UMEÅ UNIVERSITY MEDICAL DISSERTATIONS
NEW SERIES NO. 1380 ISSN 0346-6612 ISBN 978-91-7459-097-5

From the Department of Surgical and Perioperative Sciences
Anesthesiology and Intensive Care and
Umeå University, Umeå, Sweden

Methodological aspects on microdialysis sampling and measurements

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Umeå 2010

ISBN 978-91-7459-097-5

Printed in Sweden by
Print Media, Umeå, 2010

To my family

Knowledge is limited; but imagination encircles the world

Albert Einstein

ABSTRACT

Background: The microdialysis (MD) technique is widely spread and used both experimentally and in clinical practice. The MD technique allows continuous collection of small molecules such as glucose, lactate, pyruvate and glycerol. Samples are often analysed using the CMA 600 analyser, an enzymatic and colorimetric analyser. Data evaluating the performance of the CMA 600 analysis system and associated sample handling are sparse. The aim of this work was to identify sources of variability related to handling of microdialysis samples and sources of error associated with use of the CMA 600 analyser. Further, to develop and compare different application techniques of the microdialysis probes both within an organ and on the surface of an organ.

Material and Methods: Papers I and II are mainly *in vitro* studies with the exception of the No Net Flux calibration method in paper I where a pig model (n=7) was used to examine the true concentration of glucose and urea in subcutaneous tissue. Flow rate, sampling time, vial and caps material and performance of the analyser device (CMA 600) were examined. In papers III and IV normoventilated anaesthetised pigs (n=33) were used. In paper III, heart ischemia was used as intervention to compare microdialysis measurements in the myocardium with corresponding measurements on the heart surface. In paper IV, microdialysis measurements in the liver parenchyma were compared with measurements on the liver surface in association with induced liver ischemia. All animal studies were approved by the Animal Experimental Ethics Committee at Umeå University Sweden.

Results: In paper I we succeeded to measure true concentrations of glucose (4.4 mmol/L) and Urea (4.1 mmol/L) in subcutaneous tissue. Paper II showed that for a batch analyse of 24 samples it is preferred to store microdialysis samples in glass vials with crimp caps. For reliable results, samples should be centrifuged before analysis. Paper III showed a new application area for microdialysis sampling from the heart, i.e. surface sampling. The surface probe and myocardial probe (in the myocardium) showed a similar pattern for glucose, lactate and glycerol during baseline, short ischemic and long ischemic interventions. In paper IV, a similar pattern was observed as in paper III, i.e. data obtained from the probe on the liver surface showed no differences compared with data from the probe in liver parenchyma for glucose, lactate and glycerol concentrations during baseline, ischemic and reperfusion interventions.

Conclusion: The MD technique is adequate for local metabolic monitoring, but requires methodological considerations before starting a new experimental serie. It is important to consider factors such as flow rate, sampling time and handling of samples in association with the analysis device chosen. The main finding in this thesis is that analyses of glucose, lactate and glycerol in samples from the heart surface and liver surface reflect concentrations sampled from the myocardium and liver parenchyma, respectively.

Keywords: Microdialysis, liver ischemia, heart ischemia, epicardium, liver parenchyma, metabolism, CMA 600

ORIGINAL PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I Pernilla Abrahamsson, Ola Winsö.
An assessment of calibration and performance of the microdialysis system
Journal of Pharmaceutical and Biomedical Analysis 39 (2005) 730–734

II Pernilla Abrahamsson, Göran Johansson, Anna-Maja Åberg, Michael Haney, Ola Winsö.
Optimised sample handling in association with use of the CMA 600 analyser
Journal of Pharmaceutical and Biomedical Analysis 48 (2008) 940–945

III Pernilla Abrahamsson, Anna-Maja Åberg, Göran Johansson, Ola Winsö, Anders Waldenström, Michael Haney.
Detection of myocardial ischemia using surface microdialysis on the beating heart.
Clinical Physiology and Functional Imaging. Accepted for publication 2010.

IV Pernilla Abrahamsson, Anna-Maja Åberg, Ola Winsö, Göran Johansson, Michael Haney, Per-Jonas Blind.
Comparison between outcome of surface and intraparenchymatous sampling using microdialysis in an experimental liver ischemia model
Manuscript.

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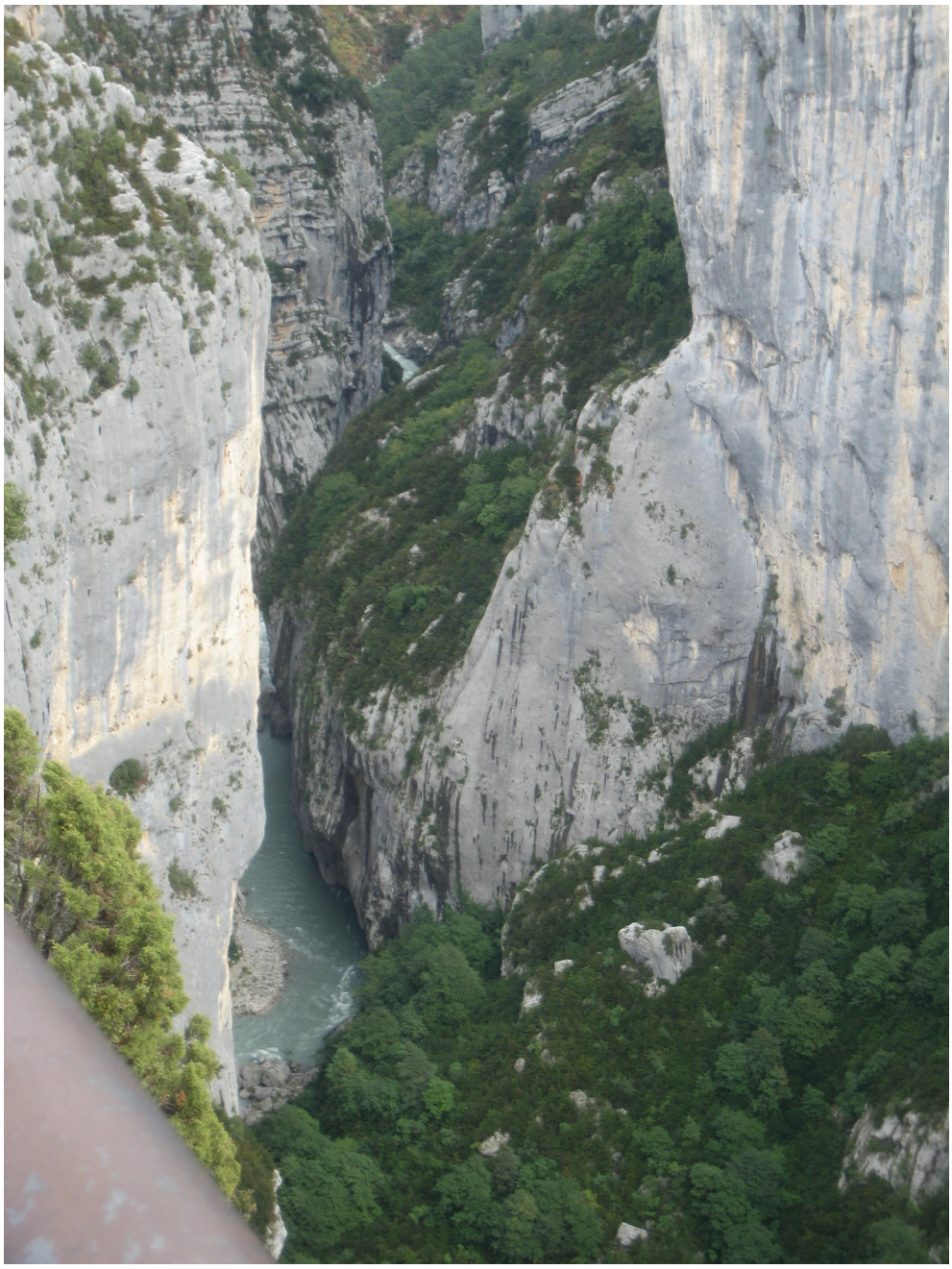


Gorges du Verdon, France 2009

INTRODUCTION

Introduction and general overview

The microdialysis (MD) technique is a relatively new method, designed and developed for measuring local metabolic changes in a specific tissue *in vivo* (1). "Micro" stands for small and dialysis for the possibility to 'capture', collect or sample substances from the fluid surrounding working cells in a vital organ, though this generally means substances with small molecular size. MD sampling aims to identify local metabolic events in a specific tissue through serial sampling, and in physiological terms this can be compared to repeated local venous blood sampling from the same area of the specific tissue. MD sampling collects substances from a specific local part of an organ, and in some organs there may be heterogeneity in their local or regional metabolism and circulations. Substances that can be analyzed are those that pass through the semi- permeable membrane and for which there is analysis methods for small volumes. The MD catheter is a small thin catheter which is easy to place within the organ of interest. Despite this invasive aspect, it is suitable even for vulnerable tissues such as the human heart (2-4). Results from MD sampling can be useful for understanding clinical problems, including help to identify patient's prosperous conditions. This thesis examines some methodological issues concerning the MD analysis method and sampling procedure. The thesis also includes the introduction of a new approach of MD sampling which is aimed to improve the processes of sampling, analysis, and use of clinical application.

Historical

The first scientific report of dialyse membrane was published in 1957. A scientist from Canada succeeded in analysing small amounts of corticosteroids in blood plasma using a dialyse membrane to collect samples which were then analysed with chromatography (5). Since then and until 2010, 13155 papers have been published in which MD was used (Pub med). In 1966 Bito et al were the first to describe MD implantation in the brain of a dog (6). Some years after, Delgado et al implanted a MD catheter in the brain of a monkey (7). In 1974, Ungerstedt published his first work on MD sampling from rat brain (8). Since then Ungerstedt has continued to develop the MD technique using different catheters and probes for MD sampling in different tissues. The first clinical paper based on sampling in subcutaneous tissue was carried out by Lönnroth and co-workers in 1987. They also showed that it is possible to determine the true concentration of glucose in subcutaneous tissue using the No Net Flux (NNF) calibration method (9). The first reports using the MD technique for studies in the human brain were presented in the early 1990´s (10, 11).

MD probe/catheter and MD pump

Probe/catheter

The MD probe (CMA 20) which we have employed includes a membrane which is composed of PolyArylEtherSulfone (PEAS). This is a synthetic material that is also employed in some clinically used haemodialysis membrane filters (12). In the pre-clinical (non-human use) probe, the perfusate flows through the inner channel, into a chamber on the probe side of the semi-permeable membrane area for 'exchange', and then exits (as dialysate) through the outer channel where the dialysate finally is collected in vials (Figure 1A). Clinically approved MD catheters have a different design where the perfusate flows through the outer channel and the semi permeable membrane, and then back (as dialysate) via the inner channel to the microvials (Figure 1B) *(CMA product catalogue)*.

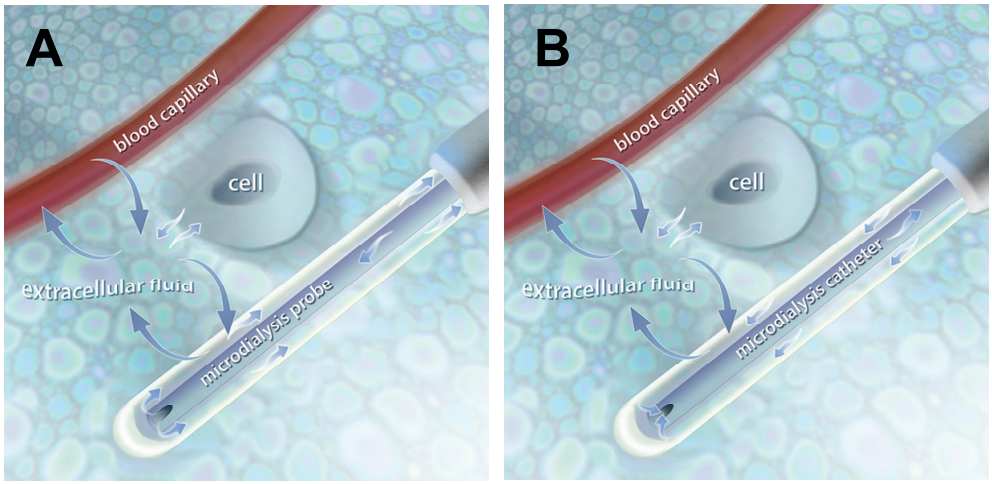


Figure 1. Microdialysis (MD) probe (panel A). The perfusate streams through an inner channel and out to the semipermiable membrane and back to the vial.
Microdialysis (MD) catheter (panel B). The perfusate streams via an outer channel and the semipermiable membrane and back to the vial via an inner channel.

Copyright Permission: CMA Microdialysis, Solna, Sweden.

Microdialysis pump

A reliable pump system for the perfusate is needed for accurate MD sampling. The perfusate syringe or injector needs to maintain a correct and constant flow rate. Incorrect or varying flow rates will alter recovery or exchange of substances across the membrane. To follow rapid changes in local tissue interstitial fluid substance concentrations, a programmable 'fraction' collector can facilitate the MD sampling procedure.

Reflection

The MD probe and catheter, and particularly the semi-permeable membrane, is fragile and can easily be damaged. In our experimental laboratory we preferably use the CMA 20 probe. Our routine includes starting a flow of perfusion fluid through the MD probe before insertion in the organ/tissue. After the experiment the MD probe is washed with distilled water, placed in a solution of distilled water, and perfused during the night. These procedures allow the possibility to continue to use the probe the next day. The MD probe function can easily be examined by testing the probe in vitro by placing it in a solution with a known concentration.

MD sampling

Diffusion- membrane

As described above, a MD catheter/probe consists of an outer semi- permeable membrane with pore sizes which are specific for each catheter type, and currently range from 20-100 KD. This allows diffusion of molecules smaller than the pore size. In the body there is continuously diffusion and transport of small molecules across cell membranes. Molecules have kinetic energy (or heat) and this leads to motion which is the basis of diffusion and the movement of molecules along concentration gradients. Diffusion is a passive process that occurs without need of specific reactions with other molecules. The diffusion rate across the MD membrane is dependent on several factors including concentration gradient, molecular size, membrane surface area and temperature. Larger molecules that theoretically would be able to pass through the semi-permeable membrane pores have a low rate of migration over the membrane. This lower recovery is due to the larger molecules moving with a slower velocity. The larger molecules do not collide or meet with the membrane as frequently as smaller molecules. Thus, it has been observed that the size of molecules that migrated through the membrane were only 25% of the actual pore size (1, 13-16).

Ringer solution is often used as perfusate since it is a physiological solution and nearly isotonic. Osmosis is the passive transport of water through a membrane. If there are two solutions with different osmotic pressures separated by a semipermiable membrane, then water will pass through the membrane to level out the concentration difference across the membrane. Therefore, the MD perfusate must be similar in its tonicity to the fluid surrounding the probe in order to prevent significant shifts of water across the MD membrane (17). The MD technique also allows introduction of endogenous and exogenous substances in the perfusion fluid, such as drugs or radioactively labelled substances (1, 13, 14, 18).

Reflection

When thinking about how MD sampling occurs, it is necessary to consider that the process of diffusion across the MD membrane is not only as simple as diffusion and pore size. Other factors may be important, and these are not necessarily well

recognised today. The membrane will not always allow substances with lower molecular weight than the pore 'size' membrane to pass through without hindrance. In this thesis, only substances with low molecular weight were analysed: glucose (180.16 g/mol=180.16 Da), lactate (90.08 g/mol=90.08 Da), pyruvate (88.06 g/mol=88.06 Da), glycerol (92.09 g/mol= 92.09 Da) and Urea (60.06 g/mol=60.06 Da).

Recovery

Influence

"Recovery" reflects the concentration in the dialysate of the substance or molecules of interest in relation to the true concentration surrounding the catheter. A high recovery means that the concentration in the microdialysate is close to the true concentration in the interstitial fluid of a specific substance of interest. Recovery in MD is dependent on many factors: perfusion flow rate, diffusion rate (the medium and the membrane), the performance of the probe (cut-off, diameter and length of the membrane, chemical interaction between substances and the membrane), and perhaps other factors. Temperature influences the diffusion rate and also the recovery. Recovery increases with 1-2% per one degree (^{o}C) of increased temperature (9, 19-21).

Dependent on these above mentioned factors, it is difficult to achieve 100 % recovery. MD facilitates measurements of relative changes in concentrations and not absolute changes (1, 13, 14, 19). Substances move from the interstitial space along a diffusion gradient, established by the perfusate as it passes through the probe. High flow rate gives high hydrostatic pressure with the result that the perfusion fluid is pressed into the interstitial fluid with lower recovery as a result. On the contrary, lower flow rates for the perfusate results in lower hydrostatic pressures in the probe channels and most of the perfusion volume returns for collection and high recovery is achieved. To establish high recovery, a long membrane, with optimal pore size and a low perfusion rate, needs to be used.

Furthermore, concerning recovery and flow rates, recovery is often presented as relative or absolute recovery (Figure 2). Relative recovery (R) reflects the ratio between the concentration in the dialysate (C_{out}) and the actual concentration from the extracellular space (surrounding the probe) (C_m) (22).

Absolute recovery (Figure 2) reflects the actual mass of a substance that is dialysed from the catheter during a specific time period (mol/unit time). Absolute recovery (A) is calculated as the product of the concentration in the dialysate (C_{out}), perfusion flow rate (FR) and the relative recovery (R) (1, 9, 13, 14, 23).

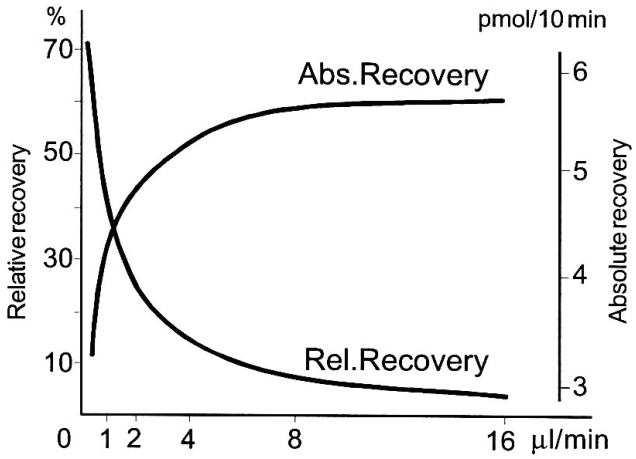


Figure 2. Example of absolute and relative recovery as a function of flow rate. CMA/10 microdialysis probe with 4 mm membrane.

Reflection

There are many other factors which influence the actual interstitial concentrations of the substances of interest, including local blood flow, metabolic rate, uptake into cells and extent of tissue vascularisation. We are interested in the rate of cellular production or consumption of a specific substance, but there is also capillary or lymphatic delivery or uptake, as well as cell transport away from the interstitium of the substances of interest. It is beyond the scope of this thesis to assess all of these cellular and local circulatory factors. But, it must be recognised that if the goal of a MD assay is to measure cellular production or consumption of a specific substance, there are many other factors which can influence interstitial concentrations.

Every new study/situation requires specific attention to optimal sampling and high or low recovery. Factors such as analytical devices, sampling volumes and measured area, protocol, fast or slow course of the physiological and pharmacological events to be studied, will be important when planning and designing the details for MD sampling.

Catheter insertion issues

Equilibrium

When a MD catheter is placed in a tissue there will be some trauma to the tissue, and this potentially can influence the results. An equilibration period is therefore needed before sample collection start.

'Equilibrium', in this case, means that the MD membrane is in contact with the interstitial fluid, and that diffusion across the membrane occurs regardless of high or low recovery (23). The time (equilibration period) to reach 'equilibrium' is most commonly 60 minutes, but there might be conditions that demand longer equilibration periods. The time to reach 'equilibrium' has been shown to be different depending on analysed substances. Flodgren et al showed the need for a longer (more than 60 minutes) equilibration period for glutamate in a patient group with muscle pain. The muscular pain group reached equilibrium after 150 minutes compared with 120 minutes for the control group (24). Other studies have shown that both in healthy and diabetic patients; a stable glucose level in MD sampling was reached first after 12 hours and was thereafter stable for 18 days after insertion of a MD catheter in subcutaneous tissue (25, 26).

Local tissue effects

The insertion of the MD probe/catheter in the tissue is thought to induce only minor damage to the tissue. During the insertion a minor bleeding might occur at the insertion point. One paper showed that an inflammatory reaction of prostaglandin E_2 and Thromboxane B_2 was present up to 4 hours after insertion of the catheter (27). Other papers showed that concentrations of ATP, glucose, adenosine, glucose, lactate, lactate/pyruvate ratio, K^+, Thromboxane B_2 and blood flow have returned to baseline already 30-60 minutes after insertion of the catheter (13, 28, 29).

Reflection

Studies showing different times for reaching equilibrium have been presented. These facts should be considered when a study protocol is designed. In our pig studies, an equilibration period of 60 minutes was enough for glucose, lactate, pyruvate and glycerol.

Assessment of absolute concentrations

While the MD technique often is used for measuring relative changes, it might be of interest to also assess the true concentration in a specific tissue. For example, if a drug delivery application is being studied, there can be a specific goal to assess the tissue or tissue interstitial concentrations for that specific agent. The most commonly used methods for this are the No Net Flux method (NNF-method) (9) and the Retrodialysis calibration method (22).

NNF- method

At least four different known concentrations of a solution (C) of interest are perfused (C_{in}) in probes placed in a specific tissue. When the solution has reach equilibrium, the concentration of the dialysate (C_{out}) is determined. The difference C_{out}-C_{in} as a function of C_{in} is plotted (figure 3).

The slope of the regression line is calculated, and the true tissue concentration is the intercept with the X-axis where the regression line crosses at Y=0. An example of calculation of an absolute (true) concentration based on the NNF calibration is shown in Figure 3. This calibration method is specific but time demanding since it requires established steady state. It might also be difficult to find the right concentrations that will reflect the true concentration in the tissue (9, 23, 30).

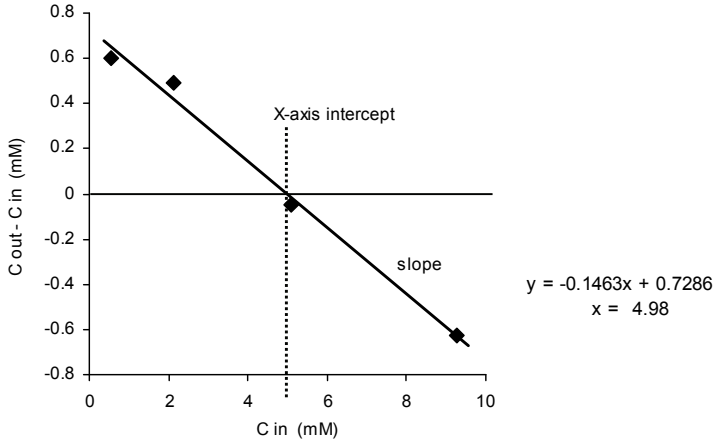


Figure 3. An example for calculating the true tissue concentration of glucose with the No Net Flux calibration method (NNF). C in = Glucose concentration in perfusate. C out = Glucose concentration in dialysate. The true tissue concentration is the intercept with the X-axis where the regression line crosses at Y=0. mM: millimol/L (Paper I)

Retrodialysis

This method is applicable for *in vivo* studies and is easy to implement and not as time demanding as the NNF- method. The diffusion/diffusibility through the membrane is assumed to be equal in both directions. An internal standard (C_{in}) is added to the perfusate and the loss of the internal standard (C_{out}) is measured and recovery (R) is calculated as a function of the loss of the standard solution. Studies have shown that the delivery retrodialysis method gave equal results in muscle and adipose tissue compared with the NNF method. To establish reliable results the internal standard must be carefully chosen so it matches the substance of interests and for minimal disturbance of the experiment (22, 30-35).

$$R = \frac{C_{in} - C_{out}}{C_{in}}$$

Analysis

The CMA 600 analyser (CMA MD, Solna, Sweden) is an automatic enzymatic and colorimetric analyser commonly used for measuring substances such as glucose, lactate, pyruvate and glycerol (Figure 4). In the analyser an enzymatic

reaction takes place with the collected substance, the reaction creates a coloured solution (quinoneimine) and the colour intensity is read at a wave length of 546 nm at 37°C. A reference method for this enzymatic system is the HPLC (High Performance Liquid chromatography) system (36). The HPLC system in our lab is used to measure many additional substances of interest, including radio-marked substrates and metabolites. In this thesis, the HPLC system has been involved as a reference method only to confirm and double-check calibration of the CMA 600 analyses.

D- **Glucose** + O_2 → gluconolactone + H_2O_2
$2H_2O_2$ + phenol + 4-amino-antipyrine →**quinoneimine** + $4H_2O$

L- **Lactate** + O_2 → pyruvate + H_2O_2
H_2O_2 + 4-chloro-phenol + 4-amino-antipyrine →**quinoneimine** + $2H_2O$+ HCL

Pyruvate + P_i + O_2 → acetylphosphate + CO_2 +H_2O_2
$2H_2O_2$ + TOOS + 4-amino-antipyrine →**quinoneimine** + $4H_2O$

Glycerol + ATP → glycerol-3-phosphate + ADP
Glycerol-3-phosphate + O2 → dihydroxyacetone phospate + H_2O_2
H_2O_2 + DCHBS + 4-amino-antipyrine → **quinoneimine**+ $2H_2O$ + HCL

Figure 4. Enzymatic reaction formulas for glucose, lactate, pyruvate and glycerol that occur in the CMA 600 analyser. Determination of the colour intensity of quinoneimine is the final step and is read by the CMA 600 analyser at a wavelength of 546 nm.

For development of the MD technique it would be an advantage if analyser systems were more optimised. An online/real-time analyse system should open for new clinical applications. A sensor for continuous glucose monitoring (SCGM1, Roche Diagnostic, Mannheim, Germany) has been tested both in animals and in diabetic and healthy humans. Glucose concentrations seem to follow the capillary concentration of glucose. This opens for new possibilities to analyse patients with diabetes but also to study other conditions in the future (26, 37-41). Other examples of online MD/realtime analysis systems are: MD-LC system (liquid chromatography), MD- ED (capillary electrophoresis) and MD- MC (mirochip electrophoresis) (42).

Glycogenolysis

Aerobic conditions

Glucose is stored in tissues as glycogen, and glycogen is broken down to glucose (glycogenolysis) when glucose availability or delivery is inadequate by other means. This pattern occurs in all tissue, but is especially prominent in skeletal

muscle and in the liver, and is facilitated by the enzyme glucose-6-phosphate. In working muscle, glycogen can remain stored as long as other substrates are available. While the liver helps to provide glucose to the bloodstream in working physiological ranges in this way, in muscle and other tissues glycogen is stored as a rapid and ready source of glucose for the cells own consumption.

Anaerobic conditions

In liver parenchyma, during total ischemia, glycogen breaks down to glucose and a high concentration of glucose occurs in the liver parenchyma (16).

Glycolysis

Aerobic conditions

Glucose is an energy source, and under aerobic conditions glycolysis breaks down glucose to pyruvate giving some limited amounts of energy to the cell. Pyruvate is further metabolised to Acetyl CoA that enters the citric acid cycle, and is combined with cellular respiration/oxidative phosphorylation the largest amount of cellular energy production (ATP generation).

Anaerobic conditions

During anaerobic glycolysis pyruvate is used as a substrate to form lactate. Lactate is a bi-product of metabolism without oxygen. Anaerobic pathway of glycolysis has a minor energy capacity compared with the aerobic glycolysis and the effectiveness decrease after a short time (16).

ß- Oxidation; free fatty acid

Aerobic conditions

Under normal conditions, free fatty acid (FFA) ß- oxidation is the main energy source for example in the heart. The end product for this FFA ß- oxidation is acetyl Co-A which is taken up into the citric acid cycle. There is very effective and efficient energy extraction from β- oxidation.

Anaerobic conditions

During ischemia, ß-oxidation of FFA do not occur, since it is dependent on availability of oxygen (16).

Glycerol

Glycerol can be produced during ß-oxidation. During prolonged ischemia (no ß-oxidation) glycerol can be released as a result of energy depletion and progressive cell cytosolic calcium overload, which increase phospholipase activity in the cell membrane. Glycerol is separated from lipids and released in this process. Glycerol in this setting is then viewed as a marker for cell membrane injury (43, 44).

AIMS

- To test the accuracy and precision of the CMA 600 analyser
- To test the sampling performance of MD probes after experimental studies
- To evaluate the NNF calibration method both *in vivo* and *in vitro*
- To identify sources of error and variability in MD sample handling and analysis steps by specifically testing the following:
    - Effects of reanalysis steps
    - Influence of storage
    - Influence of centrifugation
    - Influence of vials and caps material
- To test the performance of MD sampling on the surface of the heart
- To test the performance of MD sampling on the surface of the liver

MATERIAL AND METHODS

Animal model (I, III and IV)

In Studies I, III and IV pigs (n=40) weighing 28-45 kg were used. The pigs were kept at the University stable over night with free access to water. The study was approved by the Animal Experimental Ethics Committee at Umeå University Sweden, and was conducted in accordance with the NIH Institutional animal care and use committee guidebook (Guide for the Care and Use of Laboratory Animals, National Research Council, Washington, USA, 1996).

The pigs were premedicated with an intramuscular injection of xylazine 20 mg· ml^{-1} (Rompum vet, Bayer AB), atropine 0.05 mg·kg^{-1} (Atropin, NM, Pharma, Stockholm, Sweden) and ketamine 10 mg· kg^{-1} (Ketalar®, Pfizer, Morris Plains, New Jersey, USA). An intravenous bolus dose of pentobarbital 10 mg· kg^{-1} (Pentobarbitalnatrium, Apoteksbolaget, Stockholm, Sweden) induced anaesthesia, and for maintenance of anaesthesia; midazolam 0.3 mg· kg^{-1}· h^{-1} (Dormicum, Roche, Basel, Switzerland), pentobarbital 5 mg· kg^{-1}· h^{-1} and fentanyl 20 µg· kg^{-1}· h^{-1} (Fentanyl, Braun, Melsungen, Germany), were infused. The animals were tracheotomised (7.0 OD endotracheal tube, Rusch, Kernen, Germany) and mechanically ventilated to normoxia and normocapnia (Evita 4, Dräger, Kiel, Germany) as adjusted by intermittent arterial blood gas analysis (ABL 5, Radiometer, Denmark). Ringer´s acetate (Pharmacia- Upjohn, Sweden) was infused at 15 ml· kg^{-1}· h^{-1}.

A pressure monitoring and sampling catheter was placed in a small neck artery and a central venous catheter was inserted in the external jugular vein. Basal monitoring comprised registration of heart rate (HR), mean arterial pressure (MAP) and central venous pressure (CVP). Pressures were measured using catheters filled with isotonic saline and pressure transducers (DTX- pressure transducer, Becton Dickinson, Stockholm, Sweden) placed at the mid- axillary level. Data were continuously recorded using a computer based multi- channel signal acquisition and analysis system (Acknowledge, Biopac system Inc., CA, USA).

In Paper III a sternotomy was performed and a diagonal branch of the left anterior descending (LAD) artery was identified and a tape snare was placed around the proximal part of LAD. In Paper IV a laparotomy was made, the hepato- duodenal ligament, containing the branches of the liver artery, portal vein, and bile- duct, was identified and encircled by a cotton tape. For both Papers III and IV the ends of the tape were extracted through tight plastic tubing. Ischemia was obtained by tightening and locking the tape against the tube using a forceps.

Microdialysis

All MD probes were perfused with a modified Krebs-Ringer phosphate buffer (Fresenius Kabi, Halden, Norway). A 60 minutes period of equilibration was always allowed before starting the experimental protocol (*in vivo*). The CMA 600

analyser was used for analysis of glucose (papers I-IV), lactate (papers II-IV), pyruvate (papers II-IV), glycerol (papers II-IV) and urea (paper I). Results in paper II changed our routines concerning MD sampling and analyses. In papers III-IV the MD samples were collected in glass vials and sealed with crimp caps that were kept in place during analyse. The MD samples were always centrifuged for 30 seconds (1177 g) before analyses (Paper III and IV) (45).

Experimental protocol

Paper I

The experimental protocol consisted validation of the CMA 600 analyser, *in vitro* calibration, probe control and *in vivo* calibration using the NNF- calibration method. All samples were collected in plastic vials that were sealed with plastic caps for storage. The caps were removed before analyses which mean that vials were unsealed during analysis. During the *in vitro* calibration the influence of time and flow rate on recovery was examined. A CMA 60 catheter (CMA Microdialysis, Solna, Sweden) was placed in a glucose solution of known concentration and perfused at 1 μL/min. Sampling times were set at 10, 20 and 30 minutes. The flow rate was thereafter changed (0.3, 0.5, 1, 2 and 5 μL/min) and a constant sampling volume of 10 μL was collected.

Probe membrane function of 14 CMA 20 microdialysis probes was tested *in vitro* after experimental studies in animals. The probes were placed in a 5 mmol/L glucose solution and tested at a flow rate of 2 μL/min with a sampling time of 10 minutes. Each probe was tested *in vitro* for these consecutive sampling period of 10 minutes each.

The NNF method was performed by placing a CMA 20 probe in subcutaneous tissue (n=7). Four different Ringer solutions of glucose and Urea of known concentrations (0.5, 2, 5 and 10 mmol/L) were perfused through the probe at a flow rate of 2 μL/min for one hour/concentration. Samples were collected every 10 minutes after a 30 minutes equilibration period.

Paper II

Standardised solutions for glucose, lactate, pyruvate and glycerol, all in 'low', 'medium' and 'high' concentrations were used as 'test' samples (Figure 5). Each 'batch' comprised 24 samples (vials), and each vial was prepared with 20 μL of the respective standard solution. All samples were analysed in a CMA 600 analyser. All samples (except when specifically noted, in the case of refrigeration) were stored in the same freezer at –20^{o} C.

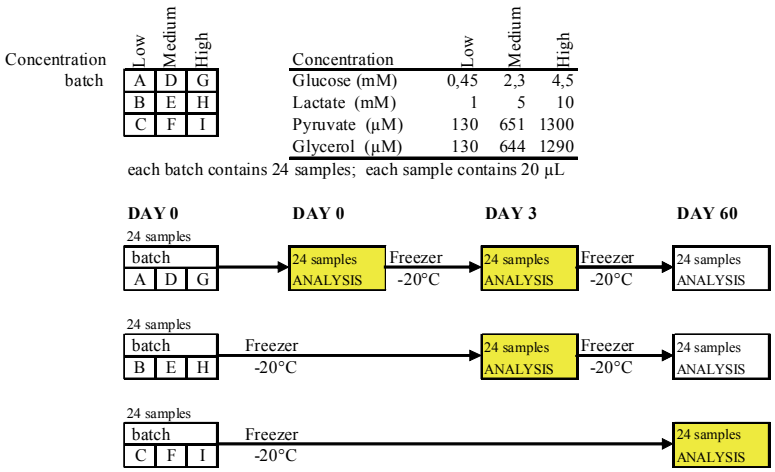


Figure 5. Protocol for sample preparation, analyses, storage and re-analyses in plastic vials. Each batch contained 24 samples with 20 µL sample volume of low, medium and high concentration of standard solution of glucose, lactate, pyruvate and glycerol, respectively.
mM: millimol/L, µM: micromol/L (Paper II)

Drift and re-analyses

All experiments were performed in batches (n=24) using plastic vials (CMA, Microdialysis, Solna, Sweden) with the same solution in each batch. The plastic vials were sealed with plastic caps for storage which were removed before analyses. Each test started with immediate analysis of batch A0. After analysis, the samples were stored in a freezer for 3 days, and then reanalysed (A3). The same samples were then stored in the freezer for 60 days, and then re-analysed a third time (A60). The same procedure was performed for the 'low', 'medium', and 'high' concentrations of each substance.

Storage at -20^{O} C

Samples were placed in plastic vials without caps. Two batches of each solution were prepared in vials on day zero and then stored in a freezer for 3 days (B3, E3 and H3) or 60 days (C60, F60 and I60) before analysis. These results were compared with analyses on day zero (A0, D0 and G0).

Centrifugation

A group of samples prepared and handled in the same way as batch B3 was centrifuged (Mini Galaxy, VWR, West Chester, PA, USA) for 30 seconds (1177 g) before analyses (K3). These samples were restored in a freezer for 60 days and then analysed again after centrifugation. These results were compared with results for analyses of B3 and B60.

Vial materials, different caps

One batch of 'low' concentration solution was prepared and placed in plastic vials with crimp caps (Chromacol, CT, USA), glass vials (Chromacol, CT, USA) with crimp caps, and microvials (CMA Microdialysis, Solna, Sweden). The vials were centrifuged (1177 g) for 30 seconds before analysis. After analysis, they were stored in a freezer for three days and then re-analysed.

Storage in plastic or glass vials and crimp cap vs microvials, freezer or refrigerator

Five batches containing 'low' concentration solution were prepared in plastic and glass vials. One batch from plastic and glass vials, both with crimp caps, was analysed on days 0, 1, 2, 3 and 14 after storage in a refrigerator (Figure 6). All samples were centrifuged before analysis. Batches with plastic vials, microvials, and glass vials were stored in a freezer for 70 days and then centrifuged and analysed.

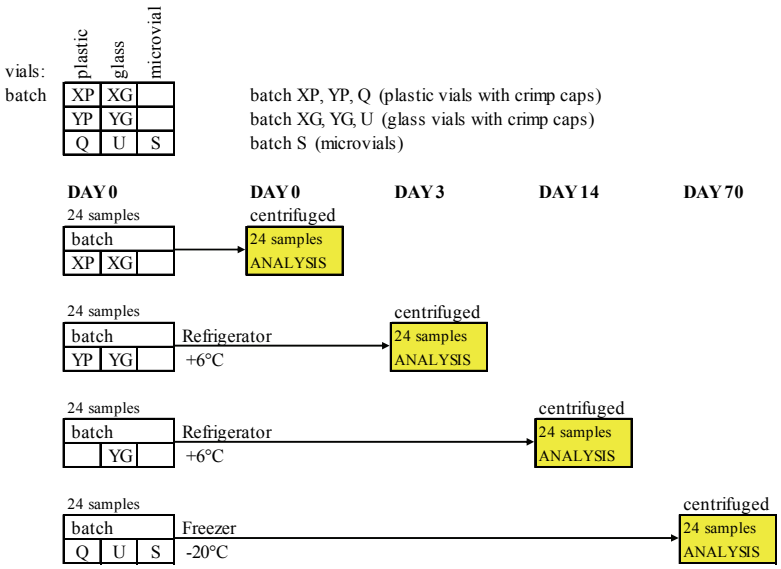


Figure 6. Protocol for sample preparation, analyses, storage and re-analyses using different vials and crimp caps. Each batch contained 24 samples with 20 µL sample volume of low concentration of standard solution of glucose, lactate, pyruvate and glycerol, respectively. (Paper II)

Paper III

MD probes were placed in the myocardial tissue (myocardial probe) supplied by the snared LAD branch. A second MD probe was placed on the epicardial surface (surface probe) in the same area as the myocardial probe (Figure 7). The probes were secured with one suture placed over the membrane and one suture placed in the fixation point. MD probes were perfused at a flow rate of 2 µL/min and

samples were collected at 10 minute intervals throughout the study protocol. After an equilibration period of 60 minutes, during which MD samples were collected and analysed, animals were randomized in two groups according to a protocol for another study. One group, (n=10) labelled protocol 1, had a 150 minutes period without any intervention (baseline) before 50 minutes of ischemia induced by tightening the snare, control group. The second group, (n=9) labelled protocol 2, had a 30 minutes baseline period before four 10 minute periods of snare occlusion, each followed by 20 minutes of reperfusion (short ischemic period), before the 50 minutes ischemic period (long ischemic period).

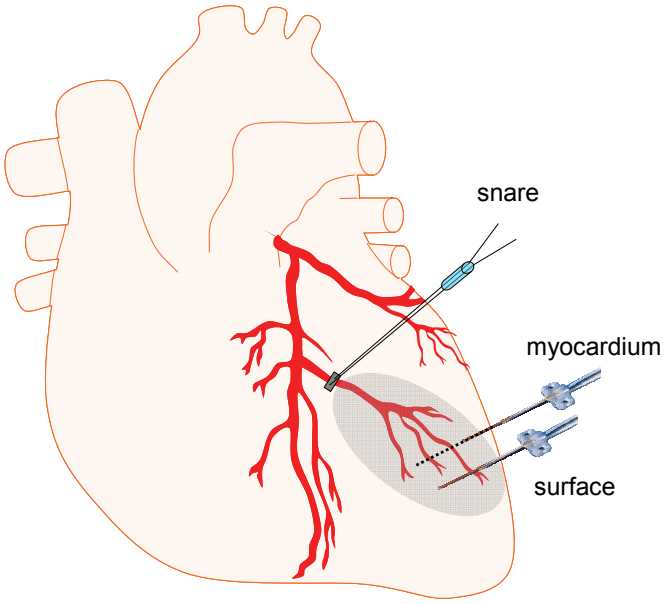


Figure 7. Schematic picture over the heart, placement of the snare over the LAD branch and the MD probe in the myocardium (dotted line) and on the surface of the heart (solid line). The ischemic area is shaded. (Paper III)

Paper IV

Four MD probes were placed on the surface of the liver (surface) and four probes (parenchyma) were placed in the liver parenchyma. Two MD probes on the liver surface and two in the liver parenchyma were perfused at a flow rate of 1 µL/min (n=13). The other two probes placed on the liver surface and in the liver parenchyma were perfused at a flow rate of 2 µL/min (n=9) (Figure 8). The MD samples were collected every 15 minutes. The protocol started with 60 minutes of equilibration followed by baseline measurement for 60 minutes, 45 minutes total occlusion of the portal vein and liver artery and therefore 60 minutes of reperfusion.

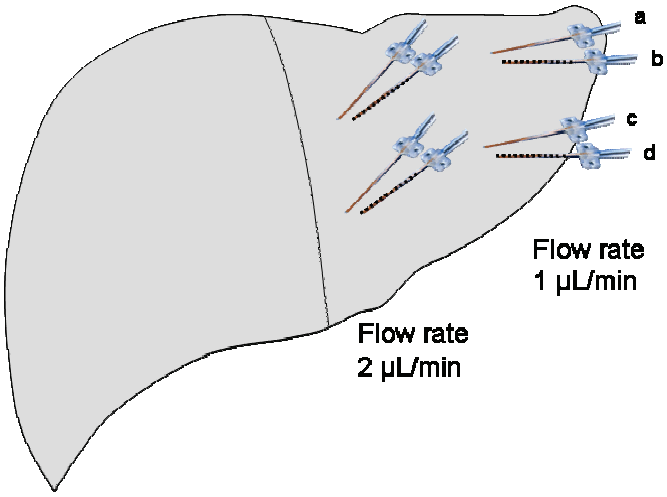


Figure 8. Schematic picture of the liver. MD probes placed on the liver surface (a, c) and MD probes placed in the liver parenchyma (b, d). The probes were perfused with a flow rate of 2 µL/min and 1 µL/min, respectively. (Paper IV)

Interventions for paper III-IV

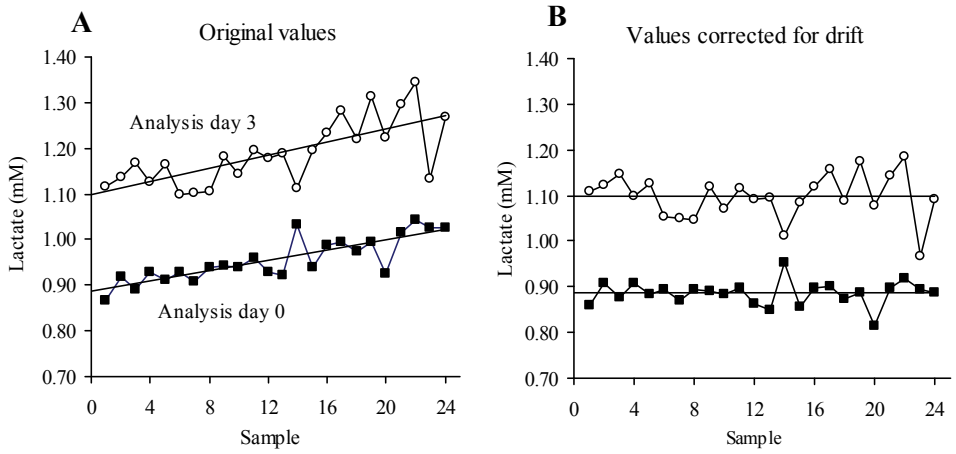
During Papers III and IV, ischemia in the heart and liver were used as interventions to compare measurements of metabolic changes in the myocardium and liver parenchyma with metabolic changes on the surface of the myocardium and liver parenchyma. Ischemia is defined as a stop in arterial blood supply into a tissue, stoppage of delivery of blood-born substrates to tissues. The tissue is not able to find energy sources enough to meet its obligatory metabolic demands. Ischemia means that energy extraction will continue for a short period without oxygen based on anaerobic glycolysis. Pyruvate is consumed producing lactate during progressive anaerobic glycolysis, and pyruvate concentrations will decrease during prolonged ischemia. An increasing pattern in Lactate/Pyruvate (L/P)- ratio is commonly observed when anaerobic glycolysis is prolonged (16, 46).

Calculations

In Papers I, III and IV paired t-tests were performed ($p< 0.05$). In Paper II, to calculate the relation between time in the analysis machine and concentration (drift), linear regression analysis was performed. An estimation of the maximal time effect on concentration (absolute drift) was presented as the difference between the Y- value of the regression line at time 0 (sample 1) and time 192 minutes (sample 24). The concentration change in percent (drift %) was calculated as:

Absolute drift / Y-intercept $_{\text{time 0}}$ x 100.

To calculate variation we subtracted each sample value from the corresponding part of the regression line. We then added the start value, Y-intercept (at time 0), to each calculated difference; this was functionally the same as placing the regression line, on the zero-level, and then adding the start value (Figure 9). This was done for both batches (Day 0 and Day 3) which enabled us to calculate the difference between mean concentrations for each batch (after correction for drift). Calculations of standard deviation (SD) and coefficient of variation (CV) for each batch were performed. Measured values are presented as mean ± SD. Independent samples T-test between groups was used for testing for differences in mean concentration between two batches. A p-value of less than 0.05 was used for statistical significance. The one-sample Kolmogorov-Smirnov test was used to determine normality in results distribution.



Linear regression: day 0 : Y=0.0056X + 0.886
day 3 : Y=0.0073X + 1.098

A	day 0	day 3		B	day 0	day 3	Conc-diff	
slope:	0.0056	0.0073		Mean concentration:	0.886	1.098	0.212	mM
drift:	0.135	0.174	mM	SD:	0.027	0.050		mM
drift%:	15%	16%		CV:	3.0	4.5		%

Figure 9. Examples of calculations made for each batch analysis of 24 samples, calculating the drift and spread of each batch and the difference in concentration between the two batches. SD = Standard deviation, CV = Coefficient of variation. (Paper II)

In Papers III and IV measured values are presented as mean and for Paper III standard error of the mean (SEM) and for Paper IV 95% confidential interval. A paired comparison to measure differences in relative changes between probes was calculated with a 95% confidential interval (Papers III and IV). The analyses were performed with the SPSS software package (version 18.0; SPSS Inc., Chicago IL, USA). In Microsoft Excel calculation and figures were accomplished.

RESULTS

Paper I

Flow rate vs. concentration

Figure 10 illustrates that glucose concentrations decreased when the flow rate was increased. The largest decrease in concentration occurred when the flow rate was increased from 2 µL/min to 5 µL/min.

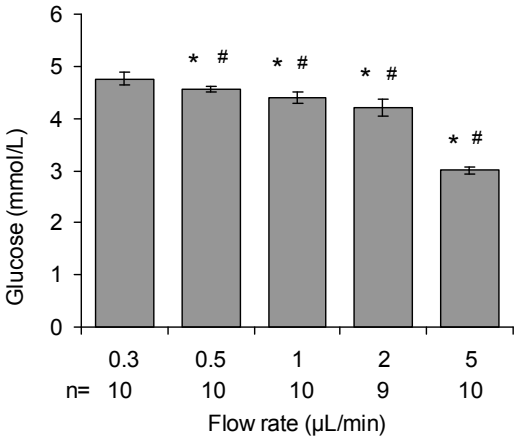


Figure 10. In vitro study. A CMA 60 catheter was placed in a glucose solution of known concentration and perfused at 0.3, 0.5, 1, 2 and 5 µL/min. Data are presented as mean and 95% confidence intervals. * $p<0.05$ using between groups T-test vs. the lowest flow rate. # $p<0.05$ vs. the previous flowrate. (Paper I)

No Net Flux calibration method

Four different glucose and urea solutions of known concentrations were perfused through a CMA 20 probe placed in subcutaneous tissue. Samples were then analysed and plotted against known concentrations. The calculated glucose concentration was 4.4 mmol/L (Figure 11A). In the same way the real concentration of urea in subcutaneous tissue was calculated and found to be 4.1 mmol/L (Figure 11B).

Further results

A pilot study (n=1) showed that the concentration of pyruvate in the pancreas parenchyma (CMA 20 probe) was about 160 µmol/L (Figure 12A) as measured by the NNF method. Figure 12B show the difficulties to determine the true concentration of urea in the liver tissue (CMA 20 probe) using the NNF calibration method. The four different concentrations that were chosen for perfusion of the liver tissue resulted in a spread pattern with no possibility to draw a regression line.

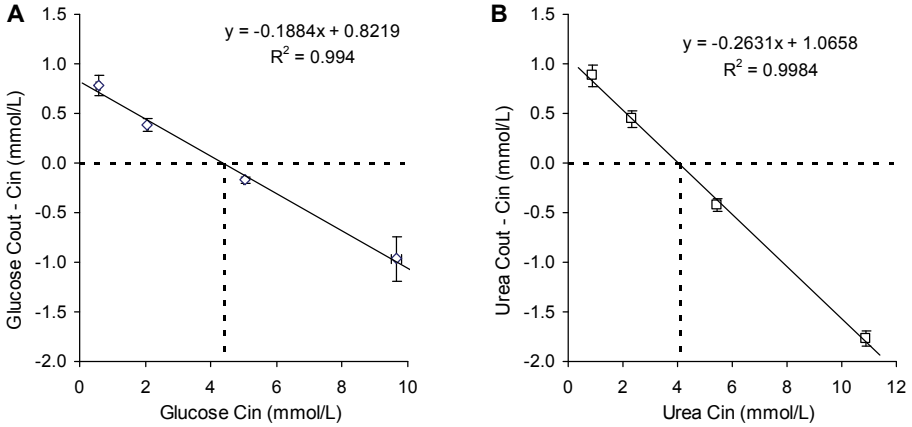


Figure 11. Determination of the true subcutaneous tissue concentration estimated by the NNF calibration method. Four different solutions with known concentrations of glucose (panel A) and urea (panel B) were used as perfusate (C in). The glucose and urea concentration was found to be 4.4 mmol/L and 4.1 mmol/L. Data are presented as mean ±SEM, n=7. (Paper I)
Calculation method is shown in figure 3.

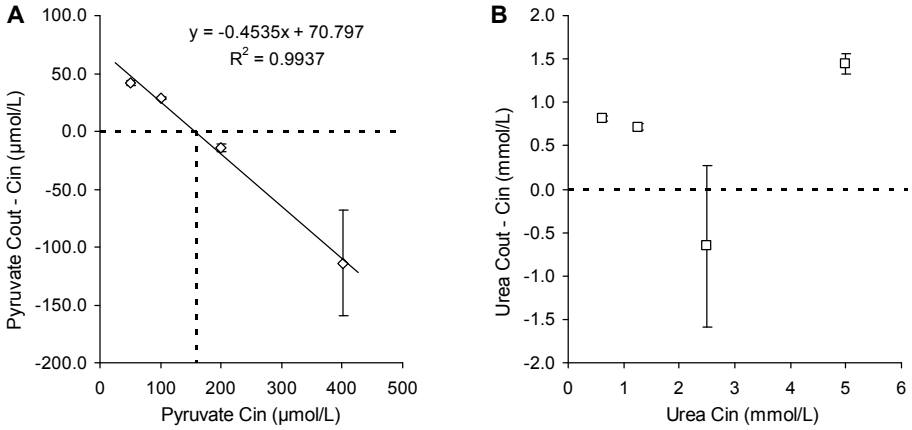


Figure 12. Pyruvate concentration in pancreas parenchyma (panel A) as estimated by the NNF calibration method. Four different solutions with known concentrations of pyruvate were used as perfusate. The true pyruvate concentration was found to be 160 µmol/L.
Panel B shows Urea concentration in the liver parenchyma. Similarly, solutions with different known concentrations of urea were used as perfusate. Since it was impossible to draw a regression line this measurement could not calculate the real urea concentration. Data are presented as mean ±SEM, n=1.

Paper II

All presented results are from samples with 'low' concentrations of glucose (0.45 mmol/L) and lactate (1.0 mmol/L).

Drift and reanalyses

The temperature inside the CMA 600 analyser was measured to be 27 °C. Programmed batch analyses of 24 samples over 192 minutes showed a difference in concentration (drift) of 12-25% for the four substances (Table 1, paper II). Figures 13A and 13B show the drift over time for glucose and lactate during analysis on the first day and after 3 days of storage in a freezer.

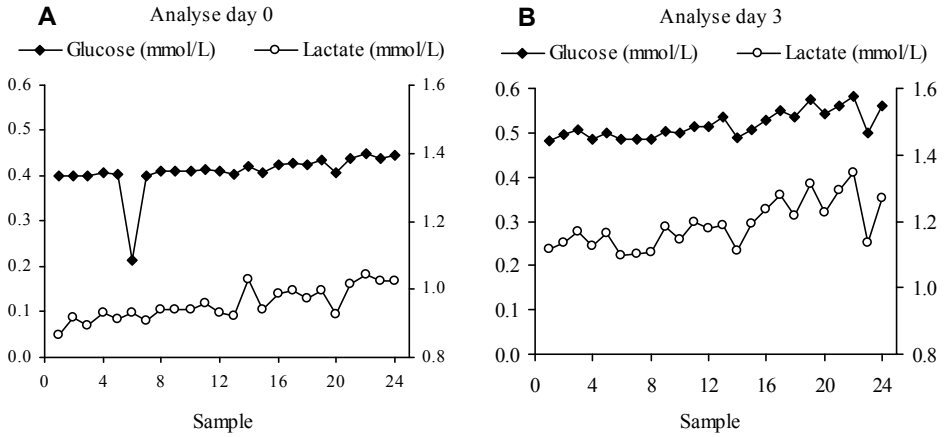


Figure 13. Drift and re-analysis. Batch analysis (24 samples) of a low standard solution of glucose (0.45 mmol/L) and lactate (1.0 mmol/L)) using plastic vials without caps. Panel A shows the samples with glucose and lactate that were analysed during a batch analysis the first day (A0) compared with panel B were the same samples were analysed after 3 days after storage in -20 °C (A3). (Paper II)

Freezer storage (-20^{O} C)

There were no differences in concentrations of glucose, lactate, pyruvate and glycerol in samples analysed after 60 days of storage in a freezer (-20^{O}C) compaired to analyses in the first day (Figures 14A and 14B).

Centrifugation

Variation between samples in one batch analyse was reduced with centrifugation (Figures 15A and 15B).

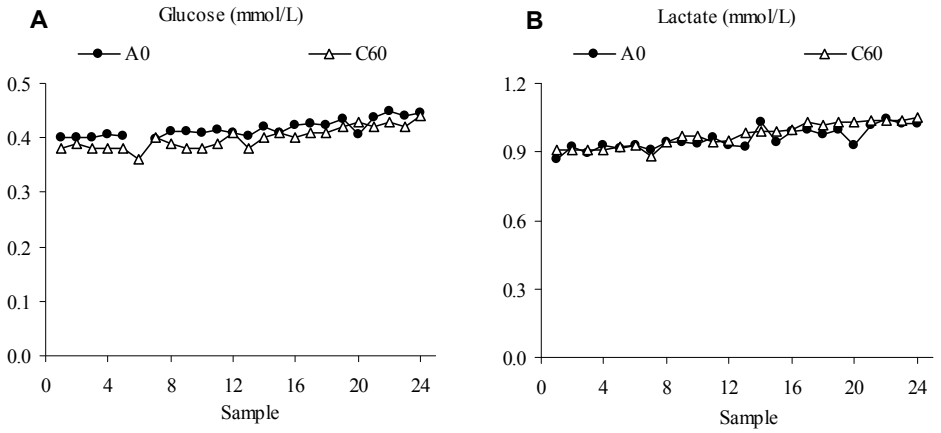


Figure 14. Storage in a freezer at -20 °C. Glucose (panel A) and lactate (panel B) analysed at day 0 (A0) and after 60 days in a freezer (C60). These figures illustrate that concentration of glucose (panel A) and lactate (panel B) were not changed after 60 days of storage. Note that concentrations and drift are similar when comparing samples analysed on day 0 and day 60. (Paper II)

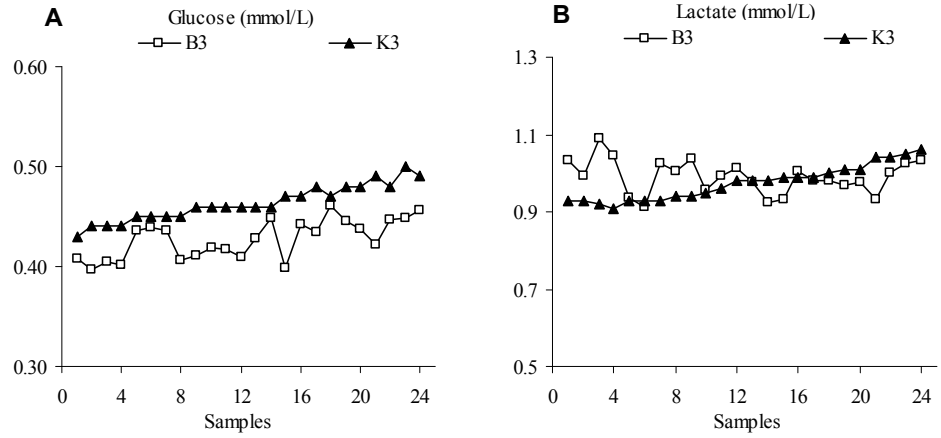


Figure 15. Centrifugation before analyses. Samples with glucose (panel A) and lactate (panel B) were analysed after 3 days in a freezer. One batch was centrifuged before analyses (K3) compared to samples that were not centrifuged (B3). (Paper II)

Vial materials, different caps

During batch analysis, on the first day the drift was similar regardless of which vials were used for analyses. After 3 days in the freezer, samples in plastic vials with crimp caps and microvials had a larger change in concentration in glucose and pyruvate compared to samples stored in glass vials with crimp caps (Table 2, paper II). The main finding in this respect was that re-analysis of samples (all) was associated with higher concentrations (Figure 16A and 16B).

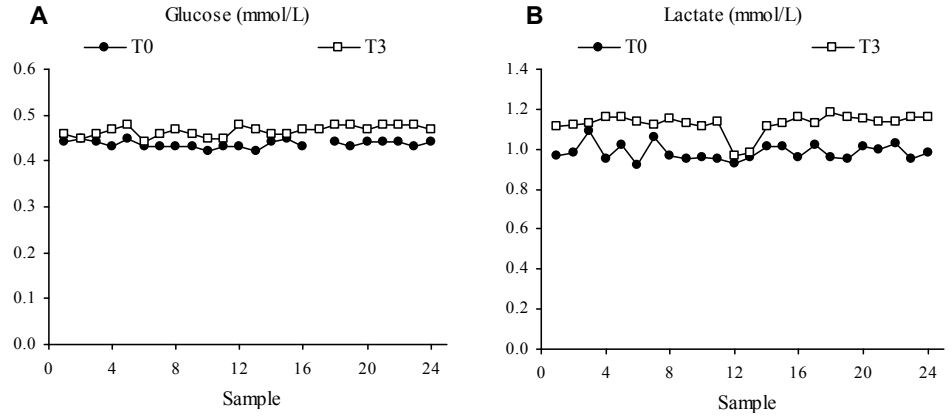


Figure 16. Vial materials, crimp caps. Analyses of solutions in glass vials sealed with crimp caps. These figures illustrate that concentrations of glucose (panel A) and lactate (panel B) were not changed during a batch analyse on day 0 (T0) and after 3 days of storage in a freezer (T3). (Paper II)

Storage in refrigerator: plastic with crimp caps or glass vials with crimp caps

Samples kept in plastic vials with crimp caps demonstrated an increased concentration during storage in a refrigerator for 3 days (Figure 17A). When samples were kept in glass vials with crimp caps glucose concentrations were unchanged during 14 days of storage in a refrigerator (Figure 17B).

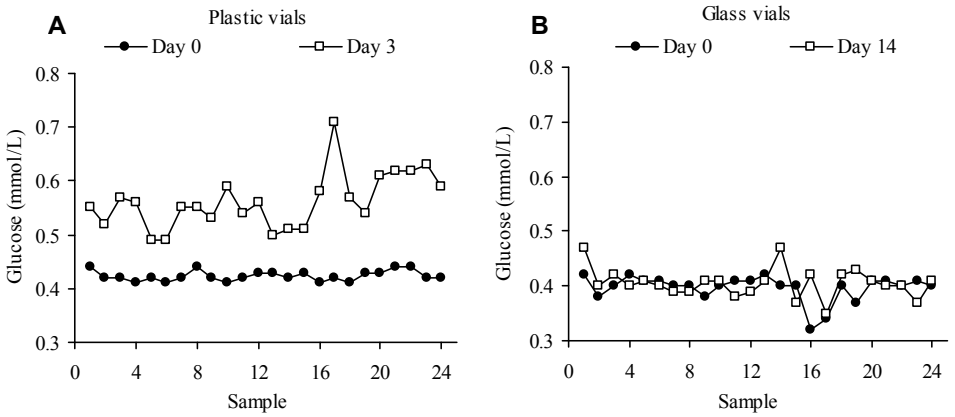


Figure 17. Different vial materials with crimp caps. Analyses of glucose. Panel A shows results from samples analysed on Day 0 and samples stored in plastic vials sealed with crimp caps in a refrigerator for three days. Panel B shows results from samples analysed on Day 0 and samples stored in glass vials sealed with crimp caps in a refrigerator for 14 days. (Paper II)

Paper III

The main finding in Paper III was that measurement of glucose, lactate, pyruvate and glycerol on the surface of the myocardium reflects relative changes in the myocardium.

Lactate

Absolute concentration

Absolute lactate concentrations during baseline were significantly higher in samples obtained from the surface probe compared with samples from the myocardial probe in both protocol 1 and 2 (Table 1).

Table 1. Absolute concentrations in microdialysate samples from a probe on the surface (S) of the heart and from a probe in the myocardium (M) during baseline (time 0), the four short ischemic periods and at the end of the long ischemic period. The baseline values were calculated as the group mean of the first two samples, short ischemia is a grouped mean over the four peak values and the long ischemia is a mean over the last sample during the ischemic period.

Substance		Baseline		Short ischemia	Long ischemia	
Protocol 1						
Lactate - S	(mM)	1.46 ± 0.59	*		5.03 ± 1.44	
Lactate - M	(mM)	1.01 ± 0.36			4.03 ± 1.11	
Glucose - S	(mM)	2.00 ± 0.67			0.42 ± 0.32	
Glucose - M	(mM)	1.76 ± 0.37			0.29 ± 0.23	
Pyruvate - S	(µM)	71.3 ± 18.7	*		92.9 ± 40.2	*
Pyruvate - M	(µM)	26.0 ± 11.9			33.7 ± 26.2	
Glycerol - S	(µM)	54.0 ± 37.5			127.8 ± 44.4	
Glycerol - M	(µM)	57.4 ± 36.8			201.3 ± 94.9	*
Protocol 2						
Lactate - S	(mM)	1.42 ± 0.34	*	2.32 ± 0.56	3.44 ± 1.22	
Lactate - M	(mM)	0.83 ± 0.23		2.08 ± 0.67	3.17 ± 1.30	
Glucose - S	(mM)	2.60 ± 0.90		1.77 ± 0.41	0.75 ± 0.73	
Glucose - M	(mM)	2.06 ± 0.48		1.27 ± 0.41	0.42 ± 0.30	
Pyruvate - S	(µM)	65.8 ± 28.0	*	102.0 ± 46.8	111.6 ± 35.3	*
Pyruvate - M	(µM)	31.2 ± 10.9		60.4 ± 18.1	51.9 ± 24.6	
Glycerol - S	(µM)	46.3 ± 35.3		38.3 ± 24.4	46.0 ± 32.7	
Glycerol - M	(µM)	39.7 ± 38.3		43.0 ± 33.7	64.6 ± 45.8	

Data are presented as mean ± 95% confidence intervals, * $p < 0.05$ using paired T-test.
S: Surface probe; M: Myocardial probe; mM: millimol/L; µM: micromol/L. Protocol 1: n=10 for all substances. Protocol 2: n=9 for lactate and glucose and n=8 for pyruvate and glycerol. (Paper III)

Relative changes

Both probes showed the same pattern with increased and decreased relative changes in concentrations during baseline, ischemic periods and reperfusion, respectively (Figures 18A-18B). A comparison between relative changes in lactate concentrations obtained from both probes revealed a similar pattern throughout the study protocol (Figures 18C-18D).

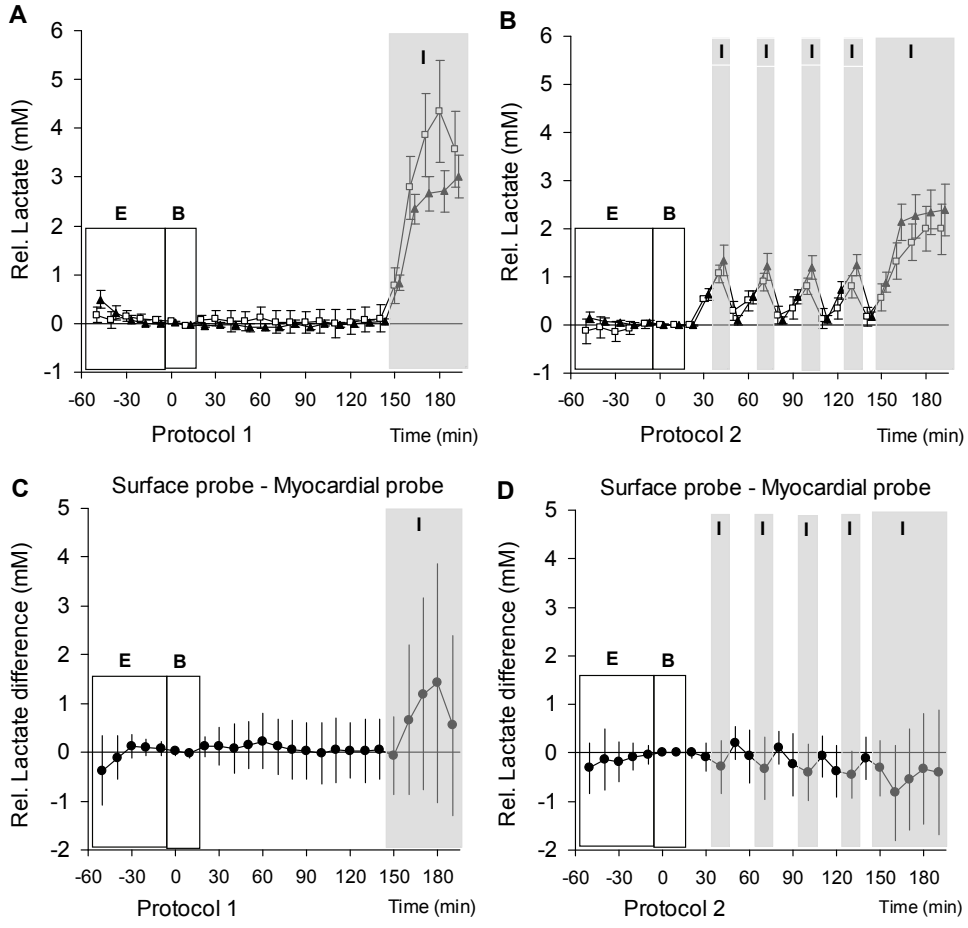


Figure 18. Lactate. Panels A and B depicts relative changes in lactate concentrations from baseline (time 0) based on data obtained from the surface probe (open squares) and the myocardial probe (filled triangles). Panels C and D depicts paired comparisons of relative changes in lactate concentrations between the surface probe and the myocardial probe at corresponding time points (filled circles). Data are presented as mean ±SEM (panels A and B) and as mean ±95% confidence intervals (panels C and D). E=Equilibration time, B=Baseline, I=Ischemia. Protocol 1, n=10 and protocol 2, n=9. mM: millimol/L (Paper III)

Glucose

Absolute concentration

There were no significant differences in absolute glucose concentration in samples from the surface probe compared with samples from the myocardial probe for both protocol 1 and 2 (Table 1).

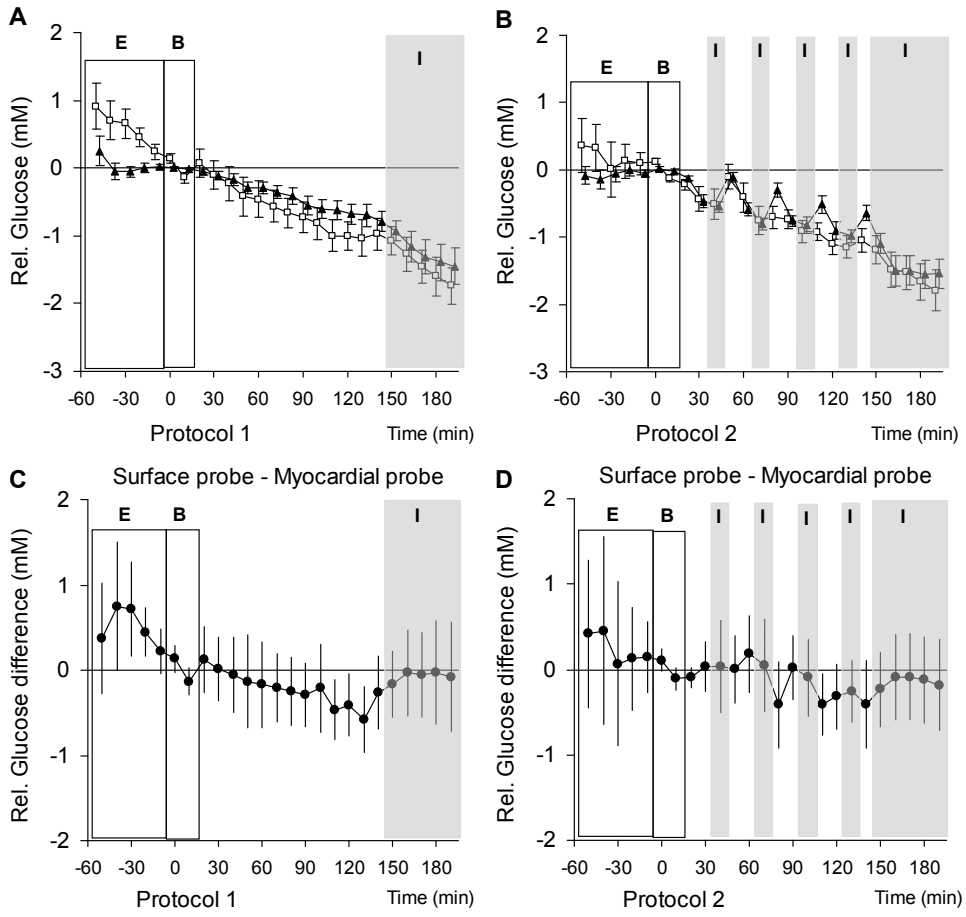


Figure 19. Glucose. Panels A and B depicts relative changes in glucose concentrations from baseline (time 0) based on data obtained from the surface probe (open squares) and the myocardial probe (filled triangles). Panels C and D depicts paired comparisons of relative changes in glucose concentrations between the surface probe and the myocardial probe at corresponding time points (filled circles). Data are presented as mean ±SEM (panels A and B) and as mean ±95% confidence intervals (panels C and D). E=Equilibration time, B=Baseline, I=Ischemia.
Protocol 1, n=10 and protocol 2, n=9. mM: millimol/L (Paper III)

Relative changes

Relative changes in glucose concentrations obtained from both probes showed a similar pattern, with no significant difference between the surface and myocardial probe during baseline, short ischemia and long ischemia. Note that the surface probe did not show the same pattern (though inverted) as the myocardial probe with decreased and then increased glucose concentrations during short ischemia periods and reperfusion (Figures 19A-19D).

Pyruvate

Absolute concentration

Absolute values for pyruvate concentrations were significantly higher in samples obtained from the surface probe during baseline and long ischemic period for both protocol 1 and 2 (Table 1).

Relative changes

There was a tendency towards higher concentrations in samples obtained from the myocardial probe during the long baseline period in protocol 1, but not during any of the other periods, i.e. ischemia and reperfusion (Figures 20A-20D).

Glycerol

Absolute concentration

Absolute glycerol concentrations in samples obtained from the myocardial probe were significantly higher than concentrations in samples from the surface probe during the long ischemic period in protocol 1 (Table 1). There were no other significant differences in absolute concentrations between samples obtained from both probes.

Relative changes

A comparison between relative changes in glycerol concentrations obtained from both probes revealed a similar pattern throughout the study (Figures 21A-21D).

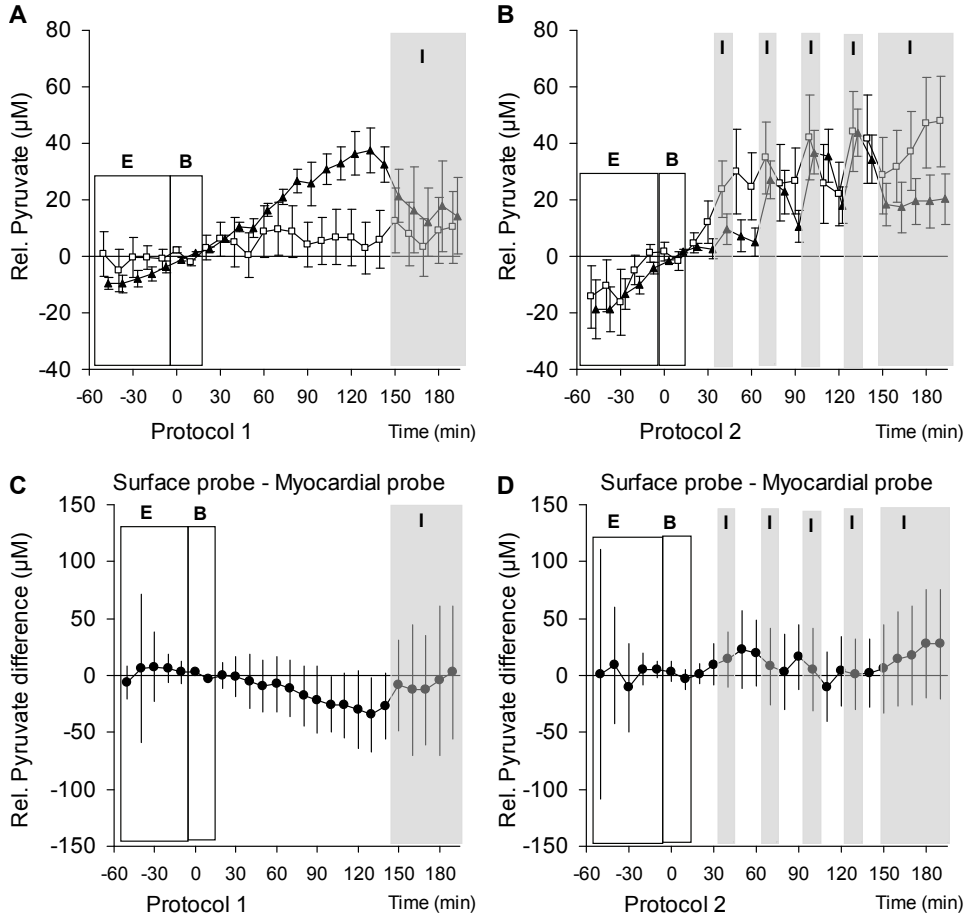


Figure 20. Pyruvate. Panels A and B depicts relative changes in pyruvate concentrations from baseline (time 0) based on data obtained from the surface probe (open squares) and the myocardial probe (filled triangles). Panels C and D depicts paired comparisons of relative changes in pyruvate concentrations between the surface probe and the myocardial probe at corresponding time points (filled circles). Data are presented as mean ±SEM (panels A and B) and as mean ±95% confidence intervals (panels C and D). E=Equilibration time, B=Baseline, I=Ischemia. Protocol 1, n=10 and protocol 2, n=8. µM: micromol/L (Paper III)

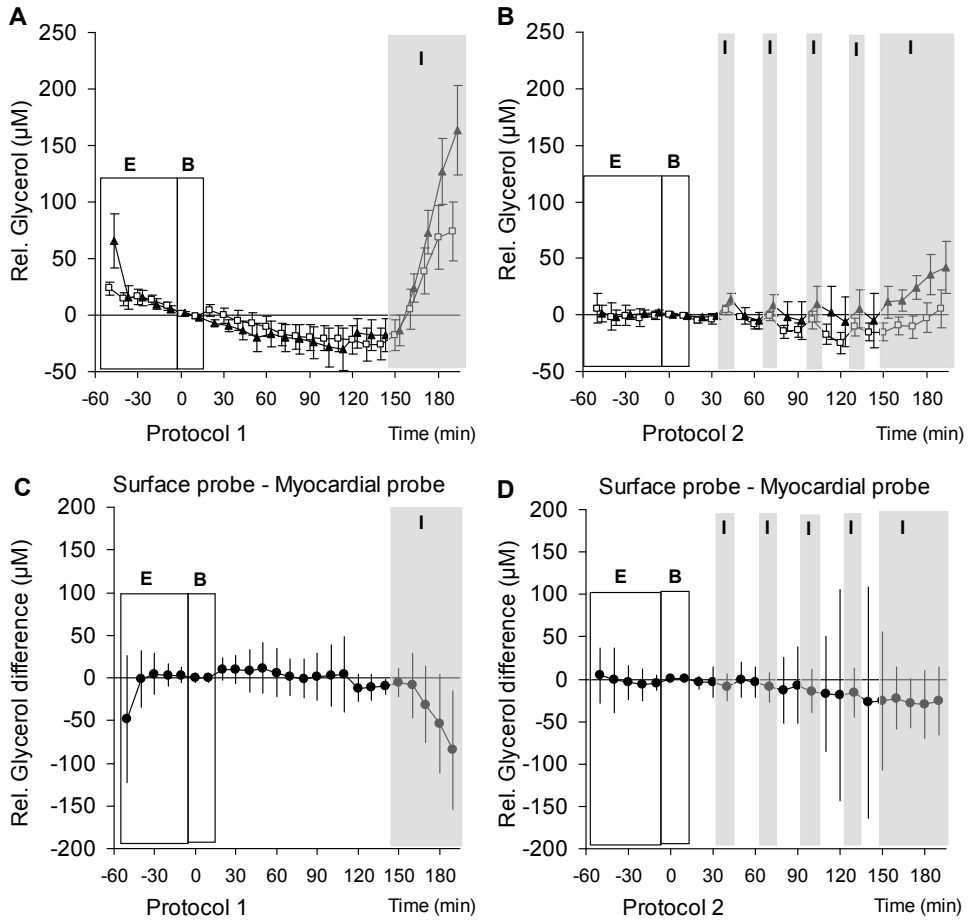


Figure 21. Glycerol. Panels A and B depicts relative changes in glycerol concentrations from baseline (time 0) based on data obtained from the surface probe (open squares) and the myocardial probe (filled triangles). Panels C and D depicts paired comparisons of relative changes in glycerol concentrations between the surface probe and the myocardial probe at corresponding time points (filled circles). Data are presented as mean ±SEM (panels A and B) and as mean ±95% confidence intervals (panels C and D). E=Equilibration time, B=Baseline, I=Ischemia. Protocol 1, n=10 and protocol 2, n=8. µM: micromol/L (Paper III)

Paper IV

Oxygen tension in the liver parenchyma fell from a mean of 41 mm Hg (range 22-55 mm Hg) to a mean of 7 mm Hg (range 0-25 mm Hg) during the period when the cotton tape encircling the portal vein and hepatic artery was kept tightened (n=9). Tissue oxygen tension remained at the attained level until the cotton tape was loosened. Thus, transient liver ischemia was achieved using the experimental model (Figure 22).

The main finding in Paper IV was that measurements of glucose, lactate and glycerol on the liver surface reflect relative changes in the liver parenchyma. MD data are presented as relative changes for the concentration of glucose, lactate, pyruvate and glycerol.

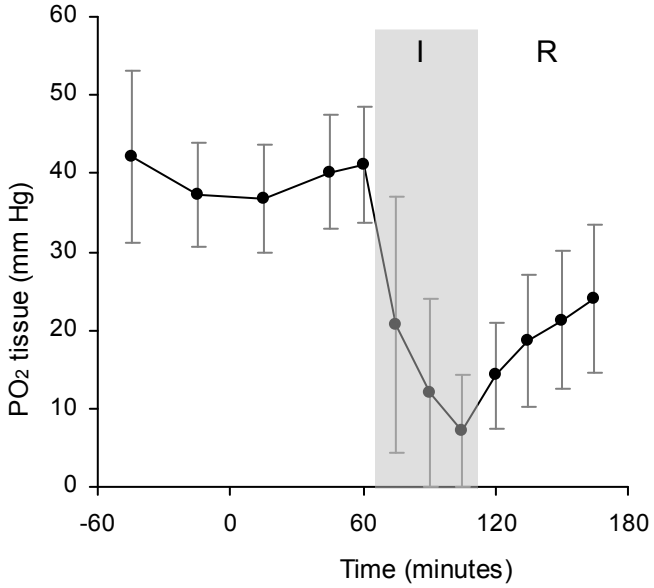


Figure 22. PO_2 tissue (mm Hg) in liver parenchyma during 45 min liver ischemia (I) and 60 min reperfusion (R). Data are presented as mean ± 95% confidence intervals, n=9. (Paper IV)

Lactate

In protocol 1 (1 µL/min), no significant differences in relative changes of lactate levels were seen between samples from surface probes and probes in liver parenchyma during equilibration, baseline, ischemia or reperfusion (Figures 23A and 23C). In protocol 2 (2 µL/min), significant differences in relative changes of lactate levels were observed in samples from the surface probes and probes in liver parenchyma during 15 to 45 min of ischemia (Figures 23B and 23D).

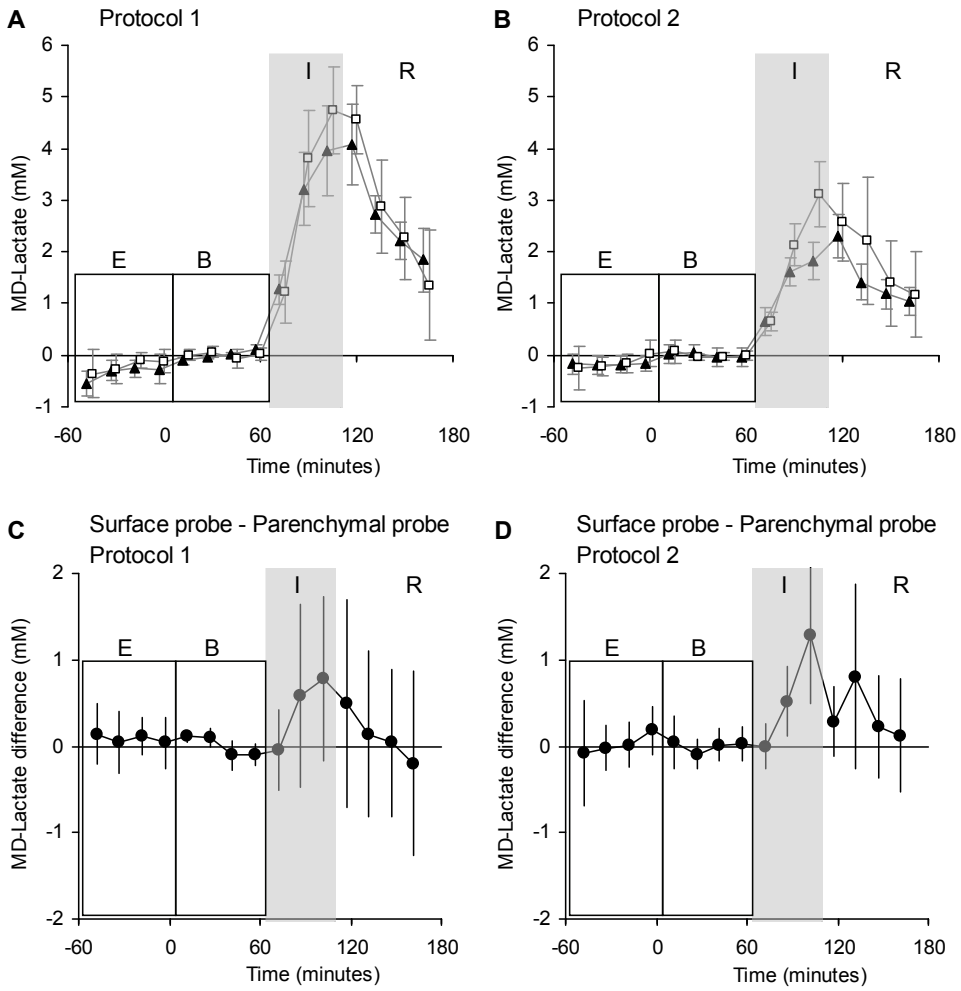


Figure 23. Lactate. Panels A-B depicts relative changes in lactate concentrations from baseline (time 0-60) based on data obtained from the liver surface probe (open squares) and the liver parenchymal probe (filled triangles). The flow rate was 1 µL/min in protocol 1, n=13 (panels A, C) and 2 µL/min in protocol 2, n=9 (panels B, D). Panels C-D depicts paired differences of relative changes in lactate concentrations between the surface probe and the parenchymal probe at corresponding time points (filled circles). E=Equilibration time, B=Baseline, I=Ischemia (shaded), R=Reperfusion. Data are presented as mean ±95% confidence intervals. mM: millimol/L (Paper IV)

Glucose

During the equilibration period, relative changes were significantly less pronounced in samples from surface probes as compared to samples from probes in liver parenchyma in protocol 2 (Figures 24B and 24D). No significant differences in relative changes in glucose levels were observed between samples from the liver surface and samples from the liver parenchyma during equilibration (protocol 1), baseline, ischemia or reperfusion for both protocols 1 and 2 (Figures 24A- 24D).

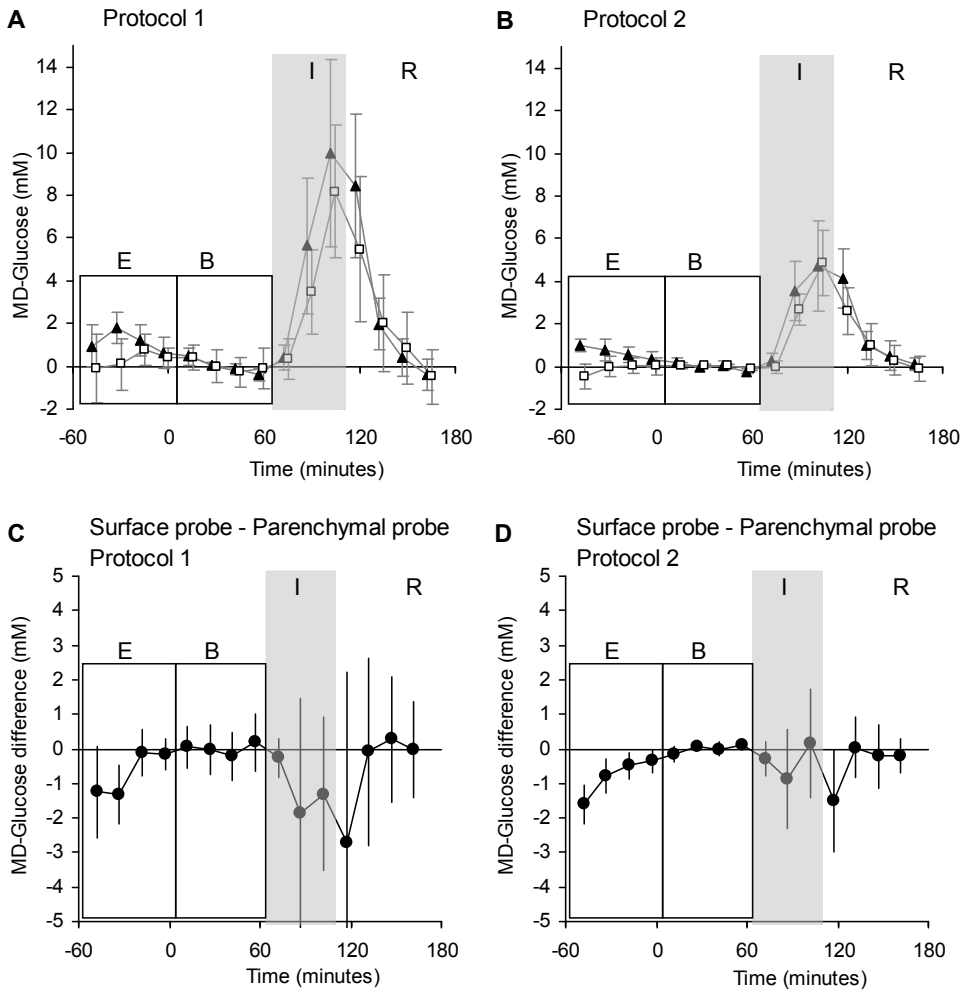


Figure 24. Glucose. Panels A-B depicts relative changes in glucose concentrations from baseline (time 0-60) based on data obtained from the liver surface probe (open squares) and the liver parenchymal probe (filled triangles). The flow rate was 1 µL/min in protocol 1, n=13 (panels A, C) and 2 µL/min in protocol 2, n=9 (panels B, D).Panels C-D depicts paired differences of relative changes in glucose concentrations between the surface probe and the parenchymal probe at corresponding time points (filled circles). E=Equilibration time, B=Baseline, I=Ischemia (shaded), R=Reperfusion. Data are presented as mean ±95% confidence intervals. mM: millimol/L (Paper IV)

Pyruvate

No significant differences in relative changes in pyruvate levels were noted in samples from surface probes and probes in liver parenchyma during equilibration, baseline or reperfusion (protocols 1 and 2) (Figures 25A-25D). During the latter phase of ischemia, relative changes were significantly more pronounced in samples from surface probes as compared to samples from probes in liver parenchyma, for protocols 1 and 2 (Figures 25A-25D).

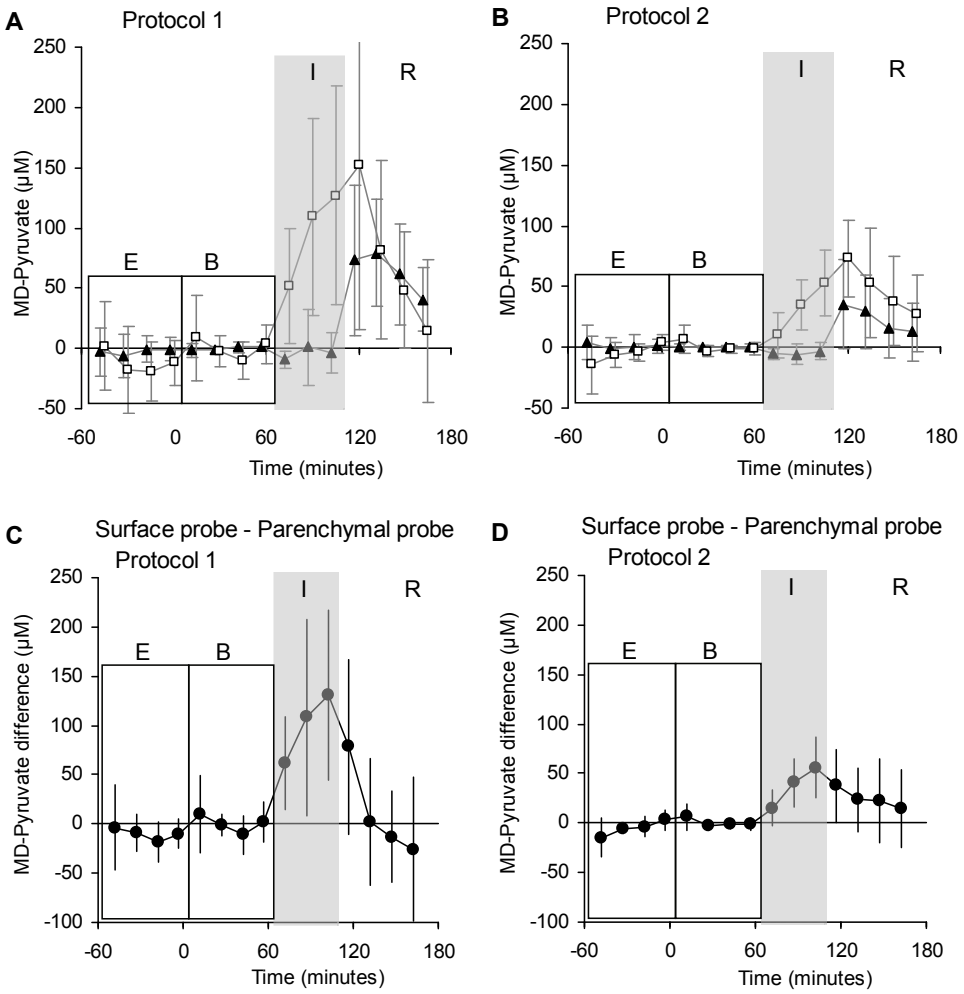


Figure 25. Pyruvate. Panels A-B depicts relative changes in pyruvate concentrations from baseline (time 0-60) based on data obtained from the liver surface probe (open squares) and the liver parenchymal probe (filled triangles). The flow rate was 1 µL/min in protocol 1, n=13 (panels A, C) and 2 µL/min in protocol 2, n=9 (panels B, D). Panels C-D depicts paired differences of relative changes in pyruvate concentrations between the surface probe and the parenchymal probe at corresponding time points (filled circles). E=Equilibration time, B=Baseline, I=Ischemia (shaded), R=Reperfusion. Data are presented as mean ±95% confidence intervals. µM: micromol/L (Paper IV)

Glycerol

During ischemic phase relative changes in glycerol levels were significantly less pronounced in samples from surface probes as compared to samples from probes in liver parenchyma, for both protocols 1 and 2 (Figures 26A-26D).

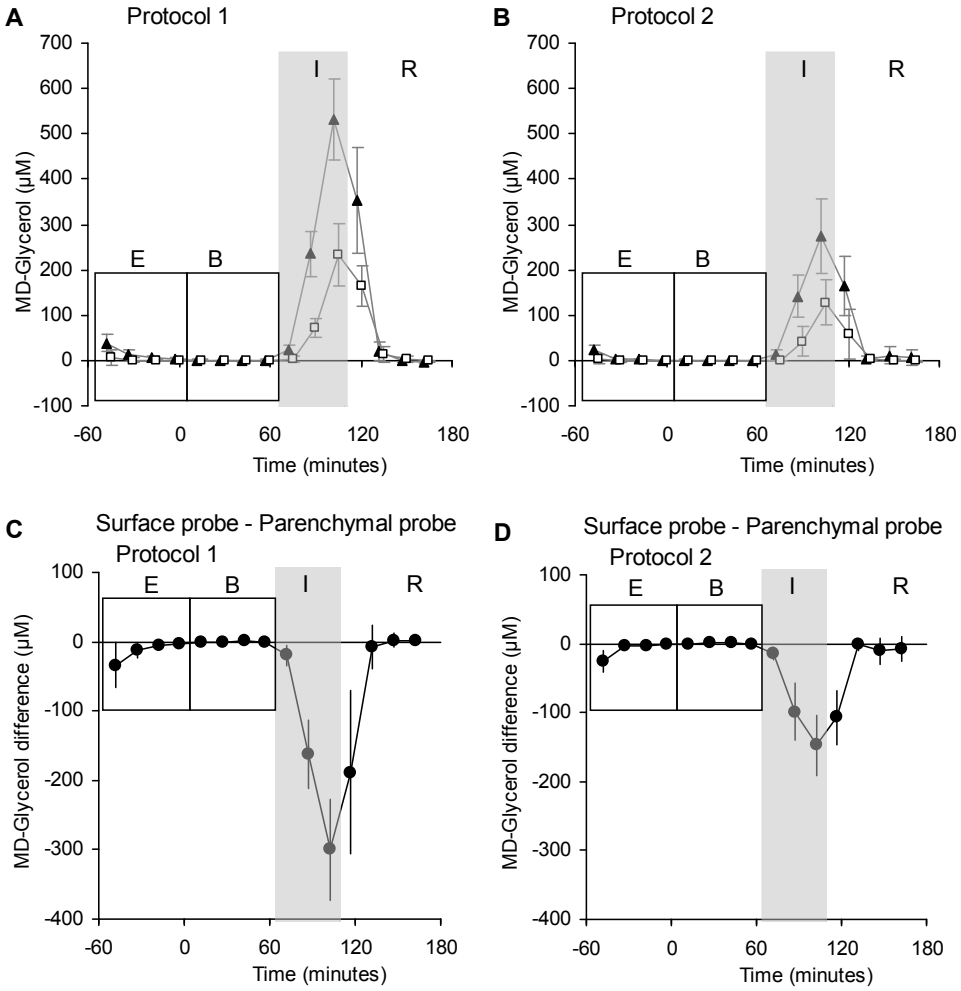


Figure 26. Glycerol. Panels A-B depicts relative changes in glycerol concentrations from baseline (time 0-60) based on data obtained from the liver surface probe (open squares) and the liver parenchymal probe (filled triangles). The flow rate was 1 µL/min in protocol 1, n=13 (panels A, C) and 2 µL/min in protocol 2, n=9 (panels B, D). Panels C-D depicts paired differences of relative changes in glycerol concentrations between the surface probe and the parenchymal probe at corresponding time points (filled circles). E=Equilibration time, B=Baseline, I=Ischemia (shaded), R=Reperfusion. Data are presented as mean ±95% confidence intervals. µM: micromol/L (Paper IV)

DISCUSSION

Microdialysis can be used to measure different conditions in several organs in the human body. The MD technique has potential for further development and more advanced clinical implementation. Many years of working with MD have been fruitful but also challenging and at times frustrating. This has for me generally concerned technical limitations in the sampling and analysis process. I have explored many technical aspects of MD with focus on those that have arisen out or our own lab group's internal critical approach to lab methodology. In this process, I recognised the need for internal validation of the methods in our hands, and also recognised that very little has been published concerning factors related to MD sampling and analytical variability. In developing this thesis, I have learned much about the MD technique, its advantages, its limitations, and have also explored a new unexpected way of MD sampling.

In the beginning, we tried to understand possible reasons for outlier or missing values that came from our analysis machine, but were not expected from the sample collection phase (animal experiments, aliquots in vials). Also, there were differences in concentrations after reanalyses of samples that have been stored in a freezer for several days. Higher concentrations with over 70% differences in concentrations were shown after reanalyses of samples from a standard solution of glucose (Figure 27). So, it was here that my search for the sources of error specifically related to MD methodology started.

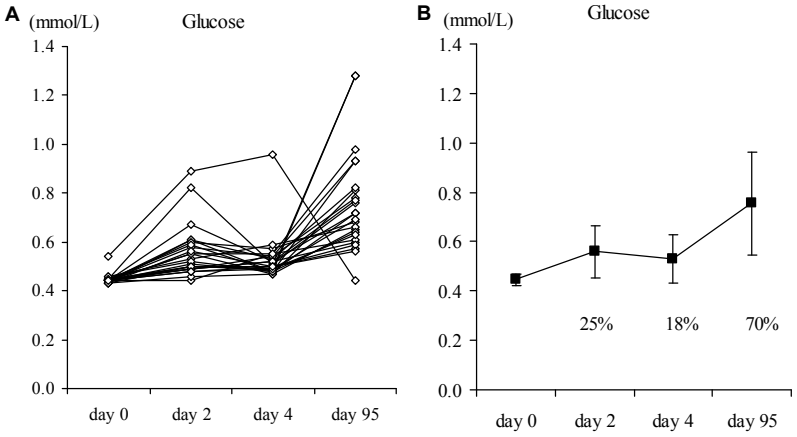


Figure 27. Glucose concentrations from a standard glucose solution. MD samples were analysed on the first day (day 0) and reanalysed after 2, 4 and 95 days of storage in -20°C. Panel A shows individual concentrations while panel B shows mean ± SEM, n=23.

Paper I

When MD samples are collected at a higher flow rate it is important to note that the MD technique only measure relative changes and not absolute concentrations.

If there is a need to know the true concentration of a substance in a specific tissue the NNF method can be used. In Paper I, the NNF method is used to try to establish the "true" concentration of glucose and urea in subcutaneous tissue. The *in vitro* experiment with the NNF method worked well, and the concentrations of the chosen substances could easily be calculated (Figures 11A, 11B and 12A). On the other hand, a pilot study showed that there can be difficulties to measure and calculate the "true" tissue concentration (Figure 12B).

Like in other studies that have described NNF method, we found this method to be extremely time-consuming and it becomes an experiment in itself (9, 13, 14). Perhaps, the NNF method is not necessary for all MD studies where relative changes provide enough information. However, it certainly would be advantageous to use a more simplified method, such as the retrodialysis method. A more simplified method would be desirable not just to find out the absolute concentration in a tissue, but also to examine if it is possible to measure a specific substance in a specific organ during a specific flow rate. The retrodialysis method is not so time demanding and it is easy to apply in all organs/tissues. Though, during pharmacological studies with the MD technique, a more advanced calibration method would be useful for understanding how the medicine is distributed in the tissue.

Paper II

Since Paper I provided some questions and ideas concerning the influence on samples of the storage and analysis steps, Paper II focused on storage and the analyser device. MD samples are stored at different temperatures (freezer, refrigerator) until they are analysed, and then during analysis held in the analyser with a temperature of 27 ^{o}C. Still, the storage and analysis steps are strongly influenced by time in the steps and by other factors. One batch containing 24 samples involve 192 minutes of storage in the analyser during analysis of four substances. During sampling with preclinical MD probes, MD samples are collected (standard) in plastic vials which are sealed with plastic caps for storage in a refrigerator or freezer. Before analysis in the CMA 600 analyser the plastic caps are removed and the vials stand open during the whole analysis time. Containing, only a small amount of solution, samples are very sensitive to exposure to air. Therefore, 192 minutes in a warm dry environment will lead to evaporation for samples, which will result in falsely high measured concentrations of the substances. The effect of evaporation can be recognised as gradual increases in concentrations of all analysed substances during the whole batch analysis step (Figures 13A and 13B).

Microvials, often used in clinical situations, have a small membrane at the top of the vials which can be penetrated with the analyser's needle. Results from analyses of samples in microvials showed no gradual increase in concentrations of

the substances through a batch analysis. The membrane at the top means that the microvials are sealed waiting to be analysed and therefore evaporation is minimal.

Since evaporation seems to affect concentrations in samples with small volumes, we decided to test glass vials with crimp caps and plastic vials with crimp caps for analyses in the CMA 600 analyser. Batch analyses with samples stored in glass vials with crimp caps and plastic vials with crimp caps showed no drift during the analysis. However, when crimp caps were not placed tightly enough around the plastic vials, storage in a refrigerator or freezer gave higher concentrations of the analysed substance. Since glass vials have been shown to give reliable results after storage in a refrigerator or freezer these vials are preferred during batch analyses. Results from Paper II led to a change in routines in our laboratory: centrifugation before analysis, glass vials with crimp caps for storage and analysis. In some cases, if the samples will be analysed by another analyser device, it might be necessary to use plastic vials. In that case the vials should be kept in the analyser for a maximum of 1 hour.

Paper III

MD sampling can be used in many preclinical and clinical situations in many different organs and with all kinds of applications. Though, the insertion of the catheter/probe means a trauma to the tissue, bleeding, blood flow disturbance and inflammatory response might occur (47, 48). This might be a limitation for the usage of the MD technique in sensitive organs. To reduce the risk of trauma, we have tried to measure metabolic changes on the heart surface. This idea came up unexpectedly during one of our other MD studies on heart muscle metabolism where a MD probe (CMA 20) relocated itself (its membrane) to the epicardium by accident. Interestingly, analysis of the 'misplaced' probe showed reasonable sampling both for concentration and pattern during ischemia and reperfusion.

The "accident" has been examined for the heart surface in paper III. An ordinary MD CMA 20 probe was used and placed on the heart surface, the probe was attached to the epicardium with a suture in the attachment point and one suture was secured over the membrane. The suture over the membrane was necessary since the probe can move during heart contractions thereby interrupting the steady contact with the epicardium. Since the suture was gently secured, the membrane was not damaged and the flow over the membrane seemed to be unaffected.

Concentrations of measured substances in the surface probe were slightly higher compared with corresponding concentrations in the myocardial probe. However, since MD measures relative changes, a relative comparison between samples from the surface probe and samples from myocardial probe is adequate showing similar changes in response to performed interventions (ischemia) for both probes when glucose, lactate and glycerol were examined. It may be counterintuitive that a surface probe that only has partial direct contact with the surface can demonstrate

the same pattern and concentrations as a probe placed within the myocardium. One theory is that a fluid layer is formed around the surface probe allowing diffusion of solutes.

Paper IV

MD has been reported to be a useful tool for measurements in association with liver transplantation and other liver conditions (49, 50). Measurements by MD on the surface of the heart and liver have not been previously described in the literature. Therefore, in Paper IV we chose to compare MD measurements on the liver surface with measurements in the liver parenchyma in an ischemic model. As in paper III ordinary conventional (CMA 20) MD probes were placed on the liver surface and in the liver parenchyma.

Compared with Paper III, where surface measurements resulted in higher concentrations, this paper showed a converted pattern with higher concentrations in samples from probes placed in the parenchyma. Maybe, our measured concentrations reflect a normal state for samples collected from the liver surface. Despite the lower concentrations in samples from the liver surface evaporation might occur and the concentrations might thereby be even lower if the conditions around the surface probe were different.

Reflections Paper III and IV

During our *in vivo* experimental studies (Paper III and IV) the thorax and abdomen were left open and only covered with a plastic film over the surgery cut. The open chest and abdomen might affect the surface of the organ and since the probe is exposed to air, the dialysate might evaporate and result in "falsely" higher concentrations of the substances. In a clinical situation, other factors may be present, and influence MD sampling from the surface of an organ. If the catheter is left in the surgery area post-operatively, the abdomen and the thorax will be closed and the surface probe/catheter design and function will need to be optimised for these conditions.

Considerations about Pyruvate

Pyruvate measurements from MD in ischemia experiments have at times been difficult to interpret. In Paper III, MD samples obtained during a longer period (hours) in the heart muscle, without interventions, showed a successive increase pyruvate concentrations. This phenomenon has also appeared in other MD studies in the myocardium (36, 51, 52). Unexpectedly, this pattern did not appear in the heart surface probe. Also, during Paper IV pyruvate measurements (as described above) appeared to behave differently dependent on the placement of the probes.

Results from pyruvate measurement in the liver parenchyma seem to differ despite probes being placed in the same way. Thus, there are reports showing

unchanged/decreased/increased pyruvate concentrations during ischemia as measured by probes in the liver parenchyma (53-57). These data should be compared with our results from MD samples collected from the liver surface.

Why the pyruvate concentration would increase when the probe is exposed to oxygen is hard to know. In order to investigate this method further, more studies have to be performed, biopsies for glycogen determination could be one possible way.

Probe/catheter design- patent

This thesis has shown that there is good reason to continue to work with new application areas for MD collection from the surface of an organ. In the future if the surface measurement is to be applied in clinical daily work, the catheter design and function need to be well validated. Since surgical incisions are always closed after surgery, clinical surface catheters will not primarily be exposed to air. The surface catheter will need some form of protection which allows specific organ sampling and reduction of influences from adjacent tissues and organs. This protection or insulation would be needed also to prevent evaporation or convection of substances from the perfusate. A new catheter design for this purpose has been developed, and a US provisional and PCT patent is pending.

Application

Surface measurements on the heart and liver have not previously been described in the MD literature. This application area opens new possibilities concerning MD measurement. Until now the MD technique has not been a routine procedure in the clinical daily work. In some specialities, MD sampling is started to be used for more routine application of patient conditions. Examples of clinical applications include liver transplantation and vascular graft vitality after surgery. Metabolic studies with MD sampling have been performed during open heart surgery both in animal and human (2-4, 58-63). Also, several studies in the animals (pig) and humans liver have been performed successfully (49, 50, 56, 57, 64). In clinical studies, the MD technique is often used in brain function studies among others to measure the metabolic changes, but also to administer drugs through the membrane to a specific tissue for example a brain tumour (65). In animals, the MD technique has, among other tissues, been applied for measurements in subcutaneous tissue (66), pancreas (67) and in the kidney (68).

Concerns about the MD technique: as a method

Despite the fact that the MD technique has great potential there are many unanswered questions. It can be difficult to compare different studies with each other, the procedure might differ between study protocols and the catheter can be placed

in different ways. This is clearly illustrated by the different results during pyruvate sampling in the liver tissue. Perhaps it is pyruvate that is difficult to measure since other substances (glucose, lactate and glycerol) seems to be more unanimous.

There are several concerns for the catheter/probe positioning in the tissue, for how long the catheter might be placed in the tissue without being affected or does the probe affect the tissue? There is a lack of studies concerning the influence of the probe/catheter implantation on tissues. Some studies have shown that the MD catheter can be placed in the tissue for a long time, in some cases for up to 3 weeks (25, 26, 37-41). Another paper, where a MD probe was implanted in a rat brain for up to 60 days showed that the tissue was affected by the implantation already after 24-48 hours. After 3 days the MD probe was invaded by macrophages (47).

Future considerations

It would be desirable to have a handbook in MD sampling in which normal conditions were described and normal concentrations of substances in different organs in both humans and animals were presented with different flow rates and catheters/probes. MD sampling is now conducted without any standard procedures. With no standardisation of routines and procedures, values from patients/animals will be difficult to compare from paper to paper.

MD sampling on the heart and liver surface seems to work well, and in the future this application should be examined even more closely. A new catheter should be developed to specifically be used for new application areas. A surface probe might lead to a new way of MD sampling, but may also help to shed light on issues concerning diffusion across the different layers in the heart muscle, liver tissue and other organs. Surface measurement is probably useful in other organs, but this needs to be further investigated. The main goal with the new surface catheter is that it could be practically useful in a number of clinical settings including for example in transplantation surgery of the heart, liver, kidney and also during heart surgery.

CONCLUSIONS

- MD sampling in a "new" organ/tissue should be preceded by a study to evaluate the possibility of diffusion across the MD membrane at a specific flow rate. To this end, a simple method as the Retrodialysis method or the more advantageous No Net Flux method could be used.

- Irrespective of chosen vials, centrifugation is necessary before MD analysis in the CMA 600 analyser.

- Plastic vials without crimp caps can be used during analysis time shorter than 1 hour.

- Plastic vials with crimp caps should not be used.

- Glass vials with crimp caps or microvials should be used during analysis in the CMA 600 analyser lasting longer than 1 hour.

- Storage in glass vials with crimp caps in a refrigerator or freezer is possible only if crimp caps have not been penetrated.

- MD sampling (lactate, glucose, pyruvate and glycerol) with a probe on the heart surface showed a similar pattern compared to a probe placed in the myocardium in an ischemic model.

- MD sampling (lactate, glucose and glycerol) with a probe on the liver surface showed a similar pattern compared to a probe placed in the liver parenchyma in an ischemic model.

- MD measurements on the surface of the liver and heart are a new way of MD sampling with reduced risk for tissue damage. This risk reduction opens for new application areas for the MD technique.

ACKNOWLEDGEMENTS

Biathlon and laboratory science is a great combination.
My supervisor, professor **Ola Winsö**, thanks for your support and for giving me the opportunity to write this thesis.

Chanterelle is easy to find, learning Italian is more difficult.
My workmate **Göran Johansson**, thanks for all your great support during all these years. Special thanks for your help throughout the work with the thesis; it would never have been possible without you.

A calm day is great for rowing along the river.
Many thanks to **Michael Haney,** my co-supervisor and workmate, for reading and discussing my works, without you this thesis would never have been possible.

During long walks you gather energy and plans for common activities.
Anna-Maja Åberg, my co-supervisor and workmate, thanks for all your support and for being my friend. The work will not be the same without you.

The surgeon who speaks Lappish, Finish, Latin and French.
To my workmate, **Per-Jonas Blind**, many thanks for giving me some important, friendly lesions in surgical technique and for your kind support.

Chasing elk is holy.
Many thanks to my workmate, **Sören Häggmark**. You have taught me so much and are always prepared to give me a hand. "Only two cuts are needed to reach trachea".

Cats are wonderful!
My former professor, **Björn Biber**, thanks for all the positive and great support you have given me during all these years.

New ideas are easy to find.
Professor, **Anders Waldenström**, thanks for believing in my new MD product.

Economist who likes being in the forest and snowboarding.
My business coach, **Bo Hammarström,** Uminova Innovation, thank for all your support during my patent process.

To all pleasant people in "**Fikarummet UKBF**", it is always nice to take a "fika" and chat with all of you.

Many thanks to **Innovationsbron**, for financial support during the verification process for the patent application.

Thanks to **Aestus International**, for good collaboration during my patent process.

Camping, parties, travels and pleasant dinners.
Thanks to all my **friends** for all the joyful moments with all of you.

Early mornings' you have been driving from Lycksele taking care of sick grandchildren.
Many thanks to my mother-in-law **Gudrun Abrahamsson** for all valuable support with the children.

Meatballs and biscuits are your speciality.
Many thanks to my wonderful grandmother, **Birgit Hedlund,** for all your love and concern for us grandchildren and great grandchildren.

The "uncrowned king" of Vasaloppet.
My younger brother **Petter Ögren,** his partner **Sofia Edler** and their wonderful children **Alfred** and **Ellen** for all pleasant company when we travel to Nice and other places. *Sofia, next time we will meet Brad Pitt.*

France (Nice) is our common dream and we can always look back to joyful and wonderful times during our travels.
Many hugs to **Staffan and Gunilla Ögren** my parents, who always support me and for just being you. *We look forward to the next autumn*.

Your fantasy has no limitations.
My beautiful and lovely daughter **Klara,** you fill my life with joy.

Building Lego and playing games full fill your days.
My wonderful and lovely son **Hugo**, a hug from you makes my day complete.

The cross- country skier, who likes to ride a bike and dreams of building his own cottage.
My lovely husband **Mattias**, thanks for your support, love and for all joyful moments with you and our children.

Populärvetenskaplig sammanfattning på svenska

Bakgrund. Mikrodialys (MD) tekniken används idag framförallt inom experimentell och klinisk forskning. Med MD tekniken kan man kontinuerligt samla och mäta små mängder av substanser lokalt i en vävnad. Exempel på dessa substanser är glukos, laktat, pyruvat och glycerol. För att analysera dessa substanser kan man exempelvis använda sig av en CMA 600 analysator. Det finns i dagsläget inte mycket litteratur som har beskrivit och utvärderat provhantering och användningen av CMA 600 analyssystemet. Syftet med denna studie har varit att identifiera eventuella felkällor vid insamling av MD prover, provhantering och användningen av CMA 600 analyssystemet. Syftet har också varit att undersöka nya möjligheter för att kunna samla MD prover på hjärtats och leverns yta.

Material och Metoder. Artikel I och II är framförallt *in vitro* studier med undantaget för No Net Flux (NNF) metoden i artikel I som användes för att kunna beräkna den sanna koncentrationen av glukos och urea i subkutan vävnad hos gris (n=7). I artikel II testades flödeshastighet, insamlingstid, provrör (vialer) och kork material samt användningen av CMA 600 analyssystemets inverkan på resultaten. I artikel III och IV genomfördes försöken på sövda och normoventilerade grisar (n=33). I artikel III användes en hjärtischemi modell, korta och långa perioder av ischemi (syrebrist) i hjärtmuskeln framkallades för att kunna jämföra känsligheten av MD mätningar i hjärtmuskeln och på hjärtats yta. I artikel IV placerades MD proberna på leverns yta och i lever parenkymet. Under försöket (efter baseline) framkallades en längre ischemi period följt av en återhämtningsperiod. Samtliga djurstudier var godkända av den Djuretiska nämnden vid Umeå Universitet.

Resultat. Resultaten av NNF kalibrering i subkutan vävnad (artikel I) gav en beräknad koncentration för glukos på 4.4 mmol/L och för urea på 4.1 mmol/L. Artikel II visar att under en serieanalys av 24 prover reduceras driften (under analystiden) då glasvialer med krymplock används jämfört med då lösningen analyseras i öppna plastvialer. För att minska spridningen mellan proverna är det viktigt att alla MD prover centrifugeras innan varje analystillfälle. Artikel III visar en ny möjlighet för MD mätningar på hjärtats yta. Proben placerad på hjärtats yta visade samma mönster för glukos, laktat och glycerol jämfört med MD mätningar gjorda i hjärtmuskeln under baseline samt under kortare och längre perioder med ischemi. Artikel IV visar god samstämmighet mellan MD mätningar i leverns parenkym och på leverns yta under hela försöket.

Konklusion. Genom MD tekniken kan man enkelt mäta lokala metabola förändringar. Det är viktigt att beakta faktorer som flödeshastighet, insamlingstid och behandlingen av proverna utifrån valet av analysutrustning och vad som skall analyseras innan en försöksserie startar. Huvudfyndet under denna avhandling är att glukos, laktat och glycerol avspeglar samma förlopp på hjärtat och leverns yta som i hjärtmuskeln respektive lever parenkymet.

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