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Continuous metabolic monitoring techniques

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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 bedside 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

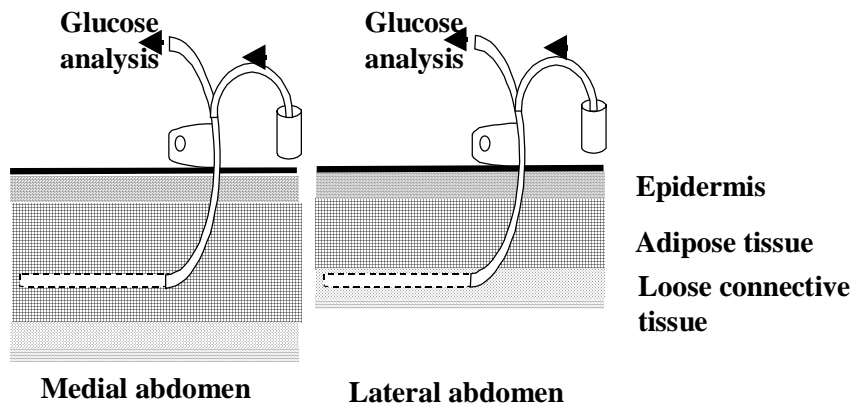


Figure 1: Position of microdialysis probes in adipose tissue (left) and in loose connective tissue (right)

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 28.4 kg/m². Skinfold thickness ranged between 13 and 40 mm.

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 interferences 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

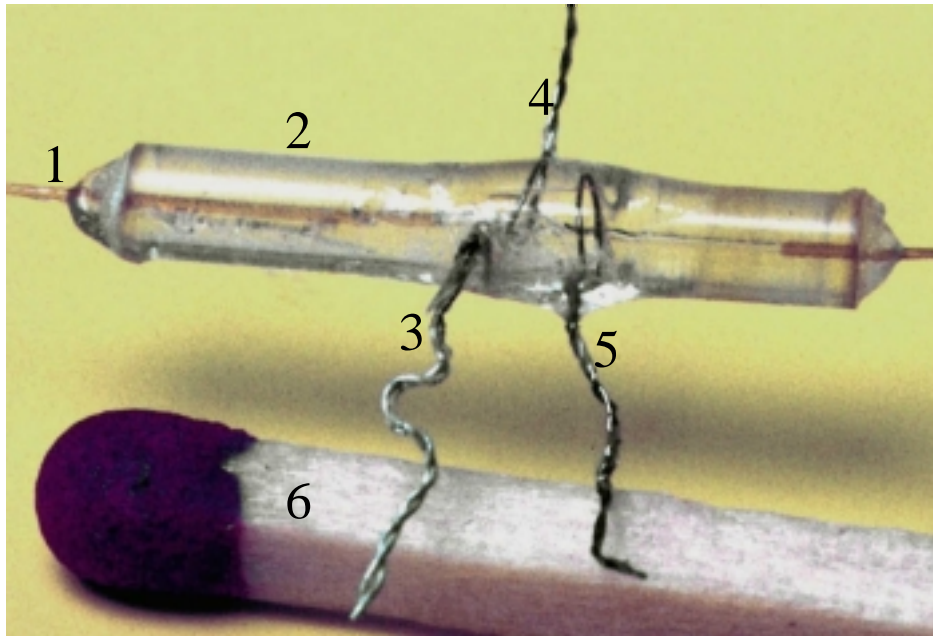


Figure 2: Photo of glucose sensor in flow-through cell

- 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 potentiostat 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

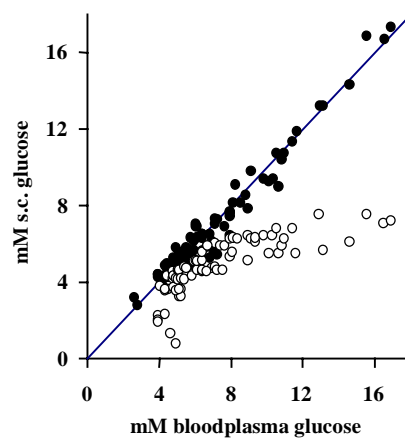
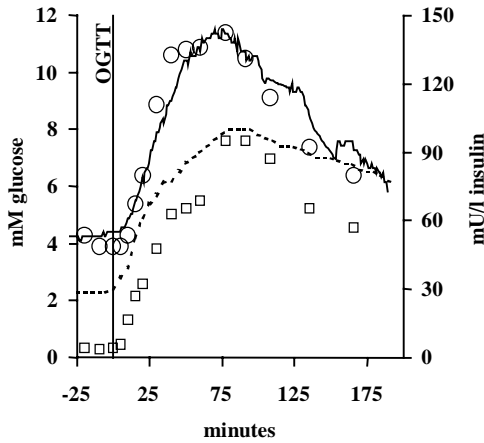


Figure 3:
 Example of an oral glucose tolerance test (OGTT | at t=0 minutes) with glucose and insulin concentrations
 o plasma glucose
 ~ connective tissue interstitial glucose
 -- adipose tissue interstitial glucose
 plasma insulin (on secondary Y-axis)

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 Gluowatch⁽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 potentiostat 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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