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Hydrophilic interaction chromatography–mass spectrometry for anionic metabolic profiling of urine from antibiotic-treated rats☆

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ABSTRACT

Hydrophilic interaction chromatography–mass spectrometry (HILIC–MS) was used for anionic metabolic profiling of urine from antibiotic-treated rats to study microbial–host co-metabolism. Rats were treated with the antibiotics penicillin G and streptomycin sulfate for four or eight days and compared to a control group. Urine samples were collected at day zero, four and eight, and analyzed by HILIC–MS. Multivariate data analysis was applied to the urinary metabolic profiles to identify biochemical variation between the treatment groups. Principal component analysis found a clear distinction between those animals receiving antibiotics and the control animals, with twenty-nine discriminatory compounds of which twenty were down-regulated and nine up-regulated upon treatment. In the treatment group receiving antibiotics for four days, a recovery effect was observed for seven compounds after cessation of antibiotic administration. Thirteen discriminatory compounds could be putatively identified based on their accurate mass, including aconitic acid, benzenediol sulfate, ferulic acid sulfate, hippuric acid, indoxyl sulfate, penicillin G, phenol and vanillin 4-sulfate. The rat urine samples had previously been analyzed by capillary electrophoresis (CE) with MS detection and proton nuclear magnetic resonance (^{1}H NMR) spectroscopy. Using CE–MS and ^{1}H NMR spectroscopy seventeen and twenty-five discriminatory compounds were found, respectively. Both hippuric acid and indoxyl sulfate were detected across all three platforms. Additionally, eight compounds were observed with both HILIC–MS and CE–MS. Overall, HILIC–MS appears to be highly complementary to CE–MS and ^{1}H NMR spectroscopy, identifying additional compounds that discriminate the urine samples from antibiotic-treated and control rats.

1. Introduction

The gut microbiota, the population of microorganisms that inhabit the gastrointestinal tract, is involved in the regulation of various metabolic pathways of the host via interactive and symbiotic microbial–host co-metabolism. Such interactions can be modulated by the diet, disease and the use of antibiotics, and contribute significantly to the metabolic phenotype of the host. The gut microbiota is a highly dynamic and expansive variable and there are several ways to study the interactions between these microbes and the host. One approach that has provided great insight into these trans-genomic interactions is the application of metabolic profiling to study biological samples from animal models of modified gut microbial states. Metabolic profiling (metabonomics/metabolomics) is the comprehensive analysis

Abbreviations: ABx-Dy, samples taken at day *y* after the use of antibiotics for *x* days; CE, capillary electrophoresis; ESI, electrospray ionization; GC, gas chromatography; HILIC, hydrophilic interaction chromatography; HPPA, hydroxyphenylpropionic acid; ^{1}H NMR, proton nuclear magnetic resonance; LC, liquid chromatography; MS, mass spectrometry; PCA, principal component analysis; QC, quality control; RSD, relative standard deviation.

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of metabolites in a biological system, providing information about the physiology and biochemical pathways of an organism [1–7]. Germ-free animal models or animals of which the microbiota is depleted through oral antibiotic treatment are typically used to study the influence of the gut microbiota on host metabolism. Urine and/or fecal samples of these animals have been analyzed using various analytical techniques, including proton nuclear magnetic resonance (^{1}H NMR) spectroscopy, gas chromatography (GC), reversed-phase liquid chromatography (LC) and capillary electrophoresis (CE) [8–15]. Most often, GC, LC and CE are coupled to mass spectrometry (MS) in order to obtain both sensitive detection and information on metabolite identification. None of the applied analytical techniques is individually capable of measuring the complete set of metabolites within a sample, since metabolites have diverse physicochemical properties and are present at concentrations that span over nine orders of magnitude [16]. However, various methodologies can provide complementary information about biochemical processes and when used in combination can increase the coverage of the metabolome, improving the understanding of microbial–host co-metabolism. All reported studies exploring the metabolic impact of orally administered antibiotics have found significant variation in urinary metabolic profiles pre- and post-exposure [8–15]. The observed differences were based on metabolites from various compound classes, detected with different analytical techniques. For instance, using ^{1}H NMR spectroscopy differences in concentration levels of amino and organic acids before and after treatment were found [8,10,12,15]. In studies applying GC and LC, the discrimination was mainly based on fluctuating levels of oligosaccharides, pyridines and purines [9]. When CE was employed, a variety of polar compounds was found to be discriminatory [14].

Hydrophilic interaction chromatography (HILIC)–MS is a metabolic profiling approach that is effective for measuring polar metabolites. Such metabolites are separated based on a combination of partitioning and electrostatic interactions with a polar stationary phase [17–19]. HILIC–MS can provide additional biochemical information following antibiotic treatment, complimentary to previously used techniques, thereby enhancing the understanding of host-microbiome co-metabolism. CE–MS has also been used to gain information about the polar components of the metabolome, but as shown before, HILIC–MS can be highly complementary [20,21]. Ibáñez and coworkers used both HILIC–MS and CE–MS in positive ionization mode to study the effect of polyphenols on the proliferation of colon cancer cells. Using HILIC–MS, 1077 features were observed, of which 214 were differentially expressed in the colon cancer cells after treatment with polyphenols. With CE–MS 2890 features were detected, of which 212 compounds were altered significantly. Of these compounds, 12 and 22 metabolites were putatively identified with HILIC–MS and CE–MS, respectively. There was no overlap in identified metabolites showing the complementarity of HILIC–MS and CE–MS [20]. Saric et al. analyzed the *Fasciola hepatica* metabolome and identified 37 and 90 metabolites when applying CE–MS and HILIC–MS in positive ionization mode, respectively. In total, 29 of these identified metabolites were found with both techniques. This implies that employing HILIC–MS in addition to CE–MS resulted in the identification of an additional 61 compounds [21].

In many metabolic profiling studies, the focus is often primarily on measuring cationogenic metabolites using positive electrospray ionization (ESI). However, a significant number of metabolites of interest is acidic and can only be ionized efficiently using negative ESI. In this study, we evaluated the applicability of HILIC–MS for anionic metabolic profiling of urine samples from control rats and those receiving the antibiotics penicillin G and streptomycin sulfate for four or eight days. Urine samples were collected at the beginning of the study (day 0) and on days four and eight. Following HILIC–MS analysis, the resulting metabolic profiles were compared using principal component analysis (PCA). Metabolites that appeared to be up- or down-regulated upon antibiotic treatment were putatively identified based on observed masses. The same rat urine samples had previously been analyzed with CE–MS and ^{1}H NMR spectroscopy to study microbial–host co-metabolism [8,14]. This gave an excellent opportunity to compare the diverse analytical techniques and to determine the complementarity and added value of HILIC–MS for metabolic profiling studies. The comparison was based on the number, identity and relative abundance of discriminatory compounds.

2. Materials and methods

2.1. Chemicals

Acetonitrile was supplied by Fluka (Steinheim, Germany) and ammonium acetate was obtained from Merck (Darmstadt, Germany). Prior to use, water was deionized and purified with a Milli-Q purification system (Millipore, Bedford, USA).

2.2. Rat urine samples

Urine samples were previously analyzed by CE–MS and ^{1}H NMR spectroscopy, and therefore have been extensively described elsewhere [8,14]. Briefly, urine samples were taken from eighteen male Wistar derived AlpkHsdRccHan:WIST rats, which were divided into three treatment groups of six rats each. The antibiotics penicillin G (2 mg/mL) and streptomycin sulfate (4 mg/mL) were provided ad libitum for 0 (AB0), 4 (AB4) or 8 days (AB8) in the rats' drinking water. Urine samples were collected overnight for 16 h on days −1 to 0 (D0), 3–4 (D4) and 7–8 (D8). All urine samples (n = 54) were randomly analyzed after dilution with acetonitrile in a proportion of 1:4, v/v and centrifugation at 10,000 rcf for 10 min. A mixture of aliquots of all urine samples was used as quality control (QC) sample and measured after every fifth run to assess the stability of the HILIC–MS system.

2.3. HILIC–MS

All rat urine samples (5 μL) were analyzed on a Waters XBridge Amide column (3.0 mm × 100 mm) with 3.5 μm particles, maintained at 45 °C during separation. HILIC–MS analyses were performed on a Shimadzu LC system (Kyoto, Japan) coupled to a time-of-flight mass spectrometer (micrOTOF, Bruker Daltonics, Bremen, Germany). Urine samples were analyzed under gradient elution with (A) 10 mM ammonium acetate (pH 6.8) in water-acetonitrile (1:1, v/v) and (B) 10 mM ammonium acetate (pH 6.8) in water-acetonitrile (1:9, v/v) at a flow rate of 0.5 mL/min. The gradient started at 100% B for 5 min, followed by a gradual decrease to 0% B in 10 min. Thereafter, the system was maintained at 100% A for 5 min, after which it was immediately switched to 100% B for reequilibration of the HILIC–MS system. A new measurement was started after 10 min at 100% B.

The mass spectrometer was operated with negative ESI, applying the following conditions: dry gas temperature, 180 °C; dry gas flow, 4 L/min, nebulizer pressure, 50 psi; ESI voltage, 2 kV. Data were acquired in the mass range m/z 50–800 with a repetition rate of 1 Hz. Recorded mass spectra were internally calibrated using sodium acetate clusters.

2.4. Data analysis

The obtained metabolic profiles were aligned using the reference peak warping function of MsXelerator software (MsMetrix, Maarssen, The Netherlands) in order to allow correct comparison