Development of sampling and bioselective techniques for on-line clinical biosensors
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Chapter 3

Methodological aspects of glucose monitoring with a slow continuous subcutaneous and intravenous ultrafiltration system in rats

As for the search for truth, I know from my own painful searching, with its many blind alleys, how hard it is to take a reliable step, be it ever so small, toward the understanding of that which is truly significant (Albert Einstein).

A method for the continuous ultrafiltration of venous blood or subcutaneous fluid is demonstrated with monitoring glucose in the living rat. Ultrafiltrate was withdrawn at a constant flow rate of approximately 100 nl min\textsuperscript{-1}. Glucose contents of the ultrafiltrates were electrochemically determined with a flow injection analysis method and a bi-enzyme reactor. After glucose loading, the time course of glucose in the ultrafiltrate from the jugular vein was virtually identical, whereas that from the subcutaneous compartment was attenuated and the peaks blunted as compared to glucose levels in concomitantly assayed arterial blood. Our study demonstrates the potential of low rate ultrafiltration for monitoring metabolism with biosensor technology \textit{in vivo}.

**Introduction**

Since their introduction in 1962 (Clark, Jr. and Lyons, 1962), biosensors have been improved, thus creating a wide variety of applications for off-line discontinuous monitoring in medicine (e.g. blood glucose, (Pickup, 1993)), industry (e.g. process control, (Renneberg et al. 1991)) and in environmental control (e.g. surface water, (Sadik and Van Emon, 1996)). Applications of biosensors for continuous \textit{in vivo} monitoring of patients -bedside or ambulant- are very limited. For such applications the sensors should not only be small, robust and easy to handle (also for minimally trained persons), but above all they must be biocompatible and give reliable performance in the complex matrix of body fluids (Reach and Wilson, 1992). When placed in blood, biosensors may cause clotting and placed in other body compartments, wound reactions and inflammation may occur (Lager et al. 1994). To avoid these complications, sampling by microdialysis and more recently by ultrafiltration (UF) has been proposed as an interface between the body and the sensor (Ash et al. 1992; Rosdahl et al. 1993; De Boer et al. 1994). Material for either sampling method is often obtained from artificial kidneys and well tested for biocompatibility. The membrane excludes cells and large molecules (proteins) in microdialysis and UF samples and may therefore be compatible with biosensor technology.

With UF body-fluid is withdrawn in a semi-permeable hollow fibre using a underpressure. Ash, Janle, Kissinger and others have developed a discontinuous subcutaneous UF-sampling technique (Ash et al. 1992; Linhares and Kissinger, 1993; Ash et al. 1993; Linhares and Kissinger, 1993). Ash et al. (1992) tested UF for 1 month in the human to monitor glucose, without significant decrease of the flow or of tissue reactions. However, these UF devices were too large for intravenous use. Recently small UF probes have been proposed (Linhares and Kissinger, 1993b; Moscone et al. 1996b). Major advantages of UF over microdialysis are, among others, the virtually 100% recovery with UF as compared to the often unknown and lower recovery of the analyte with microdialysis \textit{in vivo} (Lonnroth and Strindberg, 1995) and that no large, expensive and energy-consuming perfusion equipment are required. The currently constructed UF devices are light, cheap and small. Except for the method developed in our laboratory (Moscone et al. 1996), UF is performed by discontinuous sampling at rates of approximately 40-50 µl h\textsuperscript{-1}, approximately 1 ml day\textsuperscript{-1} (0.01 µl min\textsuperscript{-1} mm probe\textsuperscript{-1}) only (Ash et al. 1992b; Linhares and Kissinger, 1993b). Our UF-method allows continuous sampling over several days at the very constant low rate of about 100 nl min\textsuperscript{-1}. Low pulse-free filtration rates were produced with the underpressure of a disposable medical syringe provided with a fluid restriction.
Here, we explore the potential of the on-line ultrafiltration technique in vivo in anaesthetized rats. With a small probe we sample continuously in the extracellular space of subcutaneous tissue or intravenously with a flow rate of approximately 100 nl min\(^{-1}\). Glucose is used as a test analyte, thus the glucose concentration in the ultrafiltrates is compared to that in simultaneously sampled arterial blood. Glucose was detected electrochemically after enzymatic conversions in a flow injection analysis system. This approach allows us to characterize the various properties of the UF-system both in vitro and in vivo.

**Materials and methods**

**General description of the experiment**

Anaesthetized rats were provided with a femoral cannula for i.v. injection of glucose and blood sampling. An UF-probe was placed either in the jugular vein or subcutaneously. During the experiment the UF-probes were connected to the glucose detection system. The set-up of the apparatus is shown schematically in Fig. 3.1. The UF samples were withdrawn from the body by an underpressure created of a disposable syringe with a fluid restriction. This sampling system was coupled to a detection system consisting of an HPLC pump, an enzyme reactor containing horseradish peroxidase and glucose oxidase, an electrochemical detector, and a PBS buffer containing ferrocene. During the experiment, blood samples were taken manually from the femoral artery at 5-15 min intervals. The UF was analysed every 1-2.5 min by switching the position of the valve, thereby injecting ultrafiltrate into the detection system. I.p. and/or i.v. glucose injections were given to the rat to manipulate the glucose concentration artificially.

![Fig. 3.1. Set-up of the ultrafiltration sampling and analysis.](image)

**Fig. 3.1. Set-up of the ultrafiltration sampling and analysis.**

- A: air chamber
- B: buffer stock
- ECD: electrochemical detector
- Enz: enzyme reactor
- M: monovette
- Pr: probe
- Pu: pump
- R: restriction
- V: valve

41
Rats

Male Wistar rats (250-850 g, Harlan, Zeist, The Netherlands) were housed groupwise in a 12-12h light/dark regime. Food and drink were provided ad libitum. The rats were anaesthetized by an intramuscular (i.m.) injection of 0.4 mg kg$^{-1}$ body wt hypnorm (Janssen, Beerse, Belgium) and an intraperitontial (i.p.) injection of 0.24 mg kg$^{-1}$ body wt pentobarbital sodium (Sanofi, Maassluis, The Netherlands) and maintained anaesthetized with these drugs. A 0.28 mm inner diameter, 0.61 mm outer diameter polyethylene tube (Portex, Hythe, UK), filled with 500 E ml$^{-1}$ heparin in 0.9% NaCl, was inserted into the femoral artery to enable blood sampling. Using a rectal temperature probe and a heat pad, the body temperature was maintained between 36.5 and 37.5 °C. After the experiment, the probes were removed and placed in a glucose buffer and the rats were killed with an overdoses of pentobarbital sodium.

Blood glucose measurements

Blood samples were diluted ten times in 500 E ml$^{-1}$ heparin in 0.9% NaCl. After mixing, the samples were stored at -20°C for later analysis. Whole blood analysis was performed with a colorimetric method measured on the Technicon Autoanalysser, kindly made available by the Department of Animal Physiology of the University of Groningen (Anonymous, 1979). The concentrations of the glucose of the whole blood samples were increased with 15%, thus correcting for the volume of high molecular compounds (Marks, 1996).

Ultrafiltration glucose measurements

Our probe is a modified design of a previously described ultrafiltration probe (Moscone et al. 1996). We use a probe (fibers of an artificial kidney, AN69HF, acrylnitrile and sodium methallyl sulfonate copolymer, Filtral 16; Hospal Ind., Meyzieu, France, 290 µm outer diameter, 240 µm inner diameter) of 4 cm with a hand-made spring inside (stainless steel wire (Vogelsang, Hagen, Germany), D = 60 µm, 12 axial length windings cm$^{-1}$) to prevent collapsing of the fiber. This probe is connected to a 20-30 cm long fused silica tube (inner diameter 50 µm, outer diameter 150 µm, Polymicro Technologies Inc., Phoenix, Arizona, USA) screwed into a Rheodyne 7010 valve (Cotati, CA, USA). The link between the fused silica tube and the probe is made by inserting the tip of the tube into the fiber of the probe. The spring is glued to the fused silica tube, and the connection of between the fiber and the tube is closed with cyano-acrylic glue (Henkel, Nieuwegein, The Netherlands). The fiber is filled with water, and the tip of the fiber is closed with glue. The ultrafiltration flow (approximately 100 nl min$^{-1}$) is driven by the underpressure of a disposable syringe (1.2 ml Monovette, Sarstedt, Nümbrecht, Germany) by pulling and fixing the piston. One end of the restriction (a fused silica tube with a length of 4 cm, an inner diameter of 15 µm and an outer diameter of 150 µm) is glued into the syringe. The
other end of the restriction ends in a wider tube, trapping air bubbles, which otherwise obstructing the restriction. This chamber is connected to the valve with another piece of fused silica tube (50 µm diameter). The valve, switched pneumatically by a home made timer (typically, load/inject 30/30 seconds), has a loop of 20 µl which will be only partially filled with the ultrafiltrate.

Glucose is detected electrochemically using a bi-enzyme reactor in a flow injection system as described by Elekes et al. (1995). An HPLC pump (LKB 2150, Pharmacia Bromma, Sweden) pumps a ferrocene buffer, 137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 2.5 mM KH₂PO₄ (all of pro-analysis quality and purchased from Merck, Darmstadt, Germany), 0.5 mM ferrocenemonocarboxylic acid, FcA (Sigma Chemical Co, St. Louis, MO) and 0.1 vol % Kathon CG (Rhom and Haas, Croydon UK) in double quarts distilled water. The buffer (bubbled with helium to remove air) is pumped through an enzyme reactor and an electrochemical cell (Amor, Spark Holland, Emmen, The Netherlands) with a flow of 0.4 ml min⁻¹. In the enzyme reactor, 250 U Glucose oxidase, GOD (EC 1.1.3.4, grade I) and 250 U horseradish peroxidase, HRP (EC 1.11.1.7), obtained from Boehringer Mannheim (Germany), are immobilized between cellulose nitrate filters (thickness 100 µm; pore size 0.01 µm, cut-off 50 kDa, Sartorius, Göttingen, Germany). The cell is a thin layer-type cell, with a glassy carbon working electrode held at 0.00 mV relative to an Ag/AgCl reference electrode and a Teflon/carbon counter electrode connected to a digital electrochemical amperometric detector (Decade, Antec Leyden B.V., Leiden, The Netherlands).

**In vitro experiments**

Calibration curves were made by placing a probe in the ferrocene buffer with glucose concentrations of 0, 5, 10, 20 and 30 mM, changed stepwise every 20-30 min., increasing from 0 mM, and decreasing with the same steps. To determine the effect of the probe on lag-time, the spreading and the flow rate (see data processing), the calibration curve was repeated after removal of the probe from the animal. The UF was measured every minute with the ECD and the currents (in amperes) are recorded.

With the *in vitro* experiments, the possible selectivity of the membrane for water or glucose was tested, by comparing the signals with and without probe. The system delay (the lag-time) due to the volume of the connecting tube from the probe to the analysis system was calculated with the *in vitro* experiments, as well as the spreading in the system.

**In vivo experiments**

Before starting an *in vivo* experiment, the sensitivity and the lag-time of the system were determined *in vitro*. The UF system was filled with 5 or 10 mM glucose buffer and placed in the rat.
For subcutaneous measurements, the ultrafiltration probe was guided through a (with buffer filled) 16 G catheter needle (Vialon, Becton Dickinson, Meylan Cedex, France) in subcutaneous tissue on the back of the rats. Intravenous probes were inserted in the jugular vein through a hole in the vein made with a needle. The ultrafiltration samples were measured every minute. Blood samples of 50 µl were taken at a 5-15 minute interval from a canulla in the femoral artery. The glucose injections were given intraperitoneal (2.0 g kg⁻¹ body wt) or intravenous (0.3-0.4 g kg⁻¹ body wt). Five intravenous and five subcutaneous measurements were performed.

**Data processing**

**Lag-time**

The lag-time ± SEM is defined by the time interval changing the glucose concentration of the buffer to a 50 % change of signal from the initial signal to the next (see window Fig. 3.2A). This time interval is calculated by Sigmoidal-fitting of the values of the calibration curve for every single step.

**Spreading**

The spreading ± SEM, a quantitative index of the instrumental diffusion, is defined as the time between a signal change of 20 % to a signal change of 80 % (see window Fig. 3.2A). This parameter was calculated by Sigmoidal-fitting of the values of the calibration curve for every single step.

**Diffusion in the ultrafiltration probe**

The effect of the UF-probe on lag-time and spreading is determined by comparison of these parameters with and without the probe.

**Flow rate check in vivo**

The flow rate will influence both the lag-time and the amount of sample that is analysed. Both parameters will therefore provide information on the flow rate of the ultrafiltration.

The *in vivo* lag-time can be determined directly in intravenous experiments. Because the samples for the intravenous probe change immediately after an intravenous glucose injection, the increase of the signal will be influenced by the spreading and lag-time only. If the flow rate is lower than *in vitro*, a longer lag-time will be observed *in vivo*. 

44
The amount of sample injected is checked by comparison of the signal before and after transition of the probe from a buffer to the subcutaneous or venous compartment. As the tube (the approximately 30 cm long fused silica connecting the probe to the valve) is still filled with glucose buffer, a possible decrease in the flow caused by in vivo placement is seen as a decreased detection signal.

**Correlation ultrafiltrate glucose and blood sample glucose**

The correlation between intravenous ultrafiltrate glucose measurement and blood sample glucose was determined by a linear regression, after correction for the lag-time and the 15% addition in the whole blood sample glucose concentrations. The data were also compared using the Bland-Altman analysis (1986) on the relative differences of the glucose concentrations between the two methods.

The subcutaneous and the intravascular compartment are separated, but kinetically connected, so the subcutaneous measurements include a possible physiological difference in addition to (instrumental) lag-time. It is questionable whether there is a linear relationship between the glucose levels in either compartment. Therefore, no linear regression or Bland-Altman analysis has been performed on these data.

**Results**

**In vitro system evaluation**

**Sensitivity and calibration**

At the applied flow rate of 100 nl min⁻¹, 50 nl sample is injected into the detection system. The response is 29 nA mm⁻¹ for a fresh enzyme reactor, corresponding with 0.9 µA nmol⁻¹. After 4 weeks ex vivo usage this is decreased to approximately 5 nA mm⁻¹. The sensitivity during this period remained linear for physiological glucose concentrations throughout the experiment. This is checked by measuring the standard buffers before and after the in vivo experiment.

A photograph of a standard curve is shown in Fig. 3.2A. The currents of the standard curves, with and without a probe, are plotted in Fig. 3.2B and 3.2C. Regression analyses for the samples with a specific glucose concentration show a linearity for the measured concentrations (0-30 mM) with \( r > 0.99, p < 0.0001 \). There is no difference in the currents with and without probe, thus the barrier over the membrane of the probe for glucose and the buffer is similar.
Chapter 3

Lag-time and spreading

At a flow rate of 100 nl min\(^{-1}\), the lag-time of the system due to the volume between the UF-probe and the ECD is 7-15 minutes. Theoretically, for the used tube of 50 µm inner diameter, 30 cm length, this should be only 6 min (V=600 nl). The additional delay is due to the apparatus, most likely the (connection in the) valve and possible deviations in flow. The lag-time is therefore determined for every single experiment. The lag-time and the spreading in vitro are shown in Table 3.1. For the measurements with the probe, a lag-time of 9.4 ± 0.2 min is observed, whereas the spreading is 4.0 ± 0.7 min. Without the UF-probe the lag-time and spreading are 9.0 ± 0.3 and 3.2 ± 0.5 min, respectively. Thus, the probe increases the lag-time approximately half a minute, whereas the spreading increases with one minute.

Fig. 3.2. In vitro experiment A. Recorder output of standard curve (without probe). In window: Explanation of terms lag-time and spreading. B. In vitro regression curve the ultrafiltration system with probe for standard concentrations of glucose. C. In vitro regression curve the ultrafiltration system without probe for standard concentrations of glucose.
Table 3.1: In vitro standard curve.
The delay and the spreading (in parenthesis) are indicated in minutes in increasing and decreasing glucose concentrations with and without probe. See text for details and definitions.

<table>
<thead>
<tr>
<th>Glucose steps (in mM)</th>
<th>Delay (spreading) with probe in minutes</th>
<th>Delay (spreading) without probe in minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 &lt;=&gt; 5</td>
<td>9.7 (3.5)</td>
<td>9.4 (4.1)</td>
</tr>
<tr>
<td>5 &lt;=&gt; 10</td>
<td>9.1 (4.5)</td>
<td>9.6 (4.7)</td>
</tr>
<tr>
<td>10 &lt;=&gt; 20</td>
<td>9.7 (4.3)</td>
<td>9.5 (5.1)</td>
</tr>
<tr>
<td>20 &lt;=&gt; 30</td>
<td>9.2 (3.3)</td>
<td>9.3 (3.2)</td>
</tr>
</tbody>
</table>

Fig. 3.3. Intravenous ultrafiltration A. Recorder output of intravenous ultrafiltrate glucose monitoring. B, C. Examples of UF (——) and blood sample (----) glucose concentration with i.v. and i.p. glucose injections.
Chapter 3

In vivo system evaluation

Intravenous: Flow changes from in vitro $\rightarrow$ in vivo change and vice versa

Sometimes there was a significant but transient decrease of the flow rate after placing the probe intravenously, but this decrease is temporal.

Fig. 3.3 shows representative data from intravenous probes. In Fig. 3.3A, every peak represents a sample of 50 nl (for one minute) of an UF-sample. Figs. 3.3B and 3.3C shows the UF glucose concentrations together with the blood samples of two experiments. The lag-time of the UF-probe has been subtracted from the original time. Indicated are the i.v. glucose injection and the i.p. injection. When changing the glucose concentration artificially, the probe mimics the blood concentration found in the blood sample, although flattened. In high concentrations of the glucose, the UF values tend to be higher than the blood samples.

Intravenous: Comparison with sampled whole blood

Fig. 3.4A shows a linear regression with $r>0.995$, $p<0.0001$ of the blood samples and the UF for three rats. In this, values just after intravenous glucose injections (< 10 min) are omitted, because these are heavily influenced by the spreading of the system. Fig. 3.4B shows the Bland-Altman analysis on the relative concentration differences for the same measurements. The calculated 2SD (95% interval) comparing both methods is 15%.

![Graph A](image1.png)

![Graph B](image2.png)

Fig. 3.4. Mathematical analysis A. Linear regression on ultrafiltrate measurements versus blood sample glucose for three rats (indicated with different symbols) B. Bland-Altman plot of proportional differences versus the average of blood samples and UF. The proportional differences are the ratio between the difference of blood samples minus UF glucose levels and the mean value is taken as 100%.
A slow continuous ultrafiltration system

Subcutaneous: Comparison with sampled whole blood

Fig. 3.5 shows the data of two measurements with a subcutaneous probe. The glucose concentrations of the interstitium (measured with the ultrafiltrate probe) and the blood glucose (measured in the blood samples) are plotted against the time. The time axis for the UF has been corrected for the lag-time. Normally, no flow reduction was induced by the implementation of the probe subcutaneously. From the data we conclude that there is a clear difference between the subcutaneous and intravenous compartment. This is not simply a time delay, for the shape of the UF curves and intravenous curves are different.

![Graph A](image.png)

**A**

![Graph B](image.png)

**B**

Fig 3.5. A,B. Examples of glucose concentrations of interstitium UF (---) and blood (■■).  

Discussion

In this study, we shown some *in vitro* and *in vivo* experiments with continuous UF. We combined two of our previously developed techniques i.e. a slow UF technique, described previously by Moscone et al. (1996) and a flow injection analysis of small samples with a bi-enzymatically catalysed conversion of glucose, followed by electrochemical detection (Elekes et al. 1995). The *in vivo* experiments were performed in the subcutaneous and intravenous compartment. To our knowledge, this is the first study to apply UF intravenously. Not unexpectedly, glucose contents of the ultrafiltrate samples taken from blood reflected the blood levels far better compared to those of the subcutaneously obtained samples, both in temporal and quantitative aspects.

During our experiments, we kept the animals anaesthetized, although we described previously the potency of UF for monitoring circadian profiles of metabolism in freely moving animals. A problem in applying the 24h collection device is that the flow rate of the UF-probe can not be checked during the experiment as no direct connection to the glucose detection system can be made, which is the only way of checking the flow rate. Flow meters for these ultra low flow rates have yet to be developed. The applied filtration rates and the thus collected UF-volumes are too small to apply currently available swivels, which are used successfully in microdialysis.
In our set-up, the UF-sampling system is directly coupled to a flow injection assay for glucose. As a result of our analysis system, glucose peaks appear to be smoothed. Such an instrumental artefact has to be distinguished from the effects due to the configuration of the probe and to diffusion of glucose to the site of UF collection. The instrumental delay (lag-time) of the present system was about 10 min, mainly due to the valves and connecting tube to the detection unit, used for the introduction of the UF-samples into the (high flow and high-pressure) flow injection system. The UF-probes increases the lag time by about one (half) minute. The spreading was approximately one minute more than the three minutes created in the detection system. When other detection systems are combined with the UF probes in vivo, only the probe-induced lag-time has to be taken into account.

Continuous intravenous glucose measurements are performed well with the UF probe. In the experiments described here, there were two differences with the whole blood glucose sample concentration. The first difference was that the UF values are flattened, resulting in an apparent delay in fast changes. As described before, this can be prevented by using another detection system. Besides this, the fast changes shown in our experiments will hardly occur in normal physiological conditions. The second difference is that the values of the whole blood samples were somewhat lower compared to the UF samples at high concentrations of glucose. For the lower concentrations, the concentrations are perfectly similar. The bias in the high concentrations has not yet been explained. Further research, analysing serum as well as whole blood, might give insight into whether the difference is a physiological or technical effect. Because of the frequent sampling used in this experiment, we had to collect very small samples and were therefore not able to measure serum glucose.

A major point of concern is the subcutaneous concentration of glucose: we found a clear difference between blood and interstitial glucose, which was not just a time shift, as described in the literature (Fischer et al. 1994). The animals are anaesthetized during the experiments, the glucose balance and kinetics were affected, so physiological statements therefore have to be drawn carefully. However, we believe that our experiments show that the subcutaneous compartment is linked but not linearly related to the intravenous compartment. As a different profile is seen in the subcutaneous ultrafiltrate compared to the blood profile and no bleeding was seen around the probe at removal, research claiming no time difference between the subcutaneous and venous compartment (Rigby et al. 1995) may have introduced tissue damage so that glucose can diffuse directly from the vascular compartment rather than in the subcutaneous interstitium into the probe. Under steady state conditions the glucose levels are virtually the same in the subcutaneous interstitium as in blood. However, our results obtained in anaesthetized rats indicate that subcutaneous monitoring for glucose may attenuate and partially mask rapid changes in blood glucose. The concentration of glucose in the interstitial compartment is not only influenced by the concentration of the glucose in blood and the barrier between these compartments, but may also be influenced by uptake of the glucose in the surrounding cells. Further research is necessary to set up a kinetic model for this. However, whatever the kinetic model might be, the results of this research suggests that the interstitial compartment is less suitable for the control of glucose metabolism in diabetes patients with an artificial pancreas, as has often been proposed (Reach and Wilson, 1992). The issue of the relation between interstitial and blood levels of glucose clearly deserves
A slow continuous ultrafiltration system

further attention, in particular in future research in (non-anaesthetized) men. Slow UF may be helpful in such studies.

As stated in the Introduction, UF is the only technique that can give a 100% recovery of the analyte in vivo. The present study with glucose supports this claim, and shows that only the same correction as for serum has to be made to relate UF levels to whole blood glucose. UF is also suitable for studying pharmacokinetics in the living animal and perhaps men. With such applications of UF it should be recognized that only the freely circulating, not the protein bound fraction of the drug, contributes to the content of the filtrate. Accordingly, when drug levels in serum are determined as total concentrations rather than free drug concentrations, large discrepancies between drug levels of blood or serum and of UF-samples are to be expected.

In summary, we have developed a technique for continuous in vivo sampling that may have broad biomedical applications, for example in pharmacokinetics studies or metabolism research, and that can be combined with small -needle type- biosensors for ex vivo but on-line usage. We have illustrated the potency of slow continuous UF-sampling in blood and in the subcutaneous space, but there is no reason to assume that the current approach can not be applied to other compartments of the body, not only of animals, but of humans as well.

References

Chapter 3