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A UPLC–MS/MS application for profiling of intermediary energy metabolites in microdialysis samples—A method for high-throughput

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ABSTRACT

Research within the field of metabolite profiling has already illuminated our understanding of a variety of physiological and pathological processes. Microdialysis has added further refinement to previous models and has allowed the testing of new hypotheses. In the present study, a new ultra-performance liquid chromatography/electrospray-tandem mass spectrometry (UPLC-ESI-MS/MS) method for the simultaneous detection and quantification of intermediary energy metabolites in microdialysates was developed. The targeted metabolites were mainly from the citric acid cycle in combination with pyruvic acid, lactic acid, and the ATP (adenosine triphosphate) hydrolysis product adenosine along with metabolites of adenosine. This method was successfully applied to analyze the microdialysates obtained from an experimental animal study giving insight into the hitherto unknown concentration of many interstitial energy metabolites, such as succinic acid and malic acid. With a total cycle time of 3 min, injection to injection, this method permits analysis of a much larger number of samples in comparison with conventional high performance liquid chromatography/tandem mass spectrometry HPLC–MS/MS strategies. With this novel combination where microdialysis and high sensitivity UPLC–MS/MS technique is combined within cardiologic research, new insights into the intermediary energy metabolism during ischemia–reperfusion is now feasible.

1. Introduction

Targeted metabolomics has within recent years provided a more profound insight into human metabolism and has led to a better understanding of the metabolic processes taking place in e.g. normal and hypoxic tissue. The targeted approach relies on precise quantitative measurements of metabolites within a complex, heterogeneous, and dynamic biological system. The advances within this field have been driven by the continuous development of more sensitive analytical instruments in nuclear magnetic resonance spectroscopy and mass spectrometry and have led to increasing use of this approach in several scientific fields, including cardiovascular medicine [1].

Human myocardial metabolism has been the subject of intense scrutiny. This fact has influenced the development of a series of models that attempt to describe myocardial substrate utilization during health and disease [2]. In this context, ischemia–reperfusion (IR) injury, whereby the act of restoring perfusion to hypoxic tissue results in further injury, is of profound clinical interest. Myocardial infarction is a major cause of morbidity and mortality in Western countries and is predicted to be the leading cause of disease burden world-wide by 2020 [3].

While ischemia–reperfusion injury initially appears to be a relatively simple process, the underlying mechanisms leading to this enhanced tissue injury are very complex, and our understanding remains incomplete. Increasing evidence, supports the hypothesis that myocardial energetics is of major importance in ischemia–reperfusion and that strategies that manipulate myocardial energy metabolism may result in cardioprotection [4]. Obviously, methods capable of providing specific metabolic fingerprints of the cellular energy mechanisms during cardiac ischemia are of outmost importance in improving our understanding of its underlying processes.

To accomplish this goal, we set out to develop a tissue-specific microdialysis sampling method coupled with selective and sensitive UPLC–MS/MS analysis capable of monitoring the

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bioenergetic cycle (citric acid cycle, adenosine, glutamate, etc.) during ischemia, and of measuring changes in metabolism associated with protective interventions such as preconditioning. Compared to traditional blood or tissue sampling methods, microdialysis allows continuous, tissue-specific sampling. In principle, it offers the capability to monitor local, dynamic metabolic transitions associated with e.g. changes in oxygen tension. Some previous work in the field of metabolic profiling within cardiologic research has focused on profiling citric acid cycle metabolites measured in effluents or freeze-clamped tissue samples. The analytical techniques used have either been gas chromatography–mass spectrometry (GC–MS), HPLC–MS/MS or enzymatic spectrophotometric assays [5–10]. These studies focused on a select numbers of metabolites and no one has measured the complete group of citric acid cycle metabolites. Furthermore, analysis of microdialysate samples for metabolites associated with the citric acid cycle has so far been limited to pyruvic acid and lactic acid. Concentrations of these two organic acids have been measured (in microdialysates) by enzymatic reactions and analysis of resultant colorimetric products. The values found for pyruvic acid and lactic acid, both measured in pig hearts, were in the μM and mM range, respectively [11,12]. Based on a comparison of blood TCA, pyruvic acid and lactic acid concentrations, levels of the remaining carboxylic acids in the tricarboxylic acid (TCA) cycle are however expected to be even lower [13]. To our knowledge, the exact levels of these metabolites in the interstitial tissue from ischemic hearts are unknown.

The classical method of analyzing organic acids in biological samples is the use of GC–MS with derivatization of the analytes. However, when dealing with a large number of samples of very small sample volumes (2–10 μl), derivatization is preferably avoided. In comparison, LC–MS/MS offers the advantage of allowing analysis of the samples without derivatization. Previous reports have demonstrated the use of HPLC–MS/MS with matrices other than microdialysates and with detection limits in the range suitable for monitoring organic acids. However, such methods often requires run times exceeding 1 h, which is impractical for a high-throughput setup [14]. Recently, improvements in chromatography have been obtained with the use of novel reversed-phase columns equipped with smaller particle sizes, as well as with HPLC equipment capable of handling higher pressures (e.g. UPLC and UHPLC chromatographs). UPLC enables faster run time, which is a great advantage when large numbers of samples are to be analyzed [15]. To the best of our knowledge, no one has used microdialysates as sample specimen in combination with LC–MS/MS or the more novel UPLC–MS/MS analytical technique to monitor changes in levels of TCA metabolites[1],[2].

We report here a highly sensitive, UPLC–MS/MS method for the simultaneous detection and quantification of eight organic acids mainly of the citric acid cycle, glutamic acid, and four adenosine triphosphate (ATP) metabolites. This method has been successfully applied to the determination of the concentrations of these metabolites in microdialysis samples taken from an *in vivo* neonatal porcine model. This combined methodology enables the study of a vast number and range of metabolites than previously performed in microdialysates. This system is not only dynamic but also provides more detail than has previously been available about the metabolic changes that occur in the citric acid cycle during ischemia–reperfusion and how these changes are modified by interventions to reduce ischemia–reperfusion injury.

[1] Previously, the combination of microdialysis and UPLC-MS/MS has been applied in other fields see e.g. [16,17].

[2] UPLC-MS/MS was recently successfully applied to profile organic acids during fermentation [18].

2. Experimental

2.1. Reagents and materials

HPLC-grade methanol (MeOH), formic, citric, isocitric, fumaric, pyruvic, succinic, lactic, malic, and α-ketoglutaric acids, adenosine, inosine, hypoxanthine, xanthine and glutamic acid as well as internal standards $^{2}H_4$-citric, $^{2}H_4$-succinic, $^{2}H_3$-malic, $^{2}H_6$-α-ketoglutaric, $^{13}C_5$-glutamic, $^{2}H_3$-lactic, and $^{13}C_1$-pyruvic acids were obtained from Sigma–Aldrich, Denmark. The internal standards $^{15}N_2$-xanthine, $^{2}H_2$-adenosine, and $^{13}C_4$-fumaric acid were obtained from Cambridge Isotope Laboratories, Inc., USA. Water was provided by a millipore system. Krebs–Henseleit buffer (aqueous solution of: NaCl 118.5 mM; KCl 4.7 mM; $NaHCO_3$ 25.0 mM; glucose monohydrate 11.1 mM; $MgSO_4{\cdot}7H_2O$ 1.2 mM; $CaCl_2$ 2.4 mM; KH_2PO_4 1.2 mM, all obtained from Sigma–Aldrich, Denmark) was prepared and deoxygenated with (95% N_2/5% CO_2) prior to use.

2.2. Instrumentation and equipment

Sample analysis was performed on a Waters ACQUITY™ ultra-performance liquid chromatography (UPLC) system (Waters Corp., Milford, MA, USA). Detection of the analytes was carried out using a Waters Xevo™ triple quadrupole tandem mass spectrometer (Waters Corp., Manchester, UK) with a Z-spray electrospray ionization (ESI) source operating both in the positive and negative ion modes. To obtain microdialysate samples, a Univenter U820 microsampler and Univenter U801 pump, (Zejtun, Malta), were used in combination with microdialysate catheters CMA 20 Elite probe (20 kDa molecular weight cut off, 10 mm membrane length with a flow rate of 1 μl/min) (CMA, Solna, Sweden).

2.3. Method development

2.3.1. UPLC conditions

Chromatographic separation was performed at 45 °C using an Acquity HSS C_{18} column (100 mm × 2.1 mm i.d., 1.7 μm; Waters Corp., Milford, USA) equipped with an ACQUITY UPLC VanGuard Pre-Column. The following eluents were used: solvent A: H_2O, 0.2% (formic acid) (v/v); solvent B: MeOH, 0.1% (formic acid) (v/v). The gradient elution was as follows: 0–0.2 min isocratic 5% B, 0.2–1.0 min linear from 5% to 90% B, 1.0–1.5 min isocratic 90% B, and 1.5–1.8 min linear from 90% to 5% B, with 1.8–3.0 min for initial conditions of 5% B for column equilibration. The flow rate remained constant at 0.4 ml/min. A 10 μl injection volume with partial loop using needle overfill mode (PLNO) was used. Loop size 20 μl. UPLC method development was carried out using a standard aqueous stock solution of citric, isocitric, malic, fumaric, succinic, pyruvic, α-ketoglutaric, lactic, and L-glutamic acids and inosine, adenosine, hypoxanthine and xanthine (0.4 mM). Prior to analysis, calibration samples were prepared by dilution with Krebs–Henseleit buffer and final addition of internal standards. Calibration samples were prepared at concentrations of 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, and 100 μM, with additional samples of lactic acid prepared at 500 μM and 1, 5, and 10 mM. Chromatographic data were collected and analyzed with Waters MassLynx v4.1 software. Quantification was achieved for each analyte using linear regression analysis of the peak area ratio analyte/IS (weighed $1/X$) versus concentration. For analytes without IS, a regression between peak area analyte (weighed $1/X$) and concentration was used. Krebs–Henseleit buffer was used as solvent, as this medium is used in the microdialysis catheters.