Determination of Total, Free, and Esterified Short-Chain Fatty Acid in Human Serum by LC-MS/MS

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Abstract

Background: Short-chain fatty acid (SCFA) are primarily absorbed through the portal vein during lipid digestion, which is utilized as the energy source, as well as prevents type 2 diabetes and some cancers. However, reports on the determination of these SCFA in human serum are limited.

Methods: Blood samples from human subjects (n = 547, male/female = 246/301, age 58.85 ± 12.57) were collected. Saponification was applied to obtain total FA. After derivatization by 2-nitrophenylhydrazine, FA 4:0 and FA 6:0 were measured by liquid chromatography-mass spectrometry (LC-MS/MS).

Results: The developed method exhibited satisfied linearity (R^2 = 0.9996 for both). All the coefficient of variation of reproducibility and accuracy for FA 4:0 and FA 6:0 ranged in 3.0%−6.1%, with the average recoveries of 87.8%−102.4% and 92.2%−98.2%, respectively. In all the samples, the concentration of FA 4:0 (162.4 ± 76.4 μmol/L) showed significantly higher than FA 6:0 (2.0 ± 2.5 μmol/L, $P < 0.001$). Furthermore, the esterified form showed predominant in both FA 4:0 and FA 6:0 (98.2% and 82.4% of total FA, respectively). Besides, SCFA showed no significant differences in concentration with sex or age differences.

Conclusion: This developed LC-MS/MS method is convenient and reliable, which might be useful for monitoring SCFA variation in blood.

Keywords: Short-chain fatty acid (SCFA), butanoic acid (FA 4:0), caproic acid (FA 6:0), LC-MS/MS, serum

1 Introduction

Short-chain fatty acids (SCFAs), also referred to as volatile fatty acids (VFAs), are widely known as the end products of bacterial metabolites generated *via* the fermentation of dietary fibers (1). In all the SCFA in the human body, approximately 90% of the amount is absorbed in the colon and transported to the liver through the hepatic vein, while the residual amount is secreted through the feces (2,3). In the recent years, there is an increasing trend that recognizes SCFA as mediators of the local gut and systemic health (4), along with the growing studies on their positive physiological effects (5,6). Taking butyrate as an example, evidence has shown the uptake of butyrate by the colonic epithelium, disclosing it preferentially utilized by colonocytes as the primary energy source (7). After absorbed, butyrate is involved in various of therapeutic effects, such as the improvement of type 2 diabetes (4,8), the reduction of cholesterol production (2,9), the prevention of colon cancer (10), and the limit of intestinal inflammation (1). Furthermore, among SCFA, butyrate serves as the most potent promoter of intestinal regulatory T cells in vitro and the only one that showed the niacin receptor 1 (NIACR1) ligand (11).

It should be noted that the esterified fatty acid (EFA) and free fatty acid (FFA) show different physiological characteristics. For example, tricaproin (TG 6:0/6:0/6:0) induces oxygen radical production in neutrophils, while the free caproic acid (FFA 6:0) exerts no clear such effect (12). Moreover, it is known that long chain FFA can affect gene expression of macrophages, adipocytes, or endothelial cells (13), and the elevation

of its concentration is associated with most of the metabolic disorders, including impaired glucose utilization, impaired insulin secretion, dyslipidemia, pro-thrombotic, etc. (14,15). But there is a quite limited study on the effect of short-chain FFA. The bioactivity difference between EFA and FFA demands us for the attention on both of them. Thus, for SCFA determination, it is necessary to determine its not only the esterified but also the free form.

Most of the studies on SCFA determination relied on gas chromatography (GC), combined with either flame ionization detector (FID) or mass spectrometry (MS) (16–19). High-performance liquid chromatography (HPLC) is a good alternative method for SCFA analysis, of which the greatest advantage over GC is the use of lower running temperatures (17,20). In terms of HPLC, approaches with derivatization could improve the separation and sensitivity. One of the derivatization-based methods, using acidic 2-nitrophenylhydrazine hydrochloride (2-NPH·HCl) with or without saponification, has been described for the determinations of both FFA and EFA in biological materials (21). The author demonstrated that the developed method was simple, rapid, and reliable. And this method has been applied in our laboratory for determining medium-chain FA (MCFA) and long-chain FA (LCFA) of their total and free forms in various of samples, ranging from the clinical blood sample to the commercial milk (22–24). However, the strategy of HPLC with NPH-derivatization was not applicable for SCFA in our laboratory, due to the poor resolution and the interference in chromatography.

LC/MS is considered as an ideal approach for analyzing targets in the complex

matrix like biological samples, as it provides chromatographic separation along with highly sensitive and selective detection. Derivatization for LC/MS can help enhancing the ionization efficiency, as well as increasing the retention time and *m/z*, which avoid potential suppression effects. Also, an ingenious derivatization will be a strategy for controlling fragmentation in MS (25). But to the best of our knowledge, there has not been reported on SCFA determination by LC-MS/MS and NPH-derivatization yet.

Therefore, the aims of the present study are to develop a practical NPH-derived LC-MS/MS method for the determination of SCFA in the blood sample and to apply the developed method to clinical samples. The butanoic acid (FA 4:0) and caproic acid (FA 6:0) were treated as our targets, and their contents of both free and esterified forms were investigated.

2 Materials and methods

2.1 Chemicals

LC/MS grade methanol, n-hexane, and water were purchased from Wako Pure Chemical (Osaka, Japan). Ammonium acetate was purchased from Sigma-Aldrich (St. Louis, MO). Other chemicals and reagents were of analytical grade and purchased from Kanto Chemical Industry (Tokyo, Japan) unless specified.

FA 4:0, FA 6:0, and the internal standard (ISTD) undecanoic acid (FA 11:0) were purchased from Sigma-Aldrich, while 2-nitrophenyl hydrazide (NPH) of these FA were previously synthesized in our laboratory (22). The FA NPH-derivatization kit, which

contains 2-NPH·HCl, *N*-(3-Dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC·HCl), potassium phosphate buffer (pH 4.6), was provided by YMC Co., Ltd. (Kyoto, Japan).

2.2 Specimens and serum collection

Ethical approvals for this study were obtained, and informed consent was obtained from all individuals. Fasting blood sample was collected from 547 Japanese volunteers (246 males, mean age ± SD: 58.4 ± 12.6 years; 301 females, mean age ± SD: 59.1 ± 12.4 years; shown in **Figure S1 of Supplemental Material**). All of the volunteers were living in the country of Suttsu District, Hokkaido, Japan (42°47′N, 140°14′E). All of the serum was separated by centrifugation within 30 min of collection, and then stored at -80°C until tested.

2.3 Sample preparation

The serum sample preparation procedure was in according to Shrestha et al as previously reported (23). In brief, a portion of serum (25 μL for total FA, 100 μL for free FA) was spiked with the ISTD FA 11:0 (2 nmol). For total FA determination, the saponification was performed as mixing serum with 100 μL of 0.3 M KOH-EtOH, and heating at 80°C for 45 min. Then, derivatization was performed by adding 2-NPH·HCl and EDC·HCl, and incubating at 60°C for 20 min. FA-NPH derivatives were obtained by potassium phosphate buffer and n-hexane extraction, followed by vacuum dryness of the n-hexane layer. Finally, the residue was dissolved in 200 mL of methanol and stored at −80 °C before injection.

2.4 LC-MS/MS analysis

LC-MS/MS was performed using a Surveyor HPLC system and a TSQ Quantum Access MAX mass spectrometer with a heated electrospray ionization (H-ESI) probe (Thermo Fisher Scientific Inc., Waltham, MA). LC was carried out on an Ascentis® Express Phenyl-Hexyl column (5 cm × 2.1 mm I.D., 2.7 μm, Supelco, Inc., Bellefonte, PA) at 45 °C. The injection volume was set at 1.0 μL. The mobile phase consisted of 5 mM aqueous ammonium acetate (A), isopropanol (B), and methanol (C) at a flow rate of 200 μL/min. The following gradient elution was applied: 0.0−0.5 min 65% A and 35% C; 0.5–1.0 min 30% A, 20% B, 50% C; 1.0−4.5 min 5% A, 30% B, 65% C; 4.5–5.0 min 33% B, 67% C; this ratio was kept to 8.0 min; 8.0–10.0 min returned to initial gradient for re-equilibration.

The selected reaction monitoring (SRM) under negative mode was utilized for MS detection, and the main parameters were optimized. Spray voltage was set at 3000 V. Nitrogen was used as the sheath gas and the auxiliary gas (set at 50 psi and 10 psi, respectively). The vaporizer temperature and the capillary temperature was set at 350 °C and 200 °C, respectively. Collision gas (argon) pressure was set at 1.8 mTorr. Quantification was conducted by the workstation Thermo Xcalibur 2.1 software (Thermo Fisher Scientific, Inc., Waltham, MA).

2.5 Method validation

Stock solutions (200 μmol/L) of FA 4:0, FA 6:0, and FA 11:0 were prepared in methanol by measuring their dry weight on an ultrasensitive electro-balance (Cubis®

ultra micro balance, Sartorius Inc., Göttingen, Germany), and stored at −40 °C. To determine linearity, the FA 4:0 and FA 6:0 standard solutions with amounts of 0.25, 0.5, 1.5, 2.5, 5, 10 nmol and 3.125, 6.25, 10, 25, 50, 200 pmol were prepared, respectively. Each standard solution contained 2 nmol of the ISTD FA 11:0. The integration of the peak area and the plotting of each calibration curve were performed by Xcalibur. The limit of quantification (LOQ) was defined as the level of signal-to-noise ratio ≥ 10. Both reproducibility and recovery assays were tested in 6 replicated analyses for the independently prepared serum samples. Recovery was calculated with the following formula:

Recovery (%) = [(amount found − amount original)/amount spiked] × 100%.

2.6 Statistics

Statistical analysis was conducted using the unpaired two-tailed *t*-test, one-way and two-way ANOVA (using the Tukey *post-hoc* test), which was performed by Prism 6.0 (GraphPad Software, Inc., La Jolla, CA). $P < 0.05$ was considered to be statistically significant. All of the shown data are presented as the means ± standard deviation (SD). Pearson's correlation coefficient was performed with JMP 10.0 (SAS Institute Inc., Cary. NC).

3 Results and discussion

3.1 Optimization of LC-MS/MS

The optimized SRM conditions of these FA-NPH together with their acquired

chromatograms were shown in **Figure 1**. All of the three analytes showed identical sharp peaks of similar retention times in both standards mixture solution and serum sample, respectively, indicating the acceptable interfere by matrix effect. Besides, all of the analytes (ISTD included) could be eluted in less than 5 minutes, and the total run time for one injection could be controlled within 10 minutes by this method, suggesting it convenient, commercial, and capable of testing a large number of samples.

3.2 Method validation

The calibration curve was constructed by plotting the FA 4:0-NPH or FA 6:0-NPH to FA 11:0-NPH peak area ratio (y) against the amount of corresponding FA (x, nmol). Both of the calibration curves showed good linearity and acceptable ranges (for FA 4:0, $y = 0.0058x - 0.0006$, $R^2 = 0.9996$; for FA 6:0, $y = 0.1715x - 0.0004$, $R^2 = 0.9996$; shown in **Figure S2 of Supplemental Material**). The LOQ were 5 pmol and 0.12 pmol for FA 4:0 and FA 6:0, respectively.

The reproducibility and recovery of the present method were determined by analyzing the serum of volunteers added to the certain concentration of FA 4:0 and FA 6:0 in 6 replicates, respectively (shown in **Table 1**). For FA 4:0, the coefficient of variation (CV) of reproducibility and recovery were 3.1% and 5.3%, respectively, while for FA 6:0 the values were 6.1% and 3.0%, respectively, suggesting the favorable precision. The average recoveries of them were 94.7 ± 5.0% and 96.1 ± 2.9%, respectively. From these results, the developed method was confirmed to show sufficient performance to determine the two SCFA species in clinical specimens.

3.3 Comparison of the total, free, and esterified SCFA in human serum

Serum samples collected from 547 subjects were measured for FA 4:0 and FA 6:0 of both total and free forms by the developed LC-MS/MS method. And the esterified FA content was calculated as the difference total FA minus free FA. The concentrations among FA 4:0 and FA 6:0 of their total, free, and esterified form exhibited significant difference ($P < 0.05$, shown in **Figure 2**). The amount of FA 4:0 was significantly higher than that of FA 6:0 (162.39 ± 76.36 and 1.97 ± 2.51 μmol/L, respectively, $P < 0.001$), suggesting FA 4:0 the major SCFA in this study. In terms of FA forms, the average concentration of free and esterified FA 4:0 were 2.63 ± 0.83 and 159.39 ± 76.79 μmol/L, respectively, while for FA 6:0, the concentrations were 0.35 ± 0.16 and 1.62 ± 2.49 μmol/L, respectively. The results showed that the esterified form was predominant form in both FA 4:0 and FA 6:0, accounted for 98.2% and 82.4% of its corresponded total form, respectively (**Figure 2**).

Most of the previous studies on FA 4:0 and FA 6:0 quantitation were performed by GC, with the sample preparation method including distillation, organic solvent extraction, or hollow fiber supported liquid membrane extraction (16,26–28), while van Eijk et al measured SCFA *via* LC/MS with the pretreatment of methanol extraction (29). All of the above studies seemed targeting on free SCFA due to their pretreatment procedures. In our experiment, the levels of free FA 4:0 and free FA 6:0 were similar to their results (FA 4:0 varied from 0.9 ± 0.2 μmol/L to 2.5–29.6 μmol/L, and FA 6:0 ranged from 1.4−9.7 μmol/L to 4.1 ± 0.8 μmol/L).

Furthermore, in the present work, the total and esterified forms of FA 4:0 and FA 6:0 were also investigated. Based on the concentration of total, free, and esterified forms of FA 4:0 and FA 6:0, respectively, the Pearson's correlation coefficients were calculated, and the scatter plots were generated in **Figure 3**. The very strong correlation between the amount of total and its esterified form was observed (for the total and esterified FA 4:0, $r = 0.9999$, $P < 0.0001$, **Figure 3A**; for the total and esterified FA 6:0, $r = 0.9984$, $P < 0.0001$, **Figure 3B**). However, there was no correlation between free and esterified form, or total and free form ($r < 0.15$ for all in **Figure 3C−F**).

Studies showed that the elevation of plasma FFA leads to a shift from unsaturated to saturated fatty-acyl chains in membrane phospholipids, which promotes the physical attractive van der Waals interactions between phospholipid acyl chains, increasing the stiffness of both erythrocyte and endothelial membranes, and finally resulting in vascular and neurological lesions, and a various of metabolic disease (30). However, as free SCFA does not directly construct or reconstruct the cell membrane, its content variation might affect the body health through other ways, such as cell signaling or enzymatic reaction.

3.4 SCFA variation with sex and ages

The variation of SCFA between male and female of different ages (grouped as 30−39, 40−49, 50−59, 60−69, and 70−79 years old) was compared (shown in **Figure 4**). For total FA 4:0 in male, the concentration ranged from 184.47 ± 73.99 μmol/L (50−59 group) to 138.93 ± 67.12 μmol/L (70−79 group), while in female the concentration

ranged from 183.76 ± 81.08 μmol/L (40−49 group) to 142.73 ± 75.02 μmol/L (30−39 group) (**Figure 4A**). For total FA 6:0, the highest and lowest concentrations in male were presented in 60−69 group (2.05 ± 2.42 μmol/L) and 40−49 group (1.58 ± 2.15 μmol/L), respectively, while in female they were presented in 40−49 group (2.27 ± 3.37 μmol/L) and 30−39 group (1.42 ± 0.74 μmol/L), respectively (**Figure 4B**). There was no significant difference between male and female, or among different ages ($P > 0.05$ for all the comparison), suggesting the limited influence from sex and age factors to these two SCFA in the human serum. Besides, as the quite large deviations shown, it might be indicated that serum SCFA content especially for FA 6:0, varies along with individual differences, which might include multiple factors, including hormone, dietary, sports, etc. Moreover, the similar trend could be observed in free FA 4:0, free FA 6:0, esterified FA 4:0, and esterified FA 6:0 (**Figure 4C−F**).

According to the review by Lohner et al, the long-chain FA in plasma total lipid, such as FA 20:4 n-6 and FA 20:5 n-3, showed significantly lower contribution in men than in women (31). And Asciutti-Moura et al demonstrated that the older people have less circulating essential fatty acids (such as FA 18:2 n-6 and FA 20:4 n-6) due to diet and metabolism (32). These polyunsaturated FAs exert the biological actions, including maintaining cell-membrane fluidity, inhibiting inflammatory processes, decreasing secretion of proinflammatory cytokines by monocytes/macrophages, etc. (33), which are different from SCFA. That might be likely the explanation why the trend of SCFA content is not obvious in relation to sex or ages.

4 Conclusion

In the present work, a simple, precise, and accurate method for determining SCFA by LC-MS/MS was developed and applied to a large scale of human serum. The developed method might be useful for monitoring both free and esterified SCFA variation in clinical samples. For the future study, SCFA profile will be combined with biological information of subjects, to reveal the relationship between SCFA changes and lifestyle-related diseases.

Abbreviations

CV, coefficient of variation; EDC·HCl, *N*-(3-Dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride; EFA, esterified fatty acid; FA 4:0, butanoic acid; FA 6:0, caproic acid; FA 11:0, undecanoic acid; FFA, free fatty acid; HPLC, high-performance liquid chromatography; ISTD, internal standard; LCFA, long-chain fatty acid; LOD, limit of detection; LOQ, limit of quantification; MCFA, medium-chain fatty acid; NPH, 2-nitrophenyl hydrazide; SCFA, short-chain fatty acid; SD, standard deviation; SRM, selected reaction monitoring.

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Declaration of conflicting interests

The authors declare no conflict of interests.

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Ethical approval

Ethics review boards of Faculty of Health Sciences, Hokkaido University (No. 16−10) and of Graduate School of Medicine, Hokkaido University (No. 16−007) approved this study protocol.

Guarantor

S-PH

Contributorship

S-PH, and HC conducted the study design and discussion. AT, S-PH, and HC were involved in sample collection. ZC, YW, and RS performed the sample preparation. ZC, YW and ZG operated the LC-MS/MS and data process. YZ and YM advised statistics.

S-PH and HC reviewed the relevant literature. ZC wrote the first draft of the manuscript. YZ, ZG, and WY helped correcting the mistakes. All the authors reviewed and edited the manuscript, and approved the final version of the manuscript.

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Figure caption

Figure 1. The representative SRM chromatograms of FA-NPH in standards mixture solution (left) and in the serum sample (right).

Figure 2. The average concentrations of FA 4:0 and FA 6:0 with their total, free, and esterified forms. Bars without a common letter represented significantly different at the 0.001 probability level. Values above bars were presented as percentages of the corresponded total forms.

Figure 3. Pearson's correlation coefficient among the total, esterified, and free forms of FA 4:0 (A, C, E) and FA 6:0 (B, D, F).

Figure 4. Variations of FA 4:0 and FA 6:0 with total, free, and esterified forms between male and female of different ages. Sample numbers are presented as M/F (male/female).