Continuous metabolic monitoring techniques

Tiessen, Renger Garmt

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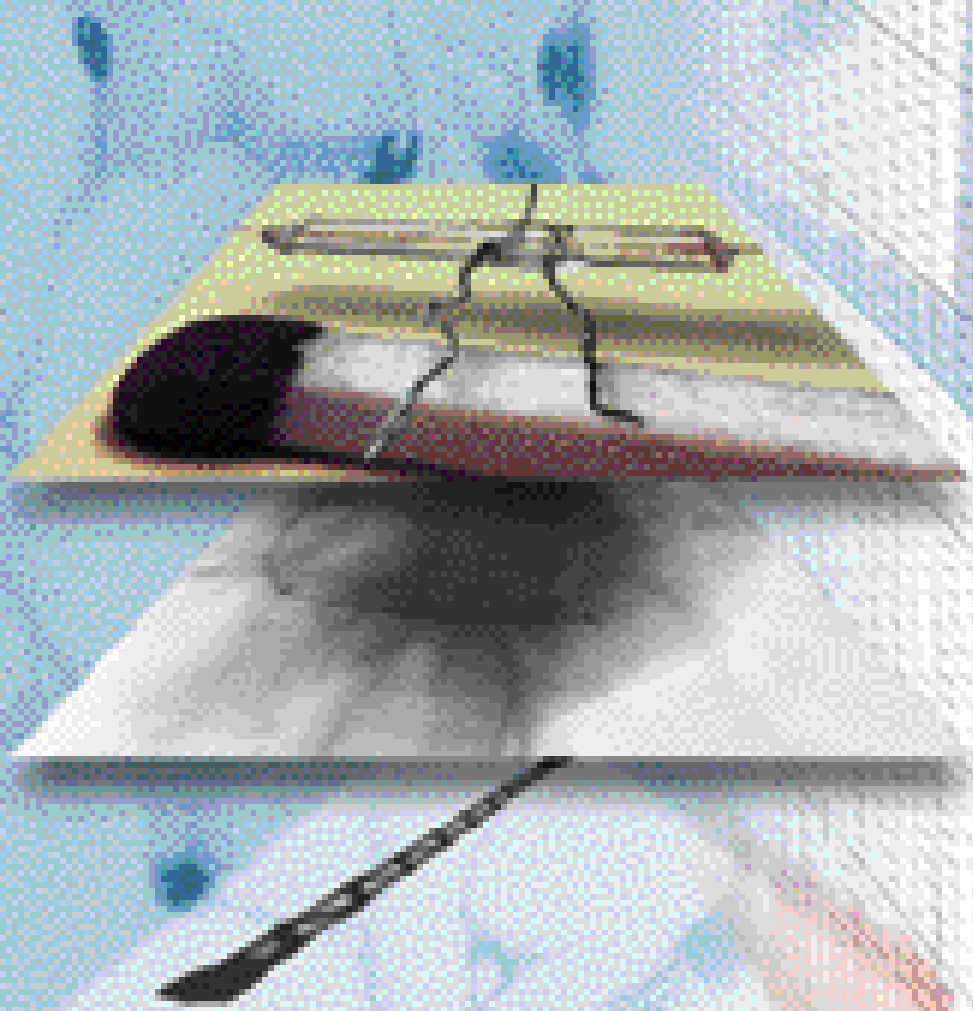

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CONTENTS

Chapter 1

General introduction

1.1 Clinical motivation to monitor metabolites

An interruption of energy supply to organs such as the brain and the heart can develop quickly into a life threatening situation. Myocardial infarction and hypoglycemic coma are two examples of such complications. These complications of atherosclerosis and diabetes mellitus patients differ in many respects, but both share the danger of the energy supply being cut off. Cellular functions can be rapidly disturbed by shortages of glucose in the brain during hypoglycemia, or accumulations of hydrogen ions during myocardial ischemia. The stores of energy in myocardial cells are exhausted within 3 to 4 seconds[(1)]. Transport of metabolites by diffusion through the fluid between cells is limited to short distances (micrometers)[(1)]. For long-distance transport the human body is dependent on blood circulation (fluid convection). Fine tuning of the uptake and release of metabolites in different organs and regulation of circulation is essential to prevent the build up of concentration differences beyond the limits acceptable for life (i.e. homeostasis). Disturbances in this vital proces can be observed often in the transporting body fluids. Already in ancient Greece, Hippocrates recognised a misbalance in the four fluids of the body, blood, phlegm, yellow bile and black bile, as a sign of disease. Much later, in 1679, the observation of sweet tasting urine led physicians to identify patients with the diagnosis diabetes mellitus (from Greek / Latin: pass through / sweet as honey). The sweet taste of urine is from glucose, one of the most important energy substrates in the organism. Normally, glucose is not lost in urine, but taken up by cells and metabolised predominantly with oxygen to carbon dioxide and water (aerobic metabolism). In the absence of oxygen, glucose is metabolised to hydrogen and lactate ions (anaerobic metabolism). Nowadays, physicians attempt to anticipate sudden pathological events by continuously monitoring patients at risk. Body fluids of critical care patients are often monitored continuously for energy metabolism substrates and products such as oxygen, carbon dioxide and hydrogen ions (pO_2, pCO_2 and pH). An arsenal of effective therapies is currently available to treat imminent energy metabolite transport derangements and treatment of acute homeostatic disturbances has proven rewarding provided a timely and accurate diagnosis is made. Apart from acute risks, homeostatic derangements also carry long-term risks, such as the late complications from frequent high glucose levels in diabetes (see paragraph below).

This thesis focuses on development of continuous glucose and lactate monitoring, because such techniques are not yet clinically available, and these metabolites are of major importance for large patient groups as diabetes

mellitus, critical care and cardiovascular patients. These three patient groups will be discussed separately in the next paragraphs, as they have different monitoring needs. The subsequent section 1.2 reviews the current state of the art of continuous glucose and lactate monitoring techniques. Section 1.3 deals with the possible backgrounds of problems arising when these techniques are applied in vivo. The aims and scope of this thesis are presented in section 1.4.

Diabetes mellitus

The number of diabetic patients in the Netherlands, with 15.8 million inhabitants, is estimated to be 450,000. Diabetes mortality amounts to 3,200 out of 136,000 deaths annually, which means diabetes is reported in 2.4 % of the population as the primary direct or indirect cause of death (CBS 1997). Diabetes is a major cause of blindness, end stage renal disease and limb amputations. There is also a 2 to 4 times increased risk of heart disease or stroke, and higher glucose levels are associated with a larger infarction area. Extensive trials have succeeded in lowering elevated blood glucose levels of diabetic patients by intensive treatment (DCCT'93[2-4], UKPDS[5]). In the DCCT trial, a reduction in microvascular complications of over 60% was achieved compared to standard treatment. The UKPDS trial showed improvements in the number of both microvascular and macrovascular complications. However, blood glucose levels were still about 40% above normal limits with the intensive treatment[2-4], and the risk of hypoglycaemia increased threefold, despite an increased frequency of blood glucose self-monitoring. This may be related among other things to the highly variable insulin levels resulting from subcutaneous injections. Therefore the risk of sudden hypoglycaemia between measurements increases with intensive treatment, thus slowing down the motivation to intensify treatment. Tight blood glucose control not only demands intensive support from a health care team, but also calls on the ability and the commitment of the patient to be continually managing his glucose levels. This means a great effort and implies, among other things, a very disciplined life style, and frequent finger pricking for the rest of the patient's life. The motivation for frequent finger pricking is not easy to sustain, possibly because high blood glucose is often not felt by the patient, who has the consequent complications usually only years later. Currently, only 33-50% of diabetic patients performs at least daily self-monitoring of blood glucose (SMBG)[6;7]. The accuracy reached through self monitoring using current devices appears also far from optimal[8;9]. The limitations of the current standard of intensive therapy can be summarised by the fact that glucose levels cannot be controlled completely , there may be long-term complications, and an overly regulated life is imposed on patients.

Improvements can be achieved by a more continuous and automated method of blood glucose monitoring. A continuous or semi-continuous monitoring technique may give a timely signal of hypo- and hyperglycaemic events. Automation may relieve the patient from tasks as finger pricking, blood sample handling (introducing inaccuracy), and, eventually, insulin dosing. The future acceptance and application of new monitoring techniques will depend on the overall advantage compared to the limitations of the existing practice. Before the general introduction of a new glucose monitoring device, this balance has to be made up. The reliability of the proposed techniques needs special attention because both the improvement of glucose control and the liberation from self-monitoring of blood glucose depend on it. The major steps in development and the present state of the art of continuous glucose monitoring techniques will be discussed in paragraph 1.2 (Sampling and sensing devices).

Myocardial ischemia

Mortality from cardiovascular diseases accounted for 50,545 deaths in the Netherlands in 1997 (CBS). Cardiovascular disease is reported as the primary direct or indirect cause in 37% of all deaths. Coronary heart diseases are responsible for the majority of deaths in this group: 26%. Yearly, there are 90,000 hospital admissions for on average 8.1 days for coronary heart disease complications (SIG 1998). The underlying disease is atherosclerosis which narrows the arteries and progressively diminishes the bloodstream, and therefore the supply of energy metabolites to the myocardial tissue. Atherosclerosis can be complicated by plugging of the arterial stricture with thrombus formation, after which myocardial tissue shows gradual changes and eventually dies (acute myocardial infarction). A patient with an acute myocardial infarct currently has a 35-50% chance to die[(10)]. However, acute insulin-glucose infusions have been shown to improve this prognosis in diabetic patients[(11)]. Intervention with defibrillation after circulatory arrest may give a 72% chance of survival[(12)]. Intravenous thrombolysis therapy has been shown to salvage the ischemic myocardium, and the faster it is applied, the more effective it is[(13)]. Therefore, secondary prevention is possible, provided early diagnosis is made. Death or myocardial infarction occurs in only 8.1% of all patients admitted to coronary care units for unstable angina[(14)]. The diagnosis in the early stages of a developing myocardial infarction depends largely on patient history and ST-segment depression[(15)], both of which may be ambiguous. For example, one-third of heart attack victims feels no chest pain, so-called “silent” ischemia[(16)], and the electrocardiogram changes may be absent in the early hours. Routinely used markers of myocardial ischemia, e.g. troponin and

creatine kinase, are released after cell death, thus these markers may be present only after several hours and can not help prevent infarction[17]. Identification of patients at risk for death or myocardial infarction is difficult with current diagnostics, yielding a sensitivity of 80% and a specificity of only 33%[14].
In the early stage of ischemia, before irreversible cell death has occurred, poorly perfused tissue shifts from aerobic to anaerobic metabolism, the latter characterised among other things by an increased lactate production. Major lactate concentration shifts may occur in a matter of minutes both after the onset and at the end of myocardial ischemia[18]. There is also a quantitative relation between the extent of ischemia in the myocard and its release of lactate[19;20]. Therefore, a lactate monitoring technique would, if available, offer the opportunity to detect a tissue oxygen deficit in time for therapeutic intervention, and thus contribute to the management of patients at cardiac risk.

Emergency medicine
Diverse other critically ill patients may also benefit from lactate monitoring. In critical care units, high lactate levels in the general circulation have a strong prognostic value[21]. Lactate levels over 5 mM (normally 0.6-2.4 mM) are associated with a poor outcome[22]. It is possible to predict organ failure from serial lactate measurements(13) and blood lactate appears to be a better prognostic factor than oxygen[22]. Lactate monitoring has been suggested to be valuable for many patients in emergency medicine: perioperatively for surgical patients[23-27], for trauma patients including battlefield medicine, for patients with head injury or cerebral ischemia[28-32], acute intestinal ischemia[33], liver ischemia[34], transplanted organ surveillance e.g. myocutaneous flaps[35], intrapartum for the foetus[36;37], and patients with septic shock[22;38;39]. Other applications include research on mental stress in healthy subjects[40], physical exercise intop althletes[41;42], and spatial and pharmacological research (e.g. in research of metformin lactacidosis, or to reduce the total blood sample volume).
In summary, large patient groups may benefit from continuous glucose and lactate monitoring devices. The future application of the proposed devices will depend on the risk, patient friendliness and reliability in comparison with concurring techniques and other therapies, e.g. pancreatic islet transplantation[43].

1.2 Sampling and sensing devices

Sensing devices

In 1956, Clark took the first step to metabolic monitoring in vivo by introducing platinum-silver/silver chloride electrodes to measure oxygen (reference). A particular oxygen concentration generates a certain electric current directly at the platinum electrode (amperometric measurement). By adding glucose oxidase enzyme, glucose concentrations can be measured indirectly, because oxygen is metabolised with glucose to hydrogenperoxide in a one to one relation[44]. Ever since, scientists and physicians have dreamt of using this technique to function as an artificial pancreas[45]. Such a technique may control glucose levels through insulin delivery guided by a glucose sensor, without need for action from the patient. Lactate oxidase enzyme can be applied in the same way to measure lactate. However, these sensors measure oxygen disappearance or hydrogenperoxide formation, so they are dependent on both the metabolite (glucose or lactate) and the oxygen concentration. In order to be independent of oxygen levels, mediators such as ferrocene have been introduced. Ferrocene is a substitute for oxygen or hydrogen peroxide by transferring electrons directly from the enzyme to the electrode. It also allows a lower voltage potential to be used for measurements, thus reducing the influence of electrically active substances such as ascorbic acid. This technical principle has been applied in flow-injection analysis[46] (see also chapter 2, figure 2). Limitation of diffusion of the metabolite and interfering substances towards the electrode has been achieved e.g. by a membrane cover[47;48] (see also chapter 5), or by physical entrapment of the enzyme in a polymer[49;50] (see also chapter 6). Thus the concentration range for linear measurement is extended and metabolite depletion at the sensor surface decreased, whilst interference is diminished. Addition of horseradish-peroxidase[46] or catalase[47] can prevent the build-up of toxic levels of hydrogen peroxide, which may denature the enzyme. Apart from the measurement of electric current (amperometry), potentiometric electrodes have also been applied. Potentiometric miniaturised sensors are e.g. ISFETs[51] (ion-sensitive field-effect transistors). A completely different sensor concept has been demonstrated by Ballerstadt et al.[52] Using a hollow fibre. In this method, changes in glucose concentration cause changes in fluorescence emission when free glucose competes with fixed glucose for affinity with a fluorophore.

Sampling devices

Hollow fibres are used in many monitoring techniques as a sampling interface between the sensor and the tissue. Hollow fibres are semi-permeable membranes in a tubular shape and a diameter of about 200-300 µm. Sampling with hollow fibres has several advantages compared to sensor implantation. Hollow fibres are easily applicable and relatively patient friendly. Hollow fibres can be inserted into tissue with a small needle, and there is no need for implantation of complex, potentially hazardous electronics or enzymes. In addition, large molecules and cellular remains are excluded by the membrane, so there is a clean matrix for measurement. Hollow fibres are often applied for microdialysis sampling. In classical microdialysis, a fluid flow through a hollow fibre partially equilibrates with the surrounding tissue, after which the sample is withdrawn and the metabolite content is measured outside the body. Many attempts have been made to determine the exact microdialysis metabolite recovery in vivo, but none of these methods are sufficiently accurate or feasible for clinical application. To obtain samples equilibrated with interstitial fluid, Rosdahl et al. lowered the microdialysis flow to 160 nanolitre per minute[53]. To prevent loss of all perfusion fluid through the membrane into the tissue, he added a colloid. Kaptein et al. employed a suction pump to lower the microdialysis flow to 100 nl/min in rats, obtaining equilibrated samples without need for colloid addition[54].

Ash et al. introduced ultrafiltration as an alternative method to microdialysis[55]. In their method, tissue fluid is ultrafiltered through a hollow-fibre membrane by underpressure. Large needles are required, because many fibres are needed to collect sufficient fluid for off-line analysis. Moscone et al. made ultrafiltration patient friendly by miniaturising the ultrafiltration probe, and introducing a light-weight disposable pump[56]. Kaptein et al. demonstrated the possibility of using ultrafiltration to continuously monitor glucose in the subcutis and the veins of rats[57]. Recently developed, light weight, nanolitre flow-sensors by Rhemrev et al.[50] make it possible to analyse glucose or lactate in a fluid flow of 30-100 nl per minute continuously,. from ultrafiltration or slow microdialysis.

Demands for monitoring

Before clinical application, new metabolic sensors will always be compared with the standard discontinuous technique. Frequent self-monitoring of blood glucose (SMBG) by finger pricking is currently the standard for diabetic patients[58]. A new glucose monitoring technique would need to have an edge over current SMBG with respect to compliance (less pain, automation),

measurement reliability and frequency, less need for selfdiscipline, and improved life expectancy, quality and duration . Essential for any improvement is device reliability, because otherwise, frequent blood sampling for calibration will equal the SMBG burden for the patient.
Blood sample lactate analysis is currently possible with bed-side devices in the operating theatre. Myocardial lactate detection is now limited to scientific research, because of the need to perform heart catheterisation for blood sampling. Analysis of blood samples of critical care patients can be done in the (remote) hospital laboratory. Lactate analysis is rarely practised, mainly due to the slow result turnaround time, preanalytic problems, and blood loss. A lactate monitoring technique would be clinically interesting if it was automated, continuous (for trending data), and operating in real-time like ECG and blood pressure monitoring.

Complications
Continuous application in patients poses specific demands to the newly proposed metabolic sensors. Generally, a device is safer and friendlier to the patient, the less invasive it is. An undisturbed, constant contact between body fluid and sensor surface is however essential for reliable measurements (see further section 1.3). Sensing and sampling devices have been proposed for intravenous, subcutaneous and transcutaneous applications. Transcutaneous devices[52;59-62] often demand skin stripping and local heating, whereas subcutaneous and intravenous devices demand insertion by needle. Pain from needle insertion depends of course on the diameter of the device, and the sensitivity of the site of the skin e.g. the arm is more sensitive than abdomen. Further complications may include damage to nearby structures at insertion (in the thin cutis of neonates), inflammation or infection (if permanent skin penetration is needed), and the risk of release of toxic (e.g. ferrocene), carcinogenic, or allergenic substances.
Experience with intravascular blood-gas monitoring and other catheters has shown that intravascular devices may cause vessel trauma, thrombus formation, blood flow cessation in the particular vessel, ischemic organ damage or loss, bacterial colonization of the catheter surface, infection, and sepsis[63]. An important secondary complication can be caused by inaccurate data, leading to misdiagnosis. Nowadays, morbidity from blood-gas sensors has become rare in critically ill patients[64;65]. Notably the appliance of an anticoagulant, such as heparin, to the surface of catheters has contributed to an improved accuracy. For out patients, such as . diabetes mellitus patients, the less invasive trans- or subcutaneous device remains the preferred option.

Device mass production
Future clinical appliance also demands that from techniques be fit for mass production. Reliable sensors require namely a standardised manufacturing process to guarantee reproducibility. Candidate techniques in this regard are e.g. thin-film sensors[47], ISFET sensors[61], the GlucoWatch® iontopheresis sensor[60], and the CGMS® needle type glucose sensor[66]. Several currently proposed lactate and glucose sensors have been shown to measure reliably in vitro, and some are already being tested in patients for in vivo accuracy (see further paragraph 1.3: Influence on measurements by device and organism). Of the currently proposed sampling techniques for glucose monitoring, ultrafiltration and ultraslow microdialysis are readily applicable in vivo, are minimally invasive, and function accurately in vitro and in blood without recovery calculations. An overview of sampling and sensing techniques currently in development for metabolic monitoring is given in table 1.

1.3 Influence on measurements by device and organism

Relation of interstitial to blood metabolite levels
Currently proposed trans- or subcutaneous glucose sensors often detect a lower concentration in tissue compared to simultaneously measured blood plasma levels, which is the reference for diabetic management. To estimate blood plasma levels, a one-point or multiple-point calibration to blood levels is necessary. This calibration factor is called “in vivo recovery” of microdialysis probes or “in vivo sensitivity” of needle type sensors. Unfortunately, this factor is rather variable, both between subjects, within subjects, and in time. To determine this factor, patients are now urged to calibrate the sensor several times a day against blood samples. To improve accuracy and patient friendliness, a better insight is urgently needed. When the relation of cutaneous and subcutaneous tissue to blood levels can be established, a more widespread application of glucose monitoring devices will become possible.

Glucose and lactate metabolism in the subcutis
Glucose and lactate are constantly metabolised and transported between tissues by the blood circulation. So, nowhere in the living organism, will there be the same concentration at one time or place, however these gradients are not necessarily steep or of long duration.

Table 1. Glucose and lactate sampling and sensing techniques

Device	Sampling interface	Detection technique	Authors
CGMS®	sc. needle membr.	g.o.d. amperometry	Mastrototaro et al.[67;68]
Glucowatch®	transdermal iontopheresis	g.o.d. amperometry	Garg et al.[60;69]
microdialysis	sc. hollow fibre diffusion	off-line	Bolinder et al.[70]
microdialysis	hollow fibre diffusion	off-line	Schoonen et al.[71-73]
needle type (gluc./lac.)	sc. needle membr.	g.o.d./l.o., amperometry	Yang et al.[49;74]
needle type (lac.)	sc. needle membr.	l.o., amperometry	Kyrolainen et al.[48]
open-flow microperfusion	sc. perforated catheter	thin-film (gluc+lac)	Schaupp et al.[75]
optical	transdermal diff. hollow fibre	fluor. competitive affinity	Ballerstadt et al.[52]
suction effusion	transcutaneous	I.S.F.E.T.	Ito et al.[51]
thin-film (gluc.+lac.)	sc. thin-film membrane	g.o.d./l.o.,catalase, amp.	Jobst et al.[47]
u.f.	hollow fibre ultrafiltration	off-line	Ash et al.[76]
u.f.	hollow fibre ultrafiltration	F.I.A. amperometry	Moscone et al.[56]
u.f.	hollow fibre ultrafiltration	flow-sensor amperometry	Rhemrev et al.[77]
u.s.m.d.	hollow fibre diffusion	F.I.A. amperometry	Kaptein et al.[57]
ultrasound	transcutaneous	g.o.d. amperometry	Kost et al.[62]

The arterio-venous differences measured over the adipose tissue of the abdominal wall are found to be very small in the late postprandial period, but increase significantly during an oral glucose load. In this situation glucose is being taken up, and lactate is being produced[78-80]. Both glucose and insulin levels influence lactate levels [81]. More lactate is found subcutaneously in lean than in obese healthy volunteers after an oral glucose load[82]. Adipose tissue also displays regional metabolic differences in lipolysis[83] and in lactate production in reaction to glucose and insulin[84]. The rate of glucose transport into cells also differs between adipose and connective tissues[85], and these are unequally distributed in the subcutis. The arterio-venous gradients mentioned above for the blood circulation constitute the minimal gradients inside adipose tissue, since glucose is exchanged freely over the capillary wall. So, the molar proportion of glucose and water will be the same on both sides of the capillary wall. The glucose concentration (mmol/l), however, will be considerably higher in the interstitium, because the protein content of interstitial fluid is much lower than in blood[1]. Differences in protein content of samples can lead to 15-20% differences in glucose concentration[86].

Abbreviations in Table 1: glucose and lactate sampling and sensing techniques

amp.	Amperometry
CGMS®:	Continuous Glucose Monitoring System®
diff.:	diffusion
F.I.A.:	Flow-Injection Analysis
fluor.:	Fluorescence
g.o.d.:	Glucose OxiDase enzyme
gluc.:	Glucose
I.S.F.E.T.:	Ion-Sensitive Field-Effect Transistor
l.o..:	Lactate Oxidase enzyme
lac.:	Lactate
membr.:	membrane
sc.:	subcutaneous
u.f.:	Ultrafiltration
u.s.m.d.:	Ultra-Slow MicroDialysis

The transit times of glucose in the organism can be identified by compartmental modelling e.g. with the minimal model[87;88]. The transit time is estimated to be a few minutes from the blood plasma, to a second, insulin independent, compartment, such as the brain, splanchnic tissues, erythrocytes and renal medulla[89]. A third, insulin dependent, compartment which equilibrates slowly is thought to exist, and presumed to be predominantly muscle and adipose tissue. Compartmental modelling is generally based on the measurement of parameters in blood; the other parameters are derived from these. The anatomical locations of the different compartments are not known with certainty; so the transit time to subcutaneous adipose tissue remains unknown from these models.

Pathophysiologic alterations in the subcutis of diabetic patients

The knowledge of physiological glucose metabolism, as discussed above, may not be applicable to diabetic patients, as they do not express a physiological insulin and glucose metabolism. In addition, there is considerable variation, between patients in such factors as insulin sensitivity. Generally, microvascular function strongly relates to insulin sensitivity [90]. The microvascular vessel anatomy and function is significantly altered in diabetis patients, as well as the diffusion in tissues [91;92].

Possible device-tissue contact interactions

Apart from the physiologic and pathophysiologic metabolism in tissues, the mere insertion and presence of a measuring device in the subcutis may well change local metabolite concentrations. The response of the organism may also influence the measurements made by the device. In particular changes around the device may especially diminish diffusion from the tissue to the device. Diffusion parameters at the contact site are the available surface area of device for sensing/sampling, diffusion distance from blood capillaries to the device, interstitial diffusion path width, and availability of medium (interstitial fluid). Furthermore, the glucose metabolism may change from predominantly aerobic to more anaerobic, switching from carbondioxide to lactate production. A comparison of the relative sizes of adipose tissue cells and implanted devices helps to illustrate these factors. The diameter of adipose cells is variable and ranges up to 100µm. The intercellular width is about 1µm. The blood circulation in a microlitre of adipose tissue is approximately 0.1-0.03 µl/min. A needle used for implanting a device has an outer diameter of around 1400-1250µm. A hollow fibre has an outer diameter of 200-600µm, and a volume of 1-8 µl. The microdialysis flow may be 0.16-10 µl/min. Other factors to consider

are that the device itself may consume more metabolites than can be restored by the circulation and organic molecules may influence the enzymatic or electric functioning of devices. Changes at the device-tissue contact site may occur as a consequence of primary cell lesions at the moment of insertion resulting in free fat or cellular remains. Primary vessel lesions at the site of insertion may result in (diffusional path lengthening, so that less fluid filtered out of the capillaries into the interstitium. In addition, hematoma may cause local volume expansion and erythrocyte metabolism, or coagulation may result in changes to the device area and surrounding cells. Hypoxia, secondary vessel trauma due to movement of a rigid device in vulnerable tissue, or inflammation may cause effusion of fluid and proteins, cellular infiltration and fibrous encapsulation. Some of these possible influences on measurements in vitro, have been documented, for example, glucose oxidase is significantly inhibited by small molecules from rabbit serum and by high molecular weight granulocyte excretion products[93]. To date there is very little information from in vivo experiments in humans.

Transcutaneous interactions

Experiments with transcutaneous glucose measurements suffer from the water resistance of skin, which, even when stripped from the corneal layer, exhibits a low and very variable permeability for interstitial fluid[94]. The transcutaneous microdialysis recovery may be one-twentieth of the glucose recovery in vitro[95]. Variable evaporation and perspiration can influence transcutaneous measurements as well[96]. Changes in the resistance due to skin water resistance will add to changes in the device-tissue contact. If the instability at the contact site remains unsolved transcutaneous devices will stay heavily dependent on finger stick blood glucose calibrations, seriously limiting the prospects of current transcutaneous devices[51;60-62].

Subcutaneous interactions

Experimental findings with subcutaneous glucose monitoring devices in vivo yield a high within subject variation coefficient of recovery or sensitivity. These differences may originate from regional differences in adipose tissue. Stallknecht found an increasing microdialysis recovery with decreasing skinfold thickness, and with increasing water content of adipose tissue[97]. Microdialysis recovery was not found to depend on blood flow. Glucose was found on average 0.8 mM lower and lactate 1.2 mM higher in adipose tissue than in blood plasma[97]. Thomas et al. found increasing recovery and less variance in subcutis from the forearm than from the abdomen[98]. Classical microdialysis however changes the biological environment by pumping fluid into the tissue (e.g. 0.1µl

of 0.3µl/min microdialysis flow is lost into tissue), by draining metabolites from the tissue, and by inducing a diffusion gradient in the surrounding tissue. This diffusion gradient may stretch for two millimetres into tissue around the probe[99]. Rosdahl et al., using equilibrated microdialysis, found lower glucose levels in muscle tissue, but similar levels in adipose tissue and blood[53].

Long-term interactions
In long-term experiments, Sharkawy found the diffusion in subcutis tissue of rats to be similar, before and after a fibrous capsule had formed around a porous implant. However, fibrous capsules around nonporous implants diminished the diffusion coefficient by one half[100-102]. Considerably reduced glucose diffusion has been observed in tumour tissue, when an only 30µm thick mesenchym layer was formed around the capillary vessels[103]. A three week experiment in healthy volunteers showed an approximately two-fold increase in microdialysis recovery the first six to nine days after subcutaneous implantation, and the estimated glucose concentration to double in the first three days[73].

Intravascular interactions
Arterial blood-gas monitoring catheters have been shown to be sensitive to a number of device-organism interactions. Measurement may be influenced by blood flow stagnation which creates gradients, and from catheter perfusion fluid diluting the blood. Measurement may also be influenced by thrombus formation, yielding declining pH and pO_2, and rising pCO_2 due to metabolism within the thrombus, and an increased response time through decreased diffusion. Other possible influences are contact with the vessel wall, with declining pO_2, and little or no deviation in pH and pCO_2[63]. Thrombus formation can be reduced by heparin coating of the catheter. However, even with anticoagulants in situ proteins in blood can still adsorb onto artificial surfaces, usually forming a monolayer. Such a thin layer may reduce and define the diffusing properties of hollow fibre membranes in artificial kidneys[104;105]. Interestingly, a disturbance in the contact between the intravascular gas sensor and with the bloodstream can be recognised by comparison of several analytes (H^+, O_2, and CO_2)[63]. These analytes are altered by cellular (an-)aerobic metabolism of glucose to CO_2 and/or lactate if the catheter becomes isolated from the blood stream as in the case of monolayer formation.

In summary, the clinical application of subcutaneous sensors is slowed down by the unknown relationship between subcutaneous measurements and blood glucose levels. Since current in vivo calibration techniques are insufficient,

frequent blood sampling is required. The differences found between subcutaneous and blood glucose levels are still subject to debate. Subcutaneous measurements have been variously interpreted to originate from blood levels in the general circulation, to reflect local tissue metabolism, or to be caused by disturbing device-tissue interactions.

1.4 Aims and scope of the thesis

The aim of this thesis was to develop and test in vivo some continuous sampling and sensing techniques for clinical monitoring of glucose and lactate. The performance of ultrafiltration and ultra-slow microdialysis equilibrated sampling techniques was determined with oral or intravenous glucose and lactate. The in vivo time-resolution was assessed with amperometric sensors placed either directly in the interstitium, in the sample flow, or in a sample flow-injection system. Interpretations of the in vivo measurements were explored studying the concurrent glucose and lactate levels in the general circulation, the effect of aerobic or anaerobic metabolism, the influence of insulin levels, different tissue types for device implantation, the temporal changes after implantation, and the effects of ischemia and reperfusion. Computation methods were developed to estimate glucose or lactate in tissues utilising intravascular glucose, lactate and insulin levels.
New ultrafiltration (ch. 2 & 4) and ultraslow microdialysis techniques (ch. 3, 6, 7) were applied for continuous equilibrated sampling in subcutaneous tissue (ch. 2, 3, 6, 7) and intravascular, in the coronary sinus (ch. 4). Application of a wearable glucose flow-sensor with a high time resolution was studied, when connected on-line with ultraslow microdialysis sampling in man (ch. 6). A novel continuous monitoring catheter was developed and studied for detection of myocardial ischemic events (ch. 4).
The performance of devices implanted intravenously (ch. 4), was evaluated in muscle, in adipose, and in connective tissue in swines (ch. 5); and in adipose and connective tissue in man (ch. 6) to find the optimal contact in vivo. A bed-side flow-injection analysis was developed and applied (ch. 2 & 3) to measure glucose and lactate simultaneously and continuously in nanolitre samples, allowing the study of glucose-lactate metabolism in tissue near an implanted device. A thin-film glucose-lactate sensor was applied directly in tissue for the same purpose (ch. 5). To explore the course of aerobic and anaerobic metabolism around a newly implanted device, the glucose and lactate concentrations were compared at the time of device insertion and 24 hours later (ch. 3). Also, the influence of insulin levels on the subcutaneous glucose

concentration (ch. 6 & 7) was studied. Computation methods were explored to estimate blood glucose levels directly from subcutaneous measurements (ch. 3) and to estimate myocardial lactate production during ischemia from venous levels (ch. 4). Finally, several kinetic models were developed and tested to identify a model for subcutaneous glucose level estimation (ch. 7).

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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, pathophysiologic 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 there 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 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]. Unfortunaley, 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

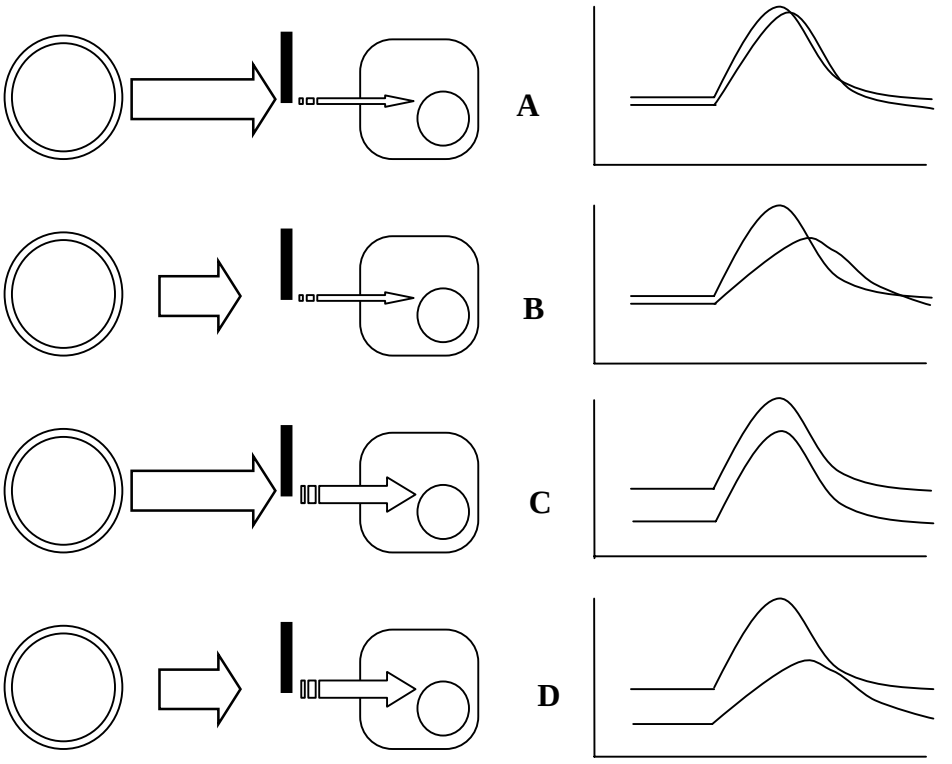

Fig. 1. four types of subcutaneous glucose curves to be expected in an OGTT. Left-hand side: schematic representation of different glucose influx (from capillaries) and efflux (to cells) from the s.c. interstitium. Right-hand side: resulting s.c. concentration curves.
A,B: glucose efflux is small compared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

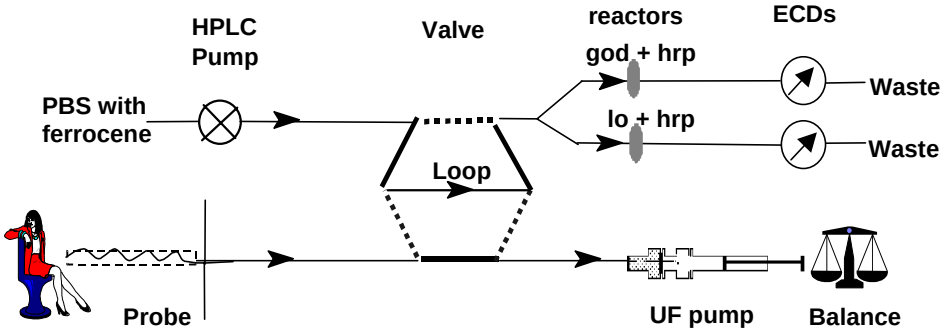

Fig. 2. Ultrafiltration sampling and analysis set-up. bottom side: sampling hollow fiber probe and UF pump on a balance; middle: sample injection valve (loop in injection position___ or in load position---); top side: detection with PBS buffer containing ferrocene, HPLC pump, splitter, two parallel enzyme 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, 340µm OD, 240µm ID, MWCO 50kD). To keep the lumen patent, the fiber was reenforced from the inside with a home-made spring of stainless steel wire(Vogelsang, Hagen, Germany), D=60µm, 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, 150µm OD, 50µm 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±1 mbar±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=150µm, ID=15µm 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 ± 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 manufactorer'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.

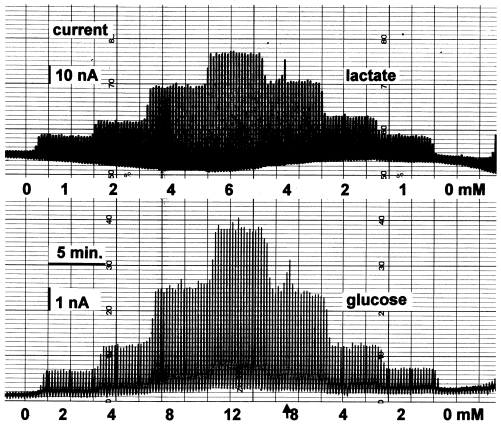

Fig. 3. Amperometric recording of calibration. Upper part: lactate. Lower part: glucose. Standard concentrations of glucose and lactate were changed every 5 minutes (↑ = artefact).

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 respons was p nA/mM lactate and q 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 x±z minutes. An artefact was produced through sample flow disturbance by roughly changing test concentrations (↑ = 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

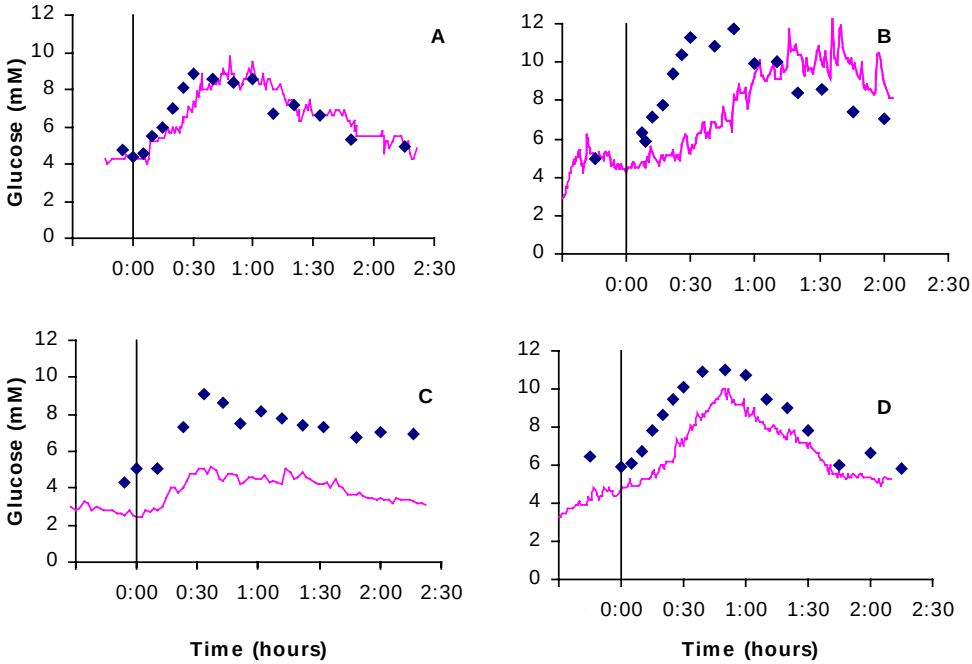

Fig. 4. OGTT (|) at zero time in four healthy volunteers. glucose levels in blood samples (◆) and in subcutaneous ultrafiltrates (❝)

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 A.
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 (B and D). In the second experiment, glucose were lower and flattened compared to intravenous (A). Lactate was higher subcutaneous (C). 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.

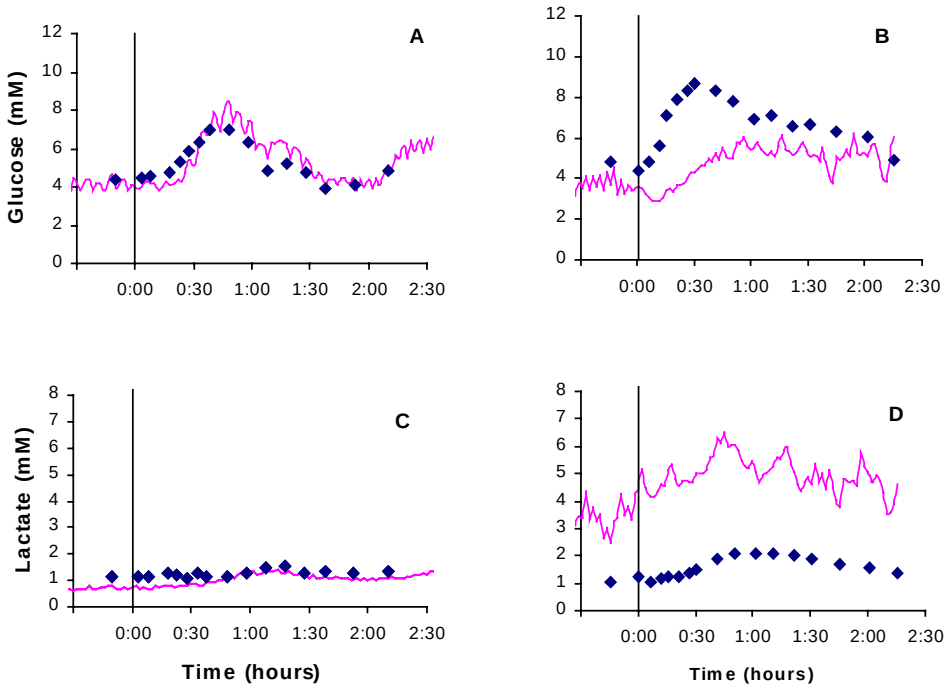

Fig. 5. OGTT (|) at zero time in two healthy volunteers (A,C and B,D). Simultaneous measurement of glucose and lactate levels in blood samples (◆) and in subcutaneous ultrafiltrates (‟)

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 mm^3 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±8% and 46±9% of blood concentration[22], Sternberg et al 72±6%[23], Stallknecht 85%[24], Lönnroth et al 91±9 %[10], and Bolinder et al reported 91±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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Chapter 3

Dynamic monitoring of glucose and lactate in the subcutis with ultraslow microdialysis

Abstract

Introduction: Subcutaneously implanted glucose sensors have been proposed for diabetes monitoring. We determined how close subcutaneous levels of glucose follow blood glucose and whether high subcutaneous lactate points to anaerobic metabolism by implantation damage at 2 subsequent days. We used slow microdialysis and a novel sensor method, to monitor glucose and lactate.
Methods: We placed microdialysis probes in the abdominal fat and applied low perfusion rates, resulting in similar concentrations of lactate and glucose in the perfusate and subcutaneous space (100% recovery). The perfusate was analyzed every 2 minutes with a biosensor. Venous glucose levels were estimated from perfusate levels and a single venous glucose assay. Oral glucose tolerance tests (OGTT) were conducted in 11 healthy volunteers and repeated the day after probe insertion.
Results: Subcutaneous glucose levels were lower than venous levels (1.47 ± 1.20 mM) at steady state and the average delay of the increase following OGTT was 7.3 ± 1.2 minutes. Lactate levels remained low in most experiments. The accuracy of the venous glucose estimates was moderate (0.85mM), both with a steady state and a non-steady state blood sample.
Conclusions: The subcutis must be considered as a kinetic compartment distinct from the vascular compartment. Subcutaneous glucose can be related to blood glucose by taking into consideration time-delay and local glucose consumption. The lactate levels indicated little implantation damage. Subcutaneous factors should be further analyzed, in particular after long-term implantation of a dialysis probe, to yield a method for accurate estimations of diabetes monitoring devices.

Abbreviations: SMBG: self monitoring of blood glucose, MD: Microdialysis, usMD: ultraslow microdialysis, OGTT: oral glucose tolerance test, sc: subcutaneous(-ly), iv: intravenous(-ly)

Introduction

Diabetes treatment goals are the prevention of acute and long-term complications [(1)]. These goals are approached by (near) normalising blood glucose levels[(2)]. Therefore current treatment is based on self monitoring of blood glucose (SMBG). SMBG has improved therapy considerably, leaving only three major drawbacks. First, a hypoglycaemic episode can not easily be foreseen because of the discontinuous nature of SMBG. Secondly, severe nocturnal or other unrecognised hypoglycaemia's interfere with attempts to normalise high glucose levels. Thirdly, only 33-50% of diabetic patients complies to perform daily SMBG[(3;4)]. Thus diabetes management will improve by the introduction of a continuous, patient friendly hypoglycaemia alerting device.

Several devices for continuous glucose monitoring have been proposed. Intravenous (iv) devices face thrombotic and infectious risks[(5)]. Alternatively, sensors placed in the abdominal subcutis carry little risk, and are easy to handle by the patient. Needle-type glucose electrodes have been applied subcutaneously (sc)[(6-8)], but suffer from a decreased signal and drift once implanted. Microdialysis (MD) could serve as an interface between a sensor and tissue. This technique obtains a relatively clean matrix for analysis because the tubular membrane is impermeable for proteins[(9;10)]. Conventional microdialysis has two major limitations. First, interstitial glucose diffusing into the probe is diluted with an unknown factor, prompting to apply lengthy calibration procedures in steady state to estimate effective interstitial levels[(11)]. Second, time-resolution is poor, because samples are most often collected for 15 minutes or longer; questioning timeliness of a sensor. The calibration procedures are clinically impractical, because non-steady-state conditions prevail in patients. Because of these limitations, several kinetic issues remain unresolved. The effective sc tissue levels are e.g. estimated between 44 and 92% of blood[(12-19)], and are possibly drifting[(19)]. Also, the distribution of glucose from blood to the subcutis appears to be delayed[(14;18)]. A possible explanation of low sc glucose could be enhanced anaerobic metabolism due to the implantation damage of the probe. If this would be the case, low sc glucose levels may be accompanied by high lactate levels.

To overcome most of the pitfalls of the studies with conventional microdialysis, we have developed a method of ultraslow microdialysis (usMD) for subcutaneous glucose and lactate monitoring. In animal studies we have shown that usMD at flow rates below 100nl/minute gives a (near) 100% recovery thus avoiding time consuming recovery calibration procedures[(20)]. We combined

usMD with a sensitive on-line detection technique of glucose and lactate, thus making our set-up suitable for fast kinetic analysis[18;21].
In the present investigation, sc glucose and lactate were monitored at steady state and during an oral glucose tolerance test (OGTT) in the abdominal subcutis of 11 healthy volunteers to measure the effective steady state and time delay. The OGTT was repeated on the day after implantation of the dialysis probe to determine short-term stability. We compared computations to estimate reference blood levels accurately, using sc levels with consideration for sc glucose consumption and time-delay. Possible clinical implications of the results are discussed with special regard for timely hypoglycemia alerting.

Methods

Oral Glucose Tolerance Test
Eighteen OGTT experiments were performed after informed consent of eleven young adult males and females with a blank history for diabetes (OGTTs in healthy volunteers as described previously[18]) . In seven subjects the OGTT was repeated on the day after insertion of the probe. Following an overnight fast, subjects sat in an easy chair from 8 a.m. till 1 p.m. For usMD was a CMA 60 microdialysis probe placed sc near the umbilicus at least 45 minutes before the start of the OGTT. The glucose and lactate concentrations were measured every two minutes with the sc usMD and in blood samples taken from a forearm vein catheter every 5 to 15 minutes. 100 g glucose dissolved in 200 ml water was ingested at zero time.

Ultraslow Microdialysis
Ultraslow Microdialysis was performed as in animal experiments published previously[20]. A CMA 60 probe consisting of a 3.0 cm long hollow fibre (Polyamide, 620 µm OD, MWCO 20 kD, CMA Microdialysis, Stockholm, Sweden) was filled up with 0.9% saline after insertion. During the experiments, the probes were connected to the system for glucose and lactate detection. The probe outlet tube was glued to a draining fused silica capillary restriction(75cm l, 150µm OD, 35µm ID, Polymicro Technologies inc., Phoenix, AZ, USA). The microdialysis flow rate was generated by the underpressure of a syringe with a fixed piston[22]. The microdialysis flow rate was on average 42 nl/min (range 30-59nl/min) as measured by weight. There was no correlation between the usMD flow rate and the sc steady-state levels ($r = 0.14$). The instrumental lag-time (time to 90% signal change) was on average 53 minutes, due to the dead

volume in wide outlet tube of the probe. The long instrumental lag-time in this set-up may simply be reduced by appropriately shortening and narrowing the tubes connecting to a wearable sensor.

Glucose and lactate analysis

We used a flow injection analysis system to detect glucose and lactate in the same usMD sample as described previously[18;21]. The system injected every 2 minutes 20nl usMD sample using an internal loop.

Two standard glucose/lactate solutions were measured before each experiment, and in vitro calibration curves were made afterwards with solutions containing 0, 1, 2, 4, 6 mM lactate and 0, 2, 4, 8, 12 mM glucose, changed stepwise every 20 minutes. The amperometric signal was linear for these lactate and glucose ranges (both $r > 0.99$, $p < 0.0001$). The 10 % to 90% spreading in the analytical system was 32±5 s (signal change from 10 to 90% after a stepwise concentration change). The spreading (10 to 90%) for the probe and analytical system together was 3-5 minutes, due to mixture in the (wide) probe tubing.

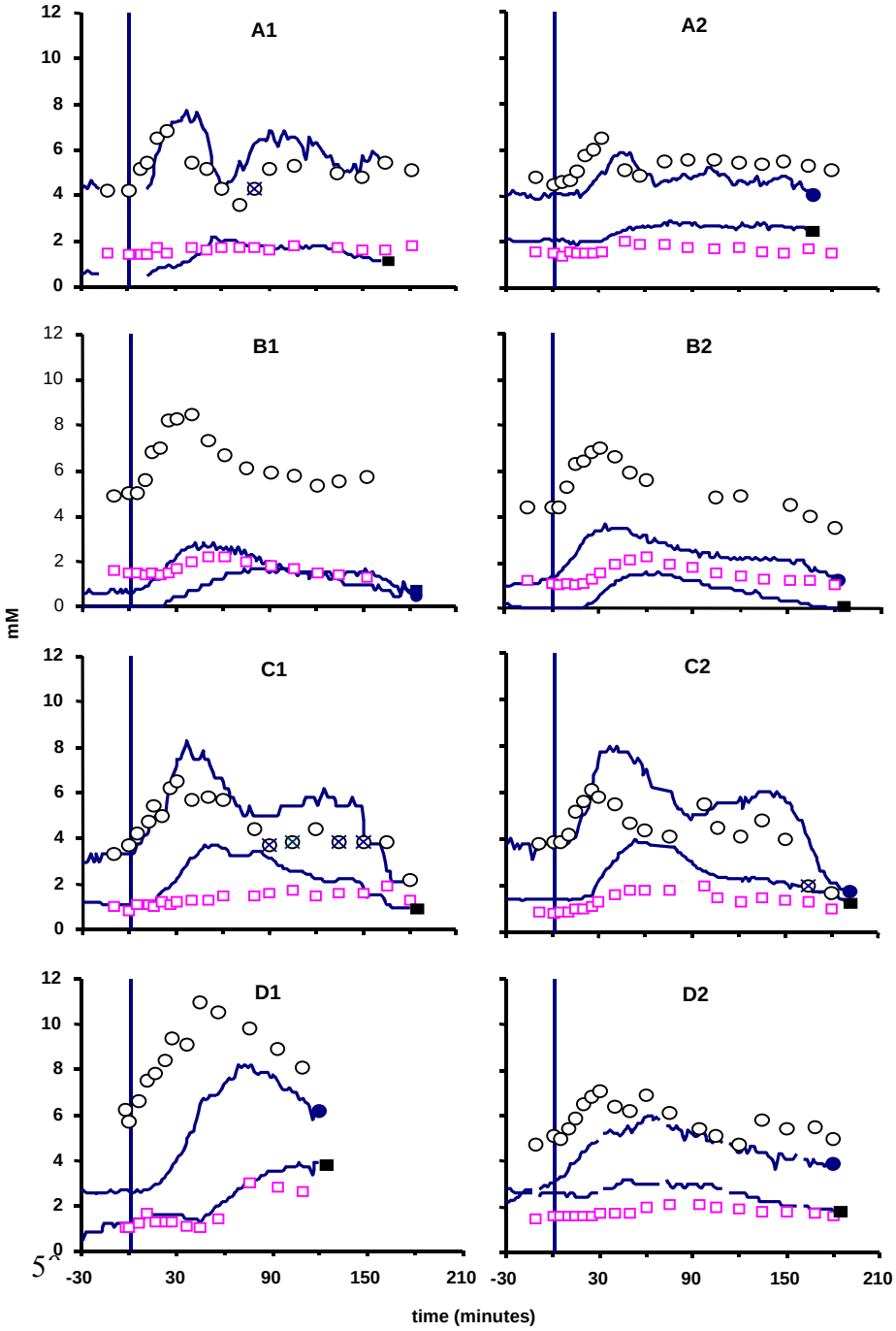
Venous sample concentrations of glucose and lactate were measured with a Vitros 750(manufacturer: Ortho-Clinical Diagnostics, Illkirch Cedex, France). Glucose molarity (mol/l blood) of whole blood is different from the molality (mol/kg water) as about 80% of the blood volume consists of water[23]. The molality of glucose is the same in whole blood, blood plasma, and the extracellular fluid of blood. In order to unite with clinical practice of molarity in full blood, we adjusted UF concentrations by -15% to make a correct comparison with full blood (in accordance with the manufacturer's report). Lactate was determined in blood plasma. No correction for plasma protein volume was done, because no corrective factor is known.

Data analysis and statistics

Glucose and lactate steady-state levels were the average ± SD of the first three iv samples (t=-15,0,5 min) and the concurrent sc levels. Iv and sc steady-state levels were compared with a paired t-test because the values appeared to have a normal distribution. A 95% confidence interval for averages (95% c.i.) was also calculated. The stability of the iv-sc steady-state differences was assessed with a paired t-test between day one and two. The relation between sc steady-state lactate concentrations and steady-state glucose iv-sc differences was tested with linear regression analysis. The iv-sc delay time was defined as the time difference of iv and sc glucose to reach 50% of the maximal glucose values following OGTT.

Computations were made to determine how accurate venous glucose levels may

be estimated from sc sensing. For these computations, we made use of only one blood sample, because we feel that a need for more than one would annihilate the clinical advantage of a sc sensor over SMBG. For the calculation of estimates we had 3 subsequent approaches. Firstly, we continued the difference at steady state,



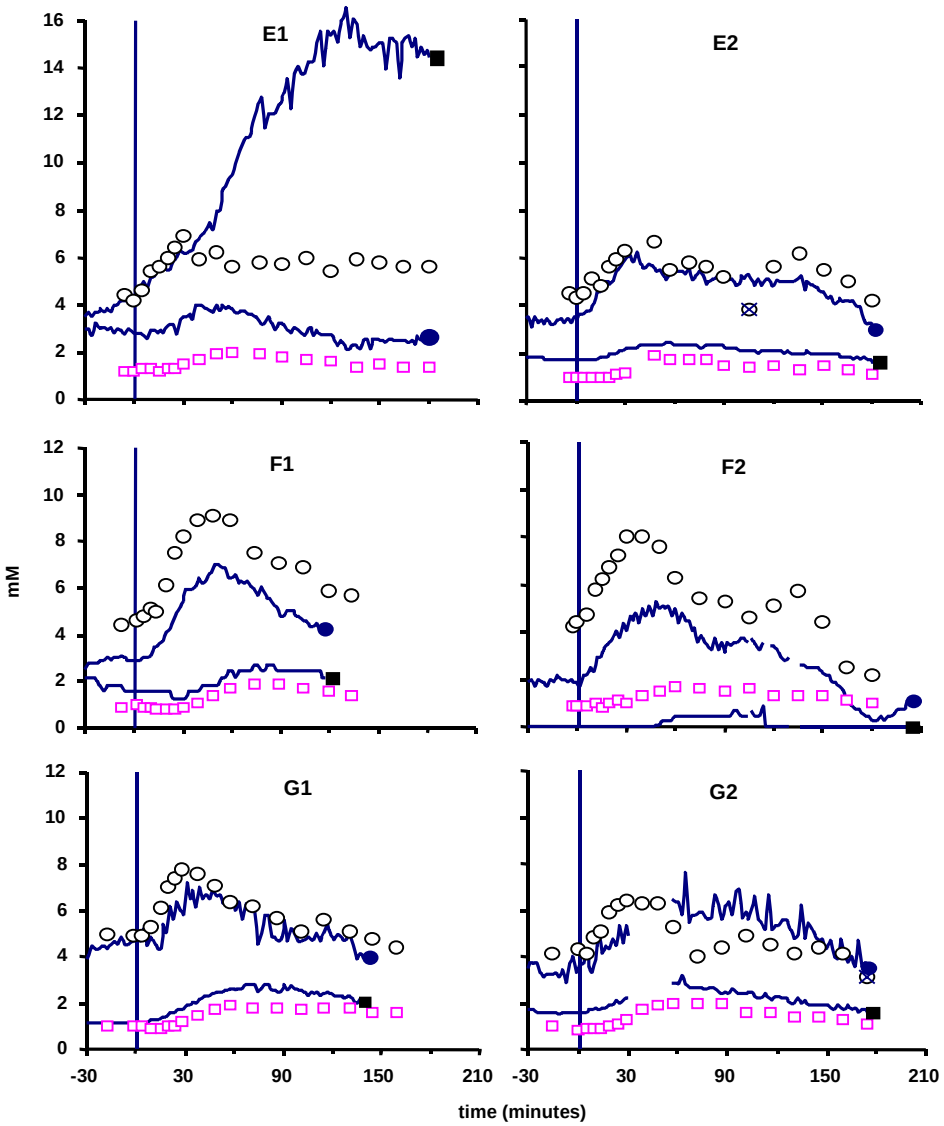
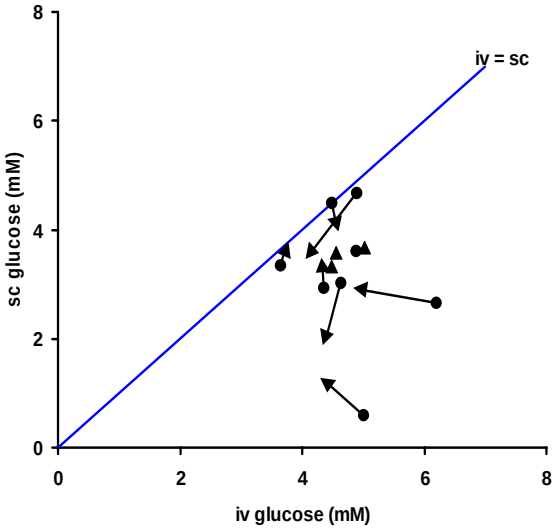


Fig. 1. Sc and iv glucose and lactate measurements during an OGTT in 7 healthy volunteers (A/G) on two successive days (day one, left; day two, right); start OGTT at zero time (|)
○ = iv glucose, top line —● = sc glucose; (in E1 is sc lactate the top line)
□ = iv lactate, bottom line —■ = sc lactate; (in E1 is sc lactate the top line)
⊗ = unacceptable iv glucose concentration estimations in the error grid analysis (iv estimations based on the sc concentrations with addition of the iv-sc difference at zero time)

so we added the difference between the sc and iv glucose concentrations at time zero to all sc values. Secondly, we made estimations departing from non-steady state, so we added the sc-iv difference at t=30 minutes. In a third approach, we calculated the best estimations possible by choosing freely a difference or rate with a time-shift delivering optimal accuracy. To evaluate the stability of the sc space 24 hours after implantation, we continued to use the t=0 and t=30 min. calculation factors of day one for the estimations on day two.
The accuracy of the estimations compared to the measured iv glucose levels was calculated with the method of residuals[24]. In this method is the accuracy the mean absolute difference between estimate and reference, and the precision is the standard deviation. We evaluated the clinical implications in an error-grid as proposed by Clarke et al.[25]. Here, estimations which fall in zone A of this grid are accurate, those in zone B are acceptable, and those in zone C, D, and E are unacceptable because the differences between reference and estimate would lead to dangerous treatment decisions. We also evaluated qualitatively the course in time through the acceptable and unacceptable zones, taking advantage of the high frequency of the sc measurements.

Results

In seven of the eleven volunteers a complete set of data was obtained. Of four persons only data of one day was collected, mainly because air bubbles in the probe blocked the downstream analysis system. This problem occurred in the first experiments only and was resolved in the course of the experiments. One volunteer appeared to have an Hb of 6.5 mM (normal 7.5-9.9 mM) (subject D in fig.1) and another had a high OGTT response 9.48 mM venous glucose was achieved after two hours (graph not shown). Both were informed and referred to their general practitioner. We evaluated the data of these three experiments as the other data.
The sc glucose concentrations in steady state versus iv levels at subsequent days showed no correlation (see fig. 2). Sc concentrations were lower than iv levels on both day one (●) and on day two (▲). We found 4.62 ± 0.53 mM iv and 3.15 ± 1.01 mM sc (mean ± SD). The iv-sc difference was 1.47±1.20 mM (95% c.i. 0.88 to 2.07 mM in a paired t-test). Expressed as a percentage sc levels were 68.8±21.9 % of iv levels. The differences were on average 1.62±1.66mM on day one and 1.35±1.10 mM on day two. The change in difference from day one to day two is -0.59 to 1.07 (95% c.i. in a paired t-test)(paired days indicated in figure 2 as arrows ●—►). In case the iv-sc



difference was small, only little

Fig. 2. Sc versus iv steady-state glucose concentrations.
● = glucose concentrations on day one
▲ = glucose concentrations on day two
●—► = day 1 and 2 measurements with same probe/person
⁄ = equality of concentrations line (iv=sc)

change in this difference was observed. Large differences were accompanied by rather big changes (n was too small for testing the significance of this trend).
Figure 1 shows the results of the seven subjects who completed the study.
The delay-time between the 50% OGTT glucose rise sc and iv was 7.3 ± 1.2 minutes (mean ± SEM). The sc curves appear to be flattened, as compared with the iv curves.
Average steady-state lactate concentrations were 1.18 mM sc, and 1.43 mM iv (95% c.i. of difference: -0.77 to 0.27mM). Again, sc-steady-state-lactate concentrations showed no correlation with steady-state-glucose-iv-sc differences (r=0.26). In most cases, lactate rose in parallel to glucose. The sc lactate concentration was well above the iv level in two volunteers on day 1 (see fig. E1, graph of second volunteer not shown).
Table 1 summarises the results of different computations reflected in the

accuracy reached and the clinical implications of the error grid analysis. This analysis is shown in figure 3 for experiment B1. The raw data, the estimates after calculation with the difference at t=0, the estimates after optimal difference and time-shift recalculation and the estimates after optimal rate and time-shift recalculation are plotted here in the error grid.

Estimates using the t=0 (steady state) difference yielded an overall accuracy and precision of 0.85 mM and 0.62 mM respectively for the 18 OGTTs. Error-grid analysis yielded 73.1% of estimates in zone A (accurate), 23.7% in zone B (acceptable), and 2.9% in zone D (unacceptable). The values in zone D are indicated with a ⊗ in figure 1.

Estimates based on the t=30 min. (non-steady state) difference yielded an accuracy and precision of 0.98 mM and 0.67 mM respectively. Error-grid analysis yielded 63.6% of estimates in zone A, 33.4% in zone B, and 2.6% in zone D.

Estimates made on the second day using the t=0 difference of the first day (testing stability of the sc-iv relation) yielded an accuracy and precision of 1.15 mM and 0.63 mM respectively. Error-grid analysis yielded 54.8% of estimates in zone A, 42.1% in zone B, and 3.2% in zone D.

Table 1. Accuracy of iv glucose concentration estimates from sc measurements analysed by the method of residuals and by the error grid method
(table on two opposite pages)

Computation method of estimates	**Analysis by the method of residuals**		
	Accuracy (mean absolute difference in mM)	Precision (SD absolute difference in mM)	Correlation
Crude sc concentration	1.73	0.65	0.67
With addition of the steady state difference of T=0 min.	0.85	0.62	0.67
With addition of non-steady state difference of T=30 min.	0.98	0.67	0.67
With addition of T=0 difference of day 1 on second day	1.15	0.63	0.64
With addition of T=30 difference of day 1 on second day	2.12	0.61	0.64
With optimal delay and addition	0.42	0.37	0.84

With optimal delay and rate	0.47	0.40	0.84

The accuracy of the estimations could be optimised to 0.42 mM by choosing an iv-sc difference of 1.20 mM mean with a time-shift of 8.7 min. mean. Error-grid analysis yielded 93.2% of estimates in zone A, 5.0% in zone B, and 1.8% in zone D.
The accuracy of the estimations could be optimised as well (to 0.47 mM) by choosing a sc-iv rate of 1.20 mean combined with a time shift of 10.8 min. mean. Error-grid analysis yielded 91.0% of estimates in zone A, 7.6% in zone B, and 1.4% in zone D.

Conclusions

The major observations of the present study are a variably lower sc steady-state glucose level and a short time-delay as well as flattening of the sc OGTT curve, as compared to iv levels. These lower sc levels are only exceptionally

during 18 OGTTs in healthy volunteers; different iv estimate computations

		Error grid analysis				
Mean added difference (mM)	Mean added delay (min.)	Zone A (%)	Zone B (%)	Zone D (%)	Reference <3.88mM (%)	Estimates (n)
		40.6	57.1	2.3	5.5	308
1.39		73.1	23.7	2.9	5.5	308
1.94		63.6	33.4	2.6	5.5	308
1.58		54.8	42.1	3.2	6.3	126
2.72		33.3	63.5	3.2	6.3	126
1.20	8.7	93.2	5.0	1.8	5.0	280
1.20	10.8	91.0	7.6	1.4	5.1	277

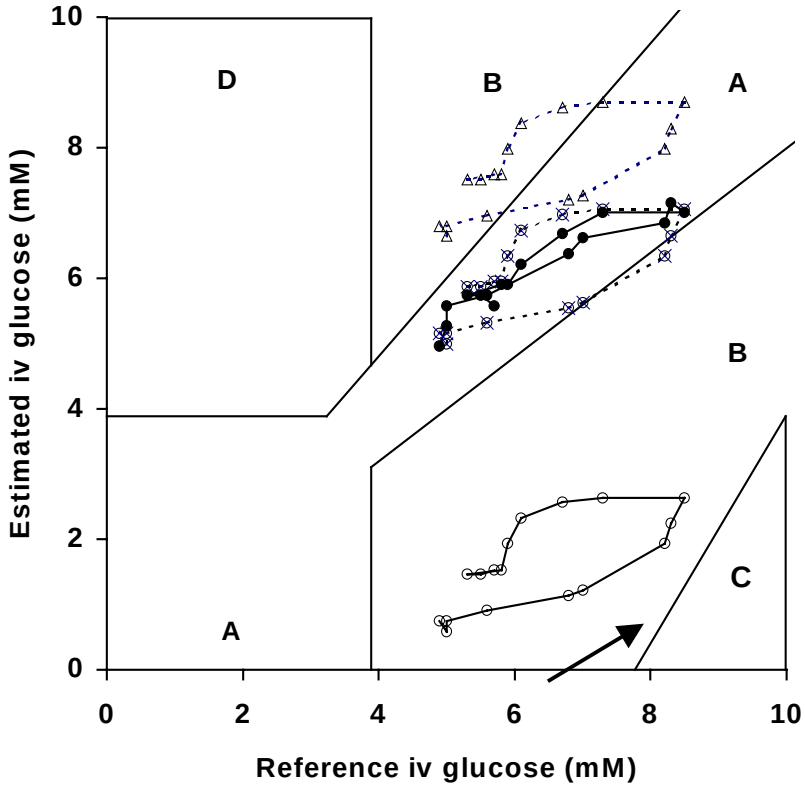


Fig. 3. Error grid analysis of the iv glucose estimations from OGTT experiment B1 in figure 1. Crude, steady-state, non-steady-state, and optimal iv glucose estimations based on the measured sc glucose concentrations are plotted against the reference iv glucose concentrations.
○ = crude sc concentrations (● is on t=0min. and on t=30min.),→ = OGTT course
⊗ = sc concentrations+4.41mM = iv estimations based on addition of the iv-sc difference at t=0 minutes, so at steady state.
Δ = sc concentrations+6.05mM = iv estimations based on addition of the iv-sc difference at t=30 minutes, so at non-steady state.
● = sc concentrations of 11 minutes later+4.36mM = optimal iv estimations based on sc time delay and addition
Error grid zones: zone A, accurate estimates; zone B, acceptable estimates; zones C and D, unacceptable iv estimates because the differences between

reference and estimate would lead to dangerous treatment decisions. Zone E is an unacceptable zone out of range of this graph.

accompanied by high sc lactate levels. Clinical accuracy of iv estimates is moderate taking into account only the difference between sc and a single blood sample at steady-state or at non-steady-state condition. The accuracy decreases on day two. By adding a time-delay sc monitoring may reach a clinically acceptable accuracy. Lowered, delayed, and flattened sc glucose levels as compared to blood levels found are in accordance with the findings of most previous investigations[12;13;16-19;26;27], and points to local glucose metabolism. The lactate levels found suggest that local anaerobic metabolism is usually not the major cause of low glucose levels in tissue around a probe. So lactate levels indicated little implantation damage.

The observed iv-sc time-delay is in accordance with values found in tissue diffusion experiments[28] and is not likely to pose a problem for the timeliness of a sc sensor hypoglycaemia warning because the development of hypoglycaemia takes most often more than 30 minutes. Here, the time-delay may (partly) be caused by the difference between iv and intra-arterial glucose levels, as seems the case e.g. in experiments A1, C1 and C2 where sc glucose levels were temporally higher than iv. A temporally higher arterial glucose concentration during OGTT has been well documented[29;30]. So, the sc glucose concentrations may thus be more closely related to arterial levels than to iv levels, at least in these cases. This consideration emphasises the relevance of the sc glucose levels found lower than iv levels in this study. Our results and the observations of others indicate that abdominal subcutis must be considered as a kinetic compartment distinct from the vascular compartment.

We tested the accuracy of some simply computed iv estimations. Firstly, the estimations were calculated from the sc levels and a single concurrent blood concentration at steady state and non-steady state. These estimations reveal only moderate accuracy, essentially because of the neglected time-delay and the neglected flattening of sc kinetics. Secondly, a recalculation was made, taking into account a time-delay and difference. Now the accuracy is near to clinical acceptance. A close look at the sc curves in fig. 1 reveals that most estimates in zone D (indicated with *) may be corrected by taking into account a time-delay and/or by the use of arterial glucose levels as a reference rather than iv levels. Unfortunately, accuracy diminishes after one day. It is thus clear that the here proposed methodology has to be applied after long-term implantation. Nevertheless, subcutaneous glucose can be related to blood glucose by taking into consideration time-delay and local glucose consumption.

In conclusion, there is perspective on clinical application of a hypoglycaemia

alerting device, utilising the now available usMD or ultrafiltration[18] techniques which give the sole opportunity to compare absolute tissue concentrations directly to blood levels with a high time-resolution. In clinical practice, we recommend to allow for sc delay and flattening when estimating iv glucose concentrations with a sc sensor. We also propose to control the calculation factor with a blood sample on day two, despite the increase of patient burden.

The remaining limitations of glucose sensing are related to the drift of local tissue kinetic factors in time. Implantation for weeks of sc MD probes is already possible without problems[19].

Future research should concern the reliability of the present approach in ultrafiltration or usMD probes implanted for a long period of time. Further, it should include diabetic patients, because their glucose tissue diffusion can differ from that of healthy subjects[28]. Such investigation may aim at a sc kinetic model, an in vivo parallel measurement of kinetic factors for continuous recalculation or a favourite anatomical place for probes. All this can lead to more accurate diabetes monitoring devices. In addition, tissue levels may even replace blood levels in future as the reference for diabetes management, because tissue levels comprise more specific metabolic information.

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Chapter 4

An ultrafiltration catheter for monitoring of venous lactate and glucose around myocardial ischemia

Abstract

Introduction: Early detection of myocardial ischemia is of major importance in critical-care medicine. Changes of lactate or glucose levels in the cardial venous efflux may be useful parameters.
Methods: We succeeded in integrating an ultrafiltration membrane in a cardiac catheter for continuous sampling. The ultrafiltrate was analyzed outside the body, resulting in a lag-time of about 24 minutes. Biosensors in a flow-injection analysis system were used for minute by minute sample analyses. The coronary sinus of pigs was catheterized to monitor the effects of 5, 15 or 45 minutes ischemia by coronary artery obstruction or myocardial stress by dobutamine infusion. A total of 27 hours was monitored.
Results: The intravascular response time was 1.33 ? 0.61 minutes (10-90%). Linear regression in vivo of blood and ultrafiltrate samples was 0.977 for lactate and 0.994 for glucose. Lactate levels rose 0.38 ? 0.10 mM above baseline within five minutes after ischemia. Reperfusion was clearly marked by a promptly peaking lactate release (max. 9.27mM). Myocardial stress by dobutamine increased glucose but not lactate levels. Once, a wall effect was noted at the catheter tip.
Conclusions: In vivo semi-continuous myocardial monitoring of absolute lactate and glucose concentrations was thus achieved by an ultrafiltration catheter. Ischemia and reperfusion can be detected very early by a lactate level rise. Further development of the ultrafiltration catheter will be focused on the diagnostic potential of lactate monitoring for patients.

Introduction

Insufficient myocardial perfusion is a major concern in critical-care medicine, because it often leads to irreversible myocardial damage. Before the damage becomes irreversible, poorly perfused tissue shifts from aerobic to anaerobic metabolism, the latter characterised among other by increased lactate production. Routinely used markers of myocardial ischemia, e.g. troponin and creatine kinase, are released only after cell death, so monitoring of these markers can not help to prevent infarction[1]. Lactate monitoring, however, may offer an opportunity to detect tissue oxygen deficit in time for preventive intervention. Ischemic myocardium releases lactate in a quantitative relation to the extent of ischemia[2;3]. Indeed, major concentration shifts may occur in a matter of minutes both after the onset and at the end of myocardial ischemia[4]. So the occlusion and reperfusion of the myocardium could be monitored by measuring the lactate efflux into the common cardiac vein, the coronary sinus. This is however not practised in the clinic, because there are no logistics for frequent blood sampling and analysis. Further, the data are neither continuous, nor instantly available for making therapeutic decisions. Moreover, to justify catheterisation for intravascular measurement, the diagnostic value of continuous lactate monitoring has yet to be proven.

Biosensors are devices that have the potential to monitor analytes in vivo continuously. There are however many requirements for in vivo application which hinder the development of an intravascular lactate sensor[5]. These requirements may be met much more easily by application of microdialysis or ultrafiltration probes as an interface between the sensor and the blood. The analyte enters such probes through a membrane inside an organ, and is subsequently transported to the sensor, which can be kept outside the individual. This separation has many technical advantages, e.g. calibration of the biosensor (if necessary) can be done without interrupting in vivo measurements[6].

Microdialysis probes directly inserted in the myocardium have been used to study myocardial metabolism in ischemia, reperfusion, and preconditioning[7-12]. Microdialysis however, has some limitations. In the first place, the precise calibration in vivo is difficult, because the diffusion inside tissue does not guarantee a constant analyte recovery[13]. If measurements are relative and instable, this precludes quantitative and reliable long-term monitoring. Secondly, the sampling time of microdialysis is usually long (5-60 minutes[7;14-17]) which is not appropriate to detect acute events. A final limitation of myocardial microdialysis is the fact that the probes can only be placed during open thorax surgery.

Recently, we described an ultrafiltration technique allowing continuous sampling and monitoring of absolute glucose concentrations in the rat jugular vein[18]. Ultrafiltration excludes compounds with a molecular weight over 20 kD, but poses no barrier for small solutes like glucose and lactate[19]. Continuously sampled blood ultrafiltrate was analyzed in our method by a biosensor placed in a flow injection analysis system.
In the present study, an ultrafiltration catheter was placed in the coronary sinus allowing myocardial monitoring without cardiac surgery. An ultrafiltration probe was integrated to this end in the tip of a double lumen cardiac catheter, in order to take simultaneously ultrafiltrate and whole blood samples. The feasibility of monitoring with this probe was evaluated for several hours in pigs, during which ischemia was induced for various periods of time by inflating a balloon in the Left Anterior Descending coronary artery (LAD). The response time of this ultrafiltration catheter was determined before an attempt to detect any prompt and major changes in lactate and glucose levels, if present during myocardial ischemic events. Next, the possibility was explored to use the data for quantification of the lactate production during myocardial ischemia. The stress of heart rate acceleration on compromised myocardium was also tested in some experiments by dobutamine infusion.

Methods

Ultrafiltration catheter
A 5F (1.78mm o.d.) Nylon blend double lumen catheter of 120 cm length (shape: Modified Cordis Multipurpose, Cordis Europa N.V., Roden, The Netherlands) was designed to simultaneously withdraw blood and blood ultrafiltrate (Figure 1). The large lumen, 0.98 mm i.d., was used as guidewire/flush/blood sample lumen. The proximal end of the large lumen was provided with a luer connector. Five side holes in the large lumen (i.d. 0.635 mm) were made in the last 4 cm of the catheter tip. The small lumen, oval with i.d. 0.83 mm x 0.35 mm, was provided with two closely adjoining fenestrations of 2 times 2 cm length at 2 mm from the distal tip, to place a hollow fiber membrane AN69HF, Hospal, Meyzieu, france; 340?m o.d.; 240?m i.d.). This hollow fiber has a MWCO of 20kD, but forms no diffusional barrier for small molecules like glucose and lactate[20]. Two marker bands, gold 18 Kt, width 1.0mm, were placed at either end of the fenestration to locate the probe on the x-ray. A helical platinum wire was placed inside the hollow fiber to support the wall of the probe. The fiber was closed and fixed with two components

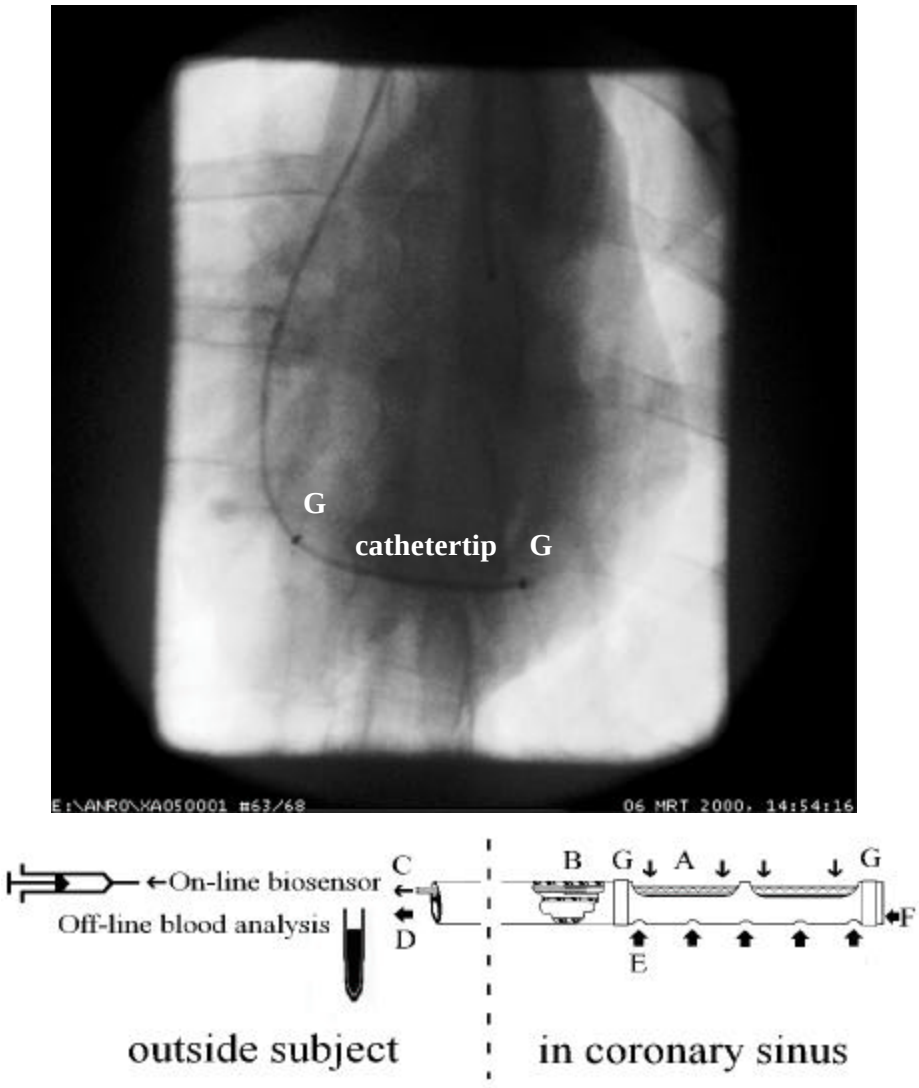

Figure 1 Schematic drawing of the ultrafiltration monitoring catheter (bottom), and x-ray of the ultrafiltration catheter in situ in the coronary sinus (top). The drawing is not to scale.

† flowdirection of continuous ultrafiltration, † flowdirection of whole blood samples A fenestration containing hollow fiber with helical spring inside, B connection of ultrafiltrate tube and fibre inside the small lumen of the catheter, C ultrafiltrate transport in polysulfon tube from catheter tip to on-line biosensor, D blood sampling via large lumen of the catheter for off-line analysis, E first of the five side holes of the large lumen, F end hole of the large lumen, G marker bands for positioning on x-ray

polyurethane adhesive (e.V. Roberts, Culver City, CA, USA) in the distal end af the catheter window. Proximal, the fiber was glued to a draining tube (polysulfon, 260ʔm o.d.; 42ʔm i.d., 145cm l.; Cordis Europa N.V., Roden, The Netherlands) which transported the sample fluid to the loop of the flow injection analysis system. The ultrafiltration flow rate was determined by the semi-vacuum of a syringe with a fixed piston (625±1 mbar±SD, 0.2 %CV, 1.2 ml s-monovette, Sarstedt, Nümbrecht, Germany) and the flowresistance of the system, as described previously[21]. The flowresistance consisted mainly of the very narrow draining tube which reduced the influence of the ultrafiltration probe membrane resistance on the flow rate. The lag-time between sampling and measurement varies with the length of the ultrafiltration draining tube. The ultrafiltrate flow generated was checked by continuously weighing the syringe.
A 9F introducer sheath (Avanti™, Cordis Europa, Roden, The Netherlands) was used to introduce the ultrafiltration catheter in the left jugular vein. To protect the tip membrane from damage while introducing, a tube was temporally placed in the valve of the introducer. From the left jugular vein, the ultrafiltration catheter was advanced, and positioned at least 5 cm within the coronary sinus. The position of the catheter was checked by contrast injection (Figure 1(top)).

Lactate and glucose analysis
The ultrafiltration catheter was connected to a 20 nanolitre loop in a flow injection analysis system. Lactate and glucose were electrochemically detected every minute before, during, and after the experiments in the continuous ultrafiltrate flow as described by Tiessen et al[22]. Calibration solutions were measured before and after each experiment by placing the catheter in standard solutions of glucose and lactate. The sensitivity in vitro of lactate and glucose sensors, respectively 8.09 and 1.66 nA/mM with both linearity $r>0.99$, $p<0.0001$, decreased over a period of days. The decrease before and after each experiment was negligible.Whole blood samples (2 ml) were withdrawn at the catheter tip every 10 minutes throughout the experiment, and every 2 or 5 minutes around anticipated quick concentration changes(Figure 1). The test tubes contained sodium fluoride and potassium oxalate to arrest coagulation and metabolism. The tubes were kept on ice until measurement on the same day. Concentrations of glucose and lactate were measured in whole blood and blood plasma after centrifugation with the Vitros 750 analyser (manufacturer: Ortho-Clinical Diagnostics, Illkirch Cedex, France) in the hospital routine laboratory. In order to make correct comparisons with whole blood glucose determinations, we adjusted the ultrafiltrate concentrations by -15% to unite with clinical practice of molarity measurement in whole blood (in accordance with the manufacturer's

report). Glucose molarity (mol/l blood) of whole blood is different from the molality (mol/kg water) as only about 80% of the blood volume consists of water[23]. Lactate was determined in blood plasma. No correction for plasma protein volume was applied, because no corrective factor is known for lactate.

Experimental procedures

All procedures were reviewed and approved by the Animal Experiments Committee of the Groningen University. Experiments were performed on six Yorkshire swines (weighing 32, 33, 34, 53, 65 and 93 kg) because of the similarity with the human cardiac anatomy. The animals were maintained on a normal diet. All procedures were performed under anaesthesia. The swines were anaesthetised using a combination of ketamine (15 mg/kg;), acepromazine (0.2 mg/kg) and atropine (0.05 mg/kg) intramuscularly. An endotracheal tube was inserted for O_2/NO_2 ventilation. Ventilation with 2 % isoflurane (Forene®, Abbott, U.S.A.) was used to ensure adequate anaesthesia throughout the experiments. Levodromaron (2mg subcutaneously) was given as analgesic at the beginning of the procedure and supplemented if necessary. Lidocaine (50mg intravenously) was given profylactically. Throughout the experiments, the condition of the animals was carefully monitored by means of continuous transcutaneous oximetry, intra-arterial blood pressure measurement, and electrocardiographic registration (ECG) of the limb leads and one modified precordial lead.

The pigs were administered initially 3mg/kg heparin intravenously, followed by 1.5mg/kg/h. The use of blood anticoagulant is known to be necessary in artificial kidneys to preserve the permeability of the membrane, which was here applied for the probe[24]. The need to prevent blood clotting was confirmed in three preparatory experiments without heparin, which resulted in poor accuracy and a response time of about 20 minutes.

To measure the response time in vivo, 0.25g/kg of glucose 40% solution in water was injected intravenously in 2 minutes time once in every experiment. 0.1 g/kg of lactate 20% solution in 0.9% saline was added to the intravenous glucose injection in one experiment.

LAD coronary artery obstruction and reperfusion (n=3)

The myocardium of three pigs were subjected to partial ischemia. To this end, a 6F left Judkins coronary guiding catheter was advanced via an introducer sheath in the carotid artery to the ostium of the left coronary artery. Cine-angiograms (26 frames per second) were made from the 30? left anterior oblique position and filmed until the venous phase was reached. A balloon catheter was advanced

through the guiding into the left anterior descending coronary artery just past the first diagonal, where the balloon was inflated temporally for 5 minutes in one pig, and for 15 and 45 minutes in two pigs. The heart rate did not change significantly during inflation (95?10 to 103?9 min^{-1}), neither did the blood pressure (79?8 to 68?7mmHg). In two instances ventricular tachycardia/fibrillation occurred at reperfusion. Electroconversion shocks were given for severe arrhythmias to re-establish sinus rhythm. The defibrillation at 10 J with surface electrodes did not interrupt the ultrafiltrate monitoring. These three experiments eventually ended with cardiac arrest after a total of 6.5, 6.5, and 8.5 hours continuous measurement.

Animal model myocardial dobutamine stress test (n=3)

Three pigs were evaluated 4 weeks after a nearly complete occlusion of the distal LAD by local injury and stent placement. The distal LAD showed at that time a strongly reduced flow (TIMI1). The angiogram of the left ventricle showed hypokinesia or akinesia in the distal part of the anterior wall with an overall good left ventricle rest function.

Dobutamine was infused with incremental doses from 2.5 ?g/kg/min to stress the myocardium by increasing the heart rate. After 3 minutes infusion of each dose, the heart rate and the blood pressure were measured, and a higher dose was started. Doses were increased until a clear drop in blood pressure was observed. The doses were increased up to 20?g/kg/min in the first pig, up to 30 ?g/kg/min in the second pig, and up to 22.5 ?g/kg/min in the third pig. On completion of each procedure, the animals were euthanised using a standard solution.

Data analysis and statistics

After a sudden change in concentration on the outside of the catheter tip, the equilibration on the inside took some time. This response followed a sigmoidal course from one level (0%) to the next level (100% equilibration). The time from 10 to 90% equilibration was calculated with sigmoidal fitting. The average (10-90%) equilibration time ± standard deviation (SD) was calculated for the twelve im- and explantations. The lag-time (average ? SD for all six experiments) was defined as the time interval from actual catheter implantation to measurement of 90% change of signal by the sensors. The response time in vivo was defined as the average time ? SD for the amperometric signal to change from 10 % to 90% after an intravenous glucose injection (n=6).

The ultrafiltrate flow rates in vitro and in vivo were calculated with the pump

weight changes every five minutes in one hour before and during the implantation (averages? SD for n=6).

The ratio of the ultrafiltrate and blood measurements after the start of the experiments was compared to the ratio near the end as an indication of stability of glucose and lactate ultrafiltrate measurements.

The correlation between whole blood glucose and coronary ultrafiltrate measurements was determined with the samples around one i.v. glucose injection every experiment. Two baseline points before the injection and all points until return to baseline were used for linear regression (42 paired measurements from 6 injections). Samples taken within two minutes before and after the injection were omitted for reasons of response time limitation. The data were also compared using the Bland-Altman analysis on the relative differences of glucose concentrations between whole blood laboratory analysis and ultrafiltrate flowinjection analysis[25]. The bias (the average difference), and the precision (the 95% confidence interval) were determined as a percentage of the reference values. The correlation between blood plasma lactate and coronary ultrafiltrate measurements was determined with paired samples around the concentration excursions from baseline after the i.v. lactate injection in one experiment (n=11) and after myocardial reperfusions in two experiments (n=35).

To detect acute ischemic changes, the lactate baseline, taken as the last 5 minutes ultrafiltrate measurements before balloon inflation, was compared to the concentration during the first 5 minutes of ischemia. The concentration shifts expressed as the average ? mM lactate and as a percentage of the baseline concentration before ischemia were compared with the Mann-Whitney Rank Sum Test. The same procedure was used for acute lactate/glucose rate changes.

Some assumptions were made in order to calculate estimations of the extracellular myocardial lactate build-up during ischemia. From the very quick lactate concentration rise after balloon deflation (see results) was deducted that this release can be considered as an intravenous lactate bolus injection with immediate distribution in the bloodplasma volume. Further, it was assumed that the body lactate clearance and production are not affected during reperfusion, and that the rate of lactate elimination from blood is linear[26]. A one-compartment behavior was assumed for the first fifteen minutes, so from t= 0 to t=15.

Next, the equation for estimations $?C_{t=x} = ?C_{t=0} * e^{-K*t=x}$ was found by least square regression (Gauss-Newton method). Thus, the differences between the measured data in the 15 minutes after balloon deflation (as far as within the response time and the precision of measurement) and the estimated data ($?C_{t=x}$)

were minimised. The ? lactate concentration at the site of origin at the time of balloon deflation (? $C_{t=0}$) was calculated by loglinear extrapolation of the estimated ? lactate elimination course (? $C_{t=x}$) for 2 or 3 minutes back to reperfusion onset at t=0. The variable K is the lactate elimination time constant with a half-live of $t_{½}$ = 0.693/K.

The total lactate release after reperfusion was determined with the area under the curve, calculated by the trapezoid method when the ? lactate concentration is plotted against time from 0 to 8. The area under the curve (AUC) was expressed in mmol?l^{-1}.?h being the amount of lactate (mmol) devided by the bloodflow in the coronary sinus(l?h^{-1}). No bloodflow measurements were available to further calculate this amount in Mols.

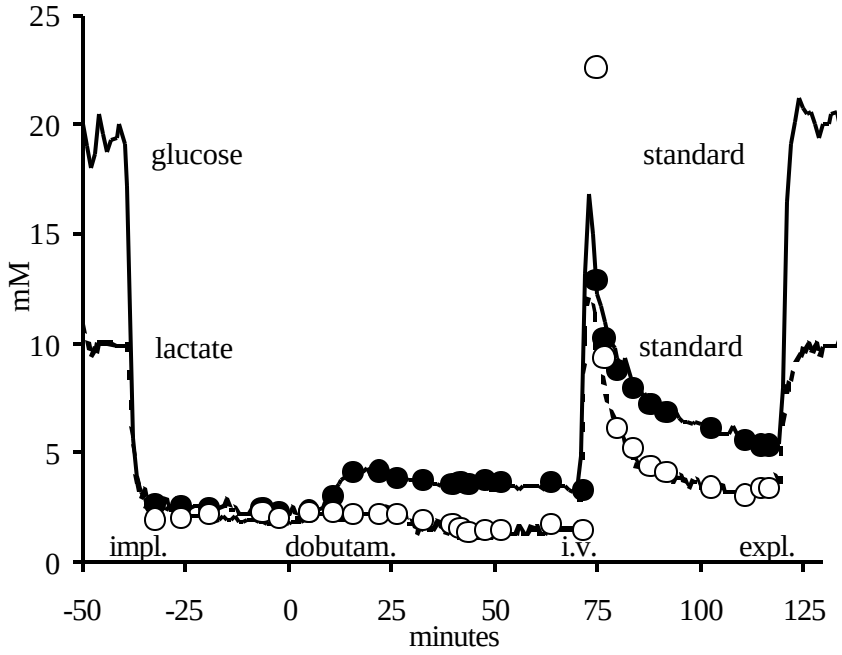

Figure 2 Typical example of an experiment. Lactate (?) and glucose (?) blood sample and lactate (---) and glucose (?) ultrafiltrate measurements. Glucose and lactate standards before implantation and after explantation of the catheter from the blood circulation. Myocardial stress by dobutamine infusion from t=0 to t=27 minutes. I.v. injection with glucose and lactate solution.

Results

In vivo performance characteristics

In total 27 hours monitoring was performed in vivo with sixty ultrafiltrate measurements per hour. The equilibration time after im- and explantation was 1.33 ? 1.00 minutes (see typical experiment in Figure 2). The response time of ultrafiltration after i.v. injections was 1.33 ? 0.61 minutes. The lag-time was on

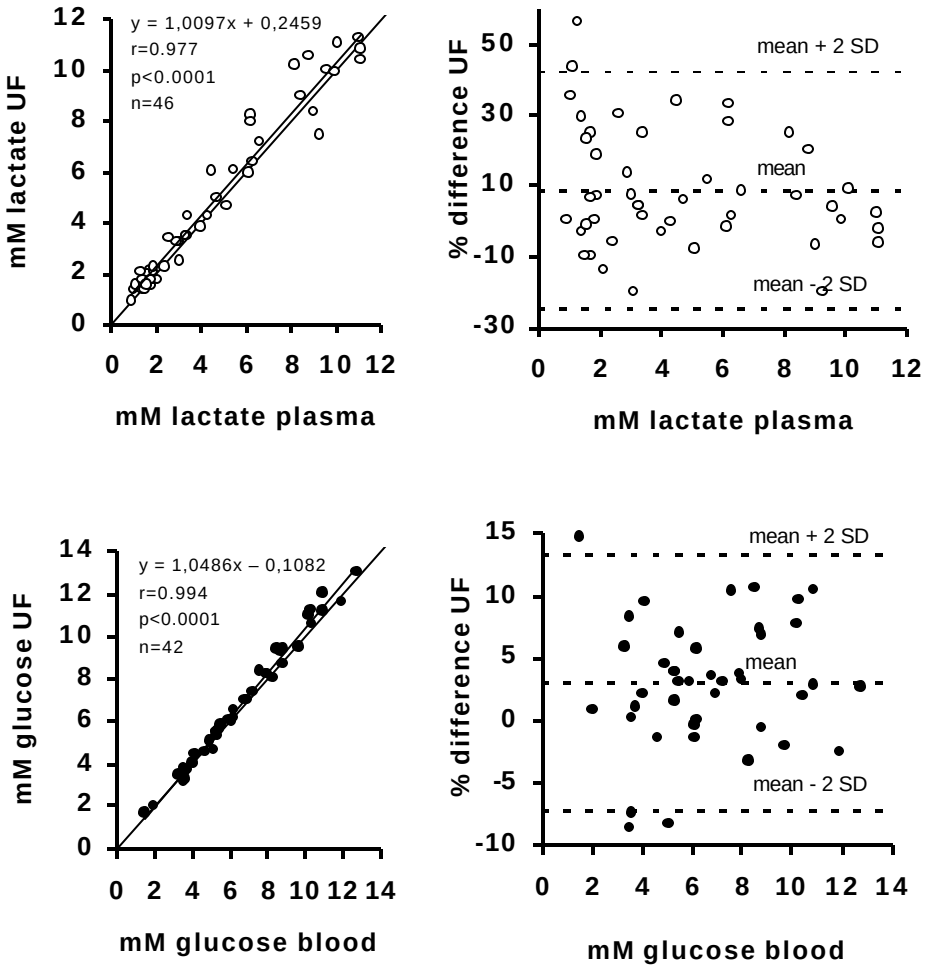

Figure 3 Regression and bias/precision plots for blood versus ultrafiltration samples. Upper panel: lactate, lower panel: glucose. The regression lines and the lines of identity are shown left. Mean (bias) and mean ? 2SD (precision) lines are shown right.

average 24.3 minutes (range 21-26 minutes). In five pigs, the catheter ultrafiltrate flow rate remained stable after introduction in the bloodstream with 85.1? 4.9 nl/min in vitro and 85.5 ? 8.2 nl/min in vivo. At the end of the experiments was the ultrafiltrate/blood concentration ratio for glucose 101 ? 9% and for lactate 88 ? 14% of the ratio at the start of the experiments ($p>0.05$). No apparent effect was seen of the electrical defibrillations on ultrafiltrate flow or measurements.

Figure 3, upper panel left shows the linear regression for lactate in the ultrafiltrates (y) and blood samples (x) which was $y = 1,0097x + 0,2459$, $r=0.977$, $p<0.0001$, $n=46$. The bias was 8.86%, the precision 33.42% (Figure 3, upper panel right). The linear regression for glucose was $y = 1,0486x – 0,1082$, $r=0.994$, $p<0.0001$, $n=42$ (Figure 3, lower panel left). The bias was 3.03%, the precision 10.22% (Figure 3, lower panel right). The regression coefficient r rose for lactate during subsequent experiments as a result of improvement of the blood sampling procedure.

Only in the second experiment declined the flow rate from 90 nl/min to 56 nl/min in the second half of the experiment lasting 6.5 hours. Blood sampling became then almost impossible. The ultrafiltration response time after an i.v. injection at 30 minutes after start of the experiment was still 1.78 minutes, the lag-time 26 minutes. The ultrafiltration response time after an i.v. glucose injection at 450 minutes after start of the experiment was estimated 0.85 minutes, but the lag-time 43 minutes. The course of the lactate concentrations in ultrafiltrate did not change significantly during and after 15 minutes balloon inflation in the LAD. The ultrafiltrate lactate levels followed an irregular course and started to rise slowly after 45 minutes balloon inflation in the LAD, while the lactate in the blood samples remained low. The ultrafiltrate/blood lactate concentration ratio rose near the end of the experiment to 324% of the start. Ultrafiltrate glucose levels decreased slowly after 45 minutes balloon inflation in the LAD while the blood levels did not alter much. The ultrafiltrate/blood glucose concentration ratio descended near the end of the experiment to 60% of the rate at the start of the experiment. The ultrafiltrate flow was immediately restored to 92 nl/min after placing the catheter from the coronary sinus into a calibration solution. The data of this experiment were excluded from further data analysis.

Coronary sinus lactate and glucose levels after LAD coronary artery obstruction and reperfusion (n=3)

After balloon inflation in the LAD, lactate levels in the coronary sinus ultrafiltrate rose by 0.38?0.10mM above baseline during the first 5 minutes of ischemia ($p<0.05$), see Figure 4. The lactate/glucose rate rose by 28?16%

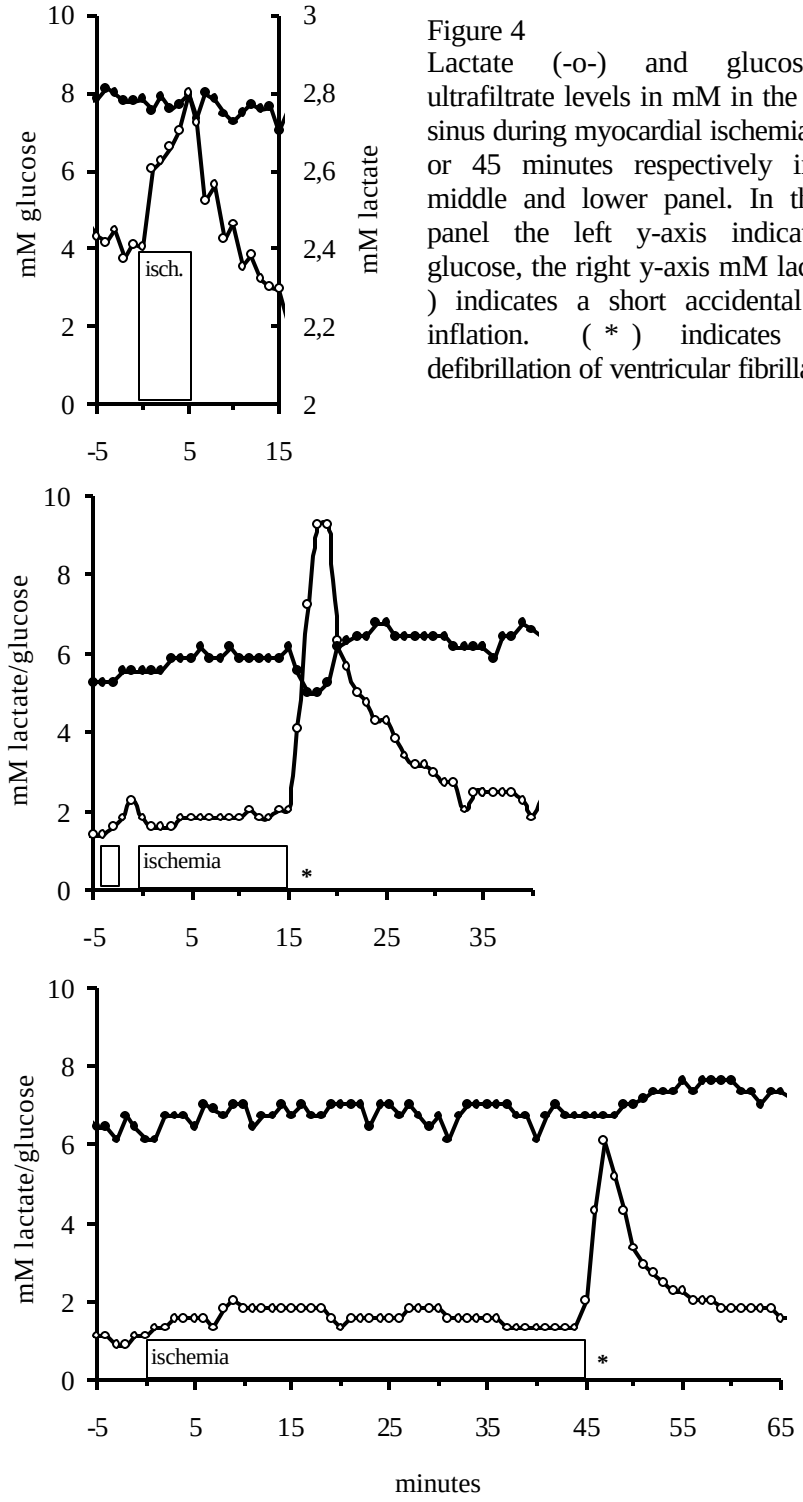

Figure 4
Lactate (-o-) and glucose (-?-) ultrafiltrate levels in mM in the coronary sinus during myocardial ischemia of 5, 15 or 45 minutes respectively in upper, middle and lower panel. In the upper panel the left y-axis indicates mM glucose, the right y-axis mM lactate. (?) indicates a short accidental balloon inflation. (*) indicates electric defibrillation of ventricular fibrillation

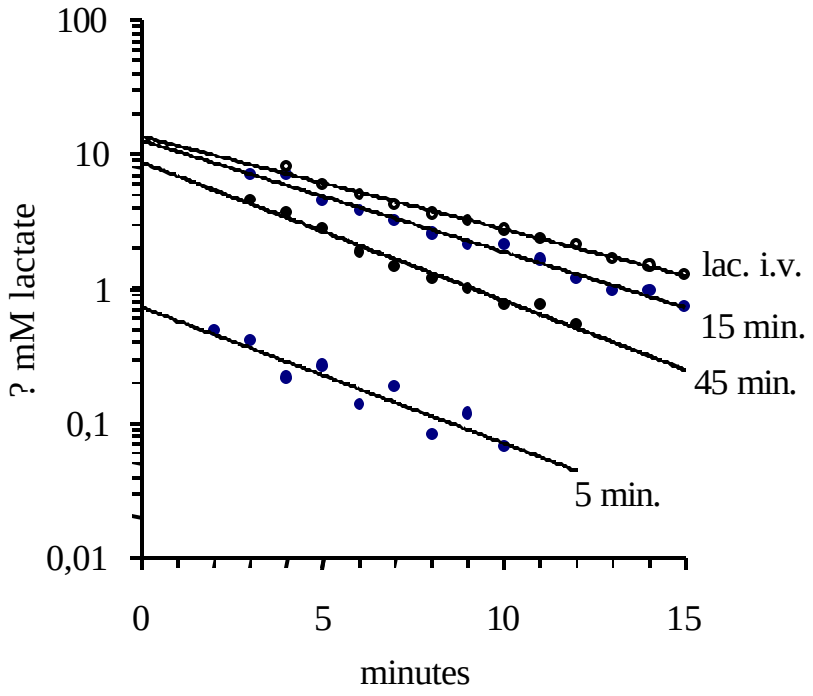

Figure 5
(?) Coronary sinus lactate concentrations (? mM above baseline) during reperfusion (onset at t = 0) after 5, 15 , or 45 minutes ischemia.
(o) Coronary sinus lactate concentrations (? mM above baseline) after intravenous lactate injection. Extrapolations are shown for best fitting lines back to the ? lactate concentration at the start of reperfusion or injection at t = 0.

(p<0.05). Arterial plasma lactate levels were similar before and after the onset of ischemia (2.1?0.4 mM, n.s.).
Immediately after balloon deflation, the lactate concentration rose sharply during reperfusion for a few minutes, reaching a maximum of 2.80 mM (116% of baseline) at 1 minute after 5 minutes ischemia, 9.27 mM (683% of baseline) at 3 minutes after 15 minutes ischemia and 6.11 mM (587% of baseline) at 3 minutes after 45 minutes ischemia (Figure 4). The total lactate releases at reperfusion were 3.77 mmol?l^{-1}?h, 1.49 mmol?l^{-1}?h, and 0.09 mmol?l^{-1}?h, respectively. The lactate plasma concentration at the moment of the intravenous injection was estimated to be 13.80 mM (R^2=0.99, $t_{½}$=4.36 min.) (Figure 5). The lactate concentrations at the onset of reperfusion were estimated to be 3.13 mM (R^2=0.88, $t_{½}$=2.97 min.),14.82 mM (R^2=0.99, $t_{½}$=3.63 min.), and 10.20 mM

(R^2=0.98, $t_{½}$=2.93min.), respectively. In some cases electrical cardioversion was necessary due to ventricle tachycardia/fibrillation at the onset of reperfusion (indicated in Figure 4 with *). Apart from the balloon manoeuvres, the lactate/glucose rate showed little change. The lactate/glucose rate rose again about 60 to 30 minutes before asystole, escorting a general deterioration of the circulation by a dying heart (diagnosed on ECG).

Lactate and glucose levels during myocardial dobutamine stress (n=3)
In the first pig, the heart rate increased from 82 to 191 at the highest dose (24 minutes after start of the dobutamine infusion). The maximal blood pressure was 123/104 mmHg at a dose of 20 ?g/kg/min. Blood pressure at the highest dose was 102/77 mmHg. In the second pig, the heart rate increased from 123 to 208 /min at the highest dose (36 minutes after start of the dobutamine infusion), with a maximal blood pressure increase from 64/46 to 120/81 at a dobutamine dose of 20?g/kg/min. Blood pressure at the highest dose was 105/50 mmHg. In the third pig (see Figure 2), the heart rate increased from 83 to 204 /min at the highest dose (27 minutes after start of the dobutamine infusion). Blood pressure increased from 95/84 to 128/108 at a dose of 20 ?g/kg/min. Blood pressure at the highest dose was 106/66 mmHg. The ECG did not show any signs of ischemia during these three experiments.
The ultrafiltrate glucose levels in the three dobutamine experiments rose gradually from a baseline at 3.84, 3.06, and 1.70 mM to a maximum of 4.88, 5.12 and 4.23 mM, at 18, 36, and 19 minutes after start of the dobutamine infusion. A similar rise was observed in the arterial blood samples. The lactate levels demonstrated no significant alteration during the dobutamine infusion test. At macroscopic evaluation the infarct areas were small and located at the apex and distal part of the interventricular septum.

Conclusions

This report describes the first application of ultrafiltration on myocardial pathophysiology. It demonstrates that an ultrafiltration catheter can sample bloodultrafiltrate continuously for hours in the coronary sinus. The combination of an intravascular ultrafiltration catheter and biosensor technology allows stable measurement of absolute lactate and glucose concentrations on a minute to minute basis. The automated detection was in good correlation with reference values in manually sampled blood. This is possibly the first study on venous efflux monitoring with such short time intervals. A significant increase in the

myocardial lactate efflux was detected within minutes after the cardial arterial flow interruptions and reperfusions performed. A quantitative estimate of the lactate efflux at reperfusion was possible. These observations suggest the existence of a tight link in time between cardiac pathologic events and metabolic shifts in the venous efflux, interesting for their potential diagnostic value.

In the present exploration, only a limited number of observations has been done, so most of our conclusions on cardiac ultrafiltration monitoring must be considered as preliminary. Once, in the second experiment described, the measurements appeared as being done outside the coronary bloodstream, an effect well known of intravascular sensors, referred to as the "wall effect" [5]. Extravasation of the catheter tip may create a diffusional barrier between the probe surface in tissue and the bloodstream. This can explain the worsened response time, the lower glucose and the increased lactate levels. Similar effects were previously noticed by us in human subcutaneous tissue[22], in intravascular rat[27] and chicken experiments, and in vitro experiments when blood cells were attached to a probe (unpublished data). The possibility of the wall effect and the need for heparinisation observed may of course limit the future application of ultrafiltration monitoring.

Various techniques, previously studied by others, have already demonstrated the feasibility of (semi-) continuous metabolic monitoring in peripheral blood. These studies applied frequent blood sampling and on-line analysis[28], microdialysis in venous shunts[6;29], and microdialysis with probes placed in peripheral veins[17;30]. Microdialysis probes have also been placed directly in myocardial tissue[7-10;15]. The lactate levels in ischemic myocardium calculated here by extrapolation were in the same range as the elevations found with interstitial space microdialysis studies (300 and 775%[9;10]). The lacking effect on lactate levels of a myocardial dobutamine challenge is also in line with the results of a previous microdialysis study[15]. There are however some important methodological differences between intravenous ultrafiltration and tissue microdialysis. Microdialysis measures in an area of tissue less than 5 mm around the probe[16], where a downward concentration gradient exists in the tissue towards the probe, descending from 100% to between 7 and 74%. This percentage can change in time, but it must be determined for calibration of microdialysis measurements. Calibration of microdialysis in vivo is a well documented, but complicated issue. Microdialysis also requires a considerable equilibration time (e.g. 90 minutes) before measurement can be started. Further is exposure of the heart required for insertion of microdialysis probes, so the technique is restricted to the operation theatre. For comparison, the present ultrafiltration catheter only needs angiography to check the position of the tip. Equilibration of the ultrafiltration

probe takes about 2 minutes for concentrations of small molecules. There is no need for in vivo calibration of ultrafiltration, because it evacuates bloodplasma water together with 100% of its small solutes, and measurements remain stable, as shown for many hours.

The limitation of microdialysis measurement to a small area around a probe in pathologic or healthy tissue may be advantageous, because one can expect the concentration changes in the coronary sinus to be diluted compared to those at the site of origin, what may diminish the sensitivity of a coronary sinus probe. Some pathologic processes may even stay hidden behind the endothelium[31]. This is however not the case for myocardial ischemia[2;3]. We extrapolated here the diminishing coronary sinus concentrations back to the concentrations at the time of release. This may be the extracellular lactate level in the ischemic tissue. So a venous application of the catheter as chosen in the present study appears sensitive enough to measure local myocardial metabolism. Besides, the entire myocardial metabolism is more often of interest than local changes, as illustrated by the afore mentioned intravascular approaches for lactate[2;3]. This underscores the advantage of the position of the present probe in the coronary sinus, so in the cardial common venous efflux.

Ultrafiltration offers new research opportunities in acute pathology through the shortening of the response time and the absolute measurements. Metabolic shifts in organs can be linked to pathologic events in time, e.g. the reopening of cardiac arteries by thrombolysis may thus be detected. Quick kinetic processes can also be studied using the absolute concentrations. Estimates can be made of the lactate concentration at the onset of reperfusion as well as the area under the curve using some assumptions on the lactate kinetic behavior. Although the datapoints showed a good fit, firm statements about onset concentrations and the area under the curve are not possible because of the limited number of experiments. In future, novel metabolic insights may be found in this way, e.g. insights in myocardial preconditioning and ventricular fibrillation mechanisms.

Future technical developments should be aimed at monitoring real-time, because a possible early detection of ischemia and reperfusion bears the potential for assisting therapeutic decision making. The lag-time of the present catheter is too long, and should be shortened by utilizing shorter catheters and by optimizing the dead volume/flow ratio or by insertion of small biosensors in the tip of the catheter. The current ultrafiltrate sampling method has indeed recently been coupled to such miniature biosensors with essentially the same results (Rhemrev et al., submitted).

The primary achievement of this study is the short response time obtained with absolute measurements with the ultrafiltration catheter and the first in vivo

observations of prompt myocardial lactate changes. Possible future benefits of such system for patients may be an early alert for acute ischemia by monitoring the moving average of lactate concentration, e.g. the reocclusion of a coronary artery. The success of therapy (e.g. of thrombolysis), defined as reperfusion of an obstructed coronary artery, may be recognizable as a sudden release of lactate from the myocardium. Metabolic monitoring may become supplementary to current ECG monitoring.

At present, there are to our knowledge as yet no intravascular sensors for monitoring metabolites available for use in patients, because this poses many demands, e.g. regarding specificity, accuracy, stability, miniaturisation, sterilisation, thrombogenity, toxicity, biocompatibility, disposability and inexpensiveness. Some of these demands may be easier to meet in future by combining biosensors with ultrafiltration sampling. It appears worthwhile to explore cardiac lactate monitoring techniques further, because it eventually may prove to have diagnostic value for patients.

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Chapter 5

Influence of implantation methods on performance of thin-film lactate and glucose micro-biosensors

Abstract

Introduction: Subcutaneously implanted lactate and glucose sensors may assist in optimising treatment in intensive care and of diabetic patients through automated, reliable and close monitoring of blood levels. Sensors are commonly implanted in subcutaneous adipose tissue, but this site yields inconsistent results.
Methods: To explore alternative implantation sites, twenty four thin-film amperometric lactate-glucose sensors were inserted in adipose tissue, striated muscle, or loose connective tissue of pigs. Sensor baselines and response times were compared, as recorded during an intravenous lactate/glucose injection.
Results: Baselines and responses were found generally close to zero in adipose and muscle tissue. In connective tissue, average lactate and glucose baseline levels ± SD were 80.5±19.1% and 90.9±15.7% of blood; and the 95% response times were 5.5±2.1 and 17.1±8.5 minutes respectively. The use of an intravenous injection of the sensor's analyte was found to be a quick way to check whether a newly implanted sensor is in close kinetic contact with blood.
Conclusions: The results indicate that subcutaneous loose connective tissue may be a better site for sensor implantation than the commonly used adipose tissue.

Introduction

A threatened energy supply to the brain or heart is an acute risk for tissue damage and eventually death because energy reserves in these tissues lasts only minutes. Such problems can occur e.g. in diabetic and intensive care patients through disturbed oxygen or glucose transport. Timely treatment of sudden ischemia or hypoglycaemia has been shown to decrease complications and death. A major problem is that many patients do not feel the impending danger, e.g. one-third of heart attack victims feels no chest pain[1], and many diabetic patients have little hypoglycaemia awareness. Tissue hypoxia is, however, indicated early in many patients by a lactate concentration increase, and has been documented to be a strong prognostic factor for complications and survival[2]. Frequent serial lactate or glucose measurement is thusfar sparsely practised, because of the heavy burden of frequent blood sampling and analysis for patients and physicians. Next, the data are not continuous, and often not instantly available for making therapeutic decisions. For further improvement of treatment of critically ill and diabetic patients, continuous subcutaneous lactate and glucose sensors combined with microdialysis have been proposed as an alternative. Automated, continuous, timely blood concentration data are advantageous over current off-line monitoring techniques. Placement of a probe subcutaneously is preferred, as such approach does not carry the risks of intravascular sensors. The first demands before clinical application of such subcutaneous sensor are of course the reliable and timely following of blood levels.

Current lactate and glucose sensors perform very accurate and with short response times in vitro[3-5]. Subcutaneously, however, a divergence from blood concentrations has been observed, e.g. with microdialysis[6;7], with ultrafiltration[8;9], and with directly implanted sensors[5;10;11]. This variable divergence is repeatedly seen, though very diverse techniques were employed for subcutaneous measurements. The reasons for this in vivo divergence have not been elucidated completely. Some of the previous results may be explained by inhibition of glucose oxidase activity by serum components, diffusional barrier formation by protein adsorption, and hydrogen peroxide consumption by granulocytes[12]. These factors, however, do not appear to inhibit thin-film sensors, which have proven to function stable and reliable in a continuous, heparinised blood stream[5]. Nevertheless, thin-film sensors combined with open-flow microperfusion suffer from the same divergence of subcutaneous concentrations from blood[13]. Possible mechanisms affecting the measurements in vivo may be related to tissue physiology or pathophysiology near the

implanted sensor. Candidate mechanisms are the damage to cells and vessels on the one hand, and local glucose to lactate metabolism in tissue on the other hand. These may impair the exchange of glucose or lactate between tissue near the sensor and the blood circulation through a barrier around the implantation wound and an increased diffusion distance to the nearest patent capillaries. The variation in structure of different tissues and the damage at implantation may explain the variable and low sensor responses, but has not yet been paid much attention.

We tested in pigs three potential subcutaneous sensor locations, to search a compartment that is in close kinetic contact with blood, because this information can be useful in later clinical investigations. Continuous, fast responding thin-film sensors[5] were placed subcutaneously in adipose, in striated muscle, and in loose connective tissue. These three tissues were chosen because they are all clinical applicable, but differ in metabolic rate and interstitial structure. The tissues' baseline was compared to the blood level, and the response time in vivo was determined for each individual sensor with an intravenous glucose/lactate injection, in an attempt to check in a quick way the sensor function. Simultaneous lactate and glucose measurements could be compared for interdependency, because the thin-film device combines both sensors next to each other.

Methods

Thin-film sensor

The characteristics of the thin-film lactate-glucose sensors employed have extensively been described previously[4;5]. These sensors consist of electrodes on the tip of a flexible polyimide carrier strip (width: 0.7mm) The distance between lactate and glucose working electrodes is 2 mm. The coated layers with either glucose or lactate oxidising enzymes produce a linear amperometric response range up to 40 mM (glucose) or 20 mM (lactate) in combination with in vitro and in serum 90% response time below 30 seconds.

Mass transfer of glucose and lactate is limited by the sensor membranes, averting exhaustion of the substrate in the sample contact layer. Possible in vivo interferents of electrodes as ascorbic acid, uric acid, and paracetamol have been found negligible. The correlation coefficient in heparinised blood was r=0.98 for glucose, and r=0.93 to r=0.99 for lactate.

Experimental procedures

Experiments were performed on four Yorkshire swines weighing 50, 38, 35, and 35 kg, each with six lactate-glucose sensors. The animals were maintained on a normal diet. All procedures were performed under anaesthesia with a combination of ketamine (15 mg/kg;), acepromazine (0.2 mg/kg) and atropine (0.05 mg/kg) given intramuscularly. An endotracheal tube was inserted for O_2/NO_2 ventilation. Ventilation with 2 % isoflurane (Forene®, Abbott, U.S.A.) was used to ensure adequate anaesthesia throughout the experiments. Levodromaron (2mg subcutaneously) was given as analgesic at the beginning of the procedure and supplemented if necessary. Lidocaine (50mg intravenously) was given prophylactically because of an experiment, separate from the present, which was performed subsequently on the same animal. Throughout the experiment, the condition of the animals was carefully monitored by means of continuous transcutaneous oximetry, intra-arterial blood pressure measurement, and electrocardiographic registration (ECG) of the limb leads and one modified precordial lead. The total time of sensors in vivo was 1.5-2 hours. To measure the response time in vivo, 0.25g/kg of glucose 40% and 0.1 g/kg of lactate 20% solution in 0.9% saline solution was injected intravenously in 2 minutes time after allowing the sensors to settle in for 45 minutes. Whole blood samples (2 ml) were withdrawn every 2, 5, or 10 minutes from a catheter in the coronary sinus vein. No heparin was used in this experiment to avoid any possible interference of anticoagulation on the measurements in tissue. The test tubes contained sodium fluoride and potassium oxalate to arrest coagulation and metabolism. The tubes were kept on ice until analysis. Concentrations of glucose and lactate were measured in whole blood and blood plasma after centrifugation with the Vitros 750 analyser (manufacturer: Ortho-Clinical Diagnostics, Illkirch Cedex, France) in the hospital routine laboratory. In order to make correct comparisons with sensor determinations, we adjusted the whole blood glucose concentrations with +15% to the molality in whole blood (in accordance with the manufacturer's report). Glucose molarity (mol/l blood) of whole blood is different from the molality (mol/kg water) as only about 80% of the blood volume consists of water[14]. The thin-film sensor measures the activity of glucose in water, hence the molality. Lactate was determined in blood plasma.

Description of the different insertion techniques

A total of twenty four lactate-glucose sensors were placed with a 1.25mm o.d. needle put through a lifted tissue fold. Each sensor was inserted from the open end into middle of the needle, then the needle was withdrawn gently, leaving

the sensor behind. In half of the experiments, 0.1ml of sterile saline was injected slowly while retracting the needle. Figure 1 shows an anatomic scheme of the different placements of the sensor. The sensors were placed either in abdominal adipose tissue (n=8), or intramuscularly in the abdominal wall (n=4), or in dermal loose connective tissue on the inside of the upper hind leg (n=12) (The latter sensors can be seen on the picture in figure 2). The sensor strips were fixated externally with tape on the skin.
Sensor electric currents in buffer with or without standards of glucose and lactate were measured before and after the experiments. The in vivo measured currents were converted to concentrations by first subtracting the baseline current in buffer from all measurements. Subsequent, the vivo measurements were calibrated with the standard curve determined before the experiment.
All procedures were reviewed and approved by the Animal Experiments Committee of the Groningen University.

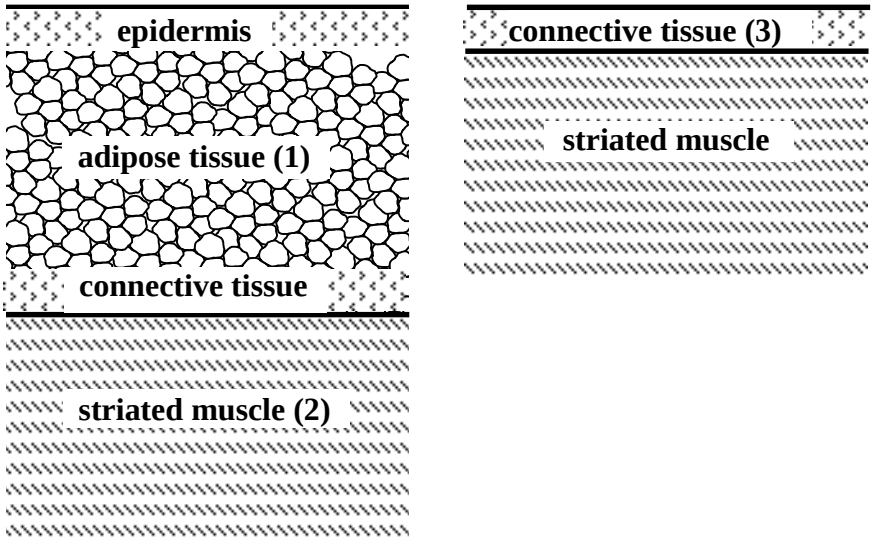

Figure 1
Scheme of anatomic layers of the abdominal wall (left) and the inside of the upper hind leg (right) in section. Sensors were inserted in adipose tissue (1), striated muscle tissue (2), and dermal loose connective tissue (3).

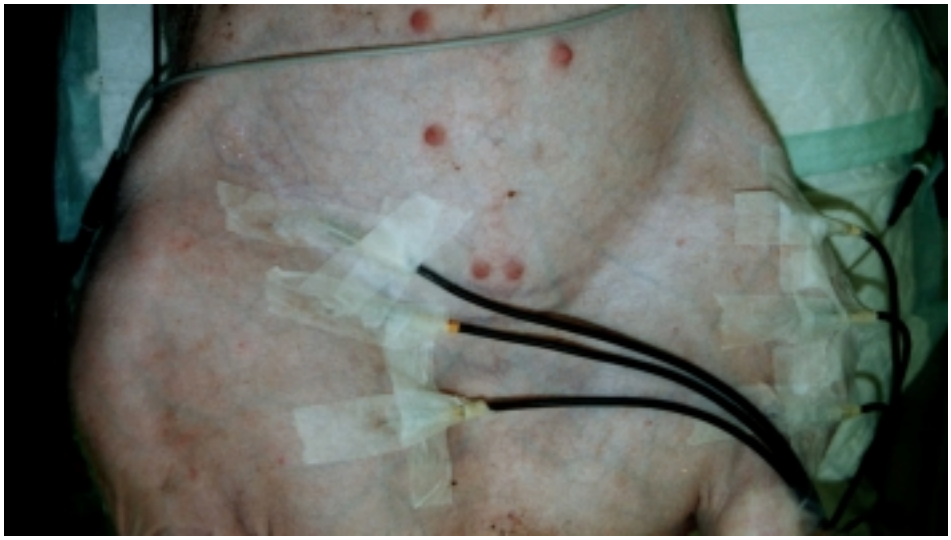

Figure 2
Six sensors placed in subcutaneous loose connective tissue on the inside of the upper hind leg.

Data presentation and statistics
The sensor data in figure three have been adjusted for presentation in graphics by presenting the datapoints as the average of nine datapoints collected in one minute in order to eliminate short wave electrical noise.
Baseline and response time results are presented as averages ± standard deviation (SD).

Results

The sensitivity of the sensors was 3.50 ± 1.84 nA/mM in vitro. The in vivo results of the experiments are summarised in table 1. Sensor baseline and response were generally close to zero in striated muscle and adipose tissue. Injection of saline together with a sensor in loose connective tissue removed the response to intravenous lactate-glucose injections.
In loose connective tissue, average sensor baselines ± SD were 80.5±19.1% and 90.9±15.7% of blood lactate and glucose levels respectively. The 95% response time was here 5.5±2.1 and 17.1±8.5 minutes. Examples of sensor responses to intravenous bolus injections are shown in figure 3. Lactate and glucose response

Table 1
Glucose and lactate baseline measurements intravenously (i.v.), and in three different tissues with sensors placed with or without injection of saline. The 95% response time to the i.v. glucose-lactate injection is also given (- indicates no response).

Tissue type	**Adipose tissue**		**Striated muscle**	**Connective tissue**	
saline injected(ml)	0	0.1	0 {0.1}	0	0.1
number of sensors	4	4	3 {1}	6	6
glucose i.v. baseline (mM)	4.15	6.70	4.15 {6.70}	3.94	3.29
sensor baseline ±SD (mM)	1.15,0,0,0	9.12,8.58,0,0	2.12,0,0 {8.40}	3.58±0.62	3.54±2.11
95% response ±SD (min.)	-,-,-,-	8,10,-,-	-,-,- {8}	17.1±8.5	-,-,-,-,-,-
lactate i.v. baseline (mM)	1.3	1.3	1.3	2.2	1.2
sensor baseline ±SD (mM)	5.48,0,0,0	4.51,4.10,0,0	1.38,0,0 {0}	1.77±0.42	1.25±1.06
95% response ±SD (min.)	-,-,-,-	10,-,-,-	-,-,- {-}	5.5±2.1	10,7,-,-,-,-

curves l.1 and g.1 are typical examples of sensors placed in adipose tissue. The fastest (sensors l.2 and g.2) and the slowest (sensors l.3 and g.3) response are shown of the sensors in connective tissue (sensors placed without saline injection).
The average intravenous levels of lactate and glucose are plotted versus connective tissue levels in figure 4. The labels near the graph indicate the sequence in time (in minutes) after the intravenous bolus injection at t = 0 minutes. The line of equality illustrates the (time-dependent) differences between both measurements. The graph indicates that interstitial glucose at baseline (-1 minute) and beyond 18 minutes after i.v. injection are at apparent steady state and near bloodplasma levels of glucose. Similarly, steady state lactate levels were close at -1 minute in the blood and interstitial compartments, but after the intravenous bolus injection, the 2 compartments equilibrated on a significantly different level.
Linear regression of in tissue response times for lactate and glucose sensors yielded $r=0.03$ ($n=6$, $p<0.05$, $\alpha=0.03$). The linear regression between subcutaneous levels of lactate and glucose was $r=0.59$ ($n=6$, $p<0.05$, $\alpha=0.21$) at $t=0$ minutes and $r=0.81$ ($n=6$, $p<0.05$, $\alpha=0.50$) at $t=45$ minutes.

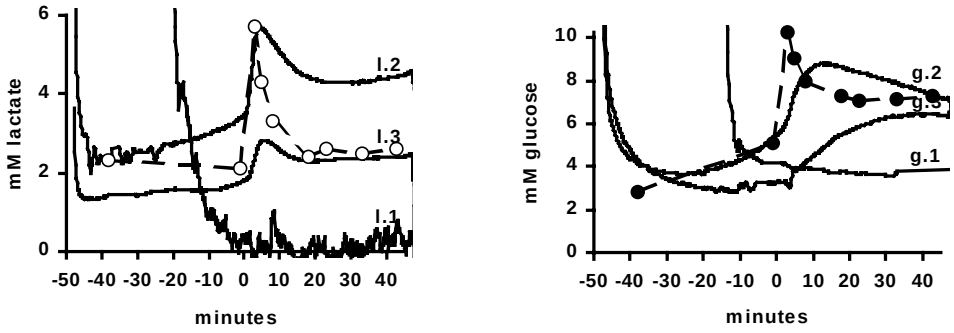

Figure 3
Lactate (left) and glucose levels (right) after an intravenous lactate and glucose bolus at t = 0 minutes. Measurements with subcutaneous sensors (~) and in venous blood samples (o lactate, and • glucose) Shown are the fastest (g.1, l.1) and slowest (g.2, l.2) responding sensors in connective tissue, and a typical sensor response in adipose tissue (g.3, l.3). The curve at the start of each graph reflects the settling of the sensor after insertion in tissue.

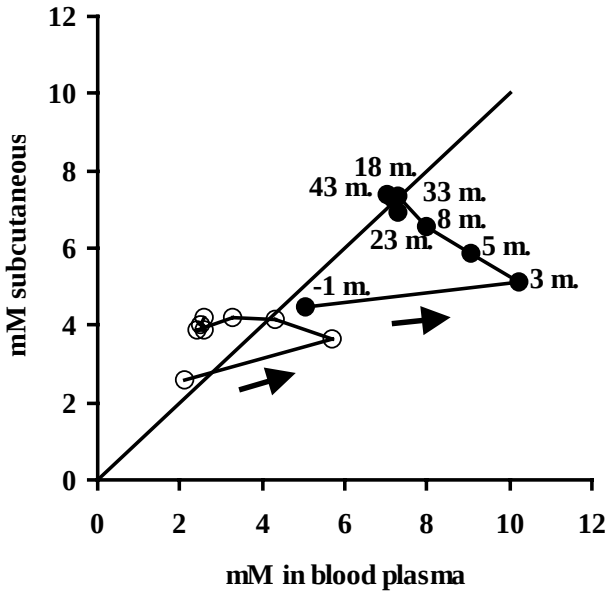

Figure 4
Lactate (o) and glucose (•) levels in venous blood versus subcutaneous connective tissue (average of six sensors). Subsequent measurements (➨) are indicated with the time in minutes (-1m. to 43 m.) after an intravenous lactate and glucose bolus at t = 0 minutes.

Conclusions

The results of this in vivo exploration of placements of a thin-film sensor in different superficial tissues indicate some promising directions for further clinical research. Adipose and striated muscle tissue placed sensors showed either zero currents or highly variable baseline levels, both without any response to intravenous lactate-glucose injections. These sensors appear kinetically separated from the intravascular compartment as they show no response. The near zero electric currents indicate that these sensors are even barely in contact with the interstitial electrolytes.

In subcutaneous loose connective tissue, sensors showed baselines close to intravascular levels and showed less variability (standard deviations lower). The proper placement of each individual sensor close to the intravascular compartment was confirmed quickly and easily with an intravenous injection, as the speed of distribution from the intravascular to the tissue compartment is reflected in the in vivo response time of each sensor. The positive and negative results of simultaneous glucose and lactate measurements parallel for different placements. Concerning a possible lactate and glucose interdependency, the subcutaneous response times showed no correlation. The t0 and t45 minutes subcutaneous glucose and lactate levels do suggest a positive correlation, although with a low power. An underlying process may explain these observations. A possible explanation is a poorer peripheral tissue perfusion due to fluid loss during the experiment. This can cause the intravascular glucose level to remain high after the bolus injection, and stimulate glucose to lactate metabolism in tissues. Whatever may be the explanation, the difference in blood and subcutis kinetics appears small for glucose, but significant for lactate.

The presented results are to our knowledge the first of a continuous lactate sensor directly in vivo. Despite the need for better monitoring of patients in critical care, few lactate sensors have been proposed so far. Off-line microdialysis experiments have been done in patients[6;15]. Further, continuous needle-type, thick-film, and thin-film sensors have been applied successfully to (on-line) ex-vivo blood and microperfusion samples[4;5;13;16]. The direct subcutaneous monitoring proposed in this study will be less of a patient burden than techniques using continuous blood sampling or heparin.

The common placement of sensors in adipose tissue is questioned by our results comparing sensor characteristics in different tissues. Discouraging results with lactate and glucose monitoring probes in the past may be due to improper placement and possibly local glucose to lactate metabolism. A quick check on

proper placement appears feasible with an intravenous injection, as demonstrated here with an extra cross check of glucose and lactate sensors for each other. This method may be useful for improving reliability of sensors, since current (subcutaneous glucose) sensors need multiple blood samples and much patience to calibrate, but these methods still gives only information on the general correlation of measurements in large groups of patients. With the here presented method, the proper placement and timeliness can be checked in principle of each single sensor placed in each individual patient. The future application of sensors may be enhanced through thus improving the reliability.

Future research can benefit from the results of this exploration. Recently, we compared microdialysis probe placement in connective tissue with adipose tissue in human volunteers, obtaining essentially the same results as in this exploration[17]. So, subcutaneous loose connective tissue appears to be the tissue of choice for sensor placement, rather than the now generally utilised periumbilical adipose tissue. The observed delays in connective tissue may be corrected in future by calculation with parameters which can be derived from the response curve to the intravenous injection (mathematical deconvolution of sensor response[18]). Whether the thin-film sensor will prove to be timely for clinical decision making, appears promising, but has to be a subject of subsequent clinical investigations. A possible interdependence of tissue glucose and lactate has to be investigated also, because these measurements may improve one other by accounting for metabolism. A possible dependency of measurements on tissue perfusion may offer additional information of interest for monitoring critically ill patients.

The present exploration has focused on the influence of anatomic placement of monitoring devices which has been paid as yet little attention. A limited number of observations has been done, so any conclusions should be drawn cautiously. Implantation procedures appear relevant for sensor functioning in vivo. Loose connective tissue was shown to be the preferential place for sensing in pigs. Future investigations for the improvement of subcutaneous sensing in patients should consider the influence of placement on monitoring.

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Chapter 6

Glucose gradient differences in subcutaneous tissue of healthy volunteers assessed with ultraslow microdialysis and a nanolitre glucose sensor

Abstract

Introduction: The abdominal subcutaneous interstitium is easily accessible for monitoring glucose for Diabetes Mellitus research and management. Available glucose sensing devices demand frequent finger prick blood sampling for calibration. Moreover, there is a controversy about the exact relationship between the levels of glucose in the subcutis and blood.

Methods: In the present study ultra-slow microdialysis was applied for subcutaneous fluid sampling, allowing continuous measurement of glucose in an equilibrated fluid with a nanolitre size sensor. The present method avoids in vivo calibration. During an oral glucose tolerance test glucose levels were measured simultaneously in blood and in adipose and loose connective tissue layers of the abdominal subcutis in seven healthy subjects.

Results: Fasting glucose levels (mM) were 2.52±0.77 in adipose tissue and 4.67±0.17 in blood, this difference increasing to 6.40±1.57 and 11.59±1.52 at maximal glucose concentration. Moreover, the kinetics of glucose in blood and adipose tissue were different. In contrast, connective tissue glucose levels differed insignificantly (4.71±0.21 fasting and 11.70±1.96 at maximum) from those in blood and correlated well (r^2=0.962).

Conclusions: Ultra-slow microdialysis combined with a nanolitre glucose sensor may become attractive to apply in intensive diabetes therapy. Frequent blood sampling for in vivo calibration can be avoided by monitoring glucose in the abdominal subcutaneous loose connective tissue, rather than in the adipose tissue.

Introduction

The abdominal subcutaneous interstitium is an easily accessible site for monitoring glucose for Diabetes Mellitus research and management. The potential of subcutaneous sampling with microdialysis for monitoring has well been recognised, and is now investigated besides implanted sensors. A major advantage of microdialysis is that fouling of the sensor surface with proteins is prevented. In classical microdialysis, glucose diffusion does not reach equilibrium, so the concentration of the analyte in the perfusate is substantially lower than that in the interstitial compartment. Several calibration methods have been proposed to estimate the actual interstitial glucose concentration, but most of these are time-consuming and require either long-term steady-state conditions or frequent blood sampling. Calibration is thus an important obstacle for widespread clinical application of glucose sensors. A solution may be the lowering of the perfusion rate in order to reach a diffusion equilibrium, so calibration becomes superfluous.

To reach this goal, an ultraslow microdialysis method (perfusion rates below 100 nl/min) was developed recently in our laboratory[(1)]. In this method is the dialysate collected by means of underpressure instead of the conventional pushing through the microdialysis dialysis probe. So no perfusion fluid enters the tissue, as does occur in classical microdialysis. due to overpressure. A constant low flow of 50 – 100 nl per minute is maintained by a disposable pump for several days. Capillary connections have to be used, to achieve an acceptable time resolution of the thus constructed glucose sensor. In previous experiments was shown that ultraslow microdialysis results in complete equilibrium of the analytes in the intercellular space and the dialysate. This was not only demonstrated in vitro, but in vivo as well. For instance, the levels of glucose measured in the effluent of ultraslow microdialysis and ultrafiltrates obtained from the rat subcutis were exactly the same[(1)].

Ultraslow sampling has recently also successfully been applied in healthy volunteers to monitor on-line subcutaneous glucose in combination with a bed-side flow-injection analysis[(2)]. For ambulant glucose monitoring, several commercial sensors have been tested in our laboratory. All of these sensors had a high cell volume, and were found to suffer from a poor time-resolution at the low perfusion rates used. Furthermore the sensitivity and/or stability were found insufficient; and air bubbles disturbed the measurements easily[(3)]. For these reasons, a miniaturised glucose sensor has been developed with a cell volume of approximately 10-20 nl. This nanolitre-sensor has been validated for accuracy, precision, linearity, selectivity and stability during in vitro and ex vivo

experiments[4;5]. In the present study this glucose sensor was connected to an ultra-slow microdialysis probe for application in human subcutis as a wearable glucose monitoring device.
Having got around the problem of in vivo calibration, we focused on the debate on the precise relationship between the subcutaneous glucose levels and glycaemia. This issue is important, because the accuracy of glucose sensors is crucial for their clinical acceptance. On the one hand, previous researchers found subcutis and blood glucose concentrations to be very close[6-9] and no influence of insulin on the level difference[10]. Others found distinct levels with the difference dependent on the insulin concentrations[11-13]. Some found both distinct and close glucose levels[2;14;15]. These previous studies used mainly in vivo calibration, measured rather discretely glucose levels in steady state than continuously during rapid changes, and were not orientated towards optimal probe placement.
The aims of the present study were to implement the combination of ultra-slow microdialysis and a new wearable glucose sensor, and to test it for continuous subcutaneous monitoring. After an initial experiment with a standard subcutaneous placement of the dialysis probe and inconclusive results, we explored glucose levels in different tissue layers as potential probe locations. Adipose and loose connective tissue were chosen because they are both easily accessible in the subcutis, but differ in metabolism and interstitial structure. A baseline and an oral glucose tolerance test (OGTT) profile was collected in blood for insulin concentrations, and in blood and on the two subcutaneous sites for glucose.

Methods

Experimental protocol
Seventeen healthy volunteers underwent an oral glucose tolerance test 16 hours after one (in first group, n=10) or two (in second group, n=7) microdialysis probes were placed (CMA 60 probes, Polyamide, 620 µm OD, MWCO 20 kD, CMA Microdialysis, Stockholm, Sweden). These probes were inserted each time by the same physician with an introducer (l=54mm, o.d.=1.4mm) through a lifted skinfold into the subcutis in the direction of the umbilicus. The first group had the probe introduced in the subcutaneous fat approximately 10cm lateral from the umbilicus. The second group had one probe inserted at 6 cm lateral from the umbilicus superficially in subcutaneous fat, and the other probe at 15

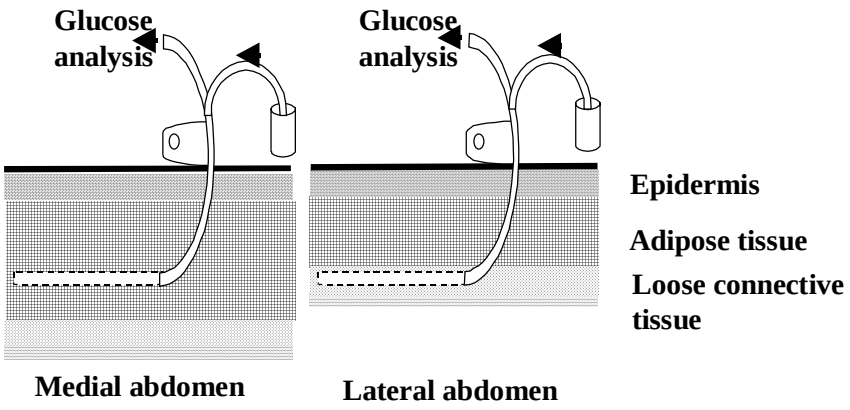

Figure 1: Position of microdialysis probes in adipose tissue (left) and in loose connective tissue (right)

cm lateral from the umbilicus into the loose connective tissue layer under the adipose tissue (figure 1).

The subject's height and weight were assessed, and the local skinfold thickness was measured with a caliper before insertions. The mean was taken of two repeated caliper measurements. The body mass index ranged from 20.5 to 28.4kg/m^2. Skinfold thickness ranged between 13 and 40mm.

Following an overnight fast, subjects ingested 100 g glucose dissolved in 200 ml water at zero time. Blood samples were taken from a forearm vein every 5 to 15 minutes during the OGTT ranging from 160 to 245 minutes. The subjects sat in an easy chair with one hand under a cloth on a heating pad in order to arteriolize the venous blood samples.

The microdialysis probes were filled 30 minutes before the OGTT with a sterile 0.9% saline solution. The inlet was then connected to a ventilated container with the same solution. The outlet of the probes were connected to the online analyses by fused silica tubing (50 cm l, 50 µm ID; Polymicro Technologies inc., Phoenix, AZ, USA) and cyanoacrylic glue (Henkel, Nieuwegein, the Netherlands). These connections and both online analysis devices were constructed in such manner to have a low dead volume, a low back pressure, and no sharp edges making air bubbles to stick to the wall. A home-made semi-vacuum syringe pump was connected to the downstream end of the microdialysate analysis devices to generate the ultraslow dialysis flow by means of underpressure as described previously[16] (1.2 ml monovette, Sarstedt, Nümbrecht, Germany). The flow rate was set at 100 nl/min. This ultraslow flow has been demonstrated previously to result in equilibrated concentrations of glucose in the probe perfusate and the interstitial space[1;17]. The time resolution

of this probe has been shown to be 3-5 minutes for a 10-90% signal change after a sudden concentration change in vitro[17]. The time resolution of the glucose detection is much shorter (about 10 seconds), and can thus be neglected.

Glucose and insulin analysis

The three simultaneous samples in this experiment (blood and two interstitial fluids) were analysed for glucose by three different methods. Glucose in plasma of arteriolized blood was measured off-line with the Vitros 750 (manufacturer: Ortho-Clinical Diagnostics, Illkirch Cedex, France). The concentration of glucose in the continuously sampled dialysate was measured online by a home-made nanolitre glucose sensor. In the first group, and from the second group in the most laterally positioned probes, the dialysate measurements were done with a flow-injection glucose analysis. Both methods have been described in detail, and compared in previous studies to the Vitros 750 glucose analysis to ensure corresponding results[2;4]. The dialysate glucose detection was calibrated with Dulbecco's buffer and a standard glucose solution prior to connection to a subcutaneous microdialysis probe.

The delay times between tissue and point of measurement were established to be 31.1±1.8 minutes. This delay was mainly determined by the volume of the internal tubes in the CMA 60 probe. The data presented are corrected for the delay times found. The insulin concentrations during the OGTT were determined in mU/l by radioimmuno assay (Pharmacia and Upjohn, Freiburg, Germany).

Nanolitre glucose sensor

The precise construction of the glucose sensor has been described in detail previously[4]. The electrodes are contained inside a flow-through cell of 10-20 nanolitre in tygon tube (ID 200 μm) (see figure 2). Measurement of glucose is performed by amperometry after enzymatic oxidation of glucose. To this end, glucose oxidase enzyme has been immobilised in a permselective membrane on the surface of a platinum wire inside the cell. The main characteristics are as follows. The cell together with pump and capillary connections weighs just under 5 g. Glucose measurements are linear up to 30 mM. Possible inferences as ascorbic acid (0.1mM) and uric acid (0.25 mM) have no significant contribution to the signal. Signal stability is at least three days, as tested with continuous measurement of both standard and serum samples. The measurement repeatability is 2-4% (the relative standard deviation of six consecutive measurements). Sera of diabetes mellitus patients measured with the validated

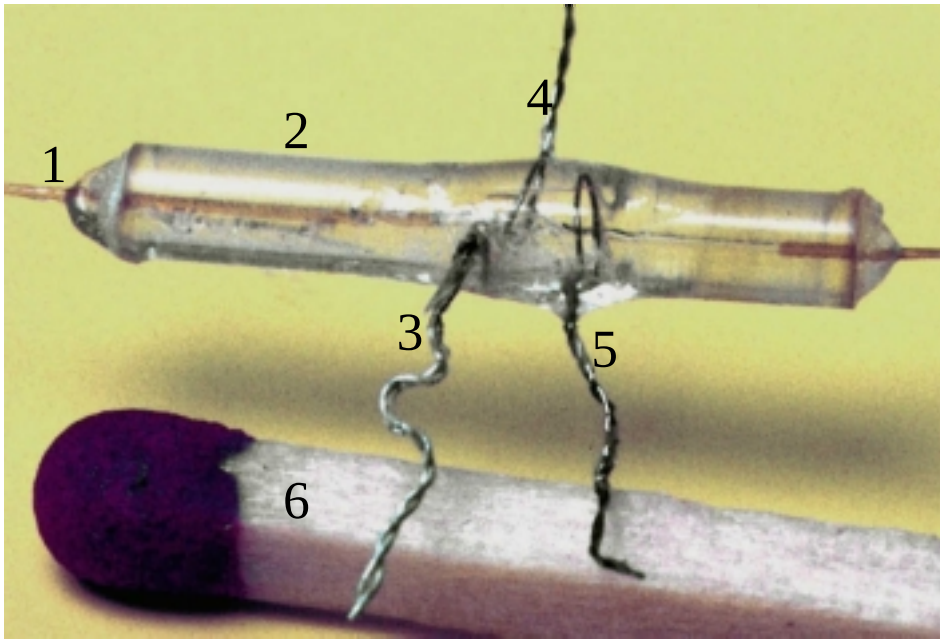

Figure 2: Photo of glucose sensor in flow-through cell
1 fused silica capillary tube
2 tygon tubing flow-through cell
3 working electrode (platinum) coated with layer containing Glucose Oxidase
4 auxiliary electrode (silver)
5 reference electrode (silver)
6 match for scale comparison

clinical laboratory technique Vitros 750 as reference (X) and with the sensor (Y) displayed a regression line Y = 0.981X + 0.1875 with a correlation coefficient of 0.9945 (n = 54). No results deviated more than 20% from the reference value.

Statistical analysis

Result values are given as means plus-minus SEM. Paired t-tests were used to test the difference between the three fasting glucose levels and between maximal glucose levels with the significance level at $p<0.05$. Normality of distribution was checked before linear regression. Accuracy and precision of the subcutaneous measurements versus arterial glucose was calculated according to Bland and Altman[18].

Analysis of potential clinical implications was done with the error grid for glucose monitoring systems proposed by Clarke et al.[19]. This grid qualifies glucose measurements as “good”, “acceptable”, or “unacceptable” for use in diabetes therapy.
Correlations were also calculated for the fasting and maximal glucose concentration in adipose interstitium as the dependent variable, and the fasting and maximal concentration of insulin, the body mass index, and the skinfold thickness at the probe site as independent variables.

Results

In the first group, 174 paired measurements were collected of plasma and subcutaneous glucose in ten OGTTs. The fasting glucose levels were 4.62±0.20 mM in plasma and 3.15±0.38 mM in subcutaneous fat ($p<0.05$). The correlation between the two measurements was $r^2=0.432$, the accuracy 1.38mM, and the precision 84.4%.
In the second group, 71 threefold glucose measurements were collected in five of seven experiments. In two of the seven experiments, there were only paired measurements, due to a technical failure of the potentiostate electric feeding to the sensor of the probe in adipose tissue. The fasting glucose levels were 4.67±0.17 mM in plasma, 4.71±0.21 mM in subcutaneous connective tissue and 2.52±0.77 mM in subcutaneous adipose tissue. (see e.g. OGTT graph in figure 3). The differences between the level in adipose tissue and plasma, and between adipose tissue and connective tissue were significant ($p<0.05$). The maximal glucose concentrations were 11.59±1.52 mM in plasma, 11.70±1.96 mM in connective tissue, and 6.40±1.57 mM in adipose tissue. The differences between these three levels were significant for adipose tissue versus plasma and adipose versus connective tissue. Fasting levels of insulin were 7.0±1.2 mU/l. Maximum levels of insulin were 79.2±13.0 mU/l.
Figure 4 shows for group two the dependence of the subcutaneous glucose concentration in two locations (y-axis) on the arterial glucose concentration (x-axis). Since the concentrations in adipose tissue were lower and not linear at curve inspection, no linear correlation coefficient,accuracy nor precision comparing adipose to arterial blood glucose was calculated. The linear correlation coefficient, accuracy and precision of measurements in connective tissue as compared to arterial glucose (n=90) were $r^2=0.962$ ($y=0.987x+0.039$), 0.43mM, and 16.8%. Analysis of potential clinical implications of the measurements in the error grid was poor for probes in adipose tissue, whereas

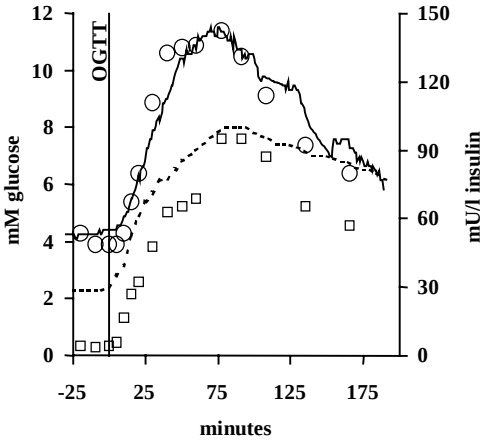

Figure 3:
Example of an oral glucose tolerance test (OGTT | at t=0 minutes) with glucose and insulin concentrations
o plasma glucose
~ connective tissue interstitial glucose
- - adipose tissue interstitial glucose
plasma insulin (on secondary Y-axis)

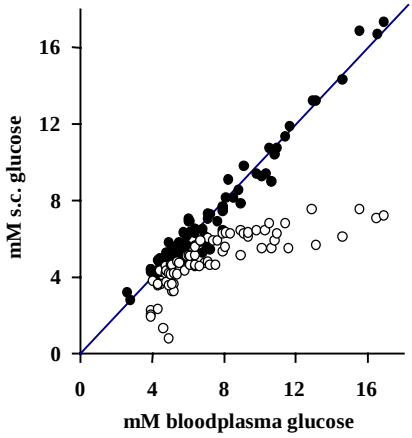

Figure 4:
Glucose concentrations compared between plasma (reference) and two subcutaneous (s.c.) tissues, shown with the line of identity (x=y).
o plasma versus adipose tissue, n=71 in five subjects.
• plasma versus connective tissue, n=90 in seven subjects.

probes in connective tissue showed 98% in zone A (good) and 2% in zone B (acceptable).
The correlation of fasting and maximum glucose concentration in adipose interstitium with the fasting concentration of insulin was r^2=0.000 and 0.510. It was 0.146 and 0.419 with the maximum concentration of insulin, 0.013 and 0.479 with the body mass index, and 0.450 and 0.470 with the skinfold thickness at the probe site. The power of these tests was between 0.02 and 0.24.

Conclusions

In the present study a new combination of ultra-slow microdialysis with a nanolitre glucose sensor was used for measurements in human subcutis. The device provided actual subcutaneous concentrations of glucose, without an in-vivo calibration. When the probe was placed in the loose connective tissue,

subcutaneous glucose levels matched plasma glucose levels so close that sensor calibration with blood samples was not necessary.
In the first study group, subcutaneous glucose levels were similar to those previously found with ultrafiltration[2]. These results together with those in rats[1] indicate that the present technique using ultraslow microdialysis realises (near) 100% recovery of glucose. These levels were consistently found to be lower than in plasma. To explain the variability of subcutaneous glucose, . we compared steady state levels and time courses of glucose obtained with probes positioned in two different subcutaneous layers in a second group of experiments. Glucose levels measured with the probe positioned in the deep subcutaneous layer of loose connective tissue were very close to arterial levels. The glucose measurements in superficial adipose tissue were lower than in blood in the fasting state, and this divergence increased during the OGTT at the maximum glucose and insulin concentrations. The correlations calculated between adipose tissue glucose levels and insulin concentrations and skinfold thickness at the probe site did not contradict influence of insulin or skinfold thickness on local glucose levels, but the correlations lacked sufficient power to draw firm conclusions.
Several others have reported variably lower glucose levels in the human subcutis as in blood, as was found here in the first group investigated. Some found also indications of adipose tissue glucose level dependence on skinfold thickness[20], level differences between adipose tissue in abdomen (thick adipose layer) and fore-arm (thin adipose layer)[21], and level differences between abdominal and gluteal adipose tissue[22]. Dependence of subcutaneous glucose levels on insulin was found both in humans[12;13] and rats[11]. Others, however, found no insulin dependence of subcutaneous glucose levels in dogs[10], and rats[23]. Possible explanation for these various results may be the positioning of the probe in the latter experiments, which was in connective, rather than adipose tissue. Removal of existing tissue-blood gradients by the in vivo calibration procedure in these experiments is another possible explanation.
In previous research, using catheterisation of abdominal adipose tissue, an increase of the glucose arterial to venous concentration gradient was found during an oral glucose tolerance test [24]. In the present study, the adipose glucose levels diverged also more from blood at higher glucose levels. Inspection of these glucose curves suggested to us a biphasic course with glucose levels fairly paralleling up to about 8, 6.5 mM (blood, adipose level). After this turning point, levels in adipose tissue increase only very little as compared to the increase in blood (see figures 3 and 4). A possible explanation is the in previous research found more than proportional increase of glucose

uptake by adipose cells at higher glucose levels, due to higher insulin levels[24]. This insulin effect is known to be mediated by GLUT4 glucose transporters, which are much more present in adipose than in connective tissue. Between both tissues, there are also differences in distance between the probe and the arterial vessels, differences in local blood flow and different physicochemical properties of the interstitium. So, the observations done in two probe locations may represent differences in tissue physiology.

The subcutaneous glucose measurements were shown to correlate well with blood levels provided the probe was placed in subcutaneous loose connective tissue. Ultra-slow microdialysis continuously provides undiluted samples, so there is no need for in vivo calibration, in contrast to most other sensors (e.g. Minimed CGMS[25] and Glucowatch[26]).The explorative results on accuracy and potential clinical implications in this study encourages us to proceed to studies in diabetic patients. Very few studies have been published thus far about what would be the best place and method of insertion of a continuous subcutaneous glucose sensor for diabetic patients. As accuracy and reliability are essential for eventual clinical application, we suggest to pay more attention to optimal subcutaneous placement of glucose sensors. In future, frequent blood sampling for sensor calibration purposes may thus become superfluous.

Recently, the present device has been made wearable, through equipment with a small potentiostate and data logger for detection and registration, which have been developed in parallel research. It may therefore become attractive to apply in intensive diabetes therapy, as the potential of long-term implantation of a dialysis probe has been demonstrated already[15].

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Chapter 7

Quantitative model of glucose transfer in subcutaneous interstitial gradients assessed with oral glucose tolerance tests and ultraslow microdialysis

Chapter 7

Abstract

Introduction: The aim of this study was to test compartmental models for subcutaneous glucose kinetics in order to find which determinants contribute, and to what extent to subcutaneous glucose levels. A secondary aim was to evaluate the possibility to reverse the algorithm found.
Methods: The data of the seven subjects in chapter 6 were used to evaluate three models. Model A is a two compartments model, using the measured arterial glucose as the linear input into the connective tissue compartment. The latter compartment has one linear glucose output. Model B is like A, but modifies the output using the arterial insulin levels. Model C is like B, but adds a third compartment between the arterial and the connective tissue compartment, and modifies also the input using the arterial insulin levels. To identify the best model, first the model parameters have been estimated by weighted nonlinear least squares. Second, to make a comparison among the models in terms of parsimony, the Akaike Information Criterion was used.
Results: The parameter precision was acceptable (coefficient of variation below 100%) and the fractional standard deviation was estimated to be below 5%. Model B showed the best fit, with model C in second place. However, the results also show parameters k3 and k4 (insulin effect) to virtually disappear in some subjects, suggesting a more simple model is then sufficient.
Conclusions: The heterogeneity of the parameters found may be a reflection of the mix in the subcutis of insulin dependent adipose and insulin independent connective tissue, and the tissue concentrations varying with the distance to the nearest capillaries.
A future reversement of the best model found will need the insulin levels as input for the algorithm. This will be possible, as the previous insulin administration will be remembered by a future artificial pancreas. However, the presented model needs additional data in future studies to be validated for this purpose.

Introduction

The fate of glucose in the interstitial fluid of tissues is of importance to better understand some pathophysiologic mechanisms in diabetes mellitus. Accordingly, interstitial fluid may inform more specific on tissue metabolism than blood, because the interstitium is usually regarded as one or more kinetically separate compartments and the place of action of insulin[1]. Major pathophysiologic phenomena localised in tissue are insulin resistance[2] and increased capillary permeability related to diabetic and hypertensive complications[3]. Further, the proper functioning of pancreatic islets transplants appears to depend not only on controlling the immunologic rejection, but also on creating a close kinetic contact with the bloodstream[4]. An interstitial glucose kinetic model may help to understand the basics of metabolism inside tissue in control conditions and of diabetic pathophysiology. Such model may also improve the interpretation of interstitial glucose sensor measurements, intended to manage diabetes without blood sampling[5-7].

Glucose kinetic models have been developed previously for the entire body, e.g. the so-called "minimal model"[1]. The interstitial fluid proper can be modelled thus far only indirectly by the input-output method (arterial-venous differences measurement)[8], by the glucose clamp method, or by thoracic duct lymph measurements[9]. All these attempts to validate the characteristics of peripheral interstitial fluid compartment in a glucose kinetics model were inevitably indirect, because of hitherto technical inaccessibility of the human interstitial compartment. Thoracic duct lymph is mostly constituted by liver and intestines, which glucose metabolism differs considerably from peripheral tissues as muscle, fat, and connective tissue. So, the important insulin effect on the peripheral tissues can be modelled little with thoracic duct lymph measurements. There is also criticism on compartmental modelling concerning the assumptions of no concentration gradients within compartments, and only single transfer rates between compartments[10].

Application of microdialysis probes placed directly in the subcutaneous interstitium for continuous sampling or implantation of glucose sensors has made local on-line measurements in principle possible. However, in current studies using classical microdialysis, sample collection time is 10 to 60 minutes, and the probe creates a concentration gradient in the surrounding tissue. Calibration needed in vivo to correct for microdialysis tissue drainage is a rather complicated and time-consuming procedure, and requires often additional blood sampling. Directly implanted amperometric glucose sensors need in vivo calibration as well. Because any physiologic glucose gradients between

subcutaneous and blood levels are thus also calibrated for, no uptake in tissue can be found, but only a rate constant for delay[7].
Recently, an ultraslow microdialysis method has been developed, which recovers completely equilibrated samples[11]. With such a continuous sampling method, glucose measurements can be done every minute using a biosensor in a coupled flow-injection analysis system[12]. So, very frequent direct measurements of absolute glucose concentrations have now become possible in the subcutaneous compartment without additional assumptions or computations. This approach allows to investigate kinetics of glucose in the subcutaneous compartment of individual subjects. In preclinical experiments in healthy volunteers to validate this method, both delays and lower glucose levels were observed in the subcutis as compared to venous blood plasma. In order to make subcutaneous sensors accurate and reliable, this difference has to be explained and, if possible, avoided or predicted. A subcutaneous kinetic model may enable to predict blood levels from subcutaneous glucose with an appropriate algorithm[7], provided the model allows inversion.
Here, we analysed glucose and insulin data from Oral Glucose Tolerance Tests (OGTTs) in seven healthy volunteers. The data were described by three models with rising complexity to select the best quantitative model to predict subcutaneous glucose levels from arteriolized blood plasma measurements, which were used as the input function for the models. We chose a new approach by comparing lumped parameters between the compartments with multiple parameters, taking into account the characteristics of two different glucose transporters: GLUT1 and GLUT4. GLUT1 plays a role in constitutive glucose uptake over the cell membrane, whereas GLUT4 can be moved by insulin from specific membrane vesicles inside the cell to its surface. Further, we individualised the parameters for each experiment, so taking into account the possibility of concentration gradients in the studied compartments. The glucose levels in the blood plasma and subcutaneous compartment were assumed to represent a homeostatic steady state immediately before the experiment. Three models were studied. In the simplest model (model A), transport from blood plasma to the interstitium (by blood filtration (solvent drag) and diffusion) was described by one linear rate. The transport from the interstitium to the blood plasma and/or into cells (by diffusion, reabsorption, and physiological glucose uptake (glucose dependent below a level of ±10 mM) was described by a second linear rate. In the model B, the second linear rate was the sum of two rates, one of these two being linearly proportionate to the blood insulin level above steady state level (insulin dependent glucose uptake). Model C is like model B with addition of an insulin dependent glucose transport from blood

plasma to the interstitium (insulin dependent glucose distribution), and with a third compartment between blood plasma and interstitium , representing the capillary blood filtration. The closest and simplest quantitative description of the set of measurements is selected as the best model.
The main question of the present study is, to what extent tissue metabolism events are isolated from blood plasma in terms of number and influence of model parameters. Relevant is especially whether insulin is of importance in explaining the observed gradient and delay in glucose transfer glucose between the compartments. Insulin constitutes namely a from glucose independent factor in diabetic patients. So insulin is potentially an uncontrollable interferent in subcutaneous glucose measurements, and thus difficult to use in an inverse model for a sensor algorithm. In the discussion we will discuss the plausibility of the model parameters found and try to match them with physiological correlates.

Methods

Experimental protocol
A 100g oral glucose tolerance test was performed on the day after probe placement in seven healthy women between 23 and 45 years old, without (family) history of diabetes. Measurements included weight, height, waist circumference (at the point of minimal abdominal girth), and skinfold thickness at the probe site. The CMA 60 microdialysis probe was inserted with an introducer (l=54mm, o.d.=1.4mm) through a lifted skinfold in the direction of the umbilicus at 15 cm from the midline. The probe was placed in the loose connective tissue layer in-between the subcutaneous adipose tissue and the muscle aponeurosis. Low probe perfusion rates (range 30-59 nl/min) were applied by a stable suction pump, resulting in equilibrated concentrations of glucose in the microdialysis perfusate and the subcutaneous space. The average 10-90% response time of the probe to sudden changes of standard concentrations post-vivo was 4.2 minutes (time for the signal to change from 10 to 90% in a sigmoidal transition from one concentration level (0%) to another (100%))(Tiessen, submitted). Concurrent with the subcutaneous measurements were blood samples taken from a cubital vein catheter every 5 to 15 minutes during the OGTT and the preceding steady state. Cubital venous blood was arteriolized by keeping the hand on a warming pad under a cloth. 100 g glucose dissolved in 200 ml water was ingested at zero time. The Medical Ethical Committee of the University Hospital of Groningen approved the experimental procedures, which were in accordance with the Declaration of Helsinki.

The microdialysis perfusate glucose was analyzed every minute in a flow-injection analysis with a biosensor as described previously[12]. Blood glucose concentrations were measured in plasma with the Vitros 750 analyser (Ortho-Clinical Diagnostics, Illkirch Cedex, France) after centrifugation of arteriolised blood. Insulin levels were determined in blood plasma by radio immuno assay (Pharmacia & Upjohn, Uppsala, Sweden).
Anthropometric data and parameters of model B were analysed using multiple linear regression, with the model parameters as the dependent variables and body mass index, waist-hip ratio and skinfold thickness as the independent variables.

Kinetic models
Model A
Glucose modelling was done by considering only the glucose measurements, i.e. plasma glucose as the known input and the subcutaneous interstitial data as the output of a linear compartment model. The first model, Model A, is shown in Fig.1 and described the glucose exchange between plasma and interstitial space by the following differential equation:

$$\dot{C}_{sc}(t) = k_{21}C_{plasma}(t) - k_{02}C_{sc}(t) \qquad C_{sc}(0) = C_{scb} \qquad (1)$$

where C_{plasma} is the plasma glucose concentration, C_{sc} is the glucose interstitial concentration and C_{scb} represents the steady state value of the subcutaneous glucose. Parameter k_{21} (min^{-1}) is the rate constant between plasma and interstitial space, parameter k_{02} (min^{-1}) is a rate constant describing both the exchange between interstitial space and plasma and the irreversible loss into the tissue.

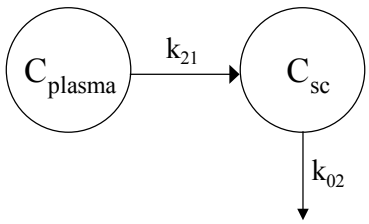

Fig.1 Model A

Considering that before the OGTT experiment one has the steady state relation:

$$k_{21}C_p - k_{21}C_{sch} = 0 \tag{2}$$

one can obtain the following steady state relation:

$$k_{21} = \frac{k_{02}C_{scb}}{C_p} \tag{3}$$

where C_p is the plasma glucose value at the basal state. Thus k_{02} was the only parameter to identify of Model A.

The second model, Model B, is shown in Fig.2. It assumes as Model A that glucose distribution is represented by two compartments. Glucose disappearance from the interstitial space is now linearly dependent on plasma insulin concentration above the basic level Ib. In particular the Model B is described by the following differential equation:

$$\dot{C}_{sc}(t) = k_{21}C_{plasma}(t) - [k_2 + k_3(I(t) - I_b)]C_{sc}(t) \qquad C_{sc}(0) = C_{sc} \tag{4}$$

where C_{sc} and C_{plasma} have the same meaning above, and I and I_b represent insulin concentration and the steady state value of the plasma insulin respectively. Considering that before the OGTT experiment one has the steady state relation:

$$\begin{aligned} k_{21}C_p - k_2C_{scb} &= 0 \\ k_{21} &= \frac{k_2C_{scb}}{C_p} \end{aligned} \tag{5}$$

where C_p has the same meaning above. Thus k_2 and k_3 were the two parameters to identify.

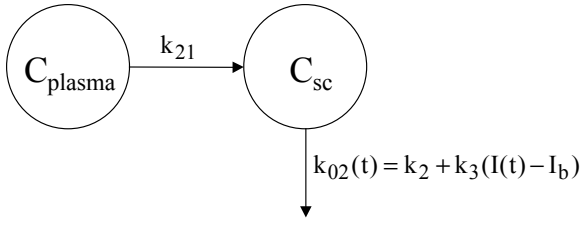

Fig.2 Model B

The third model, Model C, considers the presence of a delay between plasma glucose and interstitial glucose and of two nonlinear parameters, i.e. $k_{21}(t)$ and $k_{02}(t)$, controlled directly by the arterial plasma insulin kinetics. By introducing a third compartment, we obtained the model in Fig.3 where C_{plasma} is the plasma glucose concentration, C_r is the delayed plasma glucose concentration and C_{sc} is the glucose interstitial concentration. The differential equations describing the model are:

$$\begin{aligned} \dot{C}_{sc}(t) &= [k_1 + k_4(I(t) - I_b)]C_r(t) - [k_2 + k_3(I(t) - I_b)]C_{sc}(t) = 0 \qquad (6) \\ \dot{C}_r(t) &= k_r C_{plasma}(t) \\ C_{sc}(0) &= C_{scb} \\ C_r(0) &= C_{rb} \end{aligned}$$

where C_{scb} and C_{rb} are the steady state values of plasma glucose and delayed plasma glucose respectively. By considering that before the OGTT experiment one has the steady state relation:

$$k_1 C_p - k_2 C_{scb} = 0 \qquad (7)$$

one has:

$$k_2 = \frac{k_1 C_p}{C_{scb}} \qquad (8)$$

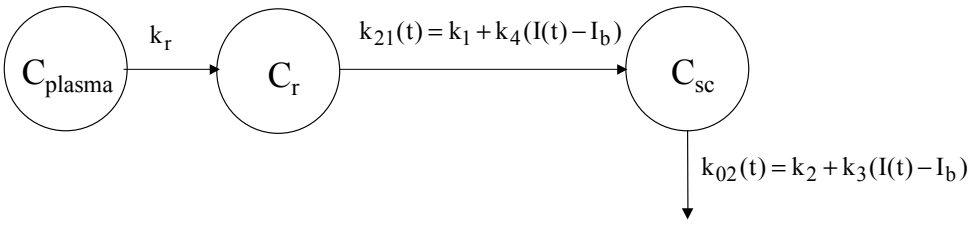

Fig.3 Model C

Model Identification

Model parameters have been estimated by weighted nonlinear least squares. Note that there is no equation for the first compartment for both the models, because $C_{plasma}(t)$ is assumed to be known and used as the input for model identification. The interstitial glucose data are described by:

$$C_{sc}^{obs}(t_j) = C_{sc}(t_j) + e(t_j) \qquad j=1, 2 \ldots N \tag{9}$$

where $e(t_j)$ is the measurement error at time t_j and N the number of data.
Measurement error was assumed to be additive, uncorrelated, gaussian, zero mean and with a constant fractional standard deviation (FSD=10% of the measurement) with an unknown proportionality factor γ:

$$\sigma^2(t_j) = \gamma\left[0.1 * C_{sc}^{obs}(t_j)\right]^2 \tag{10}$$

The scale factor γ was estimated a posteriori as:

$$\gamma = \frac{WRSS(\hat{p})}{N - P} \tag{11}$$

where $WRSS(\hat{p})$ is the value of the cost function evaluated at the minimum, i.e. for **p** equal to the vector of estimated model parameters $(\hat{\mathbf{p}})$:

$$WRSS(\hat{\mathbf{p}}) = \sum_{j=1}^{N} \frac{1}{\sigma^2}\left[C_i^{obs}(t_j) - C_i(\hat{\mathbf{p}}, t_j)\right]^2 \tag{12}$$

Note that $\gamma \approx 1$ would indicate that the assumption FSD=10% is reasonable.
The precision of parameter estimates was expressed as FSD% and obtained from the inverse of the Fisher information matrix **M** by:

$$COV(\hat{\mathbf{p}}) = \gamma \mathbf{M}^{-1} \tag{13}$$

To make a comparison among the models in terms of parsimony, the Akaike Information Criterion (AIC) was used:

$$AIC = N \ln WRSS(\hat{\mathbf{p}}) + 2P \tag{14}$$

where $WRSS(\hat{\mathbf{p}})$ is the weighted residual sum of squares, P is the number of parameters and N is the number of the data points.

Results

By using Model A, results have been obtained in all the 7 subjects. Parameter values and their CV are shown in Table 1. CV are acceptable (i.e. <100%) in all the identified subjects. Note that the mean γ value often is different from 1, consequently the fractional standard deviation, FSD, of the measurement error has to be considered less than 5% instead of our original assumption FSD=10%.

Table 1. Model A results

Subjects	k_{21} min^{-1}		k_{02} min^{-1}		**AIC**	**γ**
a	0.13	(9)	0.12	(9)	0.79	0.39
b	0.07	(5)	0.06	(5)	1.30	0.76
c	1.51	(57)	1.54	(57)	1.27	0.39
d	0.88	(75)	0.92	(75)	0.88	0.82
e	0.09	(17)	0.09	(17)	1.94	4.83
f	0.10	(6)	0.10	(6)	0.40	0.29
g	0.15	(5)	0.15	(5)	0.24	0.27
mean	0.42		0.43		0.98	1.11
SE	0.21		0.22			

The results obtained in all the original 7 by using Model B are shown in Table 2. It was not possible to estimate the parameter related with the insulin stimulation for the remaining 2 subjects, because k3 was estimated equal to zero. Parameter values and their CV are shown below. CV are acceptable (i.e.

Table 2. Model B results

Subjects	k_{21} min^{-1}		k_2 min^{-1}		k_3 L/mU/min		**AIC**	**γ**
a	0.15	(7)	0.13	(7)	0.00014	(11)	0.42	0.20
b	0.07	(5)	0.06	(5)	0.00004	(20)	1.26	0.74
c	1.44	(54)	1.48	(54)	0*	-	1.27	0.39
d	0.71	(71)	0.74	(71)	0.00009	(188)	0.87	0.82
e	0.08	(16)	0.07	(16)	0*	-	1.94	4.67
f	0.11	(6)	0.11	(6)	0.00002	(87)	0.36	0.28
g	0.15	(5)	0.15	(5)	0.00003	(35)	0.22	0.26
mean	0.39		0.39		0.00006		0.90	1.05
SE	0.20		0.20		0.00002			

* parameter estimate close to zero value

<100%) in all the identified subjects except for the k3 of the subject 'd'. FSD was estimated a posteriori to be less than 5% instead of the assumed FSD=10%. The model fit to the interstitial data resulted better than the previous ones obtained by using the Model A.
Model C parameter estimates have been obtained in 6 of the original 7 subjects. Parameter values and their precision are shown in Table 3. Parameter precision are acceptable (i.e. CV<100%) in all the identified subjects with the only exception for the k4 estimate of subject 'e'. FSD was estimated a posteriori to be less than 5% instead of the assumed FSD=10%.

Table 3. Model C results

Subjects	Cr mM/l	k_r min^{-1}	k_1 min^{-1}	k_{21} min^{-1}	k_3 L/mU/min	k_4 L/mU/min	AIC	γ
a	2.65 (10)	0.140 (14)	0.07 (8)	0.06 (8)	0.00008 (8)	0.01489 (26)	0.12	0.12
b	11.31 (3)	0.005 (12)	0.01 (10)	0.01 (10)	0.00006 (4)	0.00090 (5)	0.42	0.13
c	2.35 (80)	2.544 (78)	1.00 (39)	1.02 (39)	0* -	1.49162 (139)	1.23	0.33
d								
e	10.29 (2)	0.004 (7)	0.01 (6)	0.01 (6)	0.00006 (4)	0.00123 (4)	0.22	0.11
f	0.60 (31)	0.805 (36)	0.09 (8)	0.10 (8)	0.00003 (49)	0.57049 (121)	0.35	0.27
g	5.26 (6)	0.264 (9)	0.26 (9)	0.26 (9)	0.00008 (25)	0.00092 (50)	0.27	0.29
mean	**5.41**	**0.627**	**0.24**	**0.24**	**0.00006**	**0.34668**	**0.45**	**0.21**
SE	**1.82**	**0.402**	**0.16**	**0.16**	**0.00001**	**0.24695**		

* parameter estimate close to zero value

Correlation of anthropometric data and the parameters of model B
Regression analyses between the anthropometric data and the parameters of model B were all negative, but the tests lacked power (P=0.05, α<0.8).

Conclusions

The central goal of the present study was to determine a model closely describing the subcutaneous measurements as a prediction from blood plasma glucose levels in healthy volunteers. The successful identification of such a model, despite considerable variability of glycemia profiles, underlines the power of the here chosen new approach. The appliance of individualised parameters may have contributed to the strength of the model in the sense that it allows for physiological gradients. The use of ultraslow microdialysis to obtain absolute interstitial glucose concentrations allows modelling of glucose disposal

inside tissue, which was previously impossible[7;9]. Especially interesting is the first appliance of multiple parameters between compartments, allowing to model both glucose and insulin dependent glucose disposal. In the current study no correlations were found between the model parameters and the body measures, but this may well be a matter of increasing the number of experiments.

Results have been obtained for all three proposed models. All three models can be accepted as possible models as the assumptions of CV<100% and FSD□10% are met. The FSD can be estimated a posteriori to be less than 5% in these models.

Comparing the models in terms of parsimony, the AIC were higher for Model A fits than for Model B and Model C fits. Model B or Model C provided a slightly but significantly better fit than the Model A. Considering only Model B and Model C, the AIC were higher for Model B fits. However the lack of identifiability associated with the fits were higher for the Model C (one full non-convergence in a subject plus a parameter close to zero for Model C; only two parameter close to zero for Model B),the high CV associated with two parameters of the Model C (i.e. k4 of subjects c and f) and high variability of the estimates of k4 allow to select the Model B as the best to describe the data.

So, both models (B and C) with an insulin dependent glucose disappearance from the interstitium are better than the model (A) without insulin effects. The quantitative effect of the maximum insulin concentration (about 100mU/L) on total glucose disappearance can be estimated to be maximal ~60% in model C. This relatively small effect of insulin may be related to the low rate of glucose uptake in connective tissue as compared with adipose or muscle tissue[13]. The model parameters found here correspond also with the generally accepted assumption that transmembrane transport is the rate limiting step in the chain to glucose metabolism.

The largest impact on all models is made by the parameters which are independent from insulin. Within each experiment, the parameters k_{21} and k_{12} are almost the same as a mathematical reflection of the C_{plasma} to C_{sc} steady-state ratio being close to one in all the experiments. Between experiments, the parameters k_r, k_{21}, k_{12} and k_4 display a high (co-)variability. This variability reflects differences in speed at which the subcutaneous interstitium catches up with glucose level changes in the blood compartment. The explanation of these differences may be physiological or due to local reaction on the probe implantation. Both the mass-transfer at the blood-tissue interface, and the tissue-probe interface are generally assumed to be determined by diffusion[10]. The present data confirm this assumption, with a subdivision in diffusion and

capillary filtration (fast solvent drag transport by pressure gradient directly over the capillary wall, as represented in model C by k_r). According to Fick's law on diffusion, a concentration difference is needed for mass transfer. This implies that a concentration difference is a conditio sine qua non for glucose transport from capillaries to the tissue interstitium, but this need not be steep. During a 75g OGTT, the arterial-venous glucose level difference over abdominal wall tissues increases from 1.4% in steady state[14] to ~0.7mM at 60 minutes[8;15]. Arterial-venous differences are constituted by metabolite exchanges over the capillary wall. Consequently, the gradients inside the tissue interstitium lining the capillaries will be of at least the same extent. The observed individual kinetic parameters appear to fall within the outline of this physiological gradient, and may be explained accordingly. Using individualised parameters as performed here, enables the modelling of compartments with gradients. Another possible explanation of the variability of the k parameters might be the probe response time. There was however no correlation between k_{21} in vivo and the probe response times in vitro (R^2=0.17). So, this is not a plausible explanation. With a recently developed ultrafiltration probe, we have now reduced the response time further to 1-2 minutes by reducing the internal dead volume(submitted). Also, the response curve in vitro may be subtracted mathematically from the in vivo sensor signal, which is possible by means of deconvolution[16].

Further, it cannot be excluded that the instrumental lag-time (time from actual change of concentration to 10% signal change) was underestimated in vivo. The instrumental lag-time can only be checked independently with limited precision by weighing the nanolitre flow pump on a milligram balance.

The implantation of the probe in the subcutaneous tissue can also not be excluded as a cause for the observed differences between the arterial and the tissue interstitial compartment. The probe with a diameter of 340µm increases the interstitial volume because the interstitial width between cells can be equal or less than 1µm. A larger interstitial volume lengthens the diffusional path between neighbouring cells and surrounding capillaries, and needs more time for mass transfer to equilibrate in it self. The fluid volume near the probe may be changed as well through blood shedding, colloidal attraction of the dialysis fluid and the evacuation of the fluid for measurements. Probe implantation may further decrease local diffusion by fibrin deposits from bleeding and exudation due to introduction damage by the needle, later mechanical friction, and vessel response to environmental changes.

In the present study we placed the probe in the subcutaneous loose connective tissue instead of the usual placement in the adipose tissue, trying to diminish

blood shed and cell debris from the introductory needle and probe-tissue friction. Also, the interstitium is larger in loose connective tissue than in adipose tissue and has a better diffusional capacity[17]. The steady state glucose concentration levels in this study are kinetically much closer to the blood plasma levels and less variable as compared to previous research performed in adipose tissue[12]. We interpret this favourable difference as resulting from the difference in tissue anatomy and its effect on probe implantation and functioning.

An attempt to inverse the presented model for glucose sensor calibration purposes will encounter two difficulties. The first difficulty is the interindividual variability of the parameters modelled, the second, the absence of any moment of steady state in diabetes patients. The necessity to know the insulin levels as input for the algorithm need not be a hindrance, as the insulin administration would be remembered by a future artificial pancreas. However, additional research is needed to construct a simple, generally applicable algorithm to predict blood plasma glucose from subcutaneous glucose measurements. The range of uncertainty of the blood plasma concentration may still be indicated by the range of the model parameters. This range may be useful e.g. to set the threshold for a hypoglycaemia alarm of a subcutaneous sensor. So, more work has to be done on the development of the glucose sensor and the proposed model.

The model parameters are likely to be different in diabetes patients groups from the healthy volunteers studied here. In patients with insulin resistance or microvascular complications, differences in tissue insulin sensitivity and capillary permeability are difficult to study with current techniques[3]. Such studies may be improved with the here presented direct interstitial measurements and the proposed model. The for each patient individually assessed parameters may be of diagnostic value, and the presented model may be validated for this purpose in future studies.

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Chapter 8

Summary and final comments

Chapter 8

General introduction

The aim of this thesis was to develop and test devices for the continuous in vivo sampling and sensing of glucose and lactate, in clinical monitoring. Currently, glucose levels are not able to be constantly monitored and are therefore not well controlled by patients with diabetes mellitus. Glucose levels vary considerably, provoking serious short and long term complications. Instable cardiac and other emergency patients are at risk of sudden deterioration (as a result of tissue ischemia), which is difficult to diagnose in time for adequate treatment. Ischemia is often accompanied by an increased lactate release from the tissue in jeopardy. To allow for early diagnosis, glucose and lactate should be monitored continuously and automatically in these patients, instead of the current practice which requires frequent blood sampling and off-line analysis.
The introduction of glucose sensors in the clinic has been delayed by poorly understood differences between measurements of glucose in subcutaneous interstitial fluid and blood. Glucose sampling and sensing devices proposed thus far, have been shown to measure reliably in vitro, but, when applied subcutaneously in man, have a low level of accuracy in estimating blood levels. Also, to date there is neither a lactate sensor nor a cardiac lactate monitoring device available for patient use. The differences found between glucose levels in the subcutis and blood, necessitate to calibrate frequently with blood samples. This has precluded the clinical application of glucose sensors. These difficulties have arisen from technical shortcomings, physiological metabolism around the device, or disturbances caused by device-tissue interactions.
In the experiments described in this thesis, equilibrated sampling and sensing techniques have been developed, thus avoiding frequent in vivo calibration. These techniques have been applied to test several hypotheses on device-tissue interactions, as well as metabolism of glucose in the subcutis, and lactate in the myocardium, in order to improve the application of sensing devices for clinical monitoring.

Glucose and lactate ultrafiltration

In **chapter 2**, a newly developed ultrafiltration hollow fibre probe (length 2.5 cm, diameter 340 µm) was inserted into the abdominal subcutis in man to sample interstitial fluid at a continuous flow rate of about 47nl/min. An on-line bedside flow injection system was developed to measure glucose and lactate simultaneously once a minute using 20 nanolitre from the sample flow. This

system contained a splitter in front of parallel enzymatic conversion cells (with glucoseoxidase, lactate oxidase and horse-radish peroxidase enzymes) and electric detection cells (amperometry). The potential for monitoring using this sampling probe was tested, and the subcutaneous and blood glucose concentrations were compared for equality (zero hypothesis). Six healthy volunteers ingested 100g glucose in the fasting state and continuous equilibrated interstitial sampling with ultrafiltration was used for monitoring glucose and lactate. Glucose concentrations in the abdominal adipose subcutis were 1.06 mM (95% confidence interval 0.127-1.98) lower than in venous blood in the fasting state. The maximum glucose concentration was reached in the subcutis between one and thirty minutes later than in blood.
The finding that subcutaneous glucose levels were variable and significantly lower than in blood samples suggests that the abdominal subcutaneous adipose tissue is a kinetic compartment not directly linked to blood.

Monitoring with ultraslow microdialysis

In **chapter 3**, microdialysis (hollow fibre probe length 3.0 cm, diameter 620 µm) was applied at a continuous ultraslow flow rate of 42 nl/min to sample fluid in equilibrium with the abdominal subcutaneous adipose tissue in man. The occurrence of a shift to anaerobic metabolism in the subcutis due to insertion damage and subsequent restoration, was explored. Glucose and lactate levels were compared at probe implantation, and followed during two oral glucose tolerance tests (100 g) on two subsequent days in seven healthy subjects. Venous glucose levels were estimated using dialysate levels and a single venous glucose assay. The accuracy of this method was evaluated.
The results of this experiment were found to be similar to the ultrafiltration experiment (see chapter 2). Fasting subcutaneous glucose levels were often lower than venous levels (1.47 ± 1.20 mM) and there was a delay of 7.3 ± 1.2 minutes between venous and subcutaneous Cmax. In some subjects, the subcutaneous glucose levels were almost equal to venous levels before the glucose tolerance test, higher during the test, and again almost equal at the end. In these subjects, subcutaneous glucose levels appeared to be close to arterial levels. The low glucose levels found, were only occasionally accompanied by elevated lactate levels, which were generally still low. Shifts to anaerobic metabolism were generally not found, suggesting that this mechanism does not explain the glucose level differences found between blood and subcutaneous adipose tissue. The degree of accuracy of estimating venous glucose levels was

moderate (0.85 mM), using blood samples both from the fasting and from the non-fasting condition. The accuracy of the estimates decreased on day two after implantation.
This experiment confirms the results of the previous chapter, showing that subcutaneous adipose tissue must be considered as a kinetic compartment distinct from the vascular compartment. Adipose tissue glucose appears to be related to blood glucose in such a complex way that estimation of blood glucose using simple mathematical methods does not give sufficient accuracy for clinical application.

Monitoring myocardial ischemia

Chapter 4 describes the development of a cardiac catheter, as well as several tests to monitor lactate and glucose in the venous efflux of the myocardium, and the exploration of its diagnostic potential. A hollow fibre (length 4 cm) was built into the tip of a cardiac catheter (length 120 cm, diameter 1.78 mm) for continuous blood ultrafiltration in the coronary sinus of swines. The on-line flow-injection system outside the body detected lactate and glucose every minute with an in vivo response time of 1.33 ± 0.61 minutes (10-90%) and a lag-time of approximately 24 minutes. For a total of 27 hours the swine were monitored. The linear regression in vivo of blood and ultrafiltrate samples was 0.977 for lactate and 0.994 for glucose. Lactate levels rose 0.38 ± 0.10 mM above baseline within five minutes after the start of ischemia by obstruction of the left anterior descending coronary artery. After 5, 15 or 45 minutes obstruction, reperfusion was promptly detected as a lactate peak with the same shape as an intravenous injection (highest level 9.27 $mmol \cdot l^{-1}$). Myocardial stress induced by dobutamine infusion increased glucose but not lactate levels. Once, a wall effect, a well known technical problem with intravascular sensors, occured at the catheter tip disturbing the measurements.
The intravascular ultrafiltration catheter has demonstrated semi-continuous glucose-lactate monitoring of an organ with good accuracy. It appears feasible to detect myocardial ischemia and reperfusion almost instantly through a lactate level rise. The concentration of lactate at the time of reperfusion of the myocardium and the area under the curve can be estimated quantitatively (max. 14.82 $mmol \cdot l^{-1}$ and 3.77 $mmol \cdot l^{-1} \cdot h$ in this experiment).

Implantation of micro-biosensors

In **chapter 5,** alternative sites for glucose and lactate monitoring device implantation were explored to find a closer contact to the circulation than the subcutaneous adipose tissue. Twenty four thin-film amperometric lactate-glucose sensors (film width: 0.7 mm) were inserted in adipose tissue, striated muscle, and loose connective tissue of swines. Sensor baselines and response times were assessed in response to an intravenous lactate plus glucose bolus injection. Baselines and responses were found generally close to zero in adipose and muscle tissue. In connective tissue, lactate and glucose baseline levels were 80.5±19.1% and 90.9±15.7% of blood; and 95% of the response was reached after 5.5±2.1 and 17.1±8.5 minutes respectively. The intravenous bolus injection of the sensor's analyte was found to be a quick way to check whether a newly implanted sensor is in close kinetic contact with blood. The results suggest that a probe in subcutaneous loose connective tissue is in better contact with the circulation than in the commonly used adipose tissue.

Glucose gradients in subcutis

In **chapter 6**, glucose measurements between arteriolized blood, abdominal superficial subcutis (predominantly adipose tissue), and abdominal deep subcutis (predominantly loose connective tissue) were compared during an oral glucose tolerance test in man. Blood insulin levels, body mass index and skinfold thickness were compared with the tissue glucose levels as well. A newly developed nanolitre size glucose sensor was connected to an ultraslow microdialysis probe. Fasting glucose levels were 2.15±0.77 mM lower in adipose tissue than in blood and this difference increased to 5.19±1.57 at maximal glucose concentration after glucose challenge. In contrast, connective tissue glucose levels did not differ significantly from those in blood (-0.06±0.27 fasting and –0.11±1.35 at maximum after challenge) and correlated well (r^2 = 0.962). Between subjects, blood insulin levels, body mass index and skinfold thickness showed no obvious correlation with the tissue glucose levels. The wearable glucose sensor functioned well when connected to the ultraslow microdialysis probe. The abdominal subcutaneous loose connective tissue appears to be a more attractive option than adipose tissue for studying glucose monitoring in intensive diabetis therapy, because of its close approximation to arterial glucose levels.

Quantitative model of glucose transfer

In **Chapter 7**, kinetic models for subcutaneous glucose were tested to find which factors contribute, and to what extent, to subcutaneous glucose levels. A secondary aim was to evaluate the possibility of reversing the algorithm found.
The data from the seven subjects in chapter 6 were used to evaluate three models.
Model A is a two compartment model, using the measured arterial glucose as the linear input into the connective tissue compartment. The latter compartment has one linear glucose output. Model B is like A, but modifies the output using the arterial insulin levels. Model C is like B, but adds a third compartment between the arterial and the connective tissue compartment, and also modifies the input using the arterial insulin levels. To identify the best model, first the model parameters have been estimated by weighted non-linear least squares. Second, to make a comparison among the models in terms of parsimony, the Akaike Information Criterion was used. The parameter precision was acceptable (coefficient of variation below 100%) and the fractional standard deviation was estimated to be below 5%. Model B showed the best fit, followed by model C. However, the results also show parameters k3 and k4 (insulin effect) to virtually disappear in some subjects, suggesting a more simple model is then sufficient. The heterogeneity of the parameters found may be a reflection of the mix in the subcutis of insulin dependent adipose tissue and insulin independent connective tissue, as well as the tissue concentrations varying with the distance to the nearest capillaries.
A future reversal of the best model found will need the insulin levels as input for the algorithm. This will be possible, as the previous insulin administration will be remembered by a future artificial pancreas. However, the model presented here needs to be validated with addional data for this purpose.

Conclusions

In conclusion, the studies described in this thesis have shown that ultrafiltration and ultraslow microdialysis can readily be applied in the subcutis in man for monitoring glucose without recovery calculations. In addition, a newly developed intravascular ultrafiltration catheter can be applied in the continuous measurement of lactate from an internal organ. The little invasive sampling probes, connected on-line with bedside flow analysis or wearable nanolitre size

sensors, have shown to measure glucose and lactate accurately, and with a high time resolution in vivo.
Information has been gathered on the relation between subcutaneous and blood glucose levels, important for the clinical application of sensing devices. The application of novel equilibrated sampling techniques and a diffusion limited sensor prevented the drainage of glucose and lactate from the interstitium as is the case in classical microdialysis. These experiments have demonstrated that drainage is not the only reason for subcutis glucose levels to be lower than in blood.

Only a few sampling device-tissue contact disturbances have been observed in the present experiments. These were implantation effects in adipose tissue marked by high lactate levels from anaerobic glucose metabolism which recovered the next day, and an intravascular wall effect in the coronary sinus. A thin-film amperometric sensor has only little electrolytic contact with the interstitial fluid if inserted in adipose or muscle tissue as compared to implantation in loose connective tissue in swine.
Generally, lower interstitial glucose levels appear to be determined by the balance between the supply through diffusion from the blood capillaries and the specific tissue metabolic characteristics. The considerable variability in glucose levels measured locally in the subcutis can be interpreted as reflecting the physiological gradients naturally present in tissue due to the spatial difference between microvascular supply and cellular metabolism.
Glucose levels were found to be closer to arterial levels in deep subcutis layers (predominantly loose connective tissue) than in superficial layers (predominantly adipose tissue). Known tissue differences such as the rate of aerobic and anaerobic metabolism, the degree of insulin dependence of cellular glucose uptake, and the width of the intercellular space may well explain the difference in interstitial glucose levels between these tissues. Estimation of blood glucose levels by simple calculation from subcutaneous adipose tissue glucose is possible, but only with a limited accuracy (see chapter 3). In the attempt to identify an optimal kinetic model for subcutaneous connective tissue glucose estimation, the different model parameters illustrated the heterogeneity of the subcutis regarding glucose supply and metabolism. The subcutaneous measurements with ultraslow microdialysis sampling are, however, so close to arterial levels, that continued clinical research in this area is justified and should be aimed at the optimal positioning in tissue for long-term measurement, to avoid algorithms for estimation.

Future

The equilibrated glucose sampling technique combined with a nanolitre sensor can be combined to form a wearable device. Future subcutaneous glucose sensing may thus become a reality for diabetes patients. Continuing clinical development of lactate sensing may help to detect and quantify ischemic events in cardiac or other emergency patients at an early stage, which would help prevent secondary infarction. Sudden reperfusion of a major coronary artery releases a remarkable lactate peak, which may be recognized even by a less invasive peripheral lactate sensor.

The techniques studied here avoid direct contact of sensor material with the organism, thereby increasing biocompatibility. Application of the small, flexible hollow fibre probe thus appears interesting for long-term monitoring with ultrafiltration and ultraslow microdialysis.

Chapter 9

Technieken voor glucose en melkzuurbewaking in het lichaam

(Samenvatting voor de geïnteresseerde leek)

Doel van het onderzoek

Het doel van het onderzoek beschreven in dit proefschrift is meettechnieken van glucose (druivensuiker) en melkzuur zo aan te passen, dat ze bruikbaar zijn in het lichaam om patiënten constant te bewaken. Een glucosesensor (een techniek voor constante meting van glucose in het lichaam) is van belang voor de behandeling van suikerpatiënten. Daarnaast is constante melkzuurmeting interessant voor bewaking van het hart en andere organen.
Het onderzoek in dit proefschrift laat zien dat glucose continu bewaakt kan worden zonder berekening van de verdunning van monsters die gewoonlijk nodig is. Dit is mogelijk dankzij ultrafiltratie en evenwichtsmicrodialyse met een eenvoudig onder de huid te leggen weinig belastend dun slangetje. Van inwendige organen zoals het hart kan het melkzuur constant bewaakt worden dankzij ultrafiltratie van het bloed aan de punt van een nieuw ontwikkelde catheter. Met de doorlatende slangetjes kan precies en met een snelle respons glucose en melkzuur in het weefsel gemeten worden, omdat ze in directe verbinding staan met draagbare sensoren of meetapparatuur naast het bed (“bedside”). Precisie en een snelle respons zijn belangrijk om in noodgevallen èn op tijd te behandelen, èn de juiste behandeling in te stellen. Dat is nodig, bijvoorbeeld om een verstopping van een bloedvat van het hart op te heffen of schommelende bloedsuikers te stabiliseren. De bovengenoemde evenwichtsmicrodialyse techniek is samen met de uitwendige glucosesensor een draagbaar instrument en vormt een mogelijke toekomstige glucosesensor voor suikerpatiënten.
Een voortgezette ontwikkeling van melkzuursensoren in ziekenhuizen kan er toe leiden dat doorbloedingsstoornissen bij hart- en intensive care patiënten vroegtijdig onderkend kunnen worden. In bepaalde situaties kan zo wellicht weefselversterf en de gevolgen daarvan worden voorkomen.

Schommelende glucose bij suikerpatiënten

De glucoseconcentratie in het bloed van suikerpatiënten schommelt vaak sterk door de dag, omdat de normale reactie van het lichaam op concentratieveranderingen verstoord is. Om de glucoseconcentratie heel precies stabiel te houden, wisselt het lichaam normaliter voortdurend tussen snelle opslag van glucose tijdens het eten en afgifte daartussenin. Suikerpatiënten proberen zelf met medicijnen de glucoseconcentratie stabiel houden. Dat is moeilijk omdat een patiënt daarvoor eigenlijk continu zijn glucoseconcentratie

zou moeten controleren. Voor elke keer controleren is het echter nodig opnieuw bloed te prikken. Te weinig glucose in het bloed is gevaarlijk omdat de hersenen steeds energie halen uit glucoseverbranding met zuurstof. Een tekort aan glucose leidt snel tot bewusteloosheid (vergelijkbaar met een tekort aan zuurstof). Te veel glucose is ook gevaarlijk omdat in de loop van jaren vaak ogen, nieren, bloedvaten en zenuwen beschadigd worden. Suikerpatiënten kunnen bijvoorbeeld blind worden, kunnen eerder een hartaanval of beroerte krijgen, kunnen tenen of voeten verliezen of ernstige nierproblemen krijgen. Het voorkómen van grote schommelingen van glucose is dus belangrijk voor alle 450.000 suikerpatiënten in Nederland. Er is dan ook vraag naar een techniek voor constante bewaking die daarbij kan helpen. Die techniek moet glucose automatisch en betrouwbaar meten, zonder dat patiënten hun eigen bloed moeten (blijven) prikken voor controle.

Melkzuur voor patiëntenbewaking

Weefsels putten energie uit glucose door het met zuurstof uit het bloed om te zetten in koolzuur en water. Bij gebrek aan zuurstof wordt energie vrijgemaakt door glucose om te zetten in melkzuur. Melkzuur komt vooral vrij uit weefsels op het moment dat de doorbloeding verslechtert. Verslechterde doorbloeding van de hartspier of ander weefsel kan plotseling optreden, bijvoorbeeld bij patiënten opgenomen in het ziekenhuis met een dreigend hartinfarct (afsluiting van een kransslagvat met een stolsel) of met een ernstige bloedvergiftiging (infectie). Het bedreigde weefsel geeft dan eerst melkzuur af. Als de doorbloeding langer slecht blijft, sterft het weefsel af door energiegebrek waarna er eiwitten uit de cellen kunnen vrijkomen. Constante bewaking van melkzuur in het lichaam van de patiënt maakt wellicht behandeling mogelijk vóór afsterving van het weefsel. Een behandeling is dan eerder mogelijk dan nu. Bewaking vindt nu plaats door het af en toe prikken van bloed en het meten van vrijgekomen eiwitten in een laboratorium. Zo'n melkzuurbewaking zou andere bewakingstechnieken kunnen aanvullen, zoals van de ademhalingsgassen in het bloed en van de elektrische activiteit in het hart. In Nederland zijn ziekten van hart en vaten (vooral beroertes en hartinfarcten) de belangrijkste doodsoorzaak. Er liggen elk jaar 90.000 patiënten ongeveer acht dagen in het ziekenhuis in verband met verslechterende doorbloeding van de hartvaten.

Problemen bij meten in het lichaam

Tot nu toe is een techniek voor constante glucosemeting in het lichaam (een glucosesensor) nog niet beschikbaar voor patiënten. Er is ook nog geen melkzuursensor beschikbaar voor patiëntenbewaking. Een permanente sensor in een bloedvat is moeilijk, omdat de sensor dan niet door een patiënt zelf geplaatst kan worden en problemen kan geven door bloedstolling en infectie. Plaatsing onder de huid van de buik lijkt een aantrekkelijk alternatief.
Glucosemetingen in onderhuids weefsel en in bloed verschillen echter nogal. De momenteel voorgestelde glucosesensoren doen het goed in de reageerbuis, maar eenmaal onder de huid geplaatst is de sensor nog te onbetrouwbaar om een glucosemeting in het bloed te vervangen. Hierdoor moet er bij de huidige sensoren nog regelmatig bloed geprikt worden om de sensormetingen te ijken. Mogelijke oorzaken van de onbetrouwbaarheid zijn puur technische gebreken, een slecht contact tussen de sensor en het onderhuidse weefsel of de aard van het weefsel. Weefsel neemt immers van nature glucose uit het bloed op waardoor de glucoseconcentratie rondom een sensor kan dalen. De suiker die het bloed naar het weefsel toe heeft gebracht, is dan al door de cellen opgenomen nog voor het gemeten kan worden.
Genoemde mogelijke oorzaken en eventuele oplossingen zijn onderzocht in de hierna beschreven experimenten. Het doel is te meten zonder dat bloedafnames nodig zijn om de metingen te ijken. Hiervoor zijn nieuwe methoden ontwikkeld om kleine hoeveelheden weefselvloeistof af te nemen, zonder deze vloeistof te verdunnen. Ook zijn nieuwe miniatuurensoren ingezet met als doel het gewicht te beperken en de toekomstige bruikbaarheid van glucose- en melkzuursensoren voor patiënten te vergroten. Tot zover hoofdstuk 1.

Glucose vergeleken onder de huid en in bloed

Hoofdstuk 2 beschrijft de glucosemetingen in het onderhuids vet vergeleken met glucosemetingen in het bloed. Zes gezonde vrijwilligers namen 100 gram glucose in op de lege maag om tijdelijk de glucoseconcentratie in het lichaam te verhogen. Een doorlatend slangetje zoog een uiterst kleine hoeveelheid van de vloeistof weg die normaal aanwezig is tussen de cellen onder de huid. Dit slangetje van ongeveer 1/3 mm dik en 25 mm lang ultrafiltreerde één tienmiljoenste liter per minuut uit het onderhuidse weefsel. Buiten het lichaam mat een elektrische cel de glucoseconcentratie in de vloeistof met behulp van enzymen (een elektrochemische detector).

De concentraties glucose gemeten in het onderhuids vet waren duidelijk lager dan in het bloed. Ook was het hoogtepunt van de glucoseopname onder de huid tot wel 30 minuten later dan in bloed. Weliswaar komt er dus glucose uit het bloed in het onderhuidse vet, maar de concentratie glucose in het onderhuidse vet is niet gelijk aan die in het bloed. Het onderhuidse vet lijkt dus gescheiden van het bloed en daarom niet eenvoudig te benutten voor een glucosesensor.

Weefselbeschadiging?

Beschadiging van weefsel door het met een naald inbrengen van het meetslangetje zou een verlaagde glucoseconcentratie kunnen verklaren. De melkzuurconcentratie zou kunnen stijgen door een verslechterde doorbloeding ter plekke. Mogelijk herstellen het weefsel en de glucoseconcentratie zich weer de volgende dag. Hoofdstuk 3 beschrijft een studie van deze opties.
Zeven gezonde vrijwilligers namen twee dagen achter elkaar 100 gram glucose in op de lege maag. Via een doorlatend slangetje onder de huid van ongeveer 1/2 mm dik en 30 mm lang werd langzaam vloeistof gepompt (microdialyse). Dit pompen ging zo langzaam dat glucose voldoende tijd had om spontaan uit het omliggende weefsel in het slangetje te lopen, zonder dat de glucose in het weefsel daardoor verlaagd werd (microdialyse in evenwicht). De in hoofdstuk twee genoemde elektrochemische detector werd uitgebreid met een splitsing van de vloeistofstroom om tegelijk glucose en melkzuur te kunnen meten. Daarnaast werden glucose en melkzuur tegelijkertijd in het bloed gemeten. De glucosemetingen onder de huid werden gebruikt om de glucoseconcentratie in het bloed te schatten. Na een meting van het concentratieverschil onder de huid en in het bloed op één tijdstip werd dit verondersteld verder gelijk te blijven. Deze wijze van schatten werd getoetst op nauwkeurigheid.
De resultaten van dit experiment waren goed vergelijkbaar met het voorgaande experiment. De glucose was duidelijk lager onder de huid dan in het bloed en volgde met een vertraging van gemiddeld zeven minuten. Het melkzuur onder de huid was, op één uitzondering na, niet verhoogd. De nauwkeurigheid van de rekenmethode om de glucose te schatten in het bloed was matig en nam verder af op de tweede dag. De schattingen zouden niet nauwkeurig genoeg zijn geweest om de behandeling van suikerpatiënten op te baseren.

Melkzuurbewaking in het hart

In hoofdstuk 4 wordt een hartcatheter beschreven die continu melkzuur en glucose meet in de veneuse bloedstroom uit de hartspier (de Sinus Coronarius). Of op deze wijze mogelijk hartpatiënten bewaakt zouden kunnen worden, is verkend bij varkens. In de punt van een hartcatheter (120 cm lang, bijna 2 mm dik) werd een doorlatend slangetje van 4 cm ingebouwd voor continue ultrafiltratie van bloed. Er is in totaal 27 uur continu gemeten in de bloedbaan. Melkzuur en glucose konden heel nauwkeurig gemeten worden. Een afsluiting van een grote slagader naar het hart, was binnen vijf minuten zichtbaar door een stijging van het melkzuur. Zo'n afsluiting is vergelijkbaar met wat er gebeurt bij een hartinfarct. Het weer opheffen van de afsluiting kon ook direct herkend worden doordat er een piek melkzuur vrijkwam uit het weefsel. Dit laatste gebeurt ook wel na een hartinfarct, spontaan of door behandeling. Verder steeg bij een door medicijnen versnelde hartslag de glucose licht, terwijl het melkzuur niet veranderde. Een geconstateerde tekortkoming van deze catheter was dat er 24 minuten nodig waren voor het ultrafiltraat van de catheterpunt het meetapparaat had bereikt. Daarnaast zat de catheterpunt een keer tegen de vaatwand waardoor de meting verstoord werd.
Met deze ultrafiltratiecatheter is aangetoond dat continue en precieze melkzuur- en glucosebewaking van een orgaan mogelijk is in het afvoerend bloedvat. Het lijkt mogelijk afsluiting en heropening van een hartslagader zeer vroegtijdig te diagnosticeren door de geconstateerde melkzuurverhoging. De concentratie van melkzuur die in de hartspier is opgehoopt gedurende het afsluiten van de doorbloeding kan ook geschat worden.

De beste plek voor een sensor in het lichaam

In hoofdstuk 5 is een verkenning beschreven waar glucose- en melkzuursensoren het best in het lichaam geplaatst kunnen worden. Uit eerdere metingen in het onderhuids vetweefsel was duidelijk geworden dat er maar een matig contact bestaat met de bloedbaan (zie hoofdstukken 2 en 3). Vierentwintig thin-film sensoren voor glucose-melkzuur meting werden bij varkens geplaatst in vetweefsel, spierweefsel, of losmazig bindweefsel. Thin-film sensoren bestaan uit een filmstrookje van 0,7 millimeter breed met op de punt twee elektrochemische detectoren. Deze detectoren zijn gemaakt van flinterdunne laagjes metaal en andere stoffen die op een dragende filmstrook zijn aangebracht. Eerst werd de basisconcentratie glucose en melkzuur in het

bloed en in het weefsel bepaald. Vervolgens werd gemeten hoe snel een injectie van glucose en melkzuur in de bloedbaan in de eerder genoemde weefsels kon worden gemeten (respons). De basisconcentratie gemeten in het spier- en vetweefsel was dicht bij nul. Een respons was daar nauwelijks te meten. In het bindweefsel was de basisconcentratie maar iets lager dan die in het bloed. De respons was hier goed zichtbaar: binnen 6 minuten na injectie van melkzuur en na 17 minuten na injectie van glucose. Een injectie in de bloedbaan kan dus op een snelle en eenvoudige manier controleren of een sensor in weefsel wel in goed contact staat met het bloed. Op dit moment wordt het vetweefsel vaak gebruikt om sensoren te testen bij patiënten. Deze resultaten doen vermoeden dat sensoren in onderhuids losmazig bindweefsel in beter contact staan met de bloedbaan dan in vetweefsel.

Glucose vergeleken in vet- en bindweefsel

Hoofdstuk 6 beschrijft glucose metingen in het onderhuids vet en in het onderhuids losmazig bindweefsel bij gezonde vrijwilligers. Deze metingen werden vergeleken met de glucoseconcentratie in het bloed. Daarnaast werden de insulines in het bloed gemeten (insuline is een hormoon (signaalstof) die zorgt voor de opname van glucose in weefsels) en de dikte van de huidplooi waarin glucose werd bepaald. Verder werd de lichaamslengte en het gewicht van de vrijwilligers vastgelegd. Voor het afnemen van weefselvloeistof werd weer gebruik gemaakt van evenwichtsmicrodialyse in het weefsel (zie de beschrijving in hoofdstuk 3). De glucosemeting in de vloeistof werd buiten het lichaam gedaan met een nieuw ontwikkelde glucosesensor, een miniatuur elektrische cel met enzymen (iets kleiner dan een lucifer). De glucoseconcentratie gemeten in het vet was weer duidelijk lager dan in het bloed en was ook lager dan in het bindweefsel. De glucosewaarden gemeten in het bindweefsel was praktisch gelijk aan die gemeten in het bloed. De basisconcentratie was gelijk, maar ook de hoogste concentratie in het lichaam die bereikt werd na de inname van 100 gram glucose. Dat de glucose in het vetweefsel lager was, kon niet worden verklaard door de gemeten insulinespiegels, de lengte en het gewicht, of de huidplooidikte van de vrijwilligers. De resultaten laten zien dat de techniek van evenwichtsmicrodialyse in combinatie met de nieuwe draagbare glucosesensor een goed meetinstrument vormt om continu te meten in het lichaam. De metingen in het losmazig bindweefsel onder de buikhuid benaderen die in het bloed veel dichter dan de metingen in het vet. Deze locatie lijkt dan ook

aantrekkelijk om te benutten voor verder onderzoek naar continue glucosebewaking in suikerpatiënten.

Rekenmodellen voor de bewegingen van glucose

In hoofdstuk 7 worden testen beschreven van rekenmodellen die de bewegingen van glucose door het lichaam voorstellen als stromen tussen delen van het lichaam. Verondersteld wordt dat de concentratie binnen zo'n deel overal gelijk is. De grootte van de concentratieverschillen tussen de delen wordt bepaald door de verschillende snelheden waarmee glucose tussen de delen stroomt. Zo stroomt glucose bijvoorbeeld van de bloedbaan naar het onderhuidse weefsel toe en stroomt vanaf daar de bindweefselcellen binnen. Glucose kan enigzins vergeleken worden met water dat door sluizen tussen kanalen stroomt. De waterstroom hangt dan onder andere af van de waterhoogteverschillen (vergelijk verschillende hoogtes van de glucoseconcentratie), hoever een bepaalde sluisdeur open staat (vergelijk de mate van contact tussen het bloed "kanaal" en het onderhuids "kanaal") en hoeveel sluizen er verderop worden open gezet (vergelijk insuline dat de glucoseopname in de cel vergroot). Het doel was factoren te vinden die de concentratie glucose onder de huid bepalen. Dit gebeurde door uit verschillende rekenmodellen dat model te selecteren, dat het beste de echte meetwaarden benadert. Een tweede doel was te kijken of dit rekenmodel omgedraaid zou kunnen worden: met zo'n rekenmodel zou dan de bloedglucose geschat kunnen worden met de onderhuidse glucosemetingen (in plaats van andersom). De meetresultaten uit hoofdstuk 6 werden gebruikt om drie rekenmodellen te testen. Model A veronderstelde een evenredige snelheid waarmee glucose uit het bloed naar het onderhuidse weefsel loopt. Model B was als A, maar veronderstelde daarnaast de invloed van insuline op de glucosestroom de cellen in. Model C was als B, maar veronderstelde nog een extra onderdeel tussen de bloedbaan en het weefsel en nog meer invloed van insuline.
Om het beste rekenmodel te kiezen werd gekeken naar de verschillen tussen de echte metingen en de schattingen die elk model maakte. Daarbij werd rekening gehouden met de mate van ingewikkeldheid van elk rekenmodel, omdat het beter kunnen schatten met een model een voordeel is, maar de ingewikkeldheid van een model een nadeel.
Model B was het beste model. In enkele experimenten was het insuline effect echter zo klein, dat het eenvoudiger model A ook voldeed. Deze verschillen zijn misschien te verklaren uit het feit dat onderhuids weefsel altijd een mengsel is

van insulinegevoelig weefsel als vet en minder gevoelig weefsel als bindweefsel. Daarnaast kan een grote afstand naar de dichtstbijzijnde bloedvaten ook nog de glucoseconcentratie verkleinen. Als model B in de toekomst gebruikt gaat worden voor bloedglucose-schattingen, moet ook de concentratie insuline bekend zijn. Dit is geen probleem, omdat een toekomstige kunstmatige pancreas die insuline zal gaan toedienen. Er is hoop dat normalisering van de glucosespiegels door zo'n apparaat het diabetesprobleem grotendeels of geheel kan oplossen. Voor het zover is, zal er echter meer onderzoek nodig zijn om helemaal zeker te zijn van het voorgestelde model.

Samenvattend

Samenvattend laat het onderzoek in dit proefschrift zien dat glucoseconcentraties continu bewaakt kunnen worden zonder berekening van de verdunning van monsters die gewoonlijk nodig is. Dit is mogelijk dankzij ultrafiltratie en evenwichtsmicrodialyse met een eenvoudig onder de huid te leggen weinig belastend dun slangetje. Van inwendige organen zoals het hart kan het melkzuur constant bewaakt worden dankzij ultrafiltratie van het bloed aan de punt van een nieuw ontwikkelde catheter. Met de doorlatende slangetjes kan precies en met een snelle respons glucose en melkzuur in het weefsel gemeten worden, omdat ze in directe verbinding staan met draagbare sensoren of meetapparatuur naast het bed ("bedside"). Precisie en een snelle respons zijn belangrijk om in noodgevallen èn op tijd te behandelen, èn de juiste behandeling in te stellen. Dat is nodig, bijvoorbeeld om een verstopping in de bloedvaten van het hart op te lossen of schommelende bloedsuikers te stabiliseren.
Kennis is verzameld over het verband tussen glucose in het bloed en onderhuidse glucoseconcentraties die van belang is voor de toekomstige toepassing van sensoren bij patiënten. Door toepassing van evenwichtsmicrodialyse werd voorkomen dat zoveel glucose en melkzuur aan het weefsel onttrokken werd, dat de meting hierdoor beïnvloed werd (zoals wel gebeurt bij de klassieke microdialyse). Dit laatste is echter niet de enige oorzaak gebleken van een lagere glucoseconcentratie onder de huid vergeleken met die van het bloed.

Er zijn gedurende de verschillende experimenten een paar maal verstoringen geconstateerd in het contact van het slangetje of de sensor met het weefsel. Kort na plaatsing van het doorlatende slangetje in het onderhuids vet werd een enkele keer een verhoogde melkzuurconcentratie gevonden, die de volgende dag weer

normaal was. Verder kwam de hartcatheter éénmaal in de vaatwand wat de meting verstoorde. De thin-film sensor had bij varkens in het vet- en spierweefsel een slecht contact met de weefselvloeistof, en in het losmazig bindweefsel een veel beter contact.
De mate van verbruik van glucose is voor elk type weefsel anders. Dit kan verklaren dat de glucoseconcentratie in verscheidene weefsels lager is dan in het bloed omdat er een evenwicht is tussen de toevoer vanuit de kleinste bloedvaten en het verbruik in de cellen. Een steil verval van glucose tussen de toevoerende kleinste vaten en de verbruikende cellen in zou de grote verschillen in glucoseconcentraties gemeten binnen één weefsel kunnen verklaren.
De glucoseconcentratie in diepe onderhuidse lagen (voornamelijk bindweefsel) lag dichter bij de concentratie in de bloedbaan dan die in de oppervlakkige onderhuidse lagen (voornamelijk vet). Bekende verschillen tussen weefsels, zoals de mate van glucoseverbranding met of zonder zuurstof, de mate waarin de glucoseopname bevorderd wordt door insuline en de hoeveelheid vloeistof tussen de cellen kunnen heel goed de verschillen in glucoseconcentraties tussen de weefsels verklaren.
Het schatten van de glucoseconcentratie in de bloedbaan met behulp van onderhuidse metingen en eenvoudige correcties is mogelijk gebleken, maar met een beperkte nauwkeurigheid (zie hoofdstuk 3). Bij de poging om een optimaal rekenmodel te vinden (hoofdstuk 7) werden nogal wisselende modelkenmerken per vrijwilliger gevonden, wat goed past bij het veronderstelde steile verval van glucose en een onregelmatige opbouw van onderhuids weefsel. De metingen in bindweefsel van vrijwilligers zijn echter zo dicht bij de glucosemetingen in bloed, dat voortgezet onderzoek bij patiënten is aangewezen.

Toekomst

Wellicht kan een optimale plaatsing in weefsel worden gevonden voor metingen op lange termijn, zodat omrekeningen en schattingen overbodig blijven. De evenwichtsmicrodialyse techniek is samen met de uitwendige glucosesensor een draagbaar instrument en vormt een mogelijke toekomstige glucosesensor voor suikerpatiënten.
Een voortgezette ontwikkeling van melkzuursensoren in ziekenhuizen kan er toe leiden dat men doorbloedingsstoornissen bij hart- en intensive care patiënten vroegtijdig kan onderkennen. In bepaalde situaties kan zo wellicht weefselversterf en de gevolgen daarvan worden voorkomen. Naast bewaking in de bloedbaan kan ook een minder belastende onderhuidse melkzuursensor

nuttig zijn. Zo veroorzaakt het heropenen van een hartslagader een dermate duidelijke melkzuurpiek dat die ook onderhuids herkend zal kunnen worden.
De voor deze studies ontwikkelde technieken waarbij alleen een slangetje in het lichaam geplaatst wordt, zijn patiëntvriendelijk en veilig omdat een direct contact tussen sensormateriaal en het lichaam wordt voorkomen. De toepassing van de doorlaatbare slangetjes met ultrafiltratie en evenwichtsmicrodialyse tussen de sensor en het lichaam lijkt dus aantrekkelijk voor bewaking op lange termijn.

List of publications from the author
(1-9)

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2. **Tiessen, R.G., N.A. van-Elsacker, K.C. Vermeij, D. Oepkes, R.J. van, and M.C. Gorsira.** A fetus with a parvovirus B19 infection and congenital anomalies. *Prenat.Diagn.* 14: 173-176, 1994.

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7. **Rhemrev-Boom, R.M., M.A. Jonker, K. Venema, G. Jobst, R.G. Tiessen, and J. Korf.** On-line continuous monitoring of glucose or lactate by ultraslow microdialysis combined with a flow-through nanoliter biosensor based on poly(m-phenylenediamine) ultra-thin polymer membrane as enzyme electrode. *Analyst* 126: 1073-1079, 2001.

8. **Rhemrev-Boom R.M., Tiessen R.G., Jonker A.A., Venema K., Vadgama P, and Korf J.** A lightweight measuring device for the continuous in vivo monitoring of glucose by means of ultraslow microdialysis in combination with a miniaturised flow-through biosensor. *Clinica Chimica Acta* In press2001.

9. **Tiessen R.G., Rhemrev-Boom R.M., and Korf J.** Glucose gradient differences in subcutaneous tissue of healthy volunteers assessed with ultraslow microdialysis and a nanolitre glucose sensor. *Life Sci.* accepted: 2001.

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Renger

Stellingen behorende bij het proefschrift “Continuous metabolic monitoring techniques” Groningen, 14 november 2001

1. Fysiologische concentratiegradienten in het onderhuids vetweefsel beletten een algemeen toepasbaar algorithme om bloedplasmaglucose te schatten met onderhuidse metingen. *Dit proefschrift*
2. Monitoring van glucose in de circulatie is beter mogelijk in het onderhuids bindweefsel dan in vetweefsel omdat de fysiologische glucosegradienten daar veel kleiner zijn. *Dit proefschrift.*
3. Omdat microdialyse zich beperkt tot zeer lokale meting in het weefsel (*Kennergren C. Scand Cardiovasc J 1997 31(6):343-9*), kan ischemie en reperfusie van het myocard beter bewaakt worden door continue melkzuurmeting in de sinus coronarius. *Dit proefschrift.*
4. Niet de technische vervolmaking van implanteerbare glucosesensoren verdient meer aandacht (*Kerner W. Exp Clin Endocrinol Diabetes 2001 109 Suppl 2:S341-6*), maar de procedure van implanteren. *Dit proefschrift.*
5. Als de uitkomst van de eerste geneesmiddelen onderzoeken bij dieren goed vergelijkbaar is met die bij mensen, dan dienen de ethische normen voor het verrichten van beide vormen van onderzoek ook vergelijkbaar te zijn.
6. De overheid houdt de schone schijn op door een klein budget aan anti-rook campagnes te besteden en tegelijkertijd toe te staan dat een veelvoud van dat bedrag aan reclames wordt besteed om het roken te stimuleren.
7. Buitenaardse en paranormale verschijnselen bestaan dankzij aardse en normaal menselijke behoeften.
8. De omstandigheden voor dieren in het centraal dierenlaboratorium zijn goed, maar de omstandigheden voor de medewerkers zijn minder gunstig.
9. Politici wachten nog steeds tot de arbeidsmarkt en de financieel-economische omstandigheden in die mate verslechterd zijn, dat een aanpassing van de wet voor de arbeidsongeschikten zeer pijnlijk wordt.
10. Het stichten van een gezin en promoveren gaan goed samen.