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Differences in metabolite profiles caused by pre-analytical blood processing procedures

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Recently, the use of metabolomic analysis of human serum and plasma for biomarker discovery and disease diagnosis in clinical studies has been increasing. The feasibility of using a metabolite biomarker for disease diagnosis is strongly dependent on the metabolite's stability during pre-analytical blood processing procedures, such as serum or plasma sampling and sample storage prior to centrifugation. However, the influence of blood processing procedures on the stability of metabolites has not been fully characterized. In the present study, we compared the levels of metabolites in matched human serum and plasma samples using gas chromatography coupled with mass spectrometry and liquid chromatography coupled with mass spectrometry. In addition, we evaluated the changes in plasma metabolite levels induced by storage at room temperature or at a cold temperature prior to centrifugation. As a result, it was found that 76 metabolites exhibited significant differences between their serum and plasma levels. Furthermore, the pre-centrifugation storage conditions significantly affected the plasma levels of 45 metabolites. These results highlight the importance of blood processing procedures during metabolome analysis, which should be considered during biomarker discovery and the subsequent use of biomarkers for disease diagnosis.

[**Key words:** Metabolomics; Mass spectrometry; Serum; Plasma; Blood processing]

Clinical biomarkers are required for early disease diagnosis and assessing therapeutic responses, especially in the oncological field (1). In clinical studies, human serum or plasma are easy to obtain and are often used for biomarker discovery and disease diagnosis. Metabolomics is a large-scale approach to the acquisition of comprehensive biological information about low-molecular-weight metabolites (<1000 Da), which can provide snapshots of patients' physiological and pathological states (2). Thus, metabolomic analysis of human serum or plasma is being increasingly applied to clinical studies (3,4).

Many analytical methods based on gas chromatography coupled with mass spectrometry (GC/MS) or liquid chromatography coupled with mass spectrometry (LC/MS) have been extensively used for metabolomic analysis (5,6). These analytical techniques focus on a class of compounds, such as carbohydrates, amino acids, organic acids, lipids, or the metabolites associated with a specific pathway. Furthermore, simple metabolite extraction methods can be applied to the stable analysis of blood metabolites (7,8). In metabolomic analysis of serum or plasma, some metabolites might exhibit aberrant changes caused by their degradation, oxidation, or metabolic reactions induced by pre-analytical blood processing. In serum, varying amounts of blood cell-derived metabolites, such as sphingosine 1-phosphate, are released during the coagulation cascade (9). In addition, the hydrolysis of phospholipids, triacylglycerols, and diacylglycerols by lipases, and the hydrolysis of cholesterol esters by esterases can also occur in serum (10). Therefore, pre-analytical techniques, including serum/plasma sampling methods and storage conditions, can have important effects on data quality.

Carefully following the standard operating procedures (SOP) can reduce the extent to which bias affects the quality of metabolomic data (11). As long as the same blood preparation procedures are used, serum and plasma should generate similar results in clinical and biological studies (12,13). However, the metabolites whose levels were measured in previous studies only represent a small part of the blood metabolome. Accordingly, it is necessary to determine whether serum and plasma analyses produce similar results for a wide variety of metabolites. The storage temperature is another important factor affecting sample stability. In metabolomic analysis, storing blood tubes at 4°C has been shown to be a reliable method for reducing the variability associated with the period prior to sample pre-processing (14). However, many of the currently used SOP have limitations in terms of the intermediate storage period between sample collection and centrifugation. In the clinical setting, the time between the collection of plasma samples and their storage at cold temperatures varies (even though plasma samples should be stored immediately after blood collection).

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Furthermore, the time for which blood tubes are stored in cold transportation containers prior to centrifugation differs among clinical institutions. The influence of the intermediate storage period on the stability of metabolites should be considered during biomarker discovery and the application of biomarkers to disease diagnosis.

In the present study, we compared the levels of metabolites in matched human serum and plasma samples by using GC/MS to analyze hydrophilic metabolites and LC/MS to analyze lipids, cationic metabolites, and anionic metabolites. In addition, we evaluated the effects of the time for which plasma samples were stored at room temperature or cold temperature on plasma metabolite levels.

MATERIALS AND METHODS

Chemicals Pyridine, ultrapure water, acetonitrile (LC/MS grade), chloroform, ammonium acetate (1 M solution, high-performance liquid chromatography grade), formic acid (LC/MS grade), and acetic acid (LC/MS grade) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and methanol (LC/MS grade) was obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). Methoxyamine hydrochloride and tributylamine were purchased from Sigma–Aldrich (MO, USA). *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) was acquired from GL Sciences, Inc. (Tokyo, Japan). 2-Isopropylmalic acid, which was purchased from Sigma–Aldrich, was used as an internal standard for the GC/MS analysis of hydrophilic metabolites. Dilauroylphosphatidylcholine (PC 12:0–12:0), which was purchased from Avanti Polar Lipids (AL, USA), was used as an internal standard for the lipid analysis, whereas 2-bromohypoxanthine and 10-camphorsulfonate, which were purchased from Sigma–Aldrich, were used as internal standards for the analysis of cationic and anionic metabolites, respectively.

Samples The human samples were collected in accordance with the guidelines of Kobe University Hospital. The serum and plasma samples were prepared from blood samples using the standard venous blood sampling protocol, which has been used for many years in the clinical site. The matched serum and plasma samples that were kept at room temperature before being centrifuged were obtained from 3 healthy volunteers. In addition, plasma samples that were collected from a healthy volunteer were used to assess the influence of the duration of the cold temperature storage period on the results of metabolomic analysis, as shown in Fig. 1. To prepare the serum samples, blood samples were collected in blood tubes containing a clotting activator and separation gel and were gently mixed and kept at room temperature for more than 30 min until they had completely coagulated. To prepare the plasma samples, blood samples were collected in blood tubes containing ethylenediaminetetraacetic acid (EDTA)-2Na and were gently mixed and kept at room temperature for 0, 15, or 30 min or at a cold temperature, which was kept by using Cube Cooler (Forte Grow Medical, Tochigi, Japan), for 1, 4, or 8 h. Then, the blood tubes were centrifuged at 2270 × *g* for 10 min at 4°C, and the serum and plasma were transferred to clean tubes and stored at −80°C before being used.

Extraction procedure To extract hydrophilic metabolites prior to the GC/MS analysis, 50 μL of serum or plasma were mixed with 250 μL of methanol and 10 μL of 2-isopropylmalic acid (0.5 mg/mL) as an internal standard. Then, the mixture was shaken at 1200 rpm for 30 min at 37°C, before being centrifuged at 19,300 ×*g* for 3 min at 4°C. Two hundred and twenty-five μL of the obtained supernatant were transferred to a clean tube, before being lyophilized using a freeze dryer. For oximation, 80 μL of 20 mg/ml methoxyamine hydrochloride dissolved in pyridine were added to the tube and then sonicated for 20 min, before being shaken at 1200 rpm for 90 min at 30°C. Then, 40 μL of MSTFA were added, and then the mixture was incubated at 1200 rpm for 30 min at 37°C. After the mixture had been centrifuged at 19,300 ×*g* for 5 min at room temperature, the resultant supernatant was subjected to GC/MS.

For the lipid analysis using LC/MS, 10 μL of serum or plasma were mixed with 80 μL of methanol and 10 μL of 500 ppb PC 12:0–12:0 dissolved in methanol as an internal standard, and then the solution was centrifuged at 16,000 ×*g* for 5 min at 4°C. The resultant supernatant was subjected to LC/MS analysis.

To extract cationic and anionic metabolites prior to the LC/MS analysis, 50 μL of serum or plasma were mixed with 900 μL of a solvent mixture (methanol: water: chloroform = 2.5:1:1) containing 1 μM 2-bromohypoxanthine and 10-camphorsulfonate as internal standards. The mixture was subsequently shaken at 1400 rpm for 30 min at 37°C, before being centrifuged at 16,000 ×*g* for 3 min at 4°C. The resultant supernatant (630 μL) was transferred to a clean tube. Then, 280 μL of water were added, and the mixture was mixed well. After the mixture had been centrifuged at 16,000 ×*g* for 5 min at 4°C, 500 μL of the resultant supernatant were centrifugally filtrated through a 3-kDa filter device (Millipore, MA, USA) at

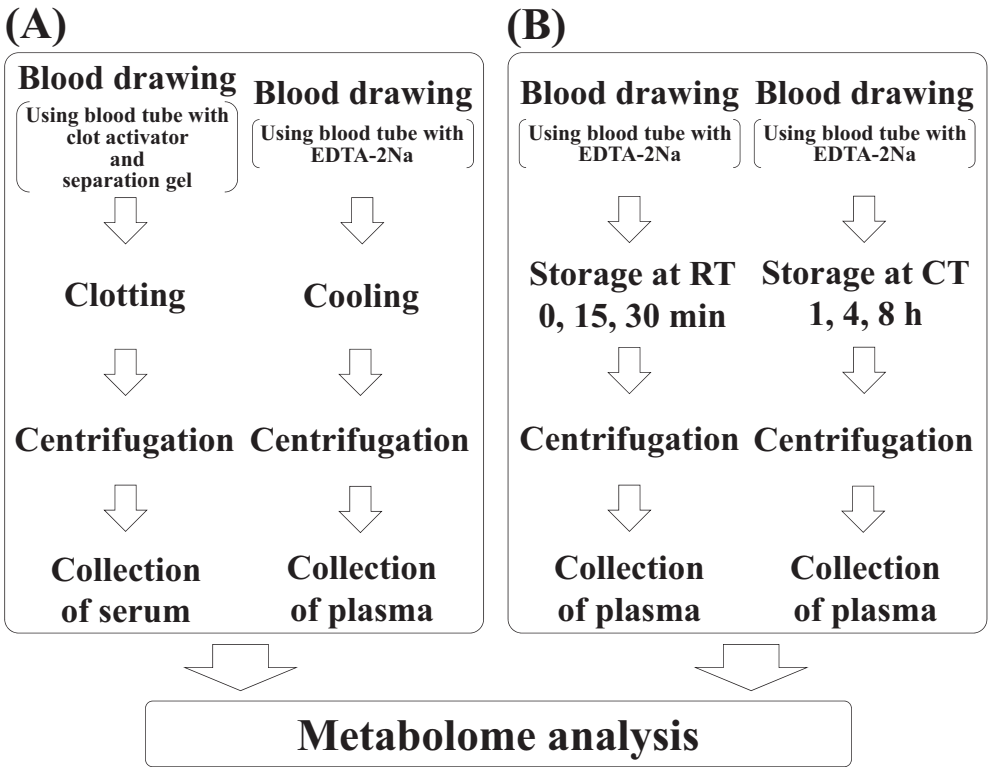

FIG. 1. Scheme of the pre-analytical blood processing. The differences in metabolite profiles caused by serum or plasma sampling were assessed (A). The changes in metabolite profiles caused by storage at room temperature (RT), which simulated the environment of blood collection during medical check-ups (during which the room temperature storage period varies), or at cold temperature (CT), which simulated blood tube storage in a cold transportation container, were assessed (B).

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