A novel and robust untargeted UPLC-MS profiling pipeline to expand tissue metabolome coverage – Optimization of chromatographic separation and tissue extraction: Application to cardiovascular disease

Panagiotis A Vorkas[1], Muzaffar A Anwar[2], Alun H Davies[2], Elizabeth J Want[1], Elaine Holmes[1]

[1] Biomolecular Medicine, Division of Computational and Systems Medicine, Department of Surgery and Cancer, Faculty of Medicine, Imperial College London, London SW7 2AZ, UK

[2] Academic Section of Vascular Surgery, Division of Surgery, Department of Surgery and Cancer, Faculty of Medicine, Imperial College London, London W6 8RF, UK

Background: Metabolic profiling studies aim towards comprehensive metabolome coverage. However, due to the vast physicochemical diversity of small molecules, multiple methods or even techniques are required to be employed to expand metabolome coverage. Additionally, the inevitable inclusion of tissue lysis and metabolite extraction steps can induce additional systematic variation. Herein, we describe a dual-extraction followed by a dual-separation pipeline for untargeted UPLC-MS profiling, utilizing both HILIC and RP chromatography. The described approach was designed and applied on tissue samples, a matrix with a remarkable amount of disease-related information, but with a small number of studies focusing on the challenges of such analyses.

Materials and Methods: A total of 26 abdominal aortic aneurysm tissues, 52 carotid stenosing plaques, 26 femoral stenosing plaques and 16 intimal thickening tissues were used. Tissue lysis and metabolite extraction was performed using bead beating and an aqueous extraction was followed by organic extraction. Aqueous extracts were analyzed by a HILIC-UPLC-MS method, while the organic extracts by a RP-UPLC-MS lipid profiling method. The QC strategy[1] was applied by interspersing 13 pooled sample injections during the analysis of the tissue extracts. Both HILIC and RP methodologies were extensively optimized to be able to perform with high separation performance and ability to handle complex tissue structures with intense lipid and protein content for several sample injections. For metabolite structural assignment, accurate m/z measurements of detected chromatographic peaks were first matched to metabolites from online MS databases (Metlin[2], HMDB[3] and Lipidmaps[4]). After an assessment of retention time and isotopic pattern, tandem MS (UPLC-MS^E and UPLC-MS/MS) fragmentation pattern was employed for further structural elucidation. Further, an authentic standard of the metabolite was run using identical UPLC-MS/MS conditions and

the detected m/z was matched to 1) the retention time, and where possible 2) the MS/MS spectrum obtained from the sample under identical experimental conditions. Matching to an authentic standard was dependent on commercial availability and was pursued for small molecules.

Results and Discussion: High reproducible peaks both chromatographically (CV% < 1%) and analytically (> 5000 reproducible peaks) were obtained. Using the described pipeline an assessment of the appropriate solvent system and order of aqueous and organic extraction were assessed. The optimal extraction system was selected based on the reproducibility of the extraction procedure, features detected and metabolome coverage for the tissue samples tested. The optimized workflow was further applied successfully on adipose and liver tissue. Structural assignments totaled identification of >250 metabolites in the tissue types tested. These included a panel of lipophilic compound classes detected by the lipid profiling method, such as phospholipids (phosphatidylcholines, lysophosphatidylcholines, phosphatidylethanolamines, lysophosphatidylethanolamines, phosphatidylserines, phosphatidylinositols and phosphatidylglycerols), sphingolipids (ceramides, sphingomyelins, phosphatidylethanolamine-ceramides, mono-, di-, tri- and tetra-hexosylceramides), long-chain free fatty acids, cholesterol and cholesteryl derivatives (cholesteryl sulfate, cholesteryl esters and oxidized cholesteryl esters), di- and triacylglycerols, carotenoids and fatty acid amides. Using the HILIC method to analyze the aqueous extracts, classes detected included purines, pyrimidines, α-aminoacids and derivatives, acylcholines, sphingosines, nicotinamide and derivatives, sugar alcohols and acylcarnitines and carnitine. Polar compounds such as creatine and creatinine, benzoic and salicylic acid, betaine, taurine and hippurate were also detected. The two methods used proved highly complementary based on the metabolites detected. These metabolites were mapped to biological pathways using the KEGG mapper[5] and Ingenuity Pathways Analysis software and were proved able to cover several pathways and biological processes such as lipid accumulation and concentration, cancer, inflammation, cell death and survival, proliferation, apoptosis, necrosis, peroxisomal disorder and cell differentiation. Finally, the application of the described pipeline on tissue samples with cardiovascular disease delivered a robust analysis with unique metabolic phenotypes for the four groups tested as demonstrated by multivariate and univariate statistics.

References:

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