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Continuous metabolic monitoring techniques

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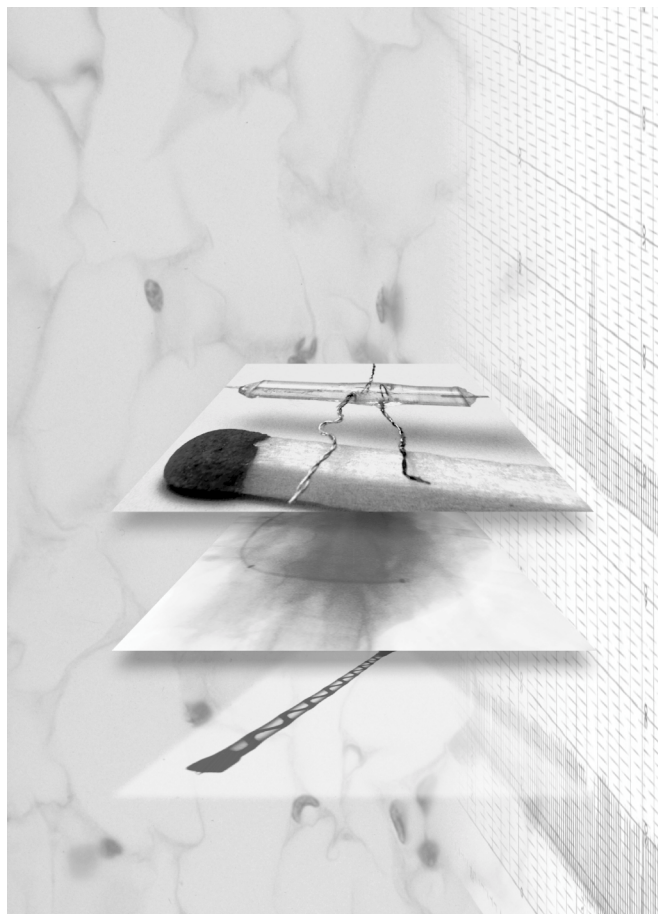
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Chapter 2

Slow ultrafiltration for continuous in vivo sampling; application for glucose and lactate in man



Abstract

Introduction: An ultrafiltration (UF) technique was developed for continuous subcutaneous (s.c.) sampling and on-line analysis of absolute glucose and lactate concentrations in tissue. The relation between subcutaneous and blood concentrations was studied in men, because a subcutaneous monitoring device would put patients on less risks than an intravascular device.

Methods: Ultrafiltrates were withdrawn continuously at a flow rate of 50-100 nl/min from a hollow fibre probe to measure glucose in the abdominal subcutis. Six healthy volunteers underwent an oral glucose tolerance test. In order to detect glucose and lactate in the same sample, a splitter was placed between the on-line flow injection valve and the parallel enzymatic conversion and electrochemical detection cells.

Results: Subcutaneous glucose concentrations were in steady state on the average 1.06 mM lower. They rose delayed and blunted as compared to blood levels. We demonstrated the ability of simultaneous lactate and glucose measurements in vivo (n=2).

Conclusions: UF makes continuous monitoring of absolute extracellular concentrations in tissue possible. We interpret the deviations of subcutaneous measurements from intravascular levels in this way that the subcutis is a kinetic compartment not directly and exclusively linked to blood. The observed differences with blood suggest that diabetes management may demand intravascular monitoring. UF combined with analysis of glucose and lactate in the same sample offers the opportunity to study pathophysiology inside tissues.

Introduction

In clinical and basic research, pathophysiological processes are often monitored by *in vivo* sampling of body fluids. Sampling of blood is most commonly utilised, because a large sample is relatively easy to obtain, and it can inform us about all organs and their interactive functions. Clinicians increasingly relying on fast biochemical analysis urge on continuous monitoring. However, continuous blood sampling poses infectious hazards and risks of internal bleedings, because the patient is often heparinised. Moreover, the wide tubes and high flow rates required to prevent obstruction can cause precious blood loss to the patient. Finally, the concentration of the analyte may change rapidly once the blood is sampled and is no longer under physiological control mechanisms.

To avoid blood sampling, monitoring *in vivo* might be done by biosensors. Since their introduction in 1962⁽¹⁾, biosensors have been improved, thus creating a wide variety of applications for off-line discontinuous monitoring in medicine e.g. for blood glucose control in medicine⁽²⁾, for process control in industry⁽³⁾, and for surface water control in the environment⁽⁴⁾. Applications of biosensors available for continuous *in vivo* monitoring of patients -bed-side or ambulant- are very limited. For such applications, the sensors should not only be safe, small, robust and easy to handle by any patient, but above all they must perform reliably in the complex matrix of body fluids⁽⁵⁾. Unfortunately, sensors directly introduced in the body face bioincompatibility problems. Sampling by microdialysis (MD) and more recently by ultrafiltration (UF) has been proposed as an interface between the body and the sensor⁽⁶⁻⁸⁾, because the material used for both sampling methods has been extensively tested for safety. UF and MD use an implanted semi-permeable membrane to filtrate or dialyse biochemical analytes from the surrounding tissue interstitium. The perfusate with the analytes is being transported by pumping and analysed outside the body. Because the membrane excludes cells and large molecules, MD and UF are able to deliver a clean matrix for measurement to a biosensor. Another advantage is the ability to monitor processes inside tissue of specific organs⁽⁶⁾. In this way, the afore-mentioned intravascular hazards are evaded.

In order to use subcutaneous MD for clinical decision making, these measurements should invariably be closely related to blood. A major problem of MD is the exact determination of the recovery of the analyte *in vivo* because it is lower and more variable than *in vitro*. The (relative) recovery is defined as the dialysate/body-fluid concentration ratio expressed as a percentage. The recovery

is assumed to be independent of the concentration of the analyte⁽⁹⁾. Sometimes, the absolute recovery is used, being the amount of analyte recovered in the dialysate during a certain time interval. Despite the development of several techniques such as ‘no-net-flux’⁽⁹⁾, ‘internal reference’⁽¹⁰⁾, and ‘various flow rates’⁽¹¹⁾, to determine the recovery in vivo, it remains difficult to estimate the absolute concentrations with MD. To follow only trends with relative measurements limits the applicability of the MD technique for monitoring patients. MD has some other drawbacks, e.g. the recovery may change after long-term implantation (possibly due to adhesion of fibrin, collagen, and cells after traumatic probe insertion), and there may be a removal of the analyte or influx of MD buffer which is not rapidly compensated. So, MD may also influence the physiology at the sampling site. An important question about the relation of subcutaneous MD to blood remains yet unanswered. Why are MD concentrations lower and more variable between probes in vivo as compared to in vitro? Are these differences entirely due to changes of the probe, or are these differences (also) reflecting actual lower tissue concentrations at the site of measurement? The latter hypothesis carries the consequence that glucose concentrations in the subcutis interstitium would be not merely dependent on intravascular concentrations but would be also dependent on local factors of the probe surrounding tissue. If that would be the case, subcutaneous glucose levels would be the result of the local equilibrium between supply from the vessels (influx) and uptake by cells (efflux). As the subcutis itself produces no glucose to buffer changes, the subcutaneous glucose levels might be more variable than in blood. In fig.1. we show four types of subcutaneous glucose curves to be expected in an OGTT. In case the glucose efflux is large compared to the influx, the glucose levels will be lower (fig.1.C,D) than when the influx is more dominant (fig.1 A,B). The equilibrium between influx and efflux may settle fast or slow, resulting in a steep follow-up curve (fig.1.A,C) or a flattened curve (fig.1.B,D). To test the hypothesis of lower glucose concentrations in tissue, a subcutaneous detection method is needed which combines measurement of absolute concentrations with a high time resolution. UF is an alternative sampling technique for MD, developed to make the analyte concentration independent from diffusion through the probe membrane. The UF technique samples interstitial fluid through a membrane by underpressure instead of diffusion. The semi-permeable membrane used for UF sampling is comparable with the microdialysis membranes, and excludes large molecules (e.g. large proteins), whereas small analytes such as glucose and lactate enter the probe together with water and ions. The technique was firstly described for batch-wise sampling^(12;13) with large

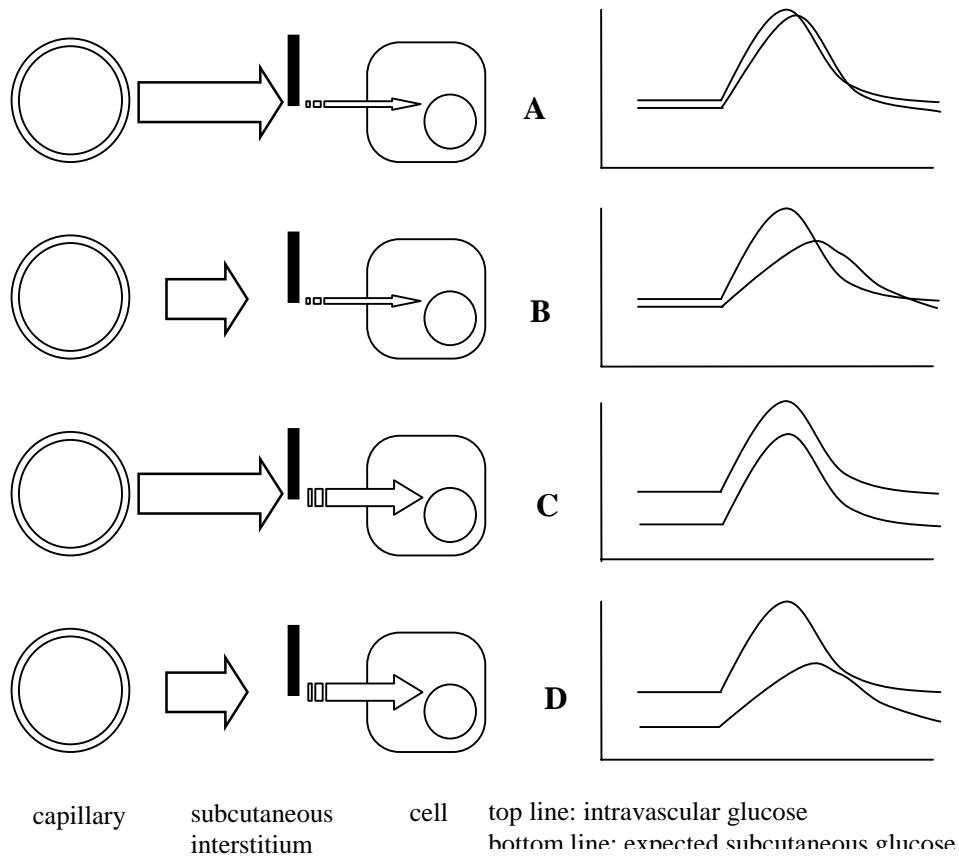


Fig. 1. four types of subcutaneous glucose curves to be expected in an OGTT. Left-hand side: schematic representation of different glucose influx (from capillaries) and efflux (to cells) from the s.c. interstitium. Right-hand side: resulting s.c. concentration curves.

A,B: glucose efflux is small compared to glucose influx;
the s.c. glucose levels will be close to i.v. levels

C,D: glucose efflux is large compared to glucose influx;
the s.c. glucose levels will be lower than i.v. levels

A,C: the equilibrium between influx and efflux settles fast,
resulting in a steep follow-up curve

B,D: the equilibrium between influx and efflux settles slow,
resulting in a flattened follow-up curve

probes and a low sampling frequency (every 10-20min). Recently, small UF probes have been proposed^(14;15). In our laboratory Moscone et al.⁽¹⁵⁾ and Kaptein et al.⁽¹⁶⁾ developed an UF technique with a 4 cm probe for continuous on-line sampling and analysis. A disposable pump (weight 5 g) produces a stable 100-300 nl/min sample flow rate for 24 hours and longer. Unlike MD, UF sample concentrations are not dependent on probe diffusion characteristics because analytes of low molecular size are almost 100% recovered by solvent drag^(16;17). Large molecules, however, may encounter hinderance of the membrane, leading to an underestimation of the analyte concentration. Discontinuous subcutaneous UF-sampling technique with large probes has been documented well^(7;12;13;17;18). The method developed in our laboratory uses small probes and allows frequent analysis^(15;16).

Here, the potential of the on-line ultrafiltration technique in vivo in human volunteers was explored to test the hypothesis of lower glucose concentrations in tissue. UF fluid was continuously withdrawn from the extracellular space in human subcutis. The UF fluid was analysed discontinuously every one or two minutes by a flow injection system detecting glucose electrochemically after enzymatic conversions. In this manner, glucose levels were continuously monitored in six volunteers who underwent an Oral Glucose Tolerance Test (OGTT). As stated above, the hypothesised lower s.c. glucose levels imply the existance of an interaction between influx and efflux parameters. These parameters would influence the levels of other metabolites in tissue as well. We tested this for lactate. The system was improved to analyse glucose and lactate levels in the same sample. Two subjects were monitored in this manner.

Methods

General description of the experiments

The experiments were performed in six volunteers. The UF probes were placed subcutaneously (sc) near the umbilicus. During the experiments, the UF-probes were connected to the glucose and lactate detection system. The ultrafiltration sampling and analysis set-up is shown in figure 2. The sampling part on the bottom side comprised a hollow fibre probe and a semi-vacuum tube as the driving force on a balance. The sample was brought into the detection part of the system by an intercalated switching loop. The detection system^(15;19) consisted of a Phosphate Buffered Saline (PBS) solution containing ferrocene, an HPLC pump, a streamsplitter, two parallel enzyme reactors containing Horse Radish Peroxidase (HRP) and either Lactate or Glucose Oxidase (LO or GOD), and two parallel ElectroChemical cells (ECDs). The ultrafiltrate was analysed

every one or two minutes by switching the position of the valve, which injected ultrafiltrate into the detection system.

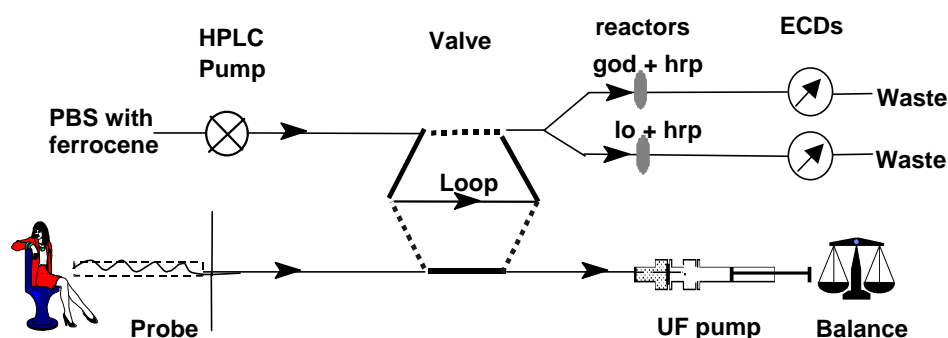


Fig. 2. Ultrafiltration sampling and analysis set-up. bottom side: sampling hollow fiber probe and UF pump on a balance; middle: sample injection valve (loop in injection position___ or in load position---); top side: detection with PBS buffer containing ferrocene, HPLC pump, splitter, two parallel enzyme reactors containing Horse Radish Peroxidase (HRP) and either Lactate or Glucose Oxidase (LO or GOD), and two parallel Electric detectors (ECDs).

Ultrafiltration

The probe consisted of a 2.5 cm long hollow fibre from an artificial kidney (AN69HF; Hospal Ind., Meyzieu, France, 340 μ m OD, 240 μ m ID, MWCO 50kD). To keep the lumen patent, the fiber was reinforced from the inside with a home-made spring of stainless steel wire (Vogelsang, Hagen, Germany), D=60 μ m, 12 axial length windings/cm. The fiber was glued with cyanoacrylate (Sicomet 40; Henkel Corp., Kankakee, IN, USA) to a draining fused silica capillary tube (20-65cm l, 150 μ m OD, 50 μ m ID, Polymicro Technologies inc., Phoenix, AZ, USA). Before closing the other end of the finer with glue, the tube and finer were filled up with 0.9% NaCl. The probe was placed in 0.9% NaCl in a disposable syringe before sterilisation with gamma irradiation (min. 25 kiloGray; Gammaster B.V., Ede, the Netherlands).

The ultrafiltration flow rate was generated by the semi-vacuum (625 \pm 1 mbar \pm SD, 0.2 %CV) of a syringe (1.2 ml monovette, Sarstedt, Nümbrecht, Germany) with a fixed piston⁽¹⁹⁾. To stabilise the ultrafiltration flow a defined capillary restriction (l=4cm, OD=150 μ m, ID=15 μ m fused silica tube; Polymicro Technologies inc., Phoenix, AZ, USA) was placed in front of the semivacuum. To avoid flow disturbance a fluid-filled bubbletrap was placed between the probe draining tube and the capillary restriction. The time

resolution of the system is limited by spreading of 3-5 minutes⁽¹⁶⁾.

The in vivo ultrafiltration flow rate was measured by weight. The subcutaneous flow rate was on average 47 nl/min (range 32-100 nl/min), as compared to 100 nl/min in vitro. When the ultrafiltrate flow changed, the instrumental lag-time and measurements were corrected accordingly.

Combined glucose and lactate analysis

The flow injection analysis system described by Elekes et al.⁽¹⁹⁾ was modified to detect glucose and lactate in the same sample. A splitter, a Lactate Oxidase with Horse Radish Peroxidase enzymatic cell, and an Electrochemical cell were added to the set-up (see fig.2). The UF sampling part was connected to the analytical part of the set-up by an intercalated valve. A Decade sampler (Antec Leyden B.V., Zoetermeer, The Netherlands) equipped with a Vici Cheminert C4 valve with a 20nl internal loop (Valco Instruments Co. Inc., Houston, USA) was used for sample injection in the detection system. The loop was partially filled. The valve injected every 15 seconds a 2 seconds collected ultrafiltrate sample in ferrocene PBS, which was being pumped at a flow rate of 0.8 ml/min (HPLC pump LC-10AD, Shimadzu, Japan) through a fifty-fifty splitter and two enzyme reactors and electrochemic detectors. The flow split proportion was approximately 50/50 and remained stable throughout the experiments. Ferrocene PBS contents were: 137 mM NaCl, 2.7mM KCl, 8mM Na₂HPO₄, 2.5 mM KH₂PO₄ (pro-analysis quality purchased from Merck, Darmstadt, Germany), 0.5 mM Ferrocenemonocarboxylic acid (Sigma Chemical Co, St. Louis, MO, USA) and 0.1 volume% Kathon CG (Rhom and Haas, Croydon, UK) in double quarts distilled water. The solution was neutralised to pH 7.4, filtered, and bubbled with Helium to remove air. The enzyme reactors contained 200 U Horseradish Peroxidase (EC 1.11.1.7) and either 200 U Glucose Oxidase (EC 1.1.3.4, grade I) or 200 U Lactate Oxidase Lyophilizate (Boehringer Mannheim, Germany) in 20 µl 0.9% NaCl immobilised between cellulose nitrate filters (pore size 0.01µm, MWCO 50kD, Sartorius, Göttingen, Germany). The electrochemical cells were of a thin layer-type, with a working electrode of glassy carbon kept at 0.00 mV relative to an Ag/AgCl reference electrode and a teflon/carbon counter electrode (Amor cell, Spark Holland, Emmen, The Netherlands). The potentiostates were a Decade (Antec Leyden B.V., Zoetermeer, The Netherlands) and an Amor amperometric detector (Spark Holland, Emmen, The Netherlands).

Calibration curves were made with solutions containing 0, 1, 2, 4, 6 mM lactate and 0, 2, 4, 8, 12 mM glucose, changed stepwise every 5 minutes, increasing from 0 mM and decreasing with the same steps. The spreading in the analytical

system due to instrumental mixture between consecutive samples was defined as the time between a 20 % and 80% amperometric signal change between concentrations. The spreading \pm SEM was calculated applying sigmoidal-fitting to the steps in the calibration curve.

Oral Glucose Tolerance Test (OGTT)

Six OGTTs were performed with healthy young males and females of normal weight. Following an overnight fast, subjects sat in an easy chair from 8 a.m. till 1 p.m. At zero time they drank 100 g glucose dissolved in 200 ml tea. An ultrafiltration probe was placed subcutaneously near the umbilicus using a 22 G cannula at approximately 45 minutes before the start of the OGTT. Glucose in ultrafiltrates was detected electrochemically using a bi-enzyme reactor in a flow injection system as described extensively by Elekes et al.⁽¹⁹⁾.

An ECA 180 Glucoanalyser (Medingen GmbH, Dresden, Germany) measured blood samples taken from a forearm vein cannula every 5 to 15 minutes. Glucose molarity (mol/L blood) of haemolysed whole blood is different from the molality (mol/kg water) as about 80% of the blood volume consists of water⁽²⁰⁾. The molality of glucose is the same in whole blood, bloodplasma, and the extracellular fluid in blood. To make a correct comparison with the UF concentrations, whole blood concentrations were increased with 17.6% in accordance to the manufacturer's report. Steady state levels were calculated as the average of the first three i.v. samples (t=-15,0,5 min) and the concurrent s.c. levels. Lactate was determined in blood plasma with the Vitros 250 (Ortho Clinical Diagnostics, Beerse, Belgium). No correction for plasma protein volume was done, because no corrective factor was available.

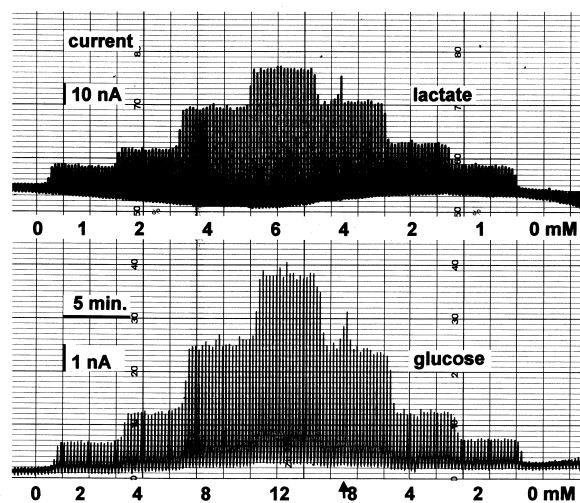


Fig. 3. Amperometric recording of calibration. Upper part: lactate. Lower part: glucose. Standard concentrations of glucose and lactate were changed every 5 minutes (\uparrow = artefact).

Results

In vitro experiments

At the applied flow rate of 100nl/min, 3.3 nl was injected 4 times per minute into the analytical system. A photograph of an amperometric recording of a calibration curve is shown in fig.3. Standard concentrations of glucose and lactate were changed every 5 minutes. The response was 4 nA/mM lactate and 0.3 nA/mM glucose. Regression analyses for the lactate and glucose concentrations with amperometric top-baseline values showed linearity for 0-6 mM lactate and 0-12 mM glucose (both $r > 0.99$, $p < 0.0001$). The spreading in the analytical system was 32 ± 5 seconds. An artefact was produced through sample flow disturbance by roughly changing test concentrations (\uparrow = artefact).

Oral Glucose Tolerance Test (OGTT)

In the steady state before the OGTT the glucose concentration was on average 1.06 mM lower subcutaneously than intravenously (95% c.i. 0.127-1.98 in a paired t-test). We found no correlation between the ultrafiltrate flow rate and these steady state levels ($r = 0.14$). Figure 4 shows the results of the OGTT in

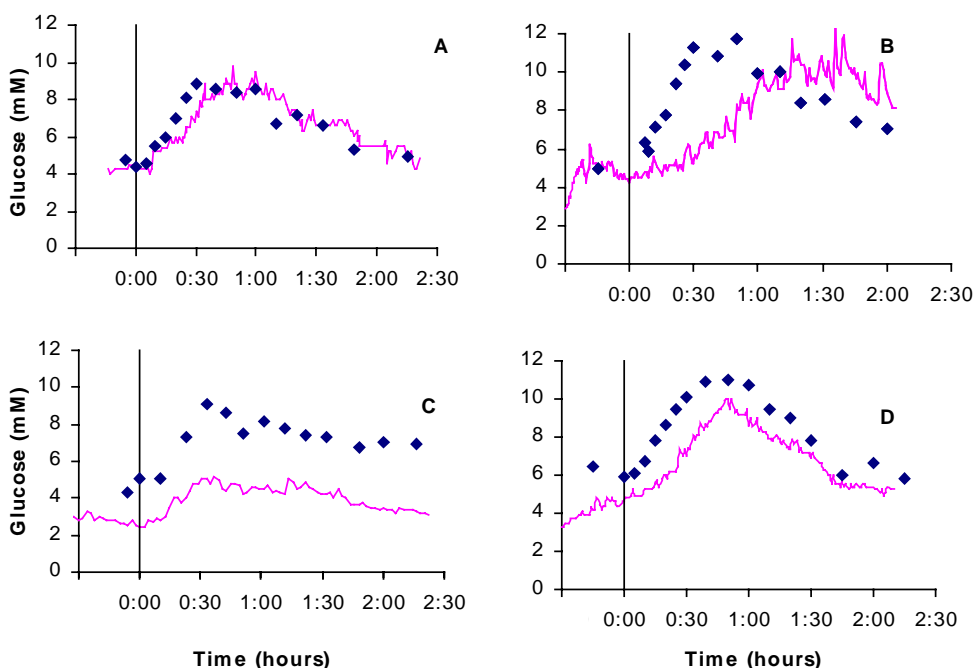


Fig. 4. OGTT (|) at zero time in four healthy volunteers. glucose levels in blood samples (◆) and in subcutaneous ultrafiltrates (◡)

four healthy volunteers (A/D). After glucose ingestion at time zero, the subcutaneous levels follow the intravenous glucose rise with various speed. The timecourse subcutaneous was not only delayed, but also less steep than intravenous. Delay times between the i.v. and s.c. maximum glucose level ranged from about one minute in C to 30 minutes in B.

Because we observed systematic differences between i.v. and s.c. levels, we thought it not appropriate to lump these data to perform regression analysis.

Combined glucose and lactate analysis in vivo

Fig.5 shows the results of two experiments (A,B and C,D) in which we measured both glucose and lactate. In one experiment, the subcutaneous lactate levels equalled blood levels where glucose levels did too (A and C). In the second experiment, glucose were lower and flattened compared to intravenous (B). Lactate was higher subcutaneous (D). So the s.c.-i.v. relation for both glucose and lactate appeared to be close in one experiment, whereas it appeared distant in the other experiment.

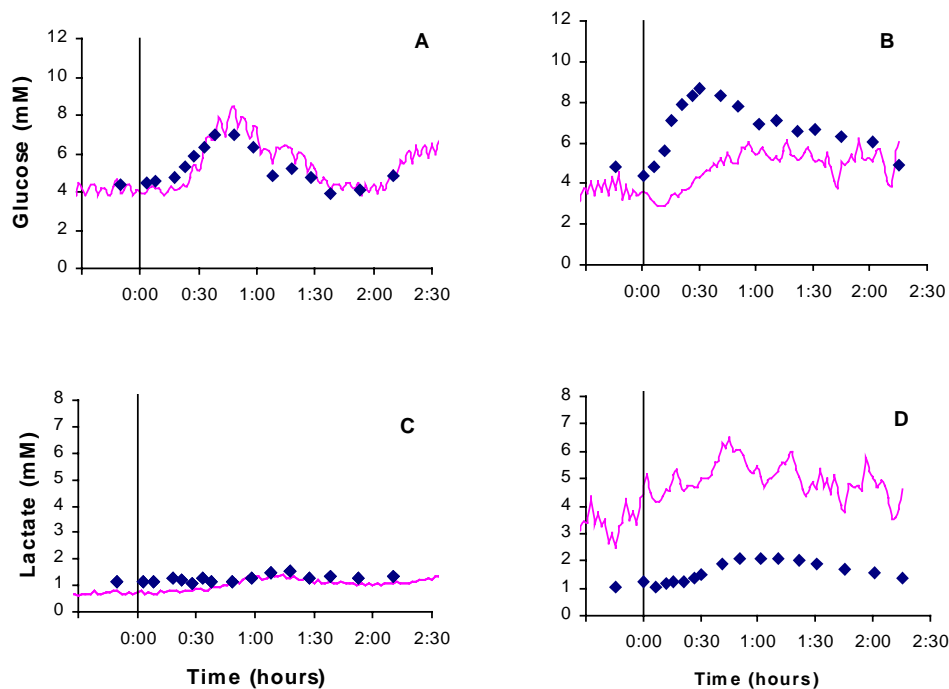


Fig. 5. OGTT (◊) at zero time in two healthy volunteers (A,C and B,D). Simultaneous measurement of glucose and lactate levels in blood samples (◆) and in subcutaneous ultrafiltrates (∗∗)

Conclusions

The present study was aimed to test the hypothesis that s.c. glucose levels are lower than i.v. levels. Two of our previously developed techniques were combined, i.e. a slow UF technique⁽¹⁵⁾ to obtain absolute concentrations, and a flow injection analysis of glucose⁽¹⁹⁾ to obtain a high time resolution. The flow injection system was further expanded in order to determine lactate and glucose in the same sample.

The simultaneous in vitro glucose and lactate analysis presented, has several advantages over previously used techniques. The linearity of the detection is elongated by dilution through injection of very small samples. The pulse-free sampling pump makes it possible to lower the sample volume, and to increase the injection frequency without pulse related problems. We created new research opportunities by introducing a splitter in the flow injection system, thus realising analysis of two metabolites in one sample.

Having the advantage of a system for frequent analysis of absolute concentrations, UF research was done in the human subcutis. Some clear differences were found between blood and subcutaneous glucose concentrations in an OGTT in men. The ultrafiltration drainage rate was compared with glucose levels in steady state and these did not show any correlation. Moreover, the fluid volume being removed equals the blood flow through 2 mm³ adipose tissue (assuming 0.03 ml/ml/min adipose tissue blood flow). This leads us to believe that UF disturbs local tissue physiology only little. The differences found in these experiments between subcutaneous glucose levels and intravenous levels suggest that these are not simply linearly related. Rather, various lower steady state concentrations, time delay, and less steep changes in the subcutis were found in these first experiments (figs.4 and 5A,B). The measured curves fit well in the expected curves (fig.1) and thus support the presented hypothesis on lower s.c. glucose concentrations. The preliminary results obtained by lactate and glucose measurements in the same sample enable to interpret both measurements together. In the first case s.c. levels of both glucose and lactate are in close relation to the i.v. levels (fig.5 A,C), compatible with curve A in fig.1. In the second case is the glucose curve (fig.5 B) compatible with curve type D in fig.1. Curve D is characterised by a high efflux of glucose into cells compared to the influx of glucose from the capillaries. Lactate concentration is higher than intravenous in this condition; as to be expected, because it is a metabolite of glucose traveling the reverse way. Previous UF-experiments in rats⁽¹⁶⁾ showed similar results as in men. The differences in rats between s.c. and i.v. glucose concentrations were recently confirmed⁽²¹⁾. Others reported human steady state s.c. concentrations in the

same range as found here; Schmidt et al reported subcutaneously $44\pm 8\%$ and $46\pm 9\%$ of blood concentration⁽²²⁾, Sternberg et al $72\pm 6\%$ ⁽²³⁾, Stallknecht 85% ⁽²⁴⁾, Lönnroth et al $91\pm 9\%$ ⁽¹⁰⁾, and Bolinder et al reported $91\pm 2\%$ ⁽²⁵⁾. These data confirm our conclusion that the s.c. glucose concentration is not directly and exclusively linked to blood. Further research may yield the various factors which modify the glucose influx and efflux, and eventually a s.c. kinetic model. Detection of both lactate and glucose in the same s.c. space may be useful to analyse local tissue metabolism. The relationship between i.v. and s.c. glucose however, seems far from direct as has been assumed previously⁽²⁵⁾. So, the extracellular space of abdominal fat tissue seems less suitable for sampling and control of glucose in diabetes patients, than has often been proposed⁽⁵⁾. Mascini⁽²⁶⁾ tested a MD probe in an other location, the forearm subcutis, with similar results results as in our abdominal experiments. Thus, a different s.c. location might not resolve the problem. We feel that to avoid complicated kinetic models, glucose sensors need to be placed intravascularly. The present study illustrates the potency of slow continuous UF sampling in subcutaneous tissue. The combination of biosensor technology and continuous in vivo sampling with UF may lead to the development of biochemical monitoring devices. Such devices will contribute to clinical and basic research e.g. the study of (patho-)physiology of energy metabolism in particular tissues.

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