Determination of Short-chain Fatty Acids in Rat and Human Feces by High-Performance Liquid Chromatography with Electrochemical Detection

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A simple method for determining short-chain fatty acids (SCFAs) in rat and human feces was developed using high-performance liquid chromatography with electrochemical detection (HPLC-ECD). A two-channel HPLC-ECD system was fabricated using an ion exclusion column and an electrochemical detector with a glassy carbon working electrode. Aqueous solutions of 0.1 mM $HClO_4$ and of ethanol containing 2-methyl-1,4-naphthoquinone served as a mobile phase and a quinone solution, respectively. Peak areas for lactic, acetic, propionic, butyric, isovaleric, and valeric acids at a detection potential of –0.9 V *vs*. an Ag/AgCl electrode showed a linear relationship with the acid amount in the range 0.1 to 40 nmol. Standard acids at 4 nmol were determined ten times with relative standard deviations (RSD) of less than 2.0%. The analytical results of healthy human feces were measured within 35 min. RSD (n = 5) in all SCFAs were less than 2.7%, and recoveries of SCFAs were more than 92%. The present method was characterized by reproducibility with the simple and rapid procedure without derivatization of analytes, and it has the potential for clinical and biomedical applications.

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Introduction

The bacteria in human colon obtain energy for growth by fermenting carbohydrates in the colonic lumen, mainly polysaccharides of plant cell walls (dietary "fiber") and some starch. The end-products include around 200 to 400 mmol day^{-1} of short-chain fatty acids (SCFAs) such as acetic, propionic, and butyric acids. Over 90% are absorbed and used by host, contributing 2 to 10% of daily energy requirements.[1,2] This is particularly important in premature babies with lactose malabsorption.[3]

An extensive disease of the bowel, surgical bypass, prolonged antibiotic therapy, total parenteral nutrition, malabsorption, immaturity[4,5] and wide variation in dietary fiber intake may all disturb this important host/bacterial symbiosis.[1,2] Since fecal SCFAs reflect colonic fermentation,[6] they are measured in studies to investigate and manipulate such disturbances. Moreover, fecal SCFA composition ratio of ulcerative colitis patients remarkably changes in comparison with that for a healthy human. In particular, the composition of lactic acid, called non-volatile fatty acid (NVFA), increased in comparison with that of acetic, propionic, and butyric acids, called volatile fatty acid (VFA), in feces of ulcerative colitis patients.[7-12] Thus, the determination of fecal SCFAs is significant to examine homeostasis, characteristics, and metabolism of intestinal bacterial flora, and it would be useful for monitoring of and diagnostic strategy against diseases in the colon.

The determination of SCFAs has been carried out by gas chromatography (GC)[13-18] and high-performance liquid chromatography with ultraviolet detection (HPLC-UV), fluorescent detection (HPLC-FL), or mass spectrometric detection (LC-MS).[19-24] The fecal analysis by GC required the complicate cleanup of the sample, and it was hard to detect NVFAs such as lactic acid. The derivatization with an appropriate chromophore before or after the column separation is often required for the determination carboxylic acids by HPLC with high sensitivity. However, the operating procedures for derivatization sometimes involves troublesome and complicated processes and can be time-consuming for disease diagnosis. In addition to the complicated sample preparation and cleanup procedures, LC-MS and GC-MS require expensive instruments. A simple, sensitive and rapid assay is thus highly desirable for the analyses in the clinical field.

Since most carboxylic acids are less active electrochemically, a few papers have so far been concerned with the use of the electrochemical detection methods in HPLC. We previously developed a new method for determining acids by voltammetry using a quinone reagent.[25] Since the method was shown to be quite sensitive and selective for acids, the method was successfully applied to the determination of the free fatty acid (FFA) content of fats and oils,[26-28] the total acid content of vinegar,[29] and the FFA concentrations of plasma.[30]

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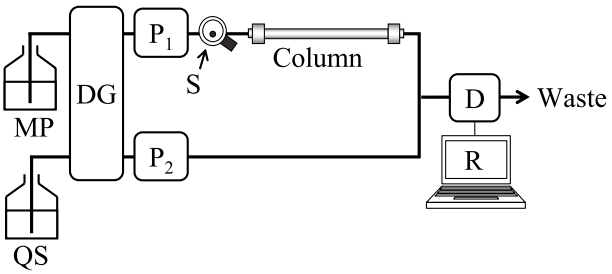

Fig. 1 Block diagram of the HPLC-ECD system. MP, Mobile phase, 0.1 mM $HClO_4$; QS, quinone solution, 6 mM VK_3 + 100 mM $LiClO_4$ in ethanol; DG, degasser; P_1 and P_2, pump; S, sample injector; column, ion exclusion column (Shim-pack SPR-H, 250 mm × 7.8 mm i.d.) with guard column (Shim-pack SPR-H (G), 50 mm × 7.8 mm i.d.); D, electrochemical detector, electrochemical cell and potentiostat; R, recorder.

In the present study, an HPLC system with electrochemical detection (HPLC-ECD) was fabricated for determining SCFAs in rat and human feces. An assessment of the HPLC-ECD method was made as an effective means for monitoring the changes of fecal SCFA contents in ulcerative colitis model rats and the day-to-day variations of fecal SCFA contents in the healthy human subjects.

Experimental

Reagents

All the chemicals used were of reagent grade. 2-Methyl-1,4-naphthoquinone (vitamin K_3, VK_3, >98.5%), $LiClO_4$ (>98.0%), $HClO_4$ (60 – 62%), ethanol (99.5%, JIS K 8101), lactic acid (analytical value: 91.7%), acetic acid (>99.7%), propionic acid (>99.0%), butyric acid (>98.0%), isovaleric acid (>98.0%), valeric acid (>98.0%), and dextran sulfate sodium (DSS, molecular weight 5000) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Other reagents and HPLC solvents were from Wako (Osaka, Japan).

HPLC system and its operation conditions

The HPLC-ECD system is shown in Fig. 1. It consists of a degasser (Model DG-980-50, Jasco, Tokyo, Japan), two pumps (Model PU-980, Jasco), a sample injector (Model 7125, Rheodyne, Cotati, CA), an ion exclusion column (Shim-pack SPR-H, 250 mm × 7.8 mm i.d., Shimadzu, Kyoto, Japan) with a guard column (Shim-pack SPR-H (G), 50 mm, × 7.8 mm i.d., Shimadzu), a column oven (Model CTO-10ASvp, Shimadzu), a potentiostat (Model 331B, Huso Electrochemical Systems, Kawasaki, Japan) and a recorder (Model 807-IT, Jasco). The commercially available electrochemical cell (Radial flow cell, BAS, Tokyo, Japan) was constructed from a glassy carbon working electrode, an Ag/AgCl reference electrode, and a stainless-steel auxiliary electrode.

An aqueous solution of 0.1 mM $HClO_4$ and an ethanol solution containing 6 mM VK_3 and 100 mM $LiClO_4$ served as the mobile phase solution and the quinone solution, respectively. The mobile phase and quinone solution were degassed by the degasser and made to flow at the rate of 0.6 and 1.0 mL min^{-1}, respectively, in each flow line. Then 20 µL of the sample solution were injected into the ion exclusion column maintained at 40°C using a column oven. The detection potential for monitoring SCFAs was set at –0.9 V *vs*. Ag/AgCl. The detection potential was decided from the measurement of the hydrodynamic voltammogram of each standard SCFAs so as to obtain the maximum current. Each acid in the mixture was monitored by measuring the peak area of a flow signal. The absolute standard curve method was used to determine fecal SCFAs.

Sample preparation

Rat: DSS was administered to five Sprague-Dawley (SD) rats (male, 6 weeks old) for 9 days with its 3% aqueous solution as drinking water. By DSS introduced *via* the drinking water, ulcerative colitis can be induced experimentally in rats[10] and mice.[31] This causes a change in the intestinal microflora population, where particularly an increase in members of the gram-negative anaerobes, including *Bacteroidaceae*, has been noted.[31] Feces specimens were taken before 3% DSS administration and once a day during administration from rats over 9 days. Rat feces before 3% DSS administration was used as control. Ten milligrams (wet weight) of rat feces were diluted with 0.2 mL of 0.1 mM $HClO_4$ containing 3% phenol, vortex-mixed for 5 min and sonicated for 10 min, and then centrifuged for 10 min at 9000*g*. The sample solution was filtered through a membrane filter (pore size, 0.45 µm). A 20-µL volume of the sample solution was injected into the HPLC system.

Human: A healthy adult subject (subject K, male, 23-years-old) was studied upon receipt of his informed consent. Before commencement of the trial, the protocol and the subject's informed consent were submitted to and approved by the Institutional Human Research Committee of Tokyo University of Pharmacy and Life Sciences. All feces specimens were obtained from the subject K. Ten milligrams (wet weight) of human feces were diluted with 0.2 mL of 0.1 mM $HClO_4$ containing 3% phenol, vortex-mixed for 1 min and sonicated for 5 min, and then centrifuged for 10 min at 9000*g*. The sample solution was filtered through a membrane filter (pore size, 0.45 µm). A 20-µL volume of the sample solution was injected into the HPLC system.

Results and Discussion

Determination of SCFAs by HPLC-ECD

In previous studies on the electrochemical reduction of VK_3 in a non-buffered ethanol solution, the addition of small amounts of acid to the solution were found to give rise to a new peak (termed the prepeak) at a more positive potential than the original reduction peak.[26] The occurrence of the prepeak is ascribed to the increased proton availability of the added acid compared to the solvent molecules, leading to the reduction potential shift to a more positive direction depending on the pK_a of the added acid.[25] The prepeak height was found to be proportional to the acetic acid concentration from 0.05 to 2.7 mM ($r = 0.999$) and propionic, butyric, and lactic acids concentrations from 0.05 to 2.5 mM ($r > 0.999$).[29] Based on these findings, acid in the eluate from the separation column in the HPLC system was detected by measuring the current height of a flow signal at the fixed potential. The choice of VK_3 as a quinone reagent for preparing the quinone solution was favorable, as it facilitated this determination owing to its reduction potential, stability and solubility in ethanol.[25]

SCFAs' determination was carried out using the ion exclusion column, the mobile phase of the 0.1 mM $HClO_4$ and the quinone solution. After a 20-µL standard acid solution aliquot was injected into the column, the eluate was mixed with quinone solution and SCFAs were detected with ECD at –0.9 V *vs*. Ag/AgCl. A typical chromatogram for a standard acid mixture

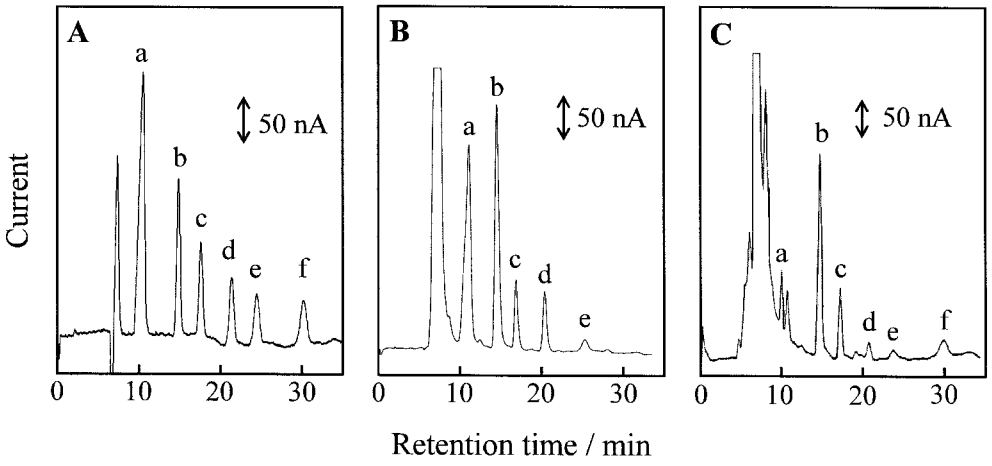

Fig. 2 Chromatograms of SCFAs obtained from (A) standard solution (4 nmol), (B) rat feces, and (C) human feces. Peaks: a, lactic acid; b, acetic acid; c, propionic acid; d, butyric acid; e, isovaleric acid; f, valeric acid. HPLC conditions: column, ion exclusion column (Shim-pack SPR-H, 250 mm × 7.8 mm i.d.) with guard column (Shim-pack SPR-H (G), 50 mm × 7.8 mm i.d.); column temperature, 40°C; mobile phase, 0.1 mM $HClO_4$; quinone solution, 6 mM VK_3 in ethanol containing 100 mM $LiClO_4$; flow rate of mobile phase, 0.6 mL min^{-1}; flow rate of quinone solution, 1.0 mL min^{-1}; applied potential, –0.9 V *vs*. Ag/AgCl.

containing lactic, acetic, propionic, butyric, isovaleric, and valeric acids at 4 nmol each is shown in Fig. 2A. Retention times of lactic, acetic, propionic, butyric, isovaleric, and valeric acids were 10.8, 15.1, 17.7, 20.6, 24.6, and 30.1 min, respectively. Resolution (R_s) between lactic and acetic acids, acetic and propionic acids, propionic and butyric acids, butyric and isovaleric acids, and isovaleric and valeric acids were 4.6, 2.8, 2.8, 3.3, and 4.1, respectively. The peak area of each acid was found to be linearly related to the acid amount injected, ranging from 0.1 to 40 nmol (r >0.999). The standard solution at 4.0 nmol was determined ten times with a relative standard deviation (RSD) of less than 2.0%, respectively. Acid detection limits (at the signal to noise ratio of 3) for a single injection of the present method was 40 pmol.

Determination of SCFAs in rat feces

The chromatogram in Fig. 2B was obtained following the injection of 20 µL sample solution from rat before DSS administration. SCFA contents in the rat feces along with SCFAs' recovery data are listed in Table 1. Each SCFA content was determined with the RSD (n = 5) of less than 3.7%. Recovery tests of each SCFA were made using each standard acid spiked in the rat feces; the results were 93 - 98% and the RSD (n = 5) was less than 3.8%, indicating that the method was given practically no influence by matrix components in the samples and was quite adequate for measuring fecal SCFAs.

Changes of NVFA to VFA ratio in rat feces during DSS administration

The NVFA (lactic acid's composition) to VFA (total of acetic, propionic, butyric, and isovaleric acids' compositions) ratio (NVFA/VFA) changes in five rats were traced before and during DSS administration by the present HPLC-ECD. Before DSS administration, the contents of lactic, acetic, propionic, butyric, and isovaleric acids in rat feces (mean ± standard error (SE) of five rats) are 11.5 ± 0.9, 23.1 ± 0.4, 3.4 ± 0.3, 1.4 ± 0.4, and 0.62 ± 0.07 µmol g^{-1}, respectively. Therefore, NVFA/VFA in rat feces before DSS administration was 0.40 ± 0.10. As shown in Fig. 3, NVFA/VFA was found to be constant, when its values before and 7 days after the administration of DSS were compared; they ranged from about 0.4 to 0.5. During DSS administration 8 days later, the contents of lactic, acetic, propionic, butyric, and isovaleric acids in rat feces (mean ± SE of five rats) are 5.1 ± 0.9, 3.9 ± 0.6, 0.42 ± 0.1, 0.35 ± 0.1, and 0.17 ± 0.06 µmol g^{-1}, respectively. Therefore NVFA/VFA in rat feces during DSS administration 8 days later was 1.06 ± 0.29. As shown in Fig. 3, NVFA/VFA was finally reached at about 1.2, 9 days later. That all these rats developed ulcerative colitis and hematochezia was also observed 8 days later.

Large bowel endoscopy is usually used to diagnose and/or inspect ulcerative colitis in human patients. Even though endoscopes can observe the diseased bowel, they are uncomfortable for patients. The NVFA/VFA measurements would offer a diagnostic clue for the ulcerative colitis as a noninvasive testing to reduce the burden for a patient. Thus, the measurements of the NVFA/VFA by the present HPLC-ECD would be applicable to diagnose ulcerative colitis.

Table 1 Contents of SCFAs in rat feces and recovery of SCFAs from rat feces spiked with those standards

SCFA	Content (n = 5)		Recovery (n = 5)		
	Amount/ µmol g^{-1}	RSD, %	Added amount[a]/ µmol g^{-1}	Recovery, %	RSD, %
Lactic	12.5	2.0	11.8	96	1.7
Acetic	20.1	1.8	18.5	98	1.2
Propionic	3.87	2.6	3.49	97	2.0
Butyric	4.16	2.3	4.20	93	1.3
Isovaleric	1.75	3.7	1.82	93	3.8

a. Five SCFAs (lactic, acetic, propionic, butyric, and isovaleric acids) at each amount were spiked to the rat feces.

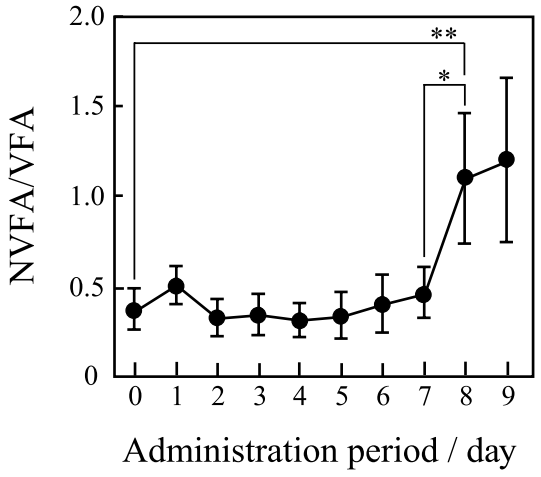

Fig. 3 Change in NVFA to VFA ratio (NVFA/VFA) in rat feces during 3% DSS administration. NVFA to VFA ratios are calculated as lactic acid's composition divided by total of acetic, propionic, butyric, and isovaleric acids' compositions. Data are plotted as mean ± SE of five rats. ∗, P <0.2; ∗∗, P <0.1 by Student's t-test.

Table 2 Contents of SCFAs in human feces and recovery of SCFAs from human feces spiked with those standards

SCFA	Content (n = 5)		Recovery (n = 5)		
	Amount/ µmol g^{-1}	RSD, %	Added amount[a]/ µmol g^{-1}	Recovery, %	RSD, %
Lactic	0.36	1.6	0.34	96	2.6
Acetic	23.3	1.9	23.9	98	2.2
Propionic	8.84	2.5	8.98	98	2.3
Butyric	2.00	2.7	1.72	92	2.6
Isovaleric	0.94	2.3	0.88	93	3.0
Valeric	1.39	2.7	1.42	98	2.6

a. Six SCFAs (lactic, acetic, propionic, butyric, isovaleric, and valeric acids) at each amount were spiked to the human feces.

Human fecal SCFA composition of day-to-day variation

The chromatogram in Fig. 2C was obtained following the injection of 20 µL sample solution from a human. The present HPLC-ECD detected lactic, acetic, propionic, butyric, isovaleric, and valeric acids in human feces. SCFA contents in the human feces along with SCFAs' recovery data are listed in Table 2. Each SCFA content was determined with the RSD (n = 5) of less than 2.7%. Recovery tests of each SCFA were made using each standard acid spiked in the human feces; the results were 92 – 98% and RSD (n = 5) was less than 3.0%, indicating that no influence on the present method was exerted by matrix components in the samples and the method was thus quite adequate for measuring fecal SCFAs.

Human feces from the subject K were collected for 7 consecutive days. Table 3 shows the day-to-day variation in the contents and compositions of SCFAs in the human feces, respectively. The compositions of fecal SCFAs in human (subject K) are different from that in SD rat because intestinal bacterial flora would be different between humans and rats. During 7 successive days, the compositions and contents of fecal SCFAs in the subject K are almost the same. Hoeverstad *et al*. reported the contents of SCFAs in the feces of 20 healthy subjects, given as µmol g^{-1} feces (wet weight), were (median and range): acetic acid, 37.4 (12.8 – 103.4); propionic acid, 12.5 (4.5 – 27.8); butyric acid, 12.4 (4.0 – 53.0); isovaleric acid, 3.2 (0.8 – 5.9) and valeric acid, 2.4 (0.6 – 3.8).[32] In addition, Garcia *et al*. reported the contents of SCFAs in the feces of 30 elderly subjects, given as µmol g^{-1} feces (wet weight), were: acetic acid, 49.5; propionic acid, 16.5; butyric acid, 19.9; isovaleric acid, 2.4 and lactic acid, 1.2.[33] The SCFA contents determined by the present HPLC-ECD are close to such reported values.

Table 3 Day-to-day variation of human fecal SCFAs contents and compositions

SCFA	Content/µmol g^{-1}	Composition, %
Lactic	0.62 ± 0.14	1.86 ± 0.52
Acetic	24.1 ± 3.12	66.2 ± 2.37
Propionic	2.92 ± 0.21	8.31 ± 0.47
Butyric	3.07 ± 0.30	8.55 ± 0.24
Isovaleric	1.90 ± 0.13	5.62 ± 0.66
Valeric	3.34 ± 0.43	9.51 ± 1.19

Human feces from the subject K were obtained during 7 successive days. Values are mean ± SE.

Conclusion

The HPLC-ECD based on the reduction of quinone was developed for determining SCFAs. The present method by the reduction of quinone have selectivity for the detection of acids, enabling simultaneous determination of lactic, acetic, propionic, butyric, isovaleric, and valeric acids within 35 min. It was found that the present method can determine the fecal SCFAs in a healthy human subject and ulcerative colitis model rats after quite simple pretreatments within 35 min. Fecal SCFAs were affected by homeostasis, characteristics, and metabolism of intestinal bacterial flora. Therefore, they are decreased by doses of antibiotics.[34-36] Moreover, fecal SCFAs contents and compositions in patients of ulcerative colitis, colonic adenoma, colon cancer, and diversion colitis are changed in comparison with those of healthy human subjects.[37,38] Thus, the present HPLC-ECD is a useful strategy for further investigation of the metabolism of SCFAs in intestinal bacterial flora of mammals and for noninvasive diagnoses for these diseases.

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