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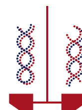

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Evaluation of a novel system for analyzing hydrophilic blood metabolites

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Metabolomics has recently been developed, and there have been a considerable number of metabolomics-based biomarker studies in the medical research field. Therefore, as a first step toward the practical use of metabolite biomarkers, a simple and quick sample preparation method involving metabolite extraction, metabolite measurement, and data analysis needs to be developed. In this study, we evaluated whether the use of simpler metabolite extraction methods would facilitate the stable analysis of hydrophilic blood metabolites during liquid chromatography/triple quadrupole mass spectrometry (LC/QqQMS)-based metabolome analysis. As a result, the anion and cation metabolites in plasma were stably analyzed via a methanol-based extraction procedure followed by ultrafiltration, and it was also confirmed that a lyophilization step was not necessary. When extraction was performed without a lyophilization step, approximately >50% and >80% of the detected metabolites had relative standard deviation values of <20% during LC/QqQMS-based anion and cation analyses, respectively. In addition, the plasma levels of L-valine, L-leucine, L-isoleucine, L-tyrosine, and L-phenylalanine were quantitatively measured using the corresponding stable isotopes; the SCLAM-2000, a fully automatic pre-treatment system for LC/MS that can be connected online to an LC/MS device; and an extraction procedure based on the simple procedure that we developed. Our findings suggest that simpler pretreatment procedures can be employed during LC/QqQMS-based metabolomics and might aid searches for metabolite biomarker candidates, the validation of metabolite biomarker candidates, and the practical use of metabolite biomarkers.

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[**Key words:** Metabolomics; Metabolite; Liquid chromatography/triple quadrupole mass spectrometry; Branched-chain amino acid; Aromatic amino acid; Fischer ratio]

Recently, metabolomics, which involves the comprehensive study of low molecular weight metabolites, has been developed and applied to a variety of research fields, such as the food science, agriculture, engineering, and medical fields. For example, in the food science field, a study examined whether metabolomics can be used to effectively assess the quality of civet coffee (1). In the case of agriculture research, metabolite fingerprinting has been employed to evaluate the quality of dried Angelica acutiloba roots (2). Regarding engineering research, the potential of metabolomics to find target genes for increasing the ethanol tolerance of *Saccharomyces cerevisiae* was investigated (3). In the medical research field, metabolomics is used to search for novel metabolite biomarkers of a variety of diseases and elucidate pathogenic mechanisms, etc., and there have been a considerable number of metabolite biomarker studies (4,5). In particular, researchers have been searching for novel metabolomic biomarkers that would aid early disease detection and the prediction of therapeutic efficacy, recurrence, and prognosis (6,7). The number of samples that are measured in such studies has recently increased. In metabolomics-based biomarker research, serum or plasma samples are often examined because it is relatively easy to collect blood from patients with a variety of diseases. Since large-scale metabolite profiling is required to search for metabolite biomarker candidates, recommended standard procedures for the large-scale metabolite profiling of serum/plasma have been reported (8,9). Moreover, as a step toward the practical use of metabolite biomarkers, a simple and quick sample preparation method involving metabolite extraction, metabolite measurement, and data analysis should be developed. In a study by Tulipani et al. (10), some blood sample preparation methods for metabolome analysis were compared, and the method speed, sample handling, compatibility to the automatic operation and applicability to the large-scale metabolomic analysis were considered in this comparison, although only partial comparisons were performed. Therefore, in this study, we evaluated whether simpler metabolite extraction methods would facilitate the stable analysis of hydrophilic blood metabolites; i.e., anionic and cationic metabolites, during liquid chromatography/triple quadrupole mass spectrometry (LC/QqQMS)-based metabolome analysis. Next, we assessed whether the plasma levels of L-valine, L-leucine, L-isoleucine, L-tyrosine, and L-phenylalanine could be quantitatively measured using the corresponding stable isotopes and the SCLAM-2000 (Shimadzu Co., Kyoto, Japan), which is a fully automatic pre-treatment system for LC/MS that can be connected online to an LC/MS device.

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MATERIALS AND METHODS

Reagents and solvents Ultrapure water (H_2O), acetonitrile (LC/MS grade), formic acid (LC/MS grade), acetic acid (LC/MS grade), and chloroform ($CHCl_3$; HPLC grade) were purchased from Wako Pure Chemical Industries (Osaka, Japan), and methanol (MeOH; LC/MS grade) was obtained from Kanto Chemical (Tokyo, Japan). Tributylamine obtained from Sigma Aldrich (MO, USA) was used as the ion-pair reagent. L-methionine sulfone, 2-bromohypoxanthine, and 10-camphorsulfonic acid, which were employed as internal standards, were purchased from Sigma Aldrich. L-valine, L-leucine, L-isoleucine, L-tyrosine, and L-phenylalanine were acquired from Sigma Aldrich. Isotopically labeled L-valine (D_8), L-leucine ($^{13}C_6$), L-isoleucine (D_{10}), L-tyrosine ($^{13}C_9$, ^{15}N), and L-phenylalanine (D_8) were purchased from Cambridge Isotope Laboratories (MA, USA). Commercially available pooled plasma (Kohjin-Bio Co., Saitama, Japan), which was collected using EDTA-Na as an anticoagulant, was utilized as human plasma, and pooled plasma with the same lot number was used for all experiments.

Method A For the LC/QqQMS-based hydrophilic (anion and cation) metabolite analysis, 10, 25, 50, and 100 μL of plasma (each N = 5) were mixed with 900 μL of a solvent mixture ($MeOH:H_2O:CHCl_3$ = 2.5:1:1) containing 1 μM 10-camphorsulfonic acid (the internal standard for the anion analysis), and 1 μM 2-bromohypoxanthine and 10 μM L-methionine sulfone (the internal standards for the cation analysis), and then the mixture was shaken at 1200 rpm for 30 min at 37°C, before being centrifuged at 16,000 ×*g* for 3 min at 4°C. Next, 630 μL of the resultant supernatant were transferred to a clean tube, and 280 μL of H_2O were added to the tube. After being mixed, the solution was centrifuged at 16,000 ×*g* for 5 min at 4°C, and 500 μL of the resultant supernatant were passed through an ultrafiltration filter (Amicon Ultra 0.5-mL centrifugal filters, Ultracel-3K; Merck Millipore, Germany) and then centrifuged at 14,000 ×*g* for 60 min at 4°C. The collected solution was dried by centrifugal concentration and lyophilization, and then reconstituted with 100 μL of H_2O. In the LC/QqQMS-based anion analysis, the resultant solution was used directly. In the LC/QqQMS-based cation analysis, a mixture of 20 μL of the resultant solution and 30 μL of H_2O was used.

The LC/MS analysis was carried out using a Nexera LC system (Shimadzu Co.) equipped with two LC-30AD pumps, a DGU-20As degasser, an SIL-30AC autosampler, a CTO-20AC column oven, and a CBM-20A control module, coupled with an LCMS-8040 triple quadrupole mass spectrometer (Shimadzu Co.) according to the method described in a previous report (11,12). The anion metabolites were separated using an octadecylsilylated silica column (InertSustain C18, 150 mm × 2.1 mm, 3 μm; GL Sciences, Tokyo, Japan), while the cation metabolites were separated using a pentafluorophenyl column (Discovery HS F5, 150 mm × 2.1 mm, 3 μm; Supelco, PA, USA) with a guard column (20 mm × 2.1 mm, 3 μm). The mobile phase for the anion metabolites was composed of A: H_2O containing 15 mM acetic acid and 10 mM tributylamine, and B: MeOH. The flow rate was 0.3 mL/min, and the column oven temperature was 35°C. The gradient program for mobile phase B was as follows: 0 min, 0%; 0.5 min, 0%; 20 min, 75%; 20.1 min, 98%; 24 min, 98%; 24.1 min, 0%; and 30 min, 0%. The mobile phase for the cation metabolites was composed of A: 0.1% formic acid in H_2O and B: acetonitrile. The flow rate was 0.3 mL/min, and the column oven temperature was 40°C. The gradient program for mobile phase B was as follows: 0 min, 0%; 7 min, 0%; 20 min, 40%; 20.1 min, 100%; 25 min, 100%; 25.1 min, 0%; and 35 min, 0%. The peak selection and integration were performed automatically using the software LabSolutions (Shimadzu Co.). All of the metabolites detected in this study were identified based on their precursor ions, specific product ions (one ion for each metabolite), and retention times acquired by analyzing authentic chemical standards using the same methods, and all data were also checked manually. The peak area value of each metabolite was normalized to that of the relevant internal standard.

Method B For the LC/QqQMS-based hydrophilic (anion and cation) metabolite analysis, 10 μL of plasma (N = 5) were mixed with 250 μL of MeOH containing 1 μM 10-camphorsulfonic acid (the internal standard for the anion analysis), and 1 μM 2-bromohypoxanthine and 10 μM L-methionine sulfone (the internal standards for the cation analysis), and then the mixture was shaken at 1200 rpm for 30 min at 37°C, before being centrifuged at 16,000 ×*g* for 3 min at 4°C. Next, the resultant supernatant was passed through an ultrafiltration filter and was then centrifuged at 14,000 ×*g* for 60 min at 4°C. The collected solution was dried via centrifugal concentration and lyophilization, before being reconstituted with 100 μL of H_2O. For the LC/QqQMS-based anion analysis, the resultant solution was used directly. For the LC/QqQMS-based cation analysis, a mixture of 20 μL of the resultant solution and 30 μL of H_2O was used. Both the LC/QqQMS-based anion and cation analyses were performed using the same methods as described in the Method A section.

Method C For the LC/QqQMS-based hydrophilic (anion and cation) metabolite analysis, 10 μL of plasma (N = 5) were mixed with 250 μL of MeOH containing 1 μM 10-camphorsulfonic acid (the internal standard for the anion analysis), and 1 μM 2-bromohypoxanthine and 10 μM L-methionine sulfone (the internal standards for the cation analysis), and then the mixture was shaken at 1200 rpm for 30 min at 37°C, before being passed through an ultrafiltration filter (Amicon Ultra 0.5-mL centrifugal filters, Ultracel-3K). The mixture was then centrifuged at 14,000 ×*g* for 60 min at 4°C, and the collected solution was subjected to LC/QqQMS-based anion and cation analyses. The LC/QqQMS-based anion and cation analyses were performed using the same methods as described in the Method A section.

TABLE 1. The MRM transitions of native and stable isotope molecules of L-valine, L-leucine, L-isoleucine, L-tyrosine, and L-phenylalanine.

Chemical name	Precursor ion (*m/z*)	Product ion (*m/z*)
L-Valine	118.1	72.15
L-Tyrosine	182.1	136.1
L-Isoleucine	132.1	86.2
L-Leucine	132.1	86.05
L-Phenylalanine	166.1	120.1
L-Valine (D_8)	126.2	80.15
L-Tyrosine ($^{13}C_9$, ^{15}N)	192.2	145.2
L-Isoleucine (D_{10})	142.25	96.15
L-Leucine ($^{13}C_6$)	138.15	91.15
L-Phenylalanine (D_8)	174.2	128.2

Manual and automatic methods The manual method was a modified version of method C. During the LC/QqQMS-based analysis of L-valine, L-leucine, L-isoleucine, L-tyrosine, and L-phenylalanine levels, 20 μL of plasma (N = 5) were mixed with 230 μL of MeOH containing 10 μM isotopically labeled L-valine, L-leucine, L-isoleucine, L-tyrosine, and L-phenylalanine as internal standards. Next, the mixture was shaken at 1200 rpm for 30 min at room temperature, before being passed through an ultrafiltration filter (Amicon Ultra 0.5-mL centrifugal filters, Ultracel-3K). The mixture was then centrifuged at 14,000 ×*g* for 60 min at 4°C, and the collected solution was subjected to the LC/QqQMS-based analysis of L-valine, L-leucine, L-isoleucine, L-tyrosine, and L-phenylalanine.

The automatic method used to analyze L-valine, L-leucine, L-isoleucine, L-tyrosine, and L-phenylalanine levels was performed using an SCLAM-2000 (Shimadzu Co.). In the SCLAM-2000, MeOH containing 10 μM isotopically labeled L-valine, L-leucine, L-isoleucine, L-tyrosine and L-phenylalanine (as internal standards) was added into the solvent container, and 20 μL of plasma (N = 5) were also applied into another tubes. By running the SCLAM-2000, 20 μL of plasma were automatically mixed with 230 μL of MeOH and the internal standards, before the resultant mixture was shaken at 1900 rpm for 30 min at room temperature. Then, the mixture was automatically subjected to suction filtration for 90 s, and the filtered solution was transferred to an SIL-30AC autosampler online, before being subjected to LC/QqQMS analysis.

The LC/MS analyses performed via the manual and automatic methods were carried out using a Nexera LC system equipped with two LC-30AD pumps, a DGU-20As degasser, an SIL-30AC autosampler, a CTO-20AC column oven, and a CBM-20A control module, coupled with an LCMS-8060 triple quadrupole mass spectrometer (Shimadzu Co.). The metabolites were separated using a pentafluorophenyl column (Discovery HS F5, 150 mm × 2.1 mm, 3 μm) and a guard column (20 mm × 2.1 mm, 3 μm). The mobile phase for the metabolites was composed of A: 0.1% formic acid in H_2O and B: acetonitrile. The flow rate was 0.25 mL/min, and the column oven temperature was 40°C. The gradient program for mobile phase B was as follows: 0 min, 0%; 2 min, 0%; 5 min, 25%; 11 min, 35%; 15 min, 95%; 20 min, 95%; 20.1 min, 0%; and 25 min, 0%. To identify L-valine, L-leucine, L-isoleucine, L-tyrosine, and L-phenylalanine, the *m/z* value and retention time of each peak were compared with those of authentic chemical standards that had been analyzed using the same analytical methods, and all data were also checked manually. The plasma concentrations of L-valine, L-leucine, L-isoleucine, L-tyrosine, and L-phenylalanine were calculated by using a standard curve derived from the peak area ratio (y-axis: native/isotope) and the concentration ratio (x-axis: native/isotope). The multiple reaction monitoring (MRM) transitions of the native and stable isotopes of L-valine, L-leucine, L-isoleucine, L-tyrosine, and L-phenylalanine are shown in Table 1.

An amino acid analysis of plasma samples with the same lot number was also performed by SRL (Tokyo Japan), and the plasma concentrations of L-valine, L-leucine, L-isoleucine, L-tyrosine, and L-phenylalanine (μM) were measured. The Fischer ratio was calculated based on the quantitative results.

RESULTS AND DISCUSSION

Serum/plasma metabolome analysis has been widely used to search for novel metabolite biomarker candidates for a variety of diseases. To work toward the practical use of metabolite biomarkers, it is necessary not only to strictly validate metabolite biomarker candidates, but also to establish procedures and systems that would allow easier (more practical) metabolite extraction. Simpler metabolite extraction procedures are important for validation studies involving large-scale metabolite analysis. Therefore, the aim of this study is to assess the utility of novel systems for analyzing hydrophilic blood metabolite levels involving simple metabolite extraction procedures.

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