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Application News

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Liquid Chromatograph Mass Spectrometry

Analysis of Short-Chain Fatty Acids/Organic Acids (3-NPH Derivatives) in Fecal Specimens from SPF and Antibiotic-Fed Mice

More than several hundred species of intestinal microbiota exist in the human intestines. Because they grow in colonies and have an appearance exactly like a field of flowers when observed under a microscope, they are also called intestinal flora. Intestinal microbiota produces so-called short-chain fatty acids, namely, acetic acid, propionic acid and butyric acid, by using indigestible saccharides as a main energy source. In recent years, reports have linked these short-chain fatty acids to autoimmune disease[(1)] by absorption in the body, and to lifestyle-related diseases, such as obesity and diabetes. In order to understand the connection between the short-chain fatty acids and disease and gain a deeper understanding of the metabolic activity of both the intestinal microbiota and the host body, there is increasing demand for quantitative analysis of the short-chain fatty acids.

Since short-chain fatty acids generally display high volatility and high hydrophilicity, LC/MS analysis by a conventional reverse-phased system is difficult. Moreover, although the derivatization method is widely used in the general GC/MS method, loss of volatile components is possible, as exsiccation of the specimen is necessary. For this reason, analysis is performed by derivatization in an aqueous solution.[(1)] In this article, analysis was conducted by derivatizing carboxylic acid with 3- nitrophenylhydrazine (3-NPH), and matching the C2 to C5 short-chain fatty acids (6 components) and organic acids such as lactic acid, pyruvic acid, succinic acid (16 components). The analysis was performed by using the registered MRM transition and analysis method (Table 1) in the LC/MS/MS method package "Short-chain fatty acids."

Although the derivatization method using 2-nitrophenylhydrazine (2-NPH) has long been known, it was applied mainly to the analysis of fatty acids. In this study, derivatization of not only short-chain fatty acids but also organic acids was performed with high efficiency by using 3-NPH rather than 2-NPH, supporting an analysis by matching short-chain fatty acids and organic acids.

The registered MRM transitions were optimized based on the 3-NPH derivative. Because 3-NPH also reacts with ketone bodies, MRM transitions derivatizing not only carboxylic acid but also the carbonyl group were set for pyruvic acid, oxaloacetic acid and other acids having a carbonyl group.

Here, analysis of the short-chain fatty acids and organic acids was performed using fecal specimens from SPF (Specific Pathogen Free) mice, in which physiological intestinal microbiota existed, and mice in which the level of intestinal microbiota was remarkably reduced by administration of an antibiotic. After collecting and weighing fecal specimens from the respective mice, the specimens were suspended in ethanol and the supernatant was recovered centrifugally separation. This supernatant was subjected to derivatization by 3-NPH. As shown in Fig. 1, the derivative was then reacted at room temperature for 30 min using pyridine as a catalyst and carbodiimide as a condensing agent. After the reaction, the product was diluted with a methanol solution containing formic acid, and simultaneous analysis was performed with a LCMS-8060.

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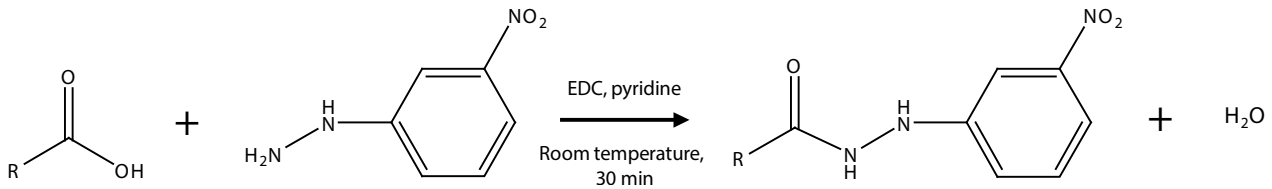

Fig. 1 Derivatization Reaction by 3-Nitrophenylhydrazine (3-NPH)

Table 1 HPLC Conditions and MS Conditions

HPLC conditions		**MS conditions (LCMS-8060)**	
Analysis column	: Mastro™ C18 (2.1 mmI.D. × 150 mmL., 3 µm, Shimadzu GLC)	Ionization method	: ESI (Positive/Negative)
Mobile phase A	: 0.1 % Formic acid-water	Nebulizer gas flow rate	: 2.0 L/min
Mobile phase B	: Acetonitrile	Drying gas flow rate	: 10.0 L/min
Time program	: Gradient	Heating gas	: 10.0 L/min
Flow rate	: 0.35 mL/min	DL temperature	: 250 °C
Injection volume	: 3 µL	Heat block temperature	: 400 °C
Column oven temperature	: 40 °C	Interface temperature	: 300 °C
		CID gas pressure	: 270 kPa

Fig. 2 shows the MRM chromatograms of the short-chain fatty acids and organic acids (3-NPH) extracted from the fecal specimens from the SPF mice and antibiotic-fed mice. Distinctive changes in the short-chain fatty acids and succinic acid can be seen in the respective fecal specimens. This suggests that the decrease in intestinal microbiota due to administration of the antibiotic is linked to a decrease in short-chain fatty acids in the fecal matter.

Next, derivatization of mixed solutions of standard substances was performed, calibration curves were prepared, and the concentrations of short-chain fatty acids and organic acids in the respective fecal specimen extracts were determined. The amount of short-chain fatty acids/organic acids (nmol/mg) per unit weight of fecal matter based on those values was plotted on a graph, as shown in Fig. 3. (These analyses were repeated n = 3 times for each specimen.) Here, the quantitative results of the analysis of 6 short-chain fatty acid components and 4 representative organic acid components among the 22 measured components are shown. From the results of the short-chain fatty acids shown by the red graphs, a large decrease in the amount of short-chain fatty acids (nmol/mg) can be observed in the antibiotic-fed mice in comparison with the SPF mice.

On the other hand, in the straight short-chain fatty acids (acetic acid, propionic acid, butyric acid, valeric acid) and the branched short-chain fatty acids (isobutyric acid, isovaleric acid) of the SPF mice, differences in the production ratios in individuals can be confirmed. This is thought to be due to differences in the intestinal flora in each individual.

In the organic acids shown by the blue graphs, a remarkably high value for succinic acid was observed in the mice given the antibiotic. Since the short-chain fatty acids and organic acids are deeply related to the activity of intestinal microbiota, extremely distinctive changes in the short-chain fatty acids and organic acids can be seen under conditions where intestinal microbiota are reduced or do not exist, for example, by administration of antibiotics or aseptic breeding.

As described above, quantitative analysis of short-chain fatty acids and organic acids can be performed by general reversed-phase LC/MS analysis by utilizing the LC/MS/MS method package "Short-chain fatty acids" for analysis of 3-NPH derivatives.

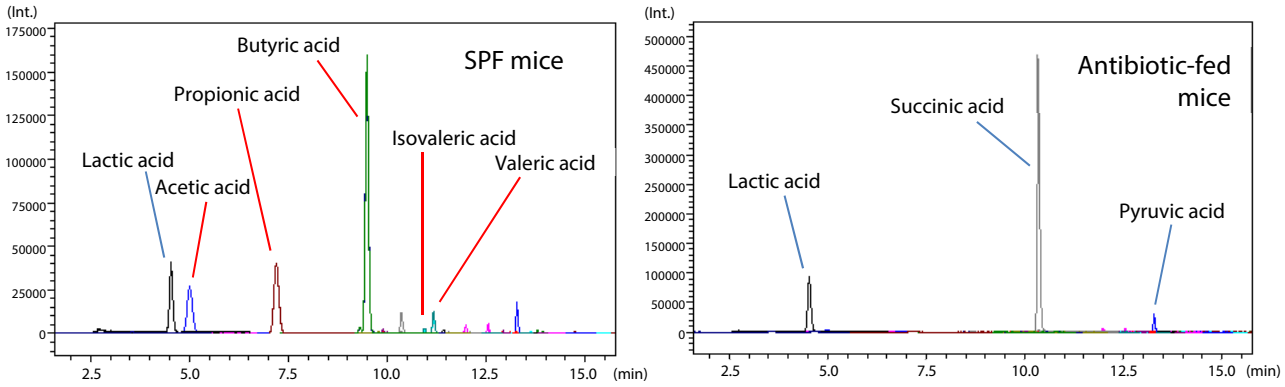

Fig. 2 MRM Chromatograms of Short-Chain Fatty Acids and Organic Acids (3-NPH) in Fecal Specimens from SPF Mice and Antibiotic-Fed Mice

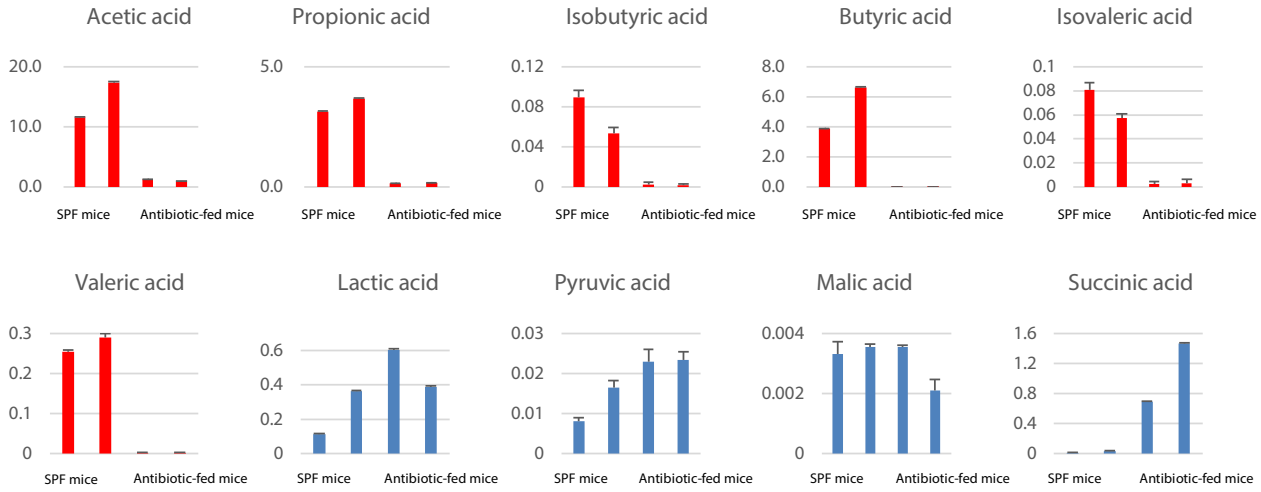

Fig. 3 Comparison of Amount of Short-Chain Fatty Acids (6 Components) and Organic Acids (4 Components) Per Unit Weight of Fecal Matter in Fecal Specimens from SPF Mice and Antibiotic-Fed Mice (Vertical Axis : nmol/mg)

* The analysis specimens introduced in this article were provided by Dr. Yuki Sugiura and Prof. Makoto Suematsu of Department of Biochemistry & integrative Medical Biology, the Keio University School of Medicine.

Reference

(1) ***Gut Microbes.*** 5(3):333-9. *2014. Characterization of the 17 strains of regulatory T cell-inducing human-derived Clostridia.* Narushima S, Sugiura Y, Oshima K, Atarashi K, Hattori M, Suematsu M, Honda K.

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