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A method for comparative metabolomics in urine using high resolution mass spectrometry

Padma Ramakrishnan, Sreenath Nair, Kannan Rangiah*

Metabolomics Facility, Centre for Cellular and Molecular Platforms, National Centre for Biological Sciences, GKVK, Bellary Road, Bangalore 560065, India

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ABSTRACT

Developing a workflow for metabolite profiling from biological fluids using mass spectrometry is imperative to extract accurate information. In this study, urine samples from smokers ($n = 10$) and non-smokers ($n = 10$) were analyzed using an ultrahigh performance liquid chromatography–high resolution mass spectrometry (UHPLC–HRMS) system. For the analysis, two different chromatographic methods [Reversed phase chromatography (RPC) and Hydrophilic interaction liquid chromatography (HILIC)], in two ionization modes (positive and negative) were used. Spiked reserpine (positive ion mode) or taurocholate (negative ion mode) were used for data extraction and normalization. Quality controls (QCs), prepared by pooling urine samples from both smokers and non-smokers (each $n = 10$), were used to assess the reproducibility of the method. The final data output from SIEVE 2.2 after applying a cut-off for QC coefficient of variation (CV) <20% and p-value <0.05 showed 165, 83, 177 and 100 unique components in RP positive/negative, HILIC positive/negative modes, respectively. Statistical analysis showed clustering of the two groups and the QCs, while the variable importance in projection (VIP) scores for the top fifteen metabolites in each of the four modes indicated the metabolites most responsible for the differences. Application of the developed workflow for comparative metabolomic analysis of urine in different diseased models will be of great use in the field of clinical metabolomics.

1. Introduction

Metabolomics is a study of the detection and quantification of small molecules (metabolome), which are the functional readout of cellular phenotype and the end products of upstream gene expression [1]. It is a cutting-edge biochemical approach at the interface of metabolic phenotype and genotype [2]. Metabolite changes have long been observed in diseased individuals either as a primary cause or a secondary indicator. The concept that individuals might have a metabolic profile that could be reflected in the makeup of their biological fluids is well known [3]. Until recently, the quantification of all possible metabolites from biological fluids has been a challenging task. New developments in high-resolution mass spectrometers (HRMS) in the past few years have enabled simultaneous detection of metabolites from biological fluids [4]. There are a growing numbers of studies using liquid chromatography–mass spectrometry (LC–MS) as a tool for biomarker discovery. The high correlation between metabolites and phenotype has created a surge of interest that is reflected in the increasing number of publications in the field of metabolomics. In the last 4–5 years LC–MS based non-targeted metabolomics has been used for clinical applications mainly as a tool for disease diagnosis [5–8].

Metabolomics has its own challenges in terms of analyzing different chemical classes of molecules and quantifying them all together in a single method. As of now analysis of metabolites in a single method is impossible, but the two major chromatographic techniques namely reversed phase chromatography (RPC) and hydrophilic interaction liquid chromatography (HILIC) are known to cover most of the metabolites in a given biological fluid [9]. There are reports using HRMS with two chromatographic columns for analysis of sera, urine and saliva samples to demonstrate the changes in metabolome of normal versus disease samples [9–11].

Abbreviations: UHPLC–HRMS, ultrahigh performance liquid chromatography–high resolution mass spectrometry; RPC, Reversed phase chromatography; HILIC, Hydrophilic interaction liquid chromatography; QCs, Quality controls; VIP, variable importance in projection; HMBD, Human Metabolome Data Base; KEGG, Kyoto Encyclopedia of Genes and Genomes; PCA, Principal component analysis; PLS-DA, partial least squares discriminant analysis; SPE, solid phase extraction; ROC, receiver operating characteristic; AUC, area under the curve; CV, coefficient of variation.

* Corresponding author at: Metabolomics Facility, Centre for Cellular and Molecular Platforms, National Centre for Biological Sciences, GKVK, Bellary Road, Bangalore, 560065, India. Fax: + 91 80 2363 6662.

E-mail addresses: kannanr@ccamp.res.in, rkannan007@gmail.com (K. Rangiah).

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Producing data from this kind of analysis is comparatively easy, whereas extraction of information from the produced data is still a challenging problem. Metabolomics data analysis relies heavily on advances in bioinformatics tools that are required for analysis and on electronic databases for metabolite identification [12]. Currently, a number of databases are available like Human Metabolome Data Base (HMDB) [13,14], Kyoto Encyclopedia of Genes and Genomes (KEGG) [15], METLIN [16], PubChem [17], MassBank [18] etc. Yet, annotation of all metabolites is quite cumbersome and an on-going process. There are many freely available (XCMS, MZmine) and vendor specific (SIEVE-Thermo Fisher, MarkerView-AB Sciex, MassLynx-Waters and Mass Profiler-Agilent) software for data extraction and analysis, but problems still exist in extracting complete metabolite information. Also, normalization of data is an issue much debated currently. This makes metabolomic analysis of biological fluids a highly complicated process. Integration of "all omics" (genomics, epigenomics, transcriptomics, proteomics, glycomics, metabolomics, microbiomics and phenomics) will be highly useful in constructing molecular networks [19], helping us understand the complex biochemical processes in a better way, but analyzing the datasets all together is a great challenge. It is therefore, necessary to establish standardized protocols of metabolite analysis from different biological fluids for all researchers in the field of metabolomics. There is a great need to improve the data quality and data mining strategies to obtain more and accurate information.

Here we have shown a workflow for urine metabolite profiling by using UHPLC–HRMS system. To demonstrate, we have taken urine samples from smokers/nonsmokers and profiled metabolites. Smokers/nonsmokers were chosen as subjects because the differentiation is well established and it seemed ideal to validate a method by obtaining expected results. Smoking is known to cause large and growing number of premature deaths, and also has direct correlation to both lung and oral cancer [20]. The advantage with urine is that, it can be collected non-invasively, is available in large quantities, has a different metabolome from blood, and sample handling is simple since there is no need to remove protein [21]. It is also widely used as a diagnostic tool in clinical practice [5–8]. One of the major considerations is the presence of large amount of salts, which can interfere with MS analysis. Care should be taken to desalt in the sample preparation step. We have used RPC and HILIC coupled with both positive and negative ionization modes for analysis. To overcome the issues in data normalization we have used reserpine (positive ion mode) or taurocholate (negative ion mode) spiked along with the samples and QCs. QCs were prepared by pooling urine samples ($n = 20$) from both smokers and non-smokers as previously shown [22]. Data analysis was done using SIEVE 2.2 software in a three step process (chromatogram alignment, component extraction and ID generation via ChemSpider by searching both HMDB and KEGG databases). A number of tobacco-related metabolites were correctly picked as differentiating components between the two sample sets.

2. Materials and methods

2.1. Materials

Reserpine, taurocholic acid (sodium salt), ammonium acetate and formic acid were procured from Sigma–Aldrich (Bangalore, India). High purity MS grade solvents (methanol and acetonitrile) were obtained from Merck Millipore (Merck Millipore India Pvt. Ltd., Bangalore). Double-distilled water for LC–MS was obtained from our in-house distillation unit. Solid phase cartridges (Strata-X 33 μ Polymeric Reversed Phase; 1 mL, 30 mg) were obtained from Phenomenex, Inc. (Hyderabad, India). Urine samples (20–40 mL) were collected from 10 healthy non-smoker and 10 healthy smoker male volunteers, aged 22–32 years. The smokers used on an average, 10–15 cigarettes per day and all samples were collected during the day time. All samples were stored at −20 °C till further analysis.

2.2. Sample preparation

Before analysis, all samples were thawed in ice, vortexed well and centrifuged at 14000 rpm for 10 min. Equal volumes of the supernatant from all 20 samples were pooled to prepare at least 5 QC samples. From the remaining supernatant of each sample and from each of the pooled QC sample, 800 μL was used for RPC (positive and negative) and 200 μL was used for HILIC (positive and negative) analysis.

2.2.1. RPC mode

Briefly, 800 μL of urine sample was acidified with 1 μL formic acid and centrifuged (14000 rpm, 5 min). The supernatant was cleaned using RP-SPE cartridges. Prior to loading, the SPE cartridge was conditioned with 1 mL methanol followed by 1 mL of water. The acidified urine was loaded onto the cartridge and allowed to bind to the column with gravity flow. It was then washed with 1 mL of acidified water (0.1% FA) twice. Metabolites were eluted using 1 mL of acetonitrile:methanol:water (40:10:1) mixture. It was then dried under vacuum and reconstituted with 80 μL of 25% methanol and used for both positive and negative ion mode analysis. For RPC positive 10 μL of reserpine (5 μg/mL), and for RPC negative 10 μL of taurocholate (25 μg/mL) were spiked to 40 μL each of the reconstituted sample and 10 μL was injected into the UHPLC–HRMS for analysis.

2.2.2. HILIC mode

Briefly, 800 μL of cold acetonitrile was added to 200 μL of each sample or QC, separately. Samples were vortexed and maintained at 4 °C for 20–30 min and centrifuged (14000 rpm for 5 min). From the resulting supernatant of each sample or QC, 200 μL was taken for HILIC positive analysis and 200 μL was taken for HILIC negative analysis. The HILIC positive samples were each spiked with 10 μL of reserpine (5 μg/mL) standard, while the HILIC negative samples were each spiked with 10 μL of taurocholate (25 μg/mL) standard. Both sets of samples were dried under vacuum and reconstituted in 50 μL of 80% acetonitrile and transferred to auto-sampler vials. For the analysis 10 μL was injected into the UHPLC–HRMS system.

2.3. UHPLC–MS

The mass spectrometer used for the metabolite analysis is a Q-Exactive Orbitrap (Thermo Fisher Scientific, San Jose, CA, USA) equipped with heated electrospray ionization (HESI) source. It also houses an HCD (higher-energy collision dissociation) cell for carrying out MS^n experiments. The Q Exactive is coupled to a Dionex UltiMate 3000 UHPLC system (Thermo Fisher Scientific, San Jose, CA, USA). This system is provided with a column oven (set at 40 °C), an auto-sampler and a thermo-controller (set at 4 °C). It uses an in-line split-loop injection design and is equipped with an external needle wash system (95% methanol) to ensure zero percent carry over problems. A total of four experiments were performed with sample analysis in RPC (positive and negative) and HILIC (positive and negative) modes. MS operating conditions for all four analyses were as follows: spray voltage, +2500 V (−2500 V for negative mode); capillary temperature, 280 °C; vaporizer temperature, 320 °C, sheath gas, 30 arbitrary units (40 for negative mode); and, auxiliary gas, 10 arbitrary units. Injector settings were as follows: 0–2 min: waste, 2–45 min: load, 45–50 min: waste.