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Simultaneous extraction of metabolome and lipidome with methyl tert-butyl ether from a single small tissue sample for ultra-high performance liquid chromatography/mass spectrometry

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ABSTRACT

A common challenge for scientists working with animal tissue or human biopsy samples is the limitation of material and consequently, the difficulty to perform comprehensive metabolic profiling within one experiment. Here, we present a novel approach to simultaneously perform targeted and non-targeted metabolomics as well as lipidomics from one small piece of liver or muscle tissue by ultra-high performance liquid chromatography/mass spectrometry (UHPLC/MS) following a methyl tert-butyl ether (MTBE)-based extraction. Equal relative amounts of the resulting polar and non-polar fractions were pooled, evaporated and reconstituted in the appropriate solvent for UHPLC/MS analysis. This mix was comparable or superior in yield and reproducibility to a standard 80% methanol extraction for the profiling of polar and lipophilic metabolites (free carnitine, acylcarnitines and FFA). The mix was also suitable for non-targeted metabolomics, an easy measure to increase the metabolite coverage by 30% relative to using the polar fraction alone. Lipidomics was performed from an aliquot of the non-polar fraction. This novel strategy could successfully be applied to one mouse soleus muscle with a dry weight of merely 2.5 mg. By enabling a simultaneous profiling of lipids and metabolites with mixed polarity while saving material for molecular, biochemical or histological analyses, our approach may open up new perspectives toward a comprehensive investigation of small, valuable tissue samples.

1. Introduction

Disturbances in liver and muscle metabolism are at the center of numerous diseases [1,2]. Metabolome and lipidome analyses can be valuable and complementary tools to study the metabolism of healthy or diseased tissue [3,4]. Metabolomic analyses are mainly performed on nuclear magnetic resonance (NMR) [5,6] and mass spectrometry-based platforms [7,8]. While NMR-based platform has the advantage of minimal sample preparation and high throughput, mass spectrometry has a higher sensitivity and chemical identification capability. If coupled with chromatography, the matrix effect from the complex samples is greatly reduced through chromatographic separation [9]. Mass spectrometry-based metabolomic analyses can be employed both to uncover novel metabolites of interest in non-targeted approaches [10,11] and to provide a means of highly sensitive quantification of specific metabolites in targeted settings [12]. Shotgun mass spectrometry [13,14], alone or in combination with liquid chromatography [15,16], is also the predominating platform employed in lipidomic analyses. Recent developments in mass spectrometry and its coupled ultra-high performance liquid chromatography technologies have enabled their broad applications in metabolomic and lipidomic analyses [17–19].

However, metabolomic and lipidomic analyses of mammalian tissue samples pose an additional challenge: the limited amount

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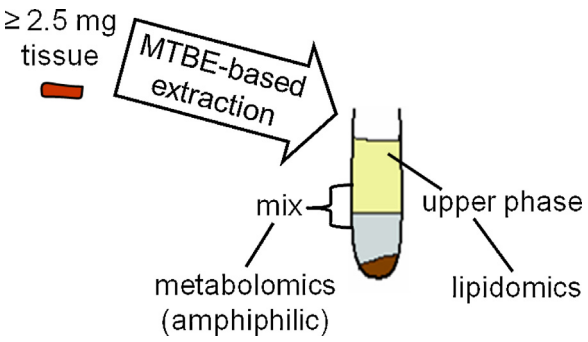

Fig. 1. Scheme depicting the MTBE-based simultaneous extraction of lipidome and metabolome from a single small tissue sample like the mouse soleus muscle. The upper non-polar fraction was used for lipidomic analysis. Equal relative volumes of the upper and lower phases were mixed to improve the analyses of hydrophilic and amphiphilic metabolites.

of tissue that can be retrieved by performing biopsies in humans and the small size of the predominating animal model, the mouse, create the ethical and practical requirement to develop efficient, tissue-saving experimental procedures. Human muscle biopsies usually have a wet weight of around 50 mg, corresponding to 15 mg dry weight [20], from which a number of analyses like quantification of mRNA and phosphoproteins, enzymatic assays, tissue embedding for histology, lipidomic and targeted or non-targeted metabolomic analyses need to be performed. Some mouse muscles, like the frequently studied soleus muscle, even have a dry weight of only 2.5 mg, making it particularly desirable to use a single small sample for the simultaneous analysis of metabolite classes that by the generally used metabolic profiling methods require different extraction procedures.

A universal procedure to extract a large number of amphiphilic metabolites with mixed polarity is 80% methanol [21]. Neutral lipids like triglycerides, however, require the use of lipophilic organic solvents. Sample extraction for lipidomics is traditionally performed using a mixture of chloroform, methanol and water, resulting in the separation of polar and non-polar compounds into two phases [22]. A recently developed sample pretreatment for lipidomics by Matyash et al. [23] introduced methyl tert-butyl ether (MTBE) as a less hazardous substitute for chloroform. MTBE has the advantage of accumulating the non-extractable pellet at the bottom, enabling easier and cleaner access to the upper non-polar and the lower polar fraction. Up to now, the complementary analyses of lipophilic and amphiphilic metabolite patterns require at least two separate extractions. This will usually lead to the consumption of a relatively large amount of tissue when two or more analyses shall be performed.

In this study, we aimed to cover different classes of metabolites with a single extraction from a small amount of tissue by developing a sample pretreatment strategy (Fig. 1) for the ultra-high performance liquid chromatography/mass spectrometry (UHPLC/MS)-based quantification of amphiphilic metabolites as well as lipids based on MTBE extraction and compared it to the classic 80% methanol-based metabolite extraction and chloroform-based lipid extraction.

2. Experimental

2.1. Chemicals and internal standards

Liquid chromatography grade solvents were purchased from Merck (Darmstadt, Germany) or TEDIA (Fairfield, OH, USA). Ammonium formate was purchased from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's phosphate-buffered saline (PBS) was from Lonza (Verviers, Belgium). Internal standards were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA), Sigma–Aldrich (Munich, Germany) and ten Brink (Amsterdam, The Netherlands).

A combination of lipid, acylcarnitine and free fatty acid (FFA) standards was added to the solvent employed in the initial extraction step. Details concerning the application of internal standards are given in the Supplementary Table S1. The lipidomics standards used were ceramide CER (d18:1/17:0), diacylglycerol DG (14:0/14:0) lysophosphatidylcholines LPC (15:0) and (19:0), phosphatidylcholines PC (17:0/17:0), and (19:0/19:0), phosphatidylethanolamine PE (17:0/17:0), sphingomyelin SM (d18:1/12:0), and triacylglycerols TG (15:0/15:0/15:0) and (17:0/17:0/17:0). Acylcarnitine standards were d_3-carnitine, d_3-acetyl- (C2:0-), d_3-butyryl- (C4:0-), d_3-hexanoyl- (C6:0-), d_3-octanoyl- (C8:0-), d_3-decanoyl- (C10:0-), d_3-dodecanoyl- (C12:0-) and d_3-hexadecanoyl- (C16:0-) carnitine. FFA standards were d_3-dodecanoic acid (C12:0) and heneicosanoic acid (C21:0). Details concerning the normalization of individual metabolites are given in the Supplementary Tables S2 (lipids), S3 (acylcarnitines) and S4 (FFA).

2.2. Liver and muscle preparation and metabolite extraction

Animal experiments were conducted in accordance with the national guidelines of laboratory animal care and approved by the local governmental commission for animal research. Tissues were quickly excised from 12 week-old male C57Bl/6J mice, washed in PBS, blotted dry and snap-frozen in liquid nitrogen. To allow weighing out identical amounts, the tissue was freeze-dried. Next, the dry tissue was finely crushed for 10 s at room temperature and 15 Hz using a TissueLyser (Qiagen, Hilden, Germany). Before and after lyophilization, tissues were stored at −80 °C. Aliquots of 10 mg were made from the pulverized liver. Due to its small size, the soleus muscle must be used completely. Freeze-dried tissue was chosen for method validation because it overcomes the difficulty of weighing out identical small amounts of fresh tissue without allowing it to thaw and prompt biochemical reactions that could alter the metabolome and lipidome, while proving to be comparable to fresh tissue in a preliminary test (data not shown). Murine liver was used for method validation.

The liquid–liquid MTBE extraction was based on a protocol recently described for lipidomics [23]. To yield a 2:1 ratio of upper (non-polar) to lower (polar) phase, the relative amount of water was increased from MTBE:methanol:H_2O (20:6:5.8) originally described by Matyash et al. [23] to MTBE:methanol:H_2O (20:6:7). This increase in water content did not negatively affect lipid recovery (Supplementary Table S2). Freeze-dried tissue samples were firstly bath sonicated for 2 min with 400 μL ice-cold 75% methanol including standards (both for quantification and linearity testing) to break up the cells. Next, 1 mL MTBE was added and the samples were shaken for 1 h at room temperature. The one-phase solvent system in this step allows for an optimal contact between extraction solvent and tissue material. Next, phase separation was induced by adding 250 μL water, letting sit for 10 min at room temperature and centrifuging for 15 min at 14,000 × *g*, 4 °C. Because of the low density and high hydrophobicity of MTBE, lipids and lipophilic metabolites are mainly extracted to the upper MTBE-rich phase, polar and semipolar metabolites are mainly extracted to the lower methanol/H_2O-rich phase and amphiphilic metabolites to both upper and lower phases. Proteins and other non-extractable cellular components accumulate at the bottom of the extraction vial, as shown in Fig. 1. The two phases were separately transferred to fresh tubes before six different aliquots were made, 2 each of upper fraction (220 μL), lower fraction (110 μL) and "mix" (220 μL upper plus 110 μL lower fraction).

For methanol extraction/protein precipitation, 1 mL ice-cold 80% methanol including internal standards was added to the tissue