid, and butyric acid produced by gut microbiota provide energy to the host. Moreover, SCFAs activate SCFA-receptors (GPR41, 43) on the cell membrane and exert epigenetic changes, such as the inhibition of histone-deacetylase activity.13–16 Therefore, the analysis of SCFAs produced by gut microbiota is very important for demonstrating causation, delineating the effects on the host due to changes in gut microbiota.

Recently, for the determination of SCFAs in feces, gas chromatography with mass spectrometric detection (GC-MS)27–29 or flame ionization detection (GC-FID)22 and HPLC with mass spectrometric detection (HPLC-MS),23–26 UV-visible spectrophotometric detection (HPLC-UV-Vis),27 or electrospray detection (HPLC-ESI)30 have been reported. In these GC methods, mass spectrometry (GC-MS) and GC-FID are used for determination SCFAs in feces. Most GC-MS and GC-FID methods for the analysis of SCFAs from feces samples require sample clean-up procedures such as liquid–liquid extraction or solid phase microextraction. These GC-MS and HPLC-MS methods require expensive instruments, and the GC-MS, GC-FID, and HPLC-MS methods are not effective for the analysis of non-volatile SCFAs such as lactic acid. The HPLC-ECD method has been used to analyze SCFAs without derivatization procedures, but only 5 species of SCFAs were analyzed.

The HPLC-UV-Vis method27 has been used to analyze SCFAs in feces based on the previously reported method by Miwa and Yamamoto. In these HPLC-UV-Vis methods, 13 species of SCFAs were derivatized with 2-nitrophenylhydrazine (2-NPH) and then separated on an octylsilyl column; however, the SCFAs were not sufficiently separated from each other.

In the present study, we developed a HPLC-Vis method for the determination of SCFAs in feces that can be widely used in biological and clinical research. To establish the present method, we applied the method previously developed by Miwa et al. to the determination of SCFAs in feces and investigated the separation of 2-NPH derivatives of SCFA in feces via reversed-phase HPLC with analytical validation.

MATERIALS AND METHODS

Chemicals and Solvents All chemicals were of analytical-reagent grade, unless stated otherwise. L-Lactic acid, isobutyric acid, n-butyric acid, 3-methylcrotonic acid, isovaleric acid, isocaproic acid, 2-ethylbutyric acid, 2-NPH-HCl, and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride were purchased from Tokyo Chemical Industry (Tokyo, Japan). Crotonic acid, tiglic acid, n-valeric acid, and

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n-caproic acid were purchased from Nakalai Tesque (Kyoto, Japan). Acetic acid, propionic acid, n-2-methylbutyric acid and HPLC-grade acetonitrile were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Reverse osmosis water (Purelite PRB-002A, Organo, Tokyo, Japan) was purified with a Purelab Ultra Analytic (Organo) instrument prior to use.

**Instrumental Conditions** The HPLC system (Shimadzu, Kyoto, Japan) consisted of two LC-10ADp pumps, a CTO-10ASVP column oven, DGU-20A3R on-line degasser, SIL-10AVP auto-injector, SPD-10AVP UV-Vis detector, SCL-10A VP, system controller, and C-R8A recorder. A TSK-gel ODS-80T (column 250 × 4.6 mm, i.d., Tosoh, Tokyo, Japan) was used at 40°C with gradient elution using (A) water (pH was maintained at 2.5 with 1 M phosphoric acid) and (B) acetonitrile as solvents. The elution program consisted of a linear gradient from 22 to 40% of B for 45 min, followed by a stepwise increase to 80% of B for 5 min, and finally a stepwise decrease to 22% of B to re-equilibrate the column for 10 min. The flow-rate was 1 mL/min. The absorbance was detected at a wavelength of 400 nm.

**Analytical Procedure** The analytical sample was prepared according to the method previously described by Miwa and Yamamoto. The mouse feces sample was homogenized in 50 volumes (v/w) of water and the homogenate was centrifuged (3000 × g, 10 min). The supernatant (100 µL) was mixed with 2-ethylbutyric acid as an internal standard (IS, 0.5 mM, in ethanol, 100 µL), 2-NPH·HCl (20 mM, in water, 200 µL), and EDC·HCl (0.25 M solution in ethanol, with an equal volume pyridine (3% (v/v), in ethanol), 400 µL). The mixture was heated at 60°C for 20 min, and potassium hydroxide (15% (w/v), 100 µL) was then added. After heating at 60°C for 15 min, the reaction mixture was mixed with phosphate buffer (1/30 M, pH 6.4)–hydrochloric acid (0.5 M) (3.8 : 0.4 (v/v), 4 mL) and the SCFA derivatives were extracted with diethyl ether (4 mL, twice). The diethyl ether layer was evaporated to dryness under a stream of nitrogen at room temperature. The residue was dissolved in methanol (200 µL) and an aliquot of 20 µL was then subjected to HPLC.

**Animal Preparation and Feces Sample** Fifteen male C57BL/6N mice, weighing 30–40 g, were obtained from SHIMIZU Laboratory Supplies (Kyoto, Japan) and housed under a 12 h light/dark cycle at 21 to 24°C for at least 1 week before the experiments. Mice were given ad libitum access to food and water before the experiments. After an acclimation period of 1 week, the 15 mice were divided into three groups of 5 mice each and were fed for 4 weeks on 3 different diets, i.e., high-fat, high-protein, and high-fiber diets. The 3 diets were purchased from Research Diets, Inc. (New Brunswick, NJ, U.S.A.).

The mice were used in the subsequent experiments. The animal studies were approved by the Research Ethics Committee of the Fukuyama University.

RESULTS AND DISCUSSION

**Chromatographic Separation** Derivatization of the SCFAs with 2-NPH proceeded under the previous conditions described by Miwa and Yamamoto. The SCFA derivatives produced by the reaction with 2-NPH were successfully separated on a reversed-phase column. The typical chromatograms obtained from a standard solution and mouse feces are shown in Fig. 1. The peaks due to thirteen species of SCFAs (retention time: i-lactic acid: 8.4; acetic acid: 9.4; propionic acid: 14.5; crotonic acid: 20.9; isobutyric acid: 21.5; butyric acid: 22.9; tiglic acid: 28.9; n-2-methylbutyric acid: 31.1; isovaleric acid: 32.9; 3-methylcrotonic acid: 33.5; n-valeric acid: 35.2; isocaproic acid: 46.7; n-caproic acid: 48.6 min) and the IS (41.1 min) were separated from each other and the reagent blank. The previous method described by Miwa and Yamamoto was used to separate the derivatives of SCFA on an octylsilyl column at pH 4.5. Although the peaks due to lactic acid and acetic acid overlapped with that of other components in the feces (peak X2 in Fig. 1(B)) during separation on the octylsilyl column (TSK-gel Octyl-80T, 250 × 4.6 mm, i.d., Tosoh), the peaks of lactic acid and acetic acid were successfully separated from peak X2 on the octadecylsilyle column (TSK-gel ODS-80Ts). When the derivatives of the SCFAs were separated at pH 4.5, the broad peak due to the reagent blank did not show a constant retention time and interfered with the analysis. Sufficient separation was obtained at pH 2.5, and the reagent blank was eluted before i-lactic acid.

Analysis of the feces of the mice fed with a high-fat diet
showed peaks of L-lactic acid, acetic acid, propionic acid, and n-butyric acid, which were separated from the peaks of the reagent blank and the other components of mouse feces (Fig. 1(B)). The other SCFAs were below the determination limit or not detected in the feces of the mice fed a high-fat diet. The peaks due to SCFAs in the mouse feces were identified by comparing the retention times.

**Linearity, Recovery, Precision, and Detection Limit**

The relationship between the SCFA/IS peak-area ratio and the amount of SCFAs was linear in the concentration range from 1–100 nmol/100 µL (0.5–50 µmol/g) (r > 0.998 for all cases). For acetic acid present at high concentration in the sample, the relationship between the peak-area ratio of acetic acid to the IS versus the amount of acetic acid was linear in the range from 1–400 nmol/100 µL (1.25–200 µmol/g) (r = 0.998).

The within-day precision was determined from 7 replicate assays in 1 d, and the day-to-day precision was determined from assays on 3 d. When the standard solution (50 nmol/100 µL) was analyzed, the within-day and day-to-day relative standard deviations of the SCFA/IS ratio were 0.9–2.8% and 1.5–8.9%, respectively.

The recovery test was performed using water or the supernatant of the homogenate (100 µL) spiked with various amounts of standard SCFAs (10, 25, and 50 nmol; for acetic acid the concentrations were 40, 100, and 200 nmol). The relationship between the SCFA/IS peak-area ratio and the amount of SCFAs was linear. The recoveries were obtained from the slope ratios of the regression equations for the analytes with/without the supernatant of the homogenate. The recoveries of the SCFAs were 90–115% (Table 1).

The detection limit (signal-to-noise ratio = 3) for the SCFAs was 20–30 pmol per injection, except in the case of L-lactic acid (55 pmol) and 3-methylcrotonic acid (109 pmol).

**Determination of SCFAs in Mouse Feces**

The SCFAs in the feces of the mice fed a high-protein diet, high-fat diet, and high-fiber diet were quantified by the present method. The feces of 5 mice for each diet group were homogenized in 1 vial, and the obtained supernatants were then analyzed. The

### Table 1. Recoveries of SCFAs

<table>
<thead>
<tr>
<th>SCFA</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Lactic acid</td>
<td>91.4</td>
</tr>
<tr>
<td>Acetate acid</td>
<td>96.3</td>
</tr>
<tr>
<td>Propionate</td>
<td>103.8</td>
</tr>
<tr>
<td>Crotonic acid</td>
<td>111.1</td>
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<tr>
<td>Isobutyrate</td>
<td>102.9</td>
</tr>
<tr>
<td>Butyrate acid</td>
<td>104.1</td>
</tr>
<tr>
<td>Tetrahydrofuran</td>
<td>103.9</td>
</tr>
<tr>
<td>n-Dodecanol</td>
<td>98.2</td>
</tr>
<tr>
<td>Isopropionate</td>
<td>109.6</td>
</tr>
<tr>
<td>3-Methylcrotonic acid</td>
<td>115.5</td>
</tr>
<tr>
<td>Valerate</td>
<td>103.5</td>
</tr>
<tr>
<td>Isovalerate</td>
<td>105.7</td>
</tr>
<tr>
<td>n-Butyrate</td>
<td>102.5</td>
</tr>
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</table>

### Table 2. Content of Analytes in Feces from Mouse and Precision

<table>
<thead>
<tr>
<th>Analyte</th>
<th>High-fat diet</th>
<th>RSD (%, n = 7)</th>
<th>High-protein diet</th>
<th>RSD (%, n = 7)</th>
<th>High-fiber diet</th>
<th>RSD (%, n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Content (µmol/g)</td>
<td>S.D.</td>
<td>Mean</td>
<td>RSD (%)</td>
<td>Content (µmol/g)</td>
<td>S.D.</td>
</tr>
<tr>
<td>L-Lactic acid</td>
<td>1.3</td>
<td>0.10</td>
<td>7.7</td>
<td>0.77</td>
<td>2.2</td>
<td>0.15</td>
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<tr>
<td>Acetate acid</td>
<td>19.9</td>
<td>0.62</td>
<td>3.1</td>
<td>0.34</td>
<td>32.5</td>
<td>0.55</td>
</tr>
<tr>
<td>Propionate</td>
<td>3.9</td>
<td>0.13</td>
<td>3.3</td>
<td>0.99</td>
<td>7.5</td>
<td>0.10</td>
</tr>
<tr>
<td>Crotonate</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Isobutyrate</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Butyrate acid</td>
<td>1.5</td>
<td>0.09</td>
<td>6.0</td>
<td>0.45</td>
<td>0.6</td>
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<td>Tetrahydrofuran</td>
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<td>&lt;0.5</td>
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<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Isopropionate</td>
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<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>3-Methylcrotonic acid</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
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<td>nd</td>
</tr>
<tr>
<td>Valerate</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
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<tr>
<td>Isovalerate</td>
<td>nd</td>
<td>nd</td>
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<td>nd</td>
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<td>nd</td>
</tr>
<tr>
<td>n-Butyrate</td>
<td>nd</td>
<td>nd</td>
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<td>nd</td>
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<td>nd</td>
</tr>
</tbody>
</table>

* a) Feces of five mice for each diet group were homogenized in one vial, and the obtained supernatants were analyzed in seven replicate assays. b) Not detected. c) Below determination limit.
content of SCFAs is given Table 2. The peaks due to L-lactic acid, acetic acid, propionic acid, isobutyric acid, n-butyric acid, tiglic acid, 2-methylbutyric acid, and isovaleric acid were detected, and the relative standard deviation for these SCFAs in each feces sample was in the range of 1.3–7.7% for seven replicate assays in 1 d.

For the high-fiber diet group, more types of SCFAs were detected than for the other groups, and the amount of these SCFAs detected was also higher. In particular, the production of DL-2-methylbutyric acid and isovaleric acid, which were not detected for the mice fed with the other diets, was observed. In contrast, these types of SCFAs were minimally produced by the high-fat and high-protein diet groups. Notably, the amount of SCFAs was also lower for the high-fat diet group. The results reveal that a high-fiber diet increased the kind and amount of SCFAs produced by gut microbiota compared to other feeds. In general, dietary fiber is metabolized by hydrolytic enzymes of gut microbiota such as β-glucosidase and is converted into SCFAs. The high-fiber diet used in this study was prepared by adding inulin, which is a water-soluble dietary fiber. Inulin is a well-known fructose-based prebiotic that has been shown to stimulate the growth of bacteria, generally considered beneficial for intestinal health. Apajalähi et al. revealed that the feed of inulin significantly increased the amount of total SCFAs. Thus, the amount of lactic acid was significantly increased, whereas that of butyric acid was minimally changed by the feed of inulin. In our study, lactic acid production increased approximately 10- to 20-fold and acetic acid production increased approximately 4- to 6-fold for the high-fiber diet group compared to the other feed groups, whereas the amount of butyric acid was almost the same as with the other feeds. Therefore, the present results are consistent with previous reports. It is known that more than 90% of the SCFAs produced by gut microbiota are derived from the metabolism of dietary fiber, whereas branched SCFAs (isobutyric acid and α-2-methylbutyric acid, etc.) are derived from proteins and amino acids. Interestingly, however, the amount of branched SCFAs increased for the high-fiber diet group in this study. This is believed to be because the metabolic production of SCFAs from proteins and amino acids was promoted throughout the colon due to changes in the gut microbiota due to the prebiotic effect of inulin in the feed. Thus, it is assumed that the amount of branched SCFAs increased for the high-fiber diet group. The kind of SCFAs produced may change depending on the change in the gut microbiota based on the amount of protein and dietary fiber in the diet. Therefore, further study along this line is required.

CONCLUSION

We established a HPLC method for the determination of SCFAs in mouse feces using 2-NPH as a pre-column derivatization reagent. The proposed method was successfully applied to the determination of thirteen species of SCFAs in mouse feces by successful separation on an octadecysilsyl column with Vis detection. The determination of SCFAs by the present method can provide useful information for biological and clinical research. Using this established method of HPLC analysis, we investigated whether different diets influence the kind and amount of SCFAs produced by gut microbiota. In current studies, this method has been used to analyze changes in SCFAs, not only in pathological models, but also before and after administration of traditional Japanese medicines. To clarify the relationship between diseases and the gut microbiota, it is of critical importance to demonstrate not only these correlations, but also the causations. The method established in this study is a useful method that can demonstrate the causation of the changes in gut microbiota and the effects on the host.

Conflict of Interest

The authors declare no conflict of interest.

REFERENCES


