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Comparison of capillary electrophoresis–mass spectrometry and hydrophilic interaction chromatography–mass spectrometry for anionic metabolic profiling of urine

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ABSTRACT

In order to assess the utility of a recently developed capillary electrophoresis–mass spectrometry (CE–MS) method for the study of anionic metabolites in urine, a comparison was made with hydrophilic interaction chromatography–MS (HILIC–MS) using negative electrospray ionization. After optimization of the HILIC conditions, a gradient employing 10 mM ammonium acetate (pH 6.8) in acetonitrile–water (5 min 90% acetonitrile followed by 90%–50% acetonitrile in 10 min) was selected, providing baseline separation of five representative anionic test metabolites. Relative standard deviations (RSDs) for HILIC retention times and peak areas were below 0.2% and 7.7%, respectively, and detection limits were in the range 0.04–2.21 μM. Metabolites in rat urine could also be analysed in a reproducible way with retention time and peak area RSDs below 0.6% and 13.6%, respectively. The CE–MS and HILIC–MS methods were compared in terms of reproducibility, sensitivity, selectivity and coverage of the anionic urinary metabolome. In general, peak area RSDs were similar whereas HILIC–MS yielded better retention-time repeatability and up to 80 times lower detection limits (expressed in injected concentration) for test metabolites as compared to CE–MS. Rat urine analysis by HILIC–MS provided detection of 1360 molecular features compared to 347 molecular features revealed with CE–MS. Of these, a number of 144 molecular features were found with both HILIC–MS and CE–MS, which showed on average 10 times higher peak areas in HILIC–MS. The HILIC retention and CE migration times of the common features were clearly not correlated. The HILIC and CE behavior of the test metabolites and 16 putatively identified common features were evaluated involving their physicochemical properties, indicating a markedly different separation selectivity, and thus significant degree of orthogonality of HILIC and CE.

1. Introduction

Elucidation and understanding of biochemical pathways require accurate and specific determination of metabolite levels in biofluids and tissues. Metabolites may exhibit very diverse physicochemical properties and can be present in a wide range of concentrations [1]. Capillary electrophoresis (CE) coupled to mass spectrometry (MS) is particularly suitable for the direct profiling of highly polar and charged metabolites as predominantly present in urine. CE can provide efficient separation of metabolites based on their charge-to-size ratios without the need for analyte derivatization [2]. Recently, we have developed a CE–MS method for the profiling of anionic metabolites applying triethylamine in the background electrolyte (BGE) and sheath liquid [3]. This new method provided significantly higher signal intensities as compared to other CE–MS methods in negative ionization mode. Urine samples from antibiotic-treated rats and control rats could be distinguished, and potential biomarkers were revealed [4]. Having this new method available, the question arises to what extent CE–MS can provide complementary information on the anionic metabolome with respect to more common liquid chromatographic (LC) techniques for metabolic profiling.

LC–MS can provide information on the quantity of low-abundant metabolites without the need for analyte derivatization. Common reversed-phase (RP) LC, however, is less suited for the analysis of highly polar compounds, and for small charged

Abbreviations: BGE, background electrolyte; CE:, capillary electrophoresis; ESI:, electrospray ionization; GC:, gas chromatography; HILIC:, hydrophilic interaction chromatography; LC, liquid chromatography; LOD, limit of detection; MS, mass spectrometry; RP:, reversed-phase; RSD, relative standard deviation; TOF, time-of-flight

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compounds, ion-pairing agents are frequently used to increase their retention [5]. Unfortunately, ion-pairing agents can cause substantial ionization suppression of compounds in MS and source contamination. Hydrophilic interaction chromatography (HILIC) allows profiling of polar compounds, providing complementary information to RPLC, as has been demonstrated by numerous metabolomics studies [6–19]. HILIC utilizes a highly organic mobile phase (often acetonitrile) containing water in combination with a polar stationary phase. Analyte separation is based on partitioning between the mobile phase and a layer of adsorbed water molecules on the stationary phase as well as electrostatic interactions with polar groups on the stationary phase [20–22]. Hence, in order to appreciate the utility of CE–MS for profiling of anionic metabolites, comparison with HILIC–MS seems indicated.

So far, only a few studies have been conducted in which both CE–MS and HILIC–MS were used for comparative and comprehensive profiling [17,23–26]. Sugimoto *et al.* applied HILIC–MS and CE–MS to analyse edamame and Japanese sake [23,24]. However, as HILIC–MS was used to profile sugars only, no conclusions could be drawn on the complementarity of HILIC–MS and CE–MS for untargeted metabolite profiling. Büscher and colleagues extensively compared CE, LC and gas chromatography (GC) methodologies, all in combination with MS [25]. Metabolites were analysed with GC–MS after two different derivatization procedures. Ion-pair RPLC and HILIC were used as LC separation modes and two CE–MS methods were employed for the analysis of cationic and anionic metabolites. A mixture of 91 test metabolites representing central carbon and energy metabolism was used for comparison. Of these test metabolites, 33 compounds could be detected by all three platforms. CE and LC showed the greatest overlap in metabolite coverage (26 compounds) and each analytical technique was capable to measure two compounds which could not be detected with the two other methodologies. The CE–MS, LC–MS and GC–MS methodologies were considered in general, that is, GC, HILIC, ion-pair RPLC and cationic and anionic CE methods were not individually compared. Moreover, the evaluation of the complementarity of the techniques for metabolic profiling was limited since it was based on a confined number of test metabolites in standard solutions [25]. Ibáñez and coworkers studied the effect of dietary polyphenols on the proliferation of colon cancer cells with CE–MS, HILIC–MS and RPLC–MS [26]. Saric *et al.* used the same techniques to analyse the metabolome of the *Fasciola hepatica* worm [17]. In both studies, a small part of the detected metabolite features was identified. There was no or a limited overlap of identified metabolites detected by CE–MS, HILIC–MS and RPLC–MS, indicating the potential complementarity of the analytical techniques [17,26]. It should be noted, however, that Ibáñez *et al.* as well as Saric *et al.* employed positive electrospray ionization (ESI) for HILIC–MS and/or CE–MS, and thus could only compare the methodologies based on detected cationogenic compounds. Still, a significant number of urinary metabolites is acidic and can only be detected using negative ESI. Therefore, in order to achieve comprehensive profiling and to evaluate the complementarity of HILIC–MS and CE–MS, particular attention should also be paid to anionic metabolites.

In the present study, we compared a previously optimized CE–MS method with HILIC–MS for anionic metabolic profiling of urine samples. We first optimized a HILIC–MS method by carrying out infusion experiments to determine and evaluate the effect of different HILIC mobile phase compositions on the signal intensities of representative anionic test metabolites. An efficient gradient HILIC–MS method applying an acetonitrile–water mobile phase containing ammonium acetate was developed and the performance was assessed in terms of sensitivity, linearity and repeatabilities of peak area and retention time. Furthermore, HILIC–MS was applied to urine analysis and the number of detected molecular features of urine components was determined. Outcomes were extensively compared with results obtained with the earlier developed CE–MS method for anionic urinary profiling [3,4]. Numbers of common and unique urinary molecular features were considered and MS responses of common features were compared. Moreover, differences in separation selectivity of CE–MS and HILIC–MS were assessed by comparing migration and retention times of test metabolites as well as putatively identified common features.

2. Materials and methods

2.1. Chemicals

Acetic acid, ammonium formate, ammonium hydroxide (25% solution), acetonitrile, formic acid, methanol, glutaric acid, hippuric acid, DL-pyroglutamic acid and uridine were obtained from Fluka (Steinheim, Germany). Sodium hydroxide and L-proline were purchased from Sigma Aldrich (Steinheim, Germany). Triethylamine was from Fisher Scientific (Loughborough, UK), piperidine was from Alfa Aesar (Karlsruhe, Germany) and ammonium acetate was supplied by Merck (Darmstadt, Germany). Water was deionized and purified with a Milli-Q purification system (Millipore, Bedford, USA) prior to use.

2.2. Test mixture and rat urine sample

Stock solutions (50 mM) of the metabolites glutaric acid, hippuric acid, proline, pyroglutamic acid and uridine were prepared in deionized water. Stock solutions of the metabolites were mixed and diluted to obtain a test mixture in which each metabolite was present at the appropriate concentration (0.25–80 μM). Test metabolite mixtures were prepared in water–acetonitrile (1:4, v/v) and water for HILIC–MS and CE–MS analyses, respectively.

A mixture of aliquots of rat urine samples provided by AstraZeneca (Department of Drug Metabolism and Pharmacokinetics, Macclesfield, UK) [27] was prepared and stored at −80 °C. Prior to analysis, the urine sample was thawed. For HILIC–MS, urine was diluted with acetonitrile in a proportion of 1:4 (v/v) and centrifuged at 10,000 rcf for 10 min in order to attain proper metabolite peak shapes (see Section 3.1). When analysed with CE–MS, dilution of urine with BGE in a proportion of 1:1 (v/v) was sufficient to achieve good CE performance [3,4].

2.3. HILIC–MS

All samples were analysed on an LC system (Shimadzu, Kyoto, Japan) coupled online via an electrospray interface to a time-of-flight (TOF) mass spectrometer (micrOTOF, Bruker Daltonics, Bremen, Germany) using a Waters XBridge™ Amide column (3.5 μm, 3.0 × 100 mm).

In the optimized HILIC–MS method, the test metabolite mixture and urine sample (5 μL; approximately 1.5% of column volume) were injected and analysed under gradient elution with mixing solvent A (10 mM ammonium acetate in water–acetonitrile (1:1, v/v)) and solvent B (10 mM ammonium acetate in water–acetonitrile (1:9, v/v)) in varying ratios at an overall flow rate of 0.5 mL/min. The gradient scheme was as follows: 0.0–5.0 min, 100% B; 5.0–15.0 min, from 100% B to 100% A; 15.0–20.0 min, 100% A; 20.1–30.0 min, 100% B. Column temperature was 45 °C during separation.

Optimal signal intensities for test metabolites were obtained using the following interface conditions: dry gas temperature, 180 °C; dry gas flow, 4 L/min, nebulizer pressure, 50 psi; ESI voltage, 2 kV. Data were acquired in negative ionization mode in the mass range *m/z* 50–800 with a repetition rate of 1 Hz.