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Assessment of capillary anion exchange ion chromatography tandem mass spectrometry for the quantitative profiling of the phosphometabolome and organic acids in biological extracts

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ABSTRACT

Metabolic profiling has become an important tool in biological research, and the chromatographic separation of metabolites coupled with mass spectrometric detection is the most frequently used approach for such studies. The establishment of robust chromatographic methods for comprehensive coverage of the anionic metabolite pool is especially challenging. In this study, the development of a capillary ion exchange chromatography (capIC) – negative ESI tandem mass spectrometry (MS/MS) workflow for the quantitative profiling of the phosphometabolome (e.g., sugar phosphates and nucleotides) is presented. The chromatographic separation and MS/MS conditions were optimized, and the precision of repetitive injections and accuracy in terms of error percentage to true concentration were assessed. The precision is excellent for a capillary flow system with an average CV% of 8.5% for a 50-fmol standard injection and in the lower 2.4–4.4% range for higher concentrations (500–7500 fmol). The limit of detection (LOD) ranges from 1 to 100 nM (5–500 fmol injected on column), and the limit of quantitation (LOQ) ranges from 1 to 500 nM (5–2500 fmol injected on column). A fast gradient method with the injection of 50% methanol in water between analytical samples is needed to eliminate carry-over and ensure optimal re-equilibration of the column. Finally, the quantitative applicability of the system was tested on real biological matrices using the constant-volume standard addition method (SAM). Extracts of the human kidney Hek293 cell line were spiked with increasing concentrations of standards to determine the concentration of each metabolite in the sample. Forty-four metabolites were detected with an average uncertainty of 4.1%. Thus, the capIC-MS/MS method exhibits excellent selectivity, sensitivity and precision for the quantitative profiling of the phosphometabolome.

1. Introduction

The metabolome is a heterogeneous collection of charged low-molecular-weight species; thus, the comprehensive profiling of metabolites is analytically challenging. Metabolite profiling by the combination of chromatographic separation and mass spectrometric detection is one of the most advanced tools in metabolomics studies, providing quantitative or qualitative information on selected metabolite groups [1]. Metabolites belonging to the phosphometabolome (i.e., nucleotides, sugar phosphates and phospho-carboxylic acids) and organic acids are intermediates in primary metabolism (glycolysis, the pentose-phosphate pathway, tricarboxylic acid) and are therefore essential in all life processes. Thus, the determination of their concentration is critical to understanding biological processes, such as primary and secondary metabolism, energy states, cell cycles, signal-transduction pathways and stress responses due to environmental perturbation [2–4].

Various analytical techniques have been developed to analyze the constituents of the phosphometabolome; however, these techniques offer varying coverage, sensitivity and selectivity. Traditionally, laborious enzymatic assays [5–9] and HPLC-ultraviolet light detection [10] have been used to determine the concentrations of one or several analytes. However, mass spectrometric (MS) detection offers superior selectivity and the simultaneous profiling of tens of metabolites. Biological extracts are complex, and a separation step is needed prior to MS detection. The phosphometabolome contains negatively charged species, which are poorly retained and separated using most popular separation techniques,

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e.g., reverse-phase (RP) LC. Capillary electrophoreses (CE)-MS is used in a few laboratories [11,12] but has not been established as a general reference methodology, probably due to its low injection volume and technical challenges for negatively charged analytes. Liquid chromatography (LC) is more widely used than any other separation technique. Porous graphitic carbon (PGC) columns have been used for the separation of nucleotides [13–15]; however, their use is limited by their shorter stable retention times caused by a low backpressure. Hydrophilic interaction liquid chromatography (HILIC) is a frequently used LC technique with applications in both the quantitative and qualitative analysis of phosphorylated metabolites [16]. HILIC is especially suitable for polar metabolites; however, like PGC, it has struggled to gain popularity in the metabolomics community because of its high retention time drift as well as the difficulty in obtaining good resolution between isobaric compounds and an overall good peak shape. Some reports indicate that the resolving power of HILIC columns is better when using high-pH mobile phases and amide stationary phase columns [17] than in traditional HILIC separations, which use low-pH and zwitterionic columns [18]. The most widely used approach for negatively charged phosphorylated metabolites by far is ion pair (IP) RP LC coupled to MS, in which a cationic component in the mobile phase (e.g., tributylamine) is used to form an ion pair with the negatively charged phosphorous metabolites, allowing separation on an RP column [19–21]. A major disadvantage of this separation technique is its low throughput, its retention time drift, the extensive cleaning procedure of the instrumentation due to the nature of the "sticky" ion-pair reagent, its limited column life and the difficulty in establishing reproducible conditions for sugar phosphate separation [14].

The analysis of small inorganic anions with ion chromatography (IC), the high-performance version of ion-exchange chromatography, and conductimetric detection was first reported by Small and co-workers [22]. Until recently, IC has received little attention in the field of metabolomics due to the limitations of conductimetric and UV detection and the high pH of the eluent. However, the development of a membrane-based electrolytic suppressor enables continuous pH neutralization and removal of non-volatile mobile-phase salts by post-column electrochemical suppression [23]. This proton–cation mobile-phase exchange makes IC separation fully compatible with MS because it prevents salts from reaching the ion source at any time during the analysis. For small anionic molecules, monolithic polystyrene stationary phases are now the most frequently used [10]. These columns are densely packed with charged sites (e.g., alkanol quaternary ammonium ions) and can often lead to irreversible binding of macromolecules [24]. However, the columns are well suited for the separation of smaller molecules (e.g., nucleotides, sugars, sugar nucleotides and organic acids) [25–27]. Van Dam and co-workers reported the analysis of sugar phosphates in *Saccharomyces cerevisiae* extracts on an analytical flow IC-ESI MS/MS system [28], while Lunn and co-workers also included nucleotides on a similar system [29]. In addition, a prototype capillary version of IC coupled to MS for the analysis of oligosaccharides has been reported [30]. Capillary IC (capIC) uses smaller sample volumes, consumes less eluent and has a lower flow rate, increasing its sensitivity. Burgess and co-workers [18] and Wang and co-workers [31] presented a reagent-free capillary anion-exchange chromatographic method coupled to orbitrap MS. Both reports demonstrated the superior resolving power of capIC compared to HILIC and reverse-phase chromatography for the phosphometabolite analysis of extracts from *Trypanosoma brucei* and several mammalian cells.

However, the quantitative performance of a capIC-MS/MS system for the detection of phosphorylated metabolites has yet to be reported. Herein, we present the development and assessment of a capIC-MS/MS method that separates and quantifies a selection of 54 metabolites from glycolysis, TCA, the pentose-phosphate pathway and the nucleotide metabolism, including a capIC-compatible sample preparation protocol of adherently growing mammalian cells. The precision and accuracy were evaluated, and the applicability of the method was tested on real biological extracts by spiking four different concentrations of an analytical standard mixture of *Hek293* cell extracts to calculate the metabolite concentration by the standard addition method (SAM).

2. Materials and methods

2.1. Chemicals

Standards were bought from Sigma–Aldrich. They were weighed out and dissolved according to the specifications given by Sigma–Aldrich, preferably in water–acetonitrile (50/50), and prepared as 100 mM or 1 mM stock solutions depending on the amount of each substance available. The initial experiments with water vs. 50% ACN-water proved that 50% ACN-water was superior to pure water for ensuring the optimal stability of di- and triphosphate nucleotides. Preliminary testing of the method revealed lower stability and the hydrolysis of triphosphates into diphosphates and monophosphates when dissolved in water only (data not shown). This phenomenon is not exclusively an ion source effect, as the triphosphate hydrolysis to mono- and di-nucleotides was also observed on the CD chromatogram.

2.2. Cultivation

The human embryonic kidney cell line *Hek293* (ATCC CRL 1573) was grown in 75-cm^2 culture flasks (#156499, Nunc) containing 15 mL of DMEM (#21063-029, Life Technologies), supplemented with 10% foetal bovine serum (#F7524, Sigma–Aldrich), 100 μg/mL gentamycin (#G1272, Sigma–Aldrich), 2.5 μg/mL amphotericin B (#A2942, Sigma–Aldrich) and 2 mM L-glutamine (#K0283, Biochrome), and incubated at 37 °C in a humidified atmosphere with 5% CO_2. The cells were then washed twice with 10 mL of phosphate-buffered saline (PBS) (#BR0014G, Oxoid) after aspiration of the growth medium, followed by enzymatic detachment by 1 mL of trypsin/EDTA solution and reconstitution in 15 mL of fresh medium. Next, 100 μL of the cell suspension was diluted by 1:10 in fresh medium before counting by a Moxi^z Cell counter, Type M cassette. In total, 8.0×10^5 cells were then seeded onto 90-mm tissue culture dishes (#734-2320, VWR) in 10 mL of fresh medium. The cells were grown for 48 h to 50–60% confluence (visually observed using a microscope) to approximately 2.4×10^6 cells on the plate prior to quenching and the extraction of intracellular metabolites.

2.3. Metabolome sampling

Sampling of the intracellular metabolome of *Hek293* cells started with the removal of the growth medium by pouring off the medium and placing the culture dish on a massive iron plate held at −80 °C. The attached cells were washed with 10 mL of cold (0 °C) 0.9% NaCl solution prepared from 18.2-MΩ de-ionized water (DI water) followed by a rapid 10-mL cold (0 °C) DI water rinse for less than 2 s. The washing solutions were carefully transferred onto the tissue culture dish with a bottle-top dispenser volumetric pipette (#613-5281, VWR) with a plastic tube connecting the dispenser and the tissue culture dish. This tube was used to direct the washing solutions onto the dish without removing the dish from the cold iron plate. Cold 55% ACN in DI water (5.5 mL, 0 °C) was added to the tissue culture dish using a bottle-top organic dispenser (#4730150, Brand). Mechanical detachment of the cells with a cell scraper was performed instantaneously after adding the extraction solvent. All visual cell materials and solvent were transferred into a 50-mL tube.