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Supercritical fluid extraction as a preparation method for mass spectrometry of dried blood spots

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ABSTRACT

The potential of supercritical fluid extraction (SFE) as a preparation method for mass spectrometry of dried blood spots (DBS) was examined. SFE is generally used for the extraction of hydrophobic compounds, but hydrophilic metabolites such as amino acids, amines, and nucleic-acid-related metabolites could be extracted by adding a low level of methanol as a modifier. Under the optimized conditions, over 200 metabolites were detected from a dried serum spot, of which over 160 metabolites could be analyzed stably (RSD <20%). These results show that SFE is an effective extraction method of metabolites with a wide range of polarity in DBS.

1. Introduction

There are many advantages of dried blood spot (DBS) sampling compared with the conventional blood sampling method, including lower sample consumption and easier handling [1–4]. The former advantage enables easier application of blood sampling to newborns and reduction of the use of experimental animals. The latter contributes to reduce costs for transport and the preservation of samples.

Owing to these advantages, DBS has been applied for newborn mass screening [5,6] and for HIV tests [7]. Furthermore, combined with mass spectrometry (MS), the application of DBS is being extended to therapeutic drug monitoring (TDM) [8–10], pharmacokinetic study [11,12], and metabolome analysis [13,14]. Although there are several problems about false positive detection such as cross-talk or in-source decay, MS is a highly sensitive and selective detection method, and is free from the problem of cross-reactivity [15,16], which is a major defect of an indirect detection method that uses antibody response. Furthermore, MS can target multiple metabolites at one analysis [17]. Generally, MS's drawback is low quantitative capability due to ion suppression, but this problem can be solved by using stable isotopes of target compounds [18]. The combination of DBS with MS is a promising strategy for biochemical analysis.

Supercritical fluid extraction (SFE) is an extraction method that has features such as rapidity, high selectivity, and low solvent consumption [19,20]. It is generally used for the extraction of hydrophobic compounds such as fat-soluble vitamins [19,21], carotenoids [22], fatty acids [23], and aliphatic hydrocarbons [24], owing to the high hydrophobicity of supercritical carbon dioxide

Abbreviations: DBS, dried blood spot; MS, mass spectrometry; TDM, therapeutic drug monitoring; SFE, supercritical fluid extraction; $SCCO_2$, supercritical carbon dioxide; LC/MS/MS, liquid chromatography/tandem mass spectrometry; BHT, dibutylhydroxytoluene; DSS, dried serum spot; DWBS, dried whole blood spot; PC, phosphatidylcholine; PE, phosphatidylethanolamine; OSE, organic solvent extraction; SFC, supercritical fluid chromatography.

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($SCCO_2$). In our previous study [25], we applied SFE as an extraction method for phospholipids in DBS. However, other metabolites including hydrophilic metabolites were not targeted in that study. Recently, on the other hand, it has been reported that hydrophilic compounds such as amino acids could be extracted from crops by SFE with a relatively large amount of modifier [26,27]. This suggests that SFE can target the wide variety of metabolites contained in DBS.

In this study, SFE's potential as a preparation method for MS using DBS was evaluated. To specify SFE's applicable range of polarity, the extracts obtained from SFE of DBS were analyzed by liquid chromatography/tandem mass spectrometry (LC/MS/MS), which target lipids (phospholipids, fatty acids, acylcarnitines, bile acids) as well as hydrophilic compounds (amino acids, amines, nucleic-acid-related metabolites). The extraction conditions were optimized and the extraction efficiency of SFE was compared to that of the commonly used organic solvent extraction to characterize SFE as a DBS extraction method.

2. Experimental

2.1. Chemicals

CO_2 used for SFE was purchased from Iwatani Corporation (Tokyo, Japan). As an extraction medium, methanol (HPLC grade) was obtained from Nacalai Tesque Inc. (Kyoto, Japan). Acetone and dibutylhydroxytoluene (BHT) used in the extraction procedure were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). For LC/MS/MS, water and acetonitrile (LC/MS grade) were purchased from Wako Pure Chemical Industries Ltd. and methanol (LC/MS grade) was obtained from Kanto Chemical Co. Inc. (Tokyo, Japan). Formic acid (LC/MS grade, Wako Pure Chemical Industries Ltd.) and ammonium acetate (1 M solution, HPLC grade, Wako Pure Chemical Industries Ltd.) were used as additives for the mobile phase. 2-Bromohypoxanthine purchased from Sigma–Aldrich (MO, USA) and dilauroylphosphatidylcholine (PC 12:0-12:0) purchased from Avanti Polar Lipids (AL, USA) were used as internal standards.

2.2. Samples

BondElut DMS card (Agilent Technologies, CA, USA) was punched using a dedicated hole puncher (3 mm, I.D.). As an antioxidant, 30 μl of 0.5% BHT (Wako Pure Chemical Industries Ltd.) in acetone (HPLC grade, Wako Pure Chemical Industries) was added to the hollowed out pieces [28]. After drying, 3 μl of human serum or whole blood from a healthy subject, including 30 μM 2-bromohypoxanthine and 2.4 μM PC 12:0-12:0 as internal standards, was dropped on the punched pieces and dried for over 2 h at room temperature. These samples are referred to as dried serum spots (DSS) and dried whole blood spots (DWBS), respectively, in this article.

2.3. Extraction conditions

In this study, a prototype SFE system made by Shimadzu Corporation (Kyoto, Japan) was used as an SFE instrument. Extraction was performed in dynamic mode. For the collection of extracts, the bubbling method was used. Time for extraction was 5 min because 5 min was sufficient for minimum flow rate condition in this study (methanol 0.3 ml/min). Other extraction conditions are shown in the main text and the figure legends. Methods for organic solvent extraction were as follows: 300 μl of methanol was added to the dried blood spot and left to stand for an hour. After centrifugation (15,000 × *g*, 4 °C, 5 min), the supernatant was collected into a new tube. The collected extracts were dried by centrifugal concentration, and reconstituted with 50 μl of water (Kanto Chemical) for hydrophilic metabolite analysis or methanol for lipid analysis.

2.4. Analytical conditions

Analyses were carried out using a Nexera LC system (Shimadzu Corp.) equipped with two LC-30AD pumps, a DGU-20As degasser, a SIL-30AC auto sampler, a CTO-20AC column oven, and a CBM-20A control module, coupled with an LCMS-8040 triple quadrupole mass spectrometer (Shimadzu Corp.). The hydrophilic 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), while lipids were separated using an octadecylsilylated silica column (Inertsustain C18, 100 mm × 2.1 mm, 3 μm, GL Sciences, Tokyo, Japan) with a guard column (10 mm × 3 mm, 5 μm). The mobile phase for hydrophilic metabolites was constituted with A: 0.1% formic acid in water 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 mobile phase for lipids consisted of A: 20 mM ammonium acetate in water and B: methanol. The flow rate was 0.4 ml/min and the column oven temperature was 40 °C. The gradient program for mobile phase B was as follows: 0 min, 80%; 13 min, 98%; 30 min, 98%; 30.1 min, 80%; and 35 min, 80%. The target metabolites of these systems are shown in Supplementary Table 1. Typical MRM chromatograms of these metabolites are shown in Supplementary Fig. 1. Hydrophilic metabolites were evaluated by peak area whereas lipids were evaluated by peak height because some lipid isomers could not be separated completely.

3. Result and discussion

3.1. Optimization of SFE conditions

First, basic conditions for extraction were investigated using methanol as a modifier. Direct collection caused splatter of the extracts. Therefore, extracts contained in the mixture of CO_2 and methanol were bubbled and trapped in methanol, at the bottom of a test tube. In order to determine what kind of metabolites can be extracted using this system, extraction of a dried serum spot (DSS) was performed under the intermediate conditions (pressure, 20 MPa; temperature, 30 °C; methanol, 0.6 ml/min; CO_2, 2.4 ml/min) and the extracts were analyzed under two different LC/MS/MS conditions that target either lipid or hydrophilic metabolites (data not shown). As a result, in addition to the formerly reported phosphatidylcholines (PC) and phosphatidylethanolamines (PEs) [25], hydrophobic metabolites such as acylcarnitines and bile acids were detected by analysis with the extracts. Furthermore, many hydrophilic metabolites such as amino acids, nucleic-acid-related metabolites, and betaines (carnitine, choline, etc.) were also extracted by SFE. These results clearly show that DSS-SFE samples contain the metabolites with a variety of polarities.

Next, parameters of SFE such as temperature, pressure, and modifier were optimized by comparing extraction efficiency of the metabolites representative of each metabolite class under the testing conditions (amino acids, isoleucine; nucleic-acid-related metabolites, hypoxanthine; betaines, choline; other hydrophilic metabolites, creatinine; bile acids, glycodeoxycholic acid; free fatty acids, linoleic acid; PC, PC 18:0-18:2/18:1-18:1; PE, PE 18:0-18:2/18:1-18:1 using DSS as samples. First, extraction temperature (30, 50, 80 °C) was investigated under the following conditions: CO_2, 2.4 ml/min; methanol, 0.6 ml/min; pressure, 20 MPa: time for extraction, 5 min. Although 80 °C was the only condition that met the supercritical conditions among these conditions [29], there was no dramatic improvement in extraction efficiency (Fig. 1A). These