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Short communication

Determination of portal short-chain fatty acids in rats fed various dietary fibers by capillary gas chromatography

Masako Murase, Yasuhiro Kimura, Yasuo Nagata*

Saga Research Institute, Otsuka Pharmaceutical Co., Higashisefuri-son, Kanzaki-gun, Saga, 842-01, Japan

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Abstract

A simple, rapid and sensitive capillary gas chromatographic method was investigated to measure portal short-chain fatty acids (SCFAs). A 200-μl sample of portal plasma was denatured with sulfosalicylic acid and then extracted with diethyl ether before the removal of protein precipitate. The resultant extract was concentrated by a transfer to 50 μl of 0.2 *M* NaOH, thus avoiding tedious further concentration steps. This reduced the sample volume to one-fourth. Since the ratio of acetic acid, a major SCFA, to other acids varies widely, ranging from 10-fold to 100-fold, acrylic and methacrylic acids were used as internal standards to simultaneously measure SCFAs having a carbon number of 2–6. As a result, good recovery (90.38–103.17%) and reproducibility (coefficient of variation 0.83–8.85%) were observed over a wide range. Furthermore, portal SCFAs in rats fed various dietary fibers were determined by the present method. We showed that the amounts not only of the major acids such as acetic acid and propionic acid, but also of the minor fermented products such as *n*-valeric acid and *n*-caproic acid, could be significantly changed by dietary manipulation. Thus, the present method is simple and reliable, and requires only a small amount of sample.

1. Introduction

Ingested dietary fibers are degraded by the intestinal microflora in the lower bowel, producing, among others, SCFAs as metabolites. SCFAs have various functions, including proliferation of colonocytes and increase of the cecal blood flow [1–5]. The determination of SCFAs in blood has been carried out by gas chromatography (GC) and gas chromatography–mass spectrometry. These methods require ethanol extraction [6–8], deproteinization by acids [9], liquid–liquid extraction [10,11], or vacuum distillation [12,13]. Since ethanol extraction and deproteinization are time-consuming because of the need to evaporate the extractants, these procedures are not very suitable to handle a large number of samples at the same time. Deproteinization possibly causes co-precipitation of the SCFAs with protein during centrifugation, resulting in a low recovery of the SCFAs. Since the ratio of acetic acid to other SCFAs varies, ranging from 10-fold to 100-fold, it could be difficult to simultaneously determine portal SCFAs having a carbon number of 2 to 6. Thus, a simple and reliable method is needed to study the relation between the functions of the various SCFAs and the amount in which they occur.

* Corresponding author.

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Thus, based on a liquid–liquid extraction procedure, which is capable of handling a number of samples at the same time and which dose not require special instrumentation, we investigated the conditions under which SCFAs can be accurately determined over a wide range using two internal standards. Finally, portal SCFAs in rats fed various dietary fibers were determined by the present method.

2. Experimental

2.1. Reagents

Sodium acetate trihydrate and sodium *n*-butyrate were purchased from Wako Pure Chemical Industries (Osaka, Japan). Sodium propionate and *n*-caproic acid were obtained from Kishida Chemical Co. (Osaka, Japan). *n*-Valeric, acrylic and methacrylic acids were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). All reagents were of analytical-reagent grade.

2.2. Apparatus and gas chromatographic condition

The gas chromatograph GC-14A (Shimadzu Co., Kyoto, Japan) was equipped with a flame ionization detector. The integrator used was a Chromatopac C-R4A (Shimadzu). The capillary column was WCOT fused-silica, 25 m × 0.32 mm I.D. and coated with FFAP-CB as the stationary phase with a desired film thickness of 0.3 μm (Chrompack EA, Middelburg, Netherlands). The temperatures of the column and injector were 130°C and 200°C, respectively. The flow-rate of the helium carrier gas was 2.6 ml/min with a split ratio of 1:9. In order to prevent phosphoric acid and non-volatile compounds in the samples from entering the capillary column, the injection port was equipped with a glass insert packed with quartz wool. The flame ionization detector was operated at 200°C, and the flow-rates of hydrogen, air and helium as make-up gas were 40, 500 and 50 ml/min, respectively. The injected sample volume for GC analysis was 1 μl, and the run time for analysis was 9 min.

2.3. Preparation of standard solutions

To calculate the concentration of the SCFAs in the samples, an internal standard solution (I.S.) containing acrylic acid at 25 μg/ml and methacrylic acid at 5 μg/ml was prepared. The standard solution mixture of the SCFAs contained 50 m*M* of acetic acid, 10 m*M* of propionic acid, 10 m*M* of *n*-butyric acid, 2 m*M* of *n*-valeric acid and 2 m*M* of *n*-caproic acid. This solution was diluted 100-fold, 50-fold, 25-fold, 12.5-fold and 6.25-fold. These solutions were designated standard solution A, B, C, D and E, respectively. To these solutions, acrylic acid equivalent to 25 μg/ml and methacrylic acid equivalent to 5 μg/ml were added as internal standards for use in examining the precision of the method.

2.4. Sample preparation

Portal plasma (200 μl) and 50 μl of I.S. were put into a round-bottomed stoppered tube in an ice-cold bath. Then, 50 μl of ice-cold 10% sulfosalicylic acid solution was added to the tube and mixed intermittently on a vortex-mixer for 30 s. The tube was allowed to stand in the ice-cold bath for 20 min. Three ml of diethyl ether were added, mixed, and centrifuged at 1500 *g* for 10 min at 4°C. The diethyl ether layer was transferred to another conical stoppered tube containing 50 μl of 0.2 *M* NaOH. After vortex-mixing and centrifugation at 1500 *g* for 30 s, the diethyl ether layer was discarded. One ml of fresh diethyl ether was added, followed by vortex-mixing and centrifugation at 1500 *g* for 30 s, and then the diethyl ether layer was discarded. This process was repeated once again. The residual organic phase was evaporated under a stream of nitrogen. A 10-μl volume of 25% phosphoric acid was added to the aqueous phase before injection, and a 1-μl aliquot of the solution was injected onto the chromatographic system.

2.5. Quantification

Calibration curves were prepared as follows. A 50-μl volume of standard solution A, B, C, D or E and 200 μl of distilled water, instead of plasma, were mixed and then treated as described in sample preparation. The peak-area ratio to acrylic acid was used for acetic and propionic acids, and that to methacrylic acid for *n*-butylic, *n*-valeric and *n*-caproic acids. The peak-area ratio was plotted against the concentration of each SCFA and applied to a linear regression curve. Plasma SCFA concentrations were determined by fitting the peak-area ratio to a calibration curve.

2.6. Reproducibility and recovery

The precision of the present method was determined using plasma and standard SCFAs with known concentrations. To 200 μl of rat portal plasma, 50 μl of I.S., standard solution B or D was added, and then treated as described above. Thus, the added standard concentrations were 0, 250 or 1000 μ*M* for acetic acid, 0, 50 or 200 μ*M* for propionic and *n*-butyric acids, and 0, 10 or 40 μ*M* for *n*-valeric and *n*-caproic acids in portal plasma used.

2.7. Animal study

Male 6-week-old Sprague–Dawley rats were obtained from Charles River Co. (Shiga, Japan). The rats were divided into 6 groups and given one of the following experimental diets for 4 weeks: fiber-free (F), 5% cellulose (C), 5% inulin (I), 5% potato-starch hydrolysate (P), 5% potato starch (S) or 5% hemicellulose-rich wheat bran (W). Cellulose was purchased from Oriental Yeast Co. (Tokyo, Japan). Inulin was purchased from Wako Pure Chemical Industries Co. Potato-starch hydrolysate and potato starch were provided by Matsutani Chemical Industries Co. (Itami, Japan). Hemicellulose-rich wheat bran was obtained from Chiba Flour Milling Co. (Chiba, Japan). The compositions of the experimental diets are shown in Table 1. The rats were individually housed in stainless steel cages in a room with controlled temperature (24 ± 0.5°C) and relative humidity (50 ± 5%); the light/dark cycle was 12 h, with light from 19:00 to 07:00. Food and water were given ad libitum.

Table 1
Composition of experimental diets

Compound	Composition (g/100g)	
	Fiber free	Fiber added
Casein[a]	22.8	21.7
Sucrose	67.1	63.7
Lard[a]	5.1	4.8
DL-Methionine[b]	0.3	0.3
AIN-76 vitamine mixture[c]	1.1	1.0
AIN-76 salt mixture[c]	3.7	3.5
Fiber[d]	–	5.0

[a] Purchased from Oriental Yeast Co.
[b] Purchased from Wako Pure Chemical Industries.
[c] American Institute of Nutrition, *J. Nutr.*, 107 (1977) 1340.
[d] Cellulose, inulin, potato-starch hydrolysate, potato starch or hemicellulose-rich wheat bran.

After 4 weeks, all animals were sacrificed at 13:00. While unfasted rats inhaled carbon dioxide, a 1-ml sample of portal blood was taken with a heparinized syringe. Blood was immediately centrifuged at 1500 *g* for 10 min at 4°C. Plasma samples were stored at −80°C until analysis.

2.8. Statistical analysis

Data were analyzed by the Tukey–Kramer test. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Chromatography

A typical chromatogram showing the separation of 5 SCFAs and the two internal standards, acrylic acid and methacrylic acid, is shown in Fig. 1. The FFAP-CB column satisfactorily separated the SCFAs and the two internal standards within 9 min. Chromatograms of plasma treated as described in sample preparation, with

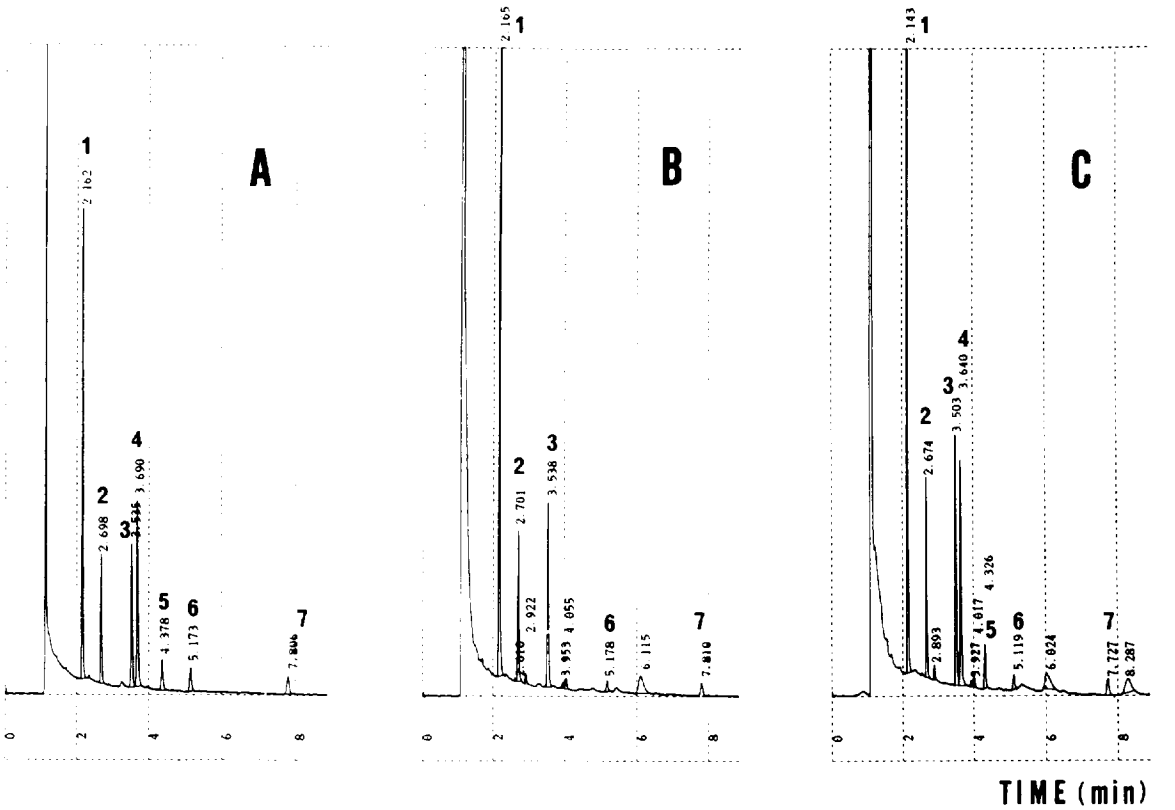

Fig. 1. Chromatograms of short-chain fatty acids. (A) Standard solution of SCFAs with internal standards. Solution B as described in Section 2.3. was used, (B) rat portal plasma without internal standards, (C) rat portal plasma with internal standards. Peaks: 1 = acetic acid; 2 = propionic acid; 3 = *n*-butyric acid; 4 = acrylic acid; 5 = metacrylic acid; 6 = *n*-valeric acid; 7 = *n*-caproic acid.

or without an internal standard, are also shown in Fig. 1. No impurities or interfering substances were found in the chromatograms.

3.2. Precision and accuracy

The reproducibility of the retention times and the peak-area ratios was investigated. A 50-μl volume of standard solution B and 10 μl of 25% phosphoric acid were mixed and injected 5 times. The retention times were remarkably stable. The coefficient of variation (C.V., %) of the peak-area ratio of each SCFA to the internal standard ranged from 1.16 to 6.30 (Table 2). The standard curves showed a good linear correlation between the peak-area ratio and the concentration. The correlation coefficient (r^2) was between 0.9967 and 0.9998 at concentrations of 125 to 2000 μM for acetic acid, 25 to 400 μM for propionic and *n*-butyric acids and 5 to 80 μM for *n*-valeric and *n*-caproic acids (Table 2). The reproducibility and recovery with standard solutions added to plasma are shown in Table 3. The resulting C.V.s for the various concentrations ranged from 0.83 to 8.85%, and the recovery varied between 90.38 and 103.17%.

3.3. Portal plasma concentration

We determined the SCFA concentrations in the portal plasma from rats fed various dietary fibers (Table 4). In group C, the amount of each SCFA was as low as that in group F. When dietary fibers were added to the diets, acetic acid and propionic acid in group I significantly increased, 2.2-fold and 7.8-fold, respectively, as

Table 2
Reproducibility of retention time and peak-area ratio and linearity in standard solution

Compound	Retention time[a] (min)	Peak-area ratio[a]	Linearity range (μM)	Correlation coefficient
Acetic acid	2.164 (0.21)	1.730 (3.32)	125–2000	0.9967
Propionic acid	2.700 (0.19)	0.568 (1.16)	25–400	0.9994
n-Butyric acid	3.537 (0.15)	3.833 (2.46)	25–400	0.9998
n-Valeric acid	5.174 (0.09)	0.833 (2.76)	5–80	0.9994
n-Caproic acid	0.883 (0.05)	0.918 (6.30)	5–80	0.9996

[a] Each value represents the mean value, and the value in parentheses is the C.V. of 5 determinations (%).

Table 3
Percentage recovery of short-chain fatty acids after diethyl ether extraction of plasma

Matrix	Concentration (μM)				
	Acetic acid	Propionic acid	*n*-Butyric acid	*n*-Valeric acid	*n*-Caproic acid
Plasma	539 (4.89)	63 (2.82)	63 (1.94)	5 (1.36)	7 (3.38)
Added	250	50	50	10	10
Plasma + B[a]	788 (2.93)	114 (4.79)	109 (0.83)	15 (4.91)	18 (8.85)
Recovery (%)	99.95	102.04	92.12	94.47	103.17
Added	1000	200	200	40	40
Plasma + D[a]	1442 (4.04)	258 (2.98)	250 (2.54)	43 (2.53)	48 (5.23)
Recovery (%)	90.38	97.41	93.36	93.14	100.68

Each value is the mean, and the value in parentheses is the C.V. of 5 determinations (%).
[a] Standard solution B or D.

Table 4
Concentration of portal SCFAs in rats fed various dietary fibers

Diet[a]	*n*[b]	Concentration (μM)				
		Acetic acid	Propionic acid	*n*-Butyric acid	*n*-Valeric acid	*n*-Caproic acid
F	5	370 ± 106^{c}	36 ± 13^{c}	14 ± 3	6 ± 2^{cd}	2 ± 1^{e}
C	6	353 ± 155^{c}	32 ± 14^{c}	16 ± 7	5 ± 2^{cd}	2 ± 1^{e}
I	6	818 ± 443^{a}	282 ± 289^{a}	34 ± 29	2 ± 4^{e}	2 ± 1^{e}
P	6	726 ± 197^{cd}	171 ± 67^{cd}	59 ± 31	3 ± 3^{e}	2 ± 1^{e}
S	6	523 ± 121^{cd}	67 ± 29^{cd}	66 ± 49	11 ± 3^{d}	19 ± 13^{d}
W	6	591 ± 132^{cd}	90 ± 58^{cd}	74 ± 48	8 ± 4^{cd}	6 ± 10^{e}

Each value is the mean ± S.D.
[a] Abbreviations used: F = fiber free; C = 5% cellulose; I = 5% inulin; P = 5% potato-starch hydrolysate; S = 5% potato starch; W = 5% hemicellulose-rich wheat bran.
[b] Number of animals used.
[c,d] Values in a row not sharing a common suffix letter are significantly different ($p < 0.05$) as determined by Tukey–Kramer test.

compared to group F. Similarly, groups P, S and W tended to show an increase in these acids, but they did not show values significantly different from those in group F. Diets containing dietary fibers other than cellulose had a tendency to show enhanced *n*-butyric acid. The levels of *n*-valeric and *n*-caproic acids were increased by feeding potato starch (group S). The *n*-caproic acid level in group S was higher than that in any other group.

Thus, the concentrations of portal *n*-valeric and *n*-caproic acids, as well as those of the major acids such as acetic and propionic acids, were influenced by dietary composition.

4. Discussion

Organic acids in blood non-specifically bind to albumin. Thus, in order to determine organic acids in blood, deproteinization with acids or organic solvents is necessary [14]. To analyze SCFAs and acetic acid, Brazier et al. [10] and Rocchiccioli et al. [11] deproteinized plasma, transferred the supernatant to tubes, and extracted the samples with organic solvent. At first, we deproteinized plasma with sulfosalicylic acid and tried to extract SCFAs from the supernatant using diethyl ether. However, we found that the recovery of *n*-valeric and *n*-caproic acids was poor, indicating the co-precipitation of SCFAs with protein (data not shown). Thus, we denatured plasma protein with sulfosalicylic acid and extracted the SCFAs with diethyl ether prior to the removal of protein precipitate, resulting in an increased recovery and good reproducibility (Table 3). Although the concentration ratio of a major acid such as acetic acid to the other acids, including *n*-valeric and *n*-caproic acids, varies in plasma samples, the use of two kinds of internal standards allowed us to simultaneously quantify SCFAs having different levels over a wide range. As shown in Table 2, the calibration curve of each SCFA was linear for the concentration found in vivo. Since this method requires neither special apparatus nor a tedious evaporation process, it is capable of handling more than 50 samples at a time. Due to the use of isothermal analysis, samples can be continuously injected. However, it should be noted that, since repeated injection of samples causes fixation of phosphoric acid on the inner wall of an insert, the glass inserts are replaced every fifty samples. Furthermore, after analysis, non-volatile compounds adsorbed onto column should be eluted by increasing the column temperature to 220°C for 1 h. Overall, the present method is simple, rapid and reliable.

SCFAs fermented from dietary fibers have divergent functions [1–5]. The cecum is a major site where fermentation takes place in rats. Quantitative analysis of SCFAs in the cecum from rats fed various dietary fibers has been previously reported [15]. However, there is little information regarding to what degree SCFAs fermented in the cecum are transported to the circulation. Using this method, we showed in the present study that the levels of SCFAs in the portal blood of rats are influenced by the type of dietary fibers ingested. Since this method enables us to measure SCFAs in a small volume of plasma, it can be useful in studying the metabolism of SCFAs in small experimental animals.

This method should provide more precise knowledge on the relation between the amounts of the SCFAs and their functions.

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