Abstract

Metabolomics allows discovery of novel therapeutics, screening drug toxicity and efficacy, and monitoring diet and environmental effects on health. Identifying metabolites from urine and plasma is essential for validating potential disease biomarkers and interrogating their affected metabolic pathways to further understand their biological implications. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis has become an essential tool for identification and quantification of metabolites in complex sample matrices due to its inherent sensitivity and selectivity. Here we report a novel sensitive workflow using a MicroEx LC coupled to a QTRAP® 6000+ mass spectrometer for qualitative and quantitative analysis of polar metabolites (Figure 1). We have implemented a single HILIC microflow LC-MS/MS method for profiling polar metabolites using multiple reaction monitoring (MRM) to achieve positive/negative polarity switching in a single injection workflow. While microflow has become increasingly popular for many applications, microflow for metabolomics has not been popular because the high-sensitivity detector requires for injecting larger volumes of samples without sacrificing chromatographic resolution. However, by simply extending the sample in an organic solvent (50% Acetonitrile, pH 9), we were able to inject up to 2 μL on to the micro-LC column, while maintaining peak shape. The ionization efficiency of the sodium adducts was increased by implementing Q1/Q2 transitions monitored from plasma/murine and MCKD cell-line metabolites covering all metabolites in all pathways with up to 60% improved sensitivity for some metabolites. The sample preparation takes ~2 hrs with an additional 1 hr for sample run and data analysis.

Materials and Methods

Sample Preparation: Human urine was diluted in water (1:4) and 100 μL of diluted urine or 100 μL of control plasma for amino acid analysis (SCIENCE) were transferred for the analysis to a clean 5-mL or 5-mL microtube (Eppendorf). 20 μL of internal standards were spiked in the tube and 800 μL of cooled acetonitrile/acetic acid (87:3) was added to extract metabolites and precipitate the proteins. Samples were vortexed and incubated at 4 °C for 30 min then centrifuged at 14,000 RPM for 10 min. An 800 μL aliquot of the supernatant which contains the extracted metabolites was transferred to a new 2-mL microtube. The sample was dried using a TurboTap evaporator to a pellet using no heat starting with 5 μL Na2SO4, gas flow for 30 min and an extra 30 min at 10 psi pressure (~1 hr). The fresh pellet was dissolved in 100 μL of HILIC sample reconstitution buffer, mixed well by vortexing and centrifuged at 14,000 RPM for 10 min. Ninety microliters of supernatant was transferred to deactivated QuEChERS (QWaters) for LC-MS/MS analysis. Injection volume was 2 μL with 5 μL replicate injections. Samples were also diluted further (1:3) to check the method sensitivity. The HILIC sample reconstitution buffer contained Acetonitrile, water, and 0.5% mobile phase A. Mobile phase A + 0.95% water, 5% acetonitrile, 200 mM ammonium acetate and 20 mM ammonium hydroxide, pH 8.0.

Analytical Liquid Chromatography: A SCIEX ExActLC™ AD HPLC system was used for the analytical flow part of the analysis. The columns used were a Luna 3 μm NH, 100 A, 1.0 mm × 250 mm (Phenomenex). Mobile phase A was 0.95% water, 5% acetonitrile, 200 mM ammonium acetate and 20 mM ammonium hydroxide, pH 8.0. Mobile phase B = 95% acetonitrile and 5% mobile phase A and 20 mM ammonium hydroxide, flow rate of 350 μL/min. Wash solvent for the autosampler was 20:80 methanol/acetone/isopropanol. Injection volume was 2 μL, and the column was kept at 40 °C. The gradient method used is listed in Table 1.

Detection: Data Processing: MultiQuattro™ 3.0.2. Software (SCIEX) was used for data analysis with MRM peak integration algorithm, gaussian smooth width of 1.0 points, RT test window of 30 min and min peak width of 8 points. Integrated peaks with minimum signal/noise ratio of 10 or more in all replicates were selected and manually validated. Samples for both microflow and traditional flow LC-MS/MS analysis were prepared on the same day to exclude variations in response due to sample flow rate. Flow rate for microflow LC-MS/MS analyses were acquired for both analytical flow and microflow LC analysis.

For each detected metabolite in urine, the lowest observed S/N (calculated by MultiQuattro™) was plotted versus the number of replicates that metabolite was detected in (Figure 2). 197 of metabolites detected with a S/N < 20 were seen in all replicates, and therefore considered to be detectable with high confidence without requiring further manual validation. Of the metabolites with the lowest S/N of 10-20, were manually validated. All of these were detected in all 5 replicates with a manually determined S/N of at least 5.

Improved Signal to Noise Ratio (S/N)

The S/N ratio for all detected metabolites using this MRM method with both analytical flow LC and microflow LC was compared. S/N ratio was improved up to 60X with an average improvement of 10X for which a solution for definition of higher multiplex validation to be identified in low concentrations and/or when sample volumes are limited.

Microflow LC-MS/MS workflow provides improved sensitivity and S/N ratio of up to 60X with an average improvement of 10X which offers a solution for definition of higher multiplex validation to be identified in low concentrations and/or when sample volumes are limited.

Microflow LC-MS/MS method provides up to 50% increase in detection of polar metabolites.

Microflow Luna-NH2 HILIC chromatography provides excellent chromatographic separation of polar, hydrophilic metabolites.

This 30 min method is a single LC-MRM targeted screening method allowing detection of 300 polar metabolites across multiple biological pathways involved in cancer, cardiovascular, neurodegenerative, Diabetes and Obesity.

Conclusions

The sensitivity and speed of the QTRAP® 6000+ with IonDrive Technology allows an efficient high throughput assay by using ± polarity switching (5 min) in a single injection sample.

MD MicroLC reduces solvent consumption and costs.

References


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