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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.

Table 1
Optimal cone voltage and collision energies for the selected SRM transitions for each metabolite.

Analyte	CV[a]/CE[b]	SRM1[c]	CV/CE	SRM2[d]	RT[e]
Metabolites analyzed using negative ion mode					
Citric acid	18/13	191.0 → 110.9	18/17	191.0 → 86.9	0.97
Isocitric acid	18/20	191.0 → 110.9	18/17	191.0 → 72.9	0.73
Pyruvic acid	20/10	86.9 → 42.9	–	–	0.78
Fumaric acid	18/8	114.9 → 70.9	–	–	1.15
Lactic acid	20/10	88.9 → 42.9	–	–	0.93
Succinic acid	18/12	116.9 → 72.9	18/12	116.9 → 98.9	1.15
α-Ketoglutaric acid	13/11	144.9 → 56.9	13/9	144.9 → 100.9	0.83
Malic acid	18/16	132.9 → 70.9	18/12	132.9 → 114.9	0.76
Metabolites analyzed using positive ion mode					
Hypoxanthine	32/18	137.0 → 94.0	32/18	137.0 → 110.0	0.89
Xanthine	30/16	153.0 → 109.9	30/14	153.0 → 135.8	1.00
Adenosine	22/52	268.1 → 118.9	22/28	268.1 → 136.0	1.16
Inosine	12/10	269.1 → 137.0	12/36	269.1 → 119.0	1.16
Glutamic acid	16/14	148.0 → 84.0	16/10	148.0 → 102.0	0.62

[a] CV, cone voltage (V).
[b] CE, collision energy (eV).
[c] SRM1 used as quantitation transition.
[d] SRM2 used as confirmation transition.
[e] RT, retention time.

2.3.2. MS tuning and optimization

The mass spectrometer was tuned for each individual analyte to obtain the maximum intensity for the precursor ions (see Table 1). When possible, analytes were monitored using two product ions. When performing the selected reaction monitoring (SRM) method, the switching valve was set to direct the column flow to waste during times where no eluting substances were being monitored (0–0.5 and 1.5–3 min). Hereby any late-eluting compounds and the column wash were diverted away from the mass spectrometer.

Nitrogen heated to 600 °C was used as the desolvation gas at a flow rate of 800 l/h. The cone and collision gas (argon) flows were set to 50 l/h and 0.15 ml/min, respectively. The source temperature remained at 150 °C.

The following parameters were used for ESI-MS analysis in negative ion/positive ion mode: capillary (kV) 0.5/3.0, extractor (V) 3/3, LM1 (low mass)/HM1(high mass) resolution 2.8/15.1, and LM2/HM2 resolution 2.8/14.8.

2.3.3. Linearity

Peak area ratios of each analyte/IS versus concentration of the calibrators ($n = 9$) were plotted. Alternatively, for analytes without IS, peak areas of each analyte were determined and plotted as a function of concentration. Data were analyzed using a linear regression (see Section 2.3.1).

2.3.4. Limits of detection and quantification

Limits of detection (LOD) values were estimated based on 3:1 signal-to-noise ratios. Standard stock solutions containing all of the analytes were diluted appropriately at the levels of these estimated values. Repeatedly analyzed standards ($n = 10$) were used to calculate the standard deviation (SD) for each of the analytes. The limit of detection and limit of quantification were calculated based on 3 × SD and 10 × SD, respectively.

2.3.5. Sample carryover

Sample carryover was evaluated by injecting relatively high concentrations (400 μM, lactic acid 25 mM) of the analytes followed by a series of blank injections to elute any residual analyte material. A wash step was included in the analytical method to flush the column with a high concentration of the organic phase (90% methanol) for 30 s. Carryover material from the injection needle was also excluded. After each injection, a needle wash was performed using a strong (acetonitrile: water, 50:50) and a weak (acetonitrile: water, 10:90) wash. The weak needle wash was also used as a seal wash on the UPLC.

2.3.6. Matrix effects

Matrix effects were studied using post-column infusion of each analyte. A 100 μM solution of analyte was infused at a flow rate of 5 μl/min, and continuous measurements of the SRM transitions for each analyte were recorded [19,20]. Simultaneously, a blank solution of water, Krebs–Henseleit buffer or a microdialysis sample was injected (10 μl) and run under the same chromatographic conditions. The injection of a microdialysis sample was included, although it contains endogenous levels of the analytes. The occurrence of ion suppression or enhancement was evaluated by visual comparison of the chromatograms.

2.3.7. Accuracy

The accuracy (bias) of the method was determined by conducting a series of recovery experiments with three pools of microdialysate samples to which analytes were added at low or high concentrations. Accuracy was calculated as the percent recovery of the added analytes.

2.3.8. Precision

The precision of this analytical method was also tested. Because each microdialysate sample contained no more than 2–10 μl, successive injections using the same solution were not possible. A pool of microdialysis samples obtained from a neonatal porcine animal experiment was prepared and aliquotted into separate microdialysis vials. Internal standard was then added to each vial. Three samples were successively analyzed on 5 days, and the inter- and intraday standard deviation was calculated using an analysis of variance (ANOVA).

2.3.9. In vivo ischemia–reperfusion model and microdialysis sampling

Microdialysate samples were obtained from an *in vivo* open chest porcine model. Danish Landrace pigs were used (age 2 ± 1 days, weight 1.1 ± 0.3 kg). The study conforms to the Danish law for animal research (Act no. 1306 of 23/11/2007 Rules in force, The Danish Ministry of Justice) and was performed in accordance with guidelines for animal care and use approved

by The Animal Experiments Inspectorate in Denmark. The surgical procedure is outlined below. Anaesthesia was induced by an intravenous injection of 0.25 ml ketamine (3.8 mg/kg) and 0.25 ml midazolam (0.8 mg/kg) administered through an ear vein. The piglet was intubated with a cuffed endotracheal tube (size 3), and ventilated with pressure control on a respirator (GE Datex-Ohmeda S5 Avance) using an inspiratory pressure of 12–16 cmH_2O, respiratory rate of 30–35 breaths/min, and PEEP pressure of 4 cmH_2O. Anaesthesia was maintained using sevoflurane (MAC 1-1.3) and continued intravenous fentanyl infusion (25–30 μg/(kg h)). Body temperature was held at 38.5–39.5 °C. Hydration was maintained with continuous intravenous infusion of a solution containing isotonic sodium, potassium, and glucose (neo-KNaG) (4 ml/(kg h)). A midline sternotomy was preformed, and the pericardium was opened. A CMA 20 Elite microdialysis probe was inserted into the free wall of the left ventricle using an introducer cannula (0.45 mm × 12 mm) and fixed with tissue glue (LiquiBand).

Samples were acquired during the baseline, regional ischemia, and reperfusion. The myocardium surrounding the catheter became ischemic after ligation of the left anterior descending artery. Reperfusion was obtained by releasing the artery ligation. The microdialysis sampling technique enables access to the metabolites in the ventricular extracellular space with minimal damage inflicted during catheter insertion [21]. To account for any bleeding or leakage from the extracellular space and any induced inflammation, a 45 min baseline period was employed after catheter insertion and prior to the start of microdialysis sampling. Samples were obtained at a 1 μl/min flow rate with a 10-min sampling interval during 60 min of stabilization, 40 min of regional ischemia, and 40 min of reperfusion.

To ensure sufficient relative recovery, a series of *in vitro* experiments were conducted at varying flow rates, and the optimal flow rate was determined. The optimal flow rate is a compromise between relative recovery and the time required to obtain sufficient sample volume.[3] The catheters were placed in an aqueous solution containing 10 μM of each of the 13 metabolites at 37 °C. During sampling, the microdialysis vials were cooled to 4 °C; afterward, they were held at −80 °C. Prior to analysis, the vials were thawed and mixed with internal standards.

The repeatability and accuracy (bias) of the microdialysis microsampler were determined by weighing 25 microdialysis vials before and after a 10-min sampling period (1 μl/min flow rate) and calculating the standard deviation of the collected volumes.

2.3.10. Microdialysis sample preparation for UPLC–MS/MS

The method required no derivatization of the analytes used. The addition of internal standards to the microdialysates was the only sample treatment. This process compensates for matrix effects and furthermore provided the proper dilution to obtain sufficient residual sample volume (10 μl) for column injection.

To minimize manual sample handling, microdialysate tubes were placed in a Waters 2-ml Square Collection Plate (96-well plate) directly applicable for UPLC–MS/MS analysis.

Fixed concentration of internal standard solution was acidified prior to use (0.4% HCOOH). The concentrations of the internal standard analogs were the following: 10.4 μM ($^2H_4$-citric acid), 8.2 μM ($^2H_4$-succinic acid), 6.7 μM ($^2H_4$-α-ketoglutaric acid), 14.5 μM ($^2H_3$-malic acid), 6.6 μM ($^{13}C_5$-Glutamic acid), 216 μM ($^2H_3$-lactic acid), 135 μM ($^{13}C_1$-pyruvic acid), 5.2 μM ($^{15}N_2$-xanthine), 74.4 nM ($^2H_2$-adenosine), and 20.0 μM ($^{13}C_4$-fumaric acid). Prior to analysis, 15 μl of the internal standard solution was added to each microdialysis vial (10 μl sample); after vortex mixing and centrifugation, samples were ready for analysis. In order to avoid any evaporation from the tubes, a vinyl sealing film for microplates was used to cover the tubes.

[3] Relative recovery reflects the equilibrium over the microfilter membrane in the catheter i.e. a 100% relative recovery corresponds to equal concentrations of a metabolite on both sides of the membrane.

3. Results and discussion

3.1. Method development

3.1.1. UPLC conditions

The aim of this study was to develop an analytical method that simultaneously detects and quantifies eight organic acids, mainly those of the citric acid cycle. This UPLC method was furthermore expanded to include the analytes hypoxanthine, xanthine, adenosine, inosine, and glutamic acid. Initially, method development was conducted using traditional HPLC–MS/MS. Good separation of the organic acids was achieved using a Waters Atlantis T3 column and an Aliance® 2695 HPLC coupled to a Waters Micromass Quattro Micro™ mass spectrometer. The limits of detection were in the μM range (e.g. citric acid, 5 μM; malic acid, 7 μM), which is comparable to previously reported methods using similar instrumentation [22]. Following the analysis of a series of microdialysis samples, however, it was evident that the sensitivity of the method was insufficient for our current purpose. Because it has recently been shown that higher sensitivity and shorter analysis time can be obtained by using columns with smaller particle sizes than those used in traditional HPLC analysis, we were encouraged to continue method development using a novel UPLC–MS/MS apparatus capable of the higher pressures generated using these novel columns. The final requirement of the method was the ability to separate the structural isomers citric acid and isocitric acid, which possess the same SRM transition 191 → 111. It has been suggested that the ion transitions 191 → 73/84 (isocitric acid/citric acid) can be used when chromatographic separation is not possible [14]. These transitions, however, are considerably lower in intensity than the 191 → 111 usually obtained, resulting in a final low sensitivity. Separation of inosine and hypoxanthine was also necessary. Inosine partially degrades to hypoxanthine in the ion source and therefore gives a false contribution to the quantification of hypoxanthine (Fig. 1). Though separation of the remaining analytes is not a necessity, it is advantageous because it allows a longer dwell time in the SRM-method and thus provides higher sensitivity. Different column sizes, flow rates, mobile phases, and gradient systems were tested during method development and optimization. These included the ACQUITY BEH C_{18}, 100 mm column, water (0.2% HCOOH), MeOH and water (0.2% HCOOH), acetonitrile (ACN) (0.1% HCOOH). ACQUITY HSS T3, 100 and 150 mm columns. Solvent combinations used were water (0.2% HCOOH), MeOH (0.1% HCOOH), and water (0.2% HCOOH), acetonitrile (0.1% HCOOH). Flow rates were 0.4 ml/min and column temperatures were 45 and 65 °C. ACQUITY BEH amide, 100 mm column, 10 mM NH_4COOH, 0.125% HCOOH (50/50 ACN/H_2O) pH 3 and 10 mM NH_4COOH, 0.125% HCOOH pH 3 (95/5 ACN/H_2O). 10 mM NH_4COOH, 0.125% HCOOH (50/50 ACN/H_2O) pH 9 and 10 mM NH_4COOH, 0.125% HCOOH pH 9 (95/5 ACN/H_2O). Flow rates were 0.6 ml/min and column temperature was 45 °C.

Following optimization, a method with sufficient analyte separation and detection limits suitable for microdialysate analysis was successfully developed. The best analyte peak shape and separation was obtained using an Acquity HSS C_{18} column (100 mm × 2.1 mm, 1.7 μm) with a gradient elu-

Table 2
Summary of regression line, limit of detection (LOD), limit of quantification (LOQ), and relative standard deviation (RSD, $n = 10$) for the metabolites analyzed using the presented method. SD was also determined.

Analyte	Curve	r^2	LOD (μM)	LOQ (μM)	Ion ratio
Citric acid	$0.38x + 0.027$	0.997	0.12	0.39	3.8
Isocitric acid	$3388x - 277$	0.996	0.07	0.24	15.6
Pyruvic acid	$46.4x + 132$	0.972	3.72	12.41	–
Fumaric acid	$2278x + 316$	0.995	0.20	0.66	–
Lactic acid	$94.5x + 4993$	0.980	5.05	16.85	–
Succinic acid	$0.16x + 0.035$	0.995	0.32	1.07	6.6
α-Ketoglutaric acid	$0.20x + 0.797$	0.996	0.76	2.54	15.6
Malic acid	$0.27x - 0.0024$	0.993	0.24	0.81	4.8
Hypoxanthine	$42572x + 393$	0.990	0.02	0.07	1.9
Xanthine	$80281x + 953$	0.982	0.02	0.05	1.1
Adenosine	$514461x + 303934$	0.980	0.03	0.10	13.9
Inosine	$230192x + 96281$	0.952	0.01	0.05	–
Glutamic acid	$0.23x - 0.007$	0.997	0.10	0.32	3.5

tion mixture of water and methanol, acidified with formic acid (Fig. 1).

3.1.2. MS tuning and optimization

The individual analytes were tuned and optimized by infusing and testing 10 μM standard solutions in combination with a 50:50% eluent flow (eluent A and B). Tuning was performed both in the positive and in the negative ion mode to maximize sensitivity. Two product ions were selected when possible. The most intense transition was used as a quantifier, and the second transition was used as qualifier. The selected SRM transitions are listed in Table 1 and the ion ratios in Table 2.

3.1.3. Microdialysis sample preparation for UPLC–MS/MS

Our goal was to develop a method that minimizes the manual handling of samples. This was achieved by programming the UPLC sample manager for direct injection from the microdialysis vials. This eliminated the labour-intensive transfer of samples to standard glass vials as well as errors associated with sample handling. Because this method is novel, it was necessary to determine the vial residual volume after injection. Though dilutions of samples are preferably avoided, dilution was necessary in order to obtain sufficient sample volume to inject 10 μl onto the column. A total amount of 25 μl was found to be the minimal usable volume; with this volume, repeat sample injections of 10 μl could be accomplished with a small relative standard deviation of 0.31% ($n = 24$). Combined with the very short analysis time of 3.0 min, this setup is suitable for analyzing a large number of samples. To ensure a sufficiently low sample pH, the internal standard solution was acidified before addition to the microdialysis sample. Analysis of samples with no added acid showed peak splitting and peak tailing of citric and α-ketoglutaric acids, features that were not observed when samples were acidified before injection. The Krebs–Henseleit solution is buffered at pH 7.4, which explains an observed difference in peak shape between aqueous standards and samples dissolved in Krebs–Henseleit solution. The pH of the microdialysis samples after the addition of formic acid was 2.9, which corresponds to a mobile phase pH of approximately 3.

3.1.4. Linearity

Calibration curves for each of the analytes in the method showed excellent linearity (weighed $1/X$) with correlation coefficients greater than 0.95 (Table 2) over the concentration range from the limits of quantification (LOQ) to 100 μM (lactic acid from 10 μM to 1 mM, adenosine from LOQ to 10 μM).

3.1.5. Limits of detection and quantification

The initial LOD values were estimated from the lowest calibration point with a signal-to-noise ratio (S/N) of around 3:1. The values ranged from 0.05 to 5 μM (inosine, hypoxanthine, adenosine, and xanthine, 0.05 μM; citric, isocitric, and malic acids, 0.1 μM; pyruvic, succinic, α-ketoglutaric, fumaric, and glutamic acids, 0.5 μM; lactic acid 5 μM). The LOD were finally calculated

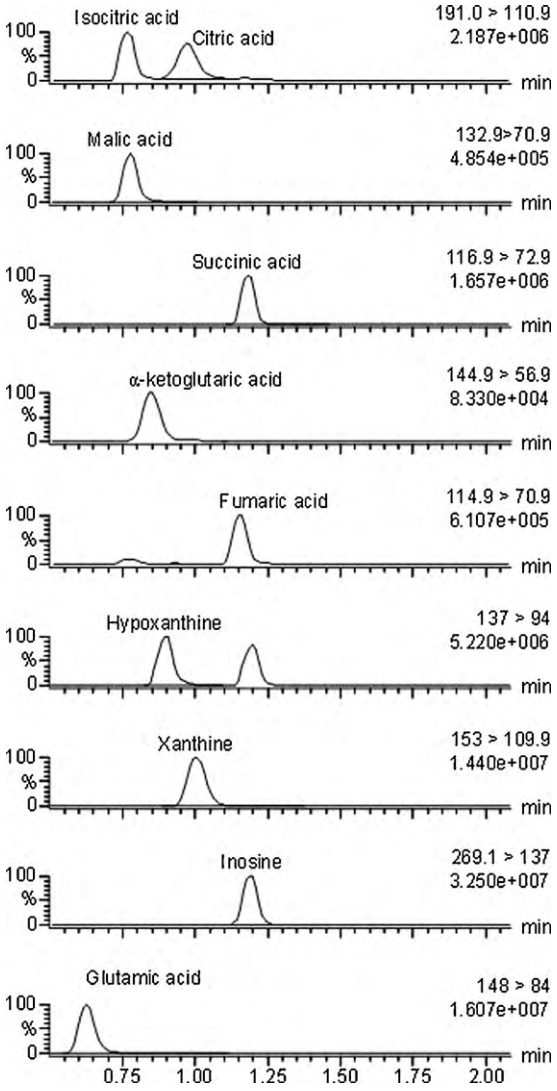

Fig. 1. UPLC–MS/MS chromatogram of selected metabolites analyzed using the presented method. The sample shown represents a mixture of all the metabolites studied in this method at 10 μM concentration (10 μl injection). Inosine and adenosine, as well as fumaric acid and succinic acid, show identical retention times (not shown).

($3 \times$ SD) using the SD of repeat injections ($n = 10$) of standards containing the analytes at the estimated LOD concentration levels. Similarly, the LOQ were calculated ($10 \times$ SD) for the analytes in the presented method. The target ion ratios were determined using pure standards and can be applied as a second criterion for positive identification of the analytes. The final LOD, LOQ, and ion ratios are summarized in Table 2. The obtained limits of detection are suitable for the current purpose, however, not competitive with a previously published HPLC-method using 80 min analysis time [14]. In a setup where e.g. hundreds of microdialysis samples are to be analysed, the importance of a short analysis time (e.g. 3 min versus 80 min), is however obvious.

3.1.6. Sample carryover

No material was detected in blank samples analyzed after injection of samples containing a relatively high concentration of analytes (400 μM, lactic acid 25 mM). This was also the case for blank samples analyzed after injection of microdialysates. The column, needle, and seal washes were concluded to be sufficient to avoid any inter-sample carryover of material.

3.1.7. Matrix effects

Matrix effects were investigated by continuous post-column infusion of 100 μM (5 μl/min) analyte solutions and by simultaneous injection and analysis of 10 μl water, Krebs–Henseleit buffer, or microdialysis sample, all diluted 1:1.5.

For some analytes, a gradual signal enhancement in the ion chromatograms was observed as the amount of organic phase was increased. Signal enhancement has previously been explained by high organic content in the mobile phase [23]. Aside from small similar variations in the ion chromatograms, no differences were observed between water, Krebs–Henseleit solution, and microdialysis sample injections. This indicates that no significant additional matrix effect from the Krebs–Henseleit buffer or microdialysis samples was detected. Notably, the occurrence of matrix effects due to the presence of larger compounds (>6–20 kDa), such as proteins and enzymes are minimized; these compounds are physically removed by the membrane in the microdialysis catheter.

3.1.8. Accuracy

The accuracy (bias) of this method was verified by calculating the recoveries based on theoretical and measured metabolite concentrations. The recoveries obtained are presented in Table 3 and are within an acceptable range for analysis of organic acids. Analytes without an internal standard (isocitric acid, hypoxanthine, and inosine) demonstrate comparable accuracy to analytes with internal standards.

3.1.9. Precision

The precision of this analytical method was tested using real microdialysis samples in order to obtain the most accurate measurements. Intraday SD (repeatability) and inter-day SD (inter-day reproducibility) were calculated by a one-way analysis of variance (ANOVA) and expressed as the relative standard deviation (RSD) of the analyte concentration in the microdialysis pool. Results from the ANOVA test are summarized in Table 4. The imprecision of this method is around 20% for all analytes measured at the endogenous level, which is acceptable for the current purpose. It should be noted, that the microdialysis pools used for accuracy and precision experimentation are not identical. Pools were individually prepared by mixing randomly selected microdialysis samples; this explains the difference in analyte concentration between the precision and accuracy experiments.

Table 3
Accuracy studies.

Analyte	Microdialysis pool I[a]			Microdialysis pool II[a]			Microdialysis pool III[a]		
	Endogenous (μM)	Recovery (%) (5 μM added)	Recovery (%) (50 μM added)	Endogenous (μM)	Recovery (%) (5 μM added)	Recovery (%) (50 μM added)	Endogenous (μM)	Recovery (%) (5 μM added)	Recovery (%) (50 μM added)
Citric acid	3.3	79	106	0.5	96	104	4.8	75	103
Isocitric acid	<LOD	119	113	<LOD	106	114	<LOD	113	114
Pyruvic acid[b]	40.9	68	120	46.1	81	97	58.7	74	92
Fumaric acid	<LOD	102	103	<LOD	97	99	<LOD	101	103
Lactic acid[b]	1709	114	71	1436	121	77	884	176	76
Succinic acid	1.3	109	103	<LOD	110	98	0.7	108	106
α-KG acid	<LOD	100	99	<LOD	<LOD	86	<LOD	<LOD	89
Malic acid	<LOD	107	103	<LOD	89	101	0.1	101	107
Hypoxanthine	0.3	135	113	12.3	69	99	3.6	107	100
Xanthine	1.2	154	106	0.7	145	108	0.8	139	97
Adenosine[c]	<LOD	156	–	<LOD	129	–	<LOD	152	–
Inosine[d]	1.7	114	114	20.0	125	92	<LOD	188	111
Glutamic acid[d]	16.0	105	107	10.2	103	106	28.2	83	92

[a] Three different microdialysis pools were used in the accuracy experiments.
[b] For lactic acid, concentrations were 1000 and 10,000 μM for low and high, respectively.
[c] Adenosine was not analyzed at high concentration due to a limited concentration span with linear detector response.
[d] For pyruvic acid, inosine, and glutamic acid, concentrations were 50 and 100 μM for low and high, respectively.

Table 4
Precision studies.

Analyte	Concentration (μM)	Precision[a] (%RSD)	Repeatability (%RSD)	Inter-day Reproducibility (%RSD)
Citric acid	2.8	20	18	10
Isocitric acid[b]	<LOD	–	–	–
Fumaric acid	1.5	24	24	6
Malic acid	4.3	15	9	12
Pyruvic acid[b]	<LOD	–	5[c]	–
Succinic acid	2.9	14	8	11
Lactic acid	505	9	8	3
α-ketoglutaric acid[b]	<LOD	–	–	–
Xanthine	0.1	20	19	7
Hypoxanthine	4.5	18	17	8
Adenosine	0.6	25	23	10
Inosine	4.6	19	17	9
Glutamic acid	9.0	16	13	9

[a] Precision is calculated as the square root of the sum of the squares of repeatability and inter-day reproducibility.
[b] Isocitric, pyruvic, and α-ketoglutaric acids were below the LOQ in the microdialysis sample pool.
[c] Repeatability of pyruvic acid was obtained from another study. From a pool of microdialysis samples, 10 individual samples were analyzed on the same day.

3.1.10. Microdialysis sampling

To obtain maximum recovery of components over the microdialysis membrane, a series of experiments were carried out using varying flow rates. As Fig. 2 shows, the highest relative recovery was obtained using a flow rate of 1 μl/min. This result correlates well with the results of previous experiments, which indicate maximum relative recovery at small flow rates through the catheter [24]. Use of a smaller flow rate would probably result in larger relative recovery; it would, however, also require a longer sampling time to obtain sufficient sample volume for the UPLC injection. Because metabolic events occur rapidly during ischemia–reperfusion, a longer sampling time would likely present inaccurate measurements of the incident. Finally, because the total amount was used for sample preparation, the precision of the microdialysis sampler was determined. The results of the precision experiments showed an average collected volume of 9.66 μl with an SD of 0.56 μl ($n = 25$), which is sufficient for the current purpose.

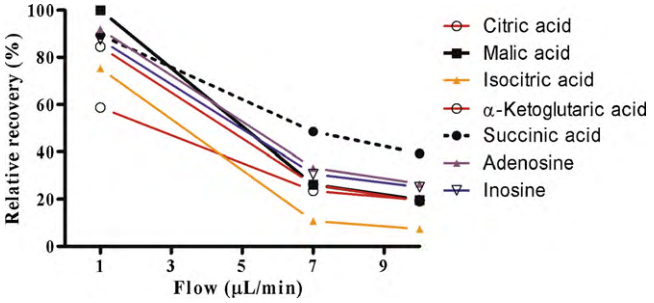

Fig. 2. Relative recoveries of selected metabolites as a function of flow rate. The catheters were placed in a 10 μM aqueous solution at 37 °C. The highest relative recovery was obtained with a flow rate of 1 μl/min.

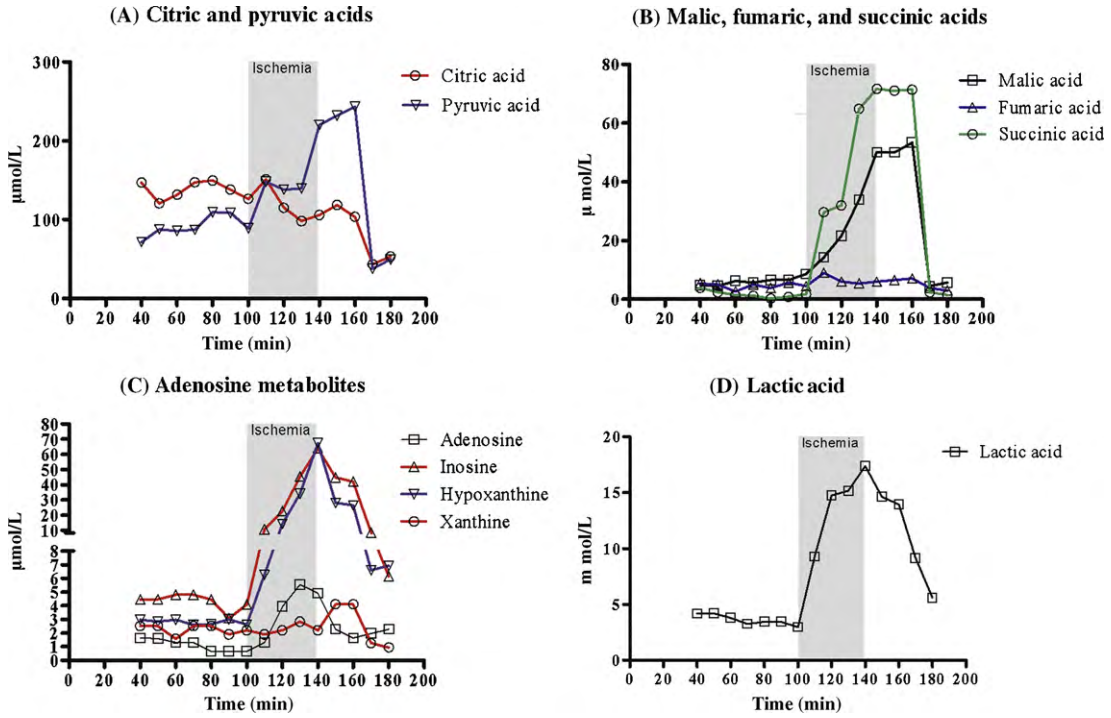

Fig. 3. Interstitial metabolites from an open chest neonatal porcine model on Danish Landrace pigs ($n = 1$). (A) Citric and pyruvic acids. (B) Malic, fumaric, and succinic acids. (C) Adenosine, inosine, hypoxanthine and xanthine. (D) Lactic acid. Isocitric acid and α-ketoglutaric acid were not detected in the present samples. These metabolites will most likely be measurable in tissue samples.

3.1.11. In vivo studies in pig hearts: microdialysis sampling coupled to UPLC–MS/MS analysis

Using our novel method, we can now quantify changes in the Krebs cycle, as well as adenosine metabolite concentrations, in microdialysis samples. The results of our first microdialysate measurements made in a neonatal pig undergoing ischemia–reperfusion are shown in Fig. 3. The method can, for the first time, document significant changes in succinic and malic acids as well as known levels of pyruvic and lactic acids, during ischemia–reperfusion. The concentrations of pyruvic and lactic acids found in the single-animal study presented here correlates well with the results of previously published studies [11,12]. Samples were obtained from the same animal before, during, and after the ischemic incident. The first samples serve as a reference for evaluating the effect of ischemia on extracellular metabolite concentrations. However, because the data presented were obtained from a single test animal, further interpretation of results must await a more comprehensive dataset.

Finally, we note that the microdialysis technique described here is versatile and can be applied to other animal models. In general, our experiments with pigs result in the detection of relatively high concentrations of metabolites. The concentrations of metabolites in the samples are influenced by the size of the catheters used as well as by the use of the heart model. When performing heart experiments on pigs, larger catheters are used compared to those used when performing heart experiments on rats or rabbits. Furthermore, the applied pig heart model is an *in vivo* model, and the metabolites are not flushed from the tissue as occurs in the retrogradely perfused *ex vivo* isolated heart model. Optimization of the catheter and membrane size, as well as the microdialytic flow and sampling time, must be carried out before other studies are undertaken.

4. Conclusions

We describe a new method for the simultaneous detection of eight organic acids, mainly those of the citric acid cycle, as well as glutamic acid and four ATP metabolites found in microdialysates. Except for the addition of internal standards, this method does not require analyte derivatization or sample preparation. This is an advantage because only small volumes are available in microdialysates (2–10 μl). The method has been successfully applied to monitor the citric acid cycle activity and adenosine metabolite concentrations during ischemia–reperfusion events in a neonatal pig heart. For the first time, we can document elevated levels of metabolites in the interstitium of a neonatal porcine heart during an ischemia–reperfusion event. The results demonstrate the feasibility of the method in acquiring select metabolite measurements at high temporal resolution in the interstitium of an animal *in vivo* model during stabilization, ischemia, and reperfusion.

Unlike methods based on the use of the traditional CMA analyzer, this method can easily be expanded to include other metabolites of interest, as well as to study the cardioprotective metabolic effects induced by e.g. remote ischemic preconditioning. Moreover, the method should be applicable for metabolic profiling in other tissue types where microdialysis can be applied.

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