Chapter 8

Twenty-four hours profiling of glucose in the subcutaneous tissue of healthy volunteers by means of a lightweight portable measuring device
Summary

A portable lightweight measuring device for continuous long-term in vivo monitoring of glucose in biological compartments is described. The measuring device consists of a flow-through glucose oxidase based biosensor of only a few nanoliters internal volume, a microdialysis probe and a disposable vacuum pump. A portable potentiostat equipped with data logging is used for detection and registration. Sampling and continuous on-line monitoring is carried out at submicroliter levels and as a consequence quantitative recoveries of glucose are achieved. Accordingly, excessive calibration procedures, as are necessary with conventional microdialysis, are avoided. The clean matrix obtained for measurements results in an improved stability and reliability of the biosensor, thereby creating the possibility to use the measuring device for long-term in vivo monitoring. The sensor is based on the amperometric detection of hydrogen peroxide after conversion of glucose by immobilised glucose oxidase. The clinical potential of this device was examined in freely moving healthy volunteers subcutaneously monitored for 24 hours. The data obtained were found to correlate well with the blood glucose levels determined throughout the day.

8.1 Introduction

Today, most biochemical parameters for the diagnosis of physiological abnormalities are measured batch-wise in discrete samples, whereas analysis takes place in the clinical laboratory. To allow rapid therapeutic intervention or to follow-up the progression of a disease, the need for methods that can measure continuously components in body fluids without the need of laboratory facilities is recognised. For instance for the home-care monitoring of diabetic patients, the ultimate goal would be the availability of a portable real-time device, which can continuously monitor glucose for long time (a week or more). Although biosensors are devices that have the potential to continuously monitor analytes in vivo, due to fouling and contamination of the electrode surface, the performance of a biosensor frequently diminishes during in vivo monitoring. For that reason, several approaches, non-invasive as well as invasive, have been proposed to overcome this problem. For instance, near-infrared detection of glucose has been proposed for non-invasive
monitoring of glucose\textsuperscript{2}. However, due to variations in sweat, changes in local blood circulation and/or interference from other body components, this technique is generally characterized as indicative. The GlucoWatch Biographer\textsuperscript{3}, a minimally invasive transcutaneous device using reverse iontophoresis as a sampling technique and an enzyme-based biosensor for analysis, can only be used for intermittent measurements. For measurements in the subcutaneous tissue, needle type biosensors with improved stability, such as the open flow microperfusion needle enzyme electrode\textsuperscript{4} and the commercial needle type biosensor CGMS\textsuperscript{5} have been presented for short-term applications. As an alternative to implanted needle glucose biosensors, microdialysis (MD) and/or ultrafiltration (UF)\textsuperscript{6,9} as a sampling interface between the body and the biosensor have been proposed. In both cases large molecules and cells are excluded by the semi-permeable membrane of the MD or UF probe and a relatively clean matrix is obtained for measurement leading to a subsequent improvement of the stability and reliability of the biosensor\textsuperscript{10,11}. Although the effect of probe implantation on the glucose measurements is still a matter of concern, characteristic features of these minimally invasive techniques is the stability and repeatability of the probe in living tissue and long operational period, which makes these techniques suitable for long-term monitoring. A major drawback of conventional MD is that the concentration of the analyte of interest is always lower in the dialysate than in the sampled interstitial fluid. To determine the nominal \textit{in vivo} concentration of the analyte of interest, complicated calibration methods have to be applied\textsuperscript{12}. A more straightforward method is, however, performing MD at extremely low flow rates\textsuperscript{13,14}. With current MD devices a near quantitative recovery is obtained at extremely low microdialysis flow rates (< 300 nl.min\textsuperscript{-1}) as the sample is able to equilibrate with the interstitial fluid. However, data obtained during a four hours oral glucose tolerance test in healthy volunteers, frequently showed a lower glucose content in the dialysate compared to blood. Although the effect of probe implantation cannot be fully neglected\textsuperscript{15}, data obtained during similar studies in our group clearly demonstrated the importance of the location of the MD probe\textsuperscript{16}. During these studies it was demonstrated that subcutaneous glucose measurements were shown to correlate better with blood levels provided that the probe was placed in subcutaneous loose connective tissue instead of adipose tissue. They found that glucose levels measured in the deep subcutaneous layer of loose connective tissue were very close to arterial levels, whereas glucose contents in adipose tissue were lower than in blood. Due to higher insulin levels they saw an increased glucose uptake by adipose cells at higher glucose levels. These insulin levels are mediated by GLUT4 glucose transporters, and are known to be more present in adipose than in connective tissue.
To allow, however, continuous *in vivo* monitoring at these low perfusion flow rates, the need for a small and low dead volume biosensor was recognised. For this reason, a flow-through biosensor with an internal volume of 10 – 20 nanoliter, as described elsewhere in detail, was developed by us for the continuous *in vivo* monitoring of glucose. The biosensor is based upon the amperometric detection of hydrogen peroxide after conversion of glucose by the immobilised enzyme glucose oxidase. Immobilisation of the enzyme was achieved by electropolymerisation of m-phenylenediamine, basically because immobilisation of biomolecules in these closed micro-channels can only be performed via this way. An additional advantage is that thanks to the self-controlling film thickness of this non-conducting polymer during electropolymerisation, biosensors can reproducibly be

*Figure 1:*

The measuring device comprised of a buffer reservoir (A), a MD probe (B), the biosensor (C) and a semi-vacuum pump (D). The biosensor is connected (E) to a home-made portable potentiostat. The dimensions of the miniature measuring device can be derived from the paperclip in the picture.
manufactured. With this biosensor, a lightweight device is composed as demonstrated in figure 1. The measuring device comprises a commercial available MD probe, the biosensor and a semi-vacuum pump as reported earlier18 and weighs less than 5 gram. The homemade portable potentiostat used for detection and registration of the analytical results is equipped with rechargeable battery and a data-logger and can be easily worn by attaching the instrument to a belt with the provided clip. Previous, this device has been validated during ex vivo experiments and some limited short-term in vivo tests19. However, in order to investigate the potential of the lightweight portable measuring device more thoroughly, in vivo monitoring of glucose in subcutaneous tissue of freely moving healthy volunteers was performed for a more extended period of time (24 hours). In this paper, the results of these in vivo experiments will be presented.

8.2 Materials and methods

8.2.1 Apparatus

For the production of the miniaturised biosensors a model DECADE electrochemical detector (Antec Leyden, Leiden, The Netherlands) and a model 22 syringe pump (Harvard Apparatus, Kent, United Kingdom) is used to pump the monomer solution through the flow-through cell during electropolymerisation. Signal output during electropolymerisation is recorded with a model BD112 flatbed recorder (Kipp & Zonen, Delft, The Netherlands). During the in vivo studies, detection is carried out at +0.5 V vs. Ag/AgCl and data is collected with a model Dextralert™ (Analytic Devices, Zeist, The Netherlands) portable potentiostat.

8.2.2 Materials

The enzyme glucose oxidase from Aspergillus niger (EC 1.1.3.4., grade I) is obtained from Boehringer Mannheim (Almere, the Netherlands). D(+) glucose for standard solutions and 1,3-phenylenediamine for the permselective membrane is purchased from Sigma Chemical Co. (St. Louis, MO). The composition of the carrier solution during microdialysis is a sterile 0.9% saline solution. Standard solutions of glucose are prepared by diluting the stock solution of glucose (50 mmolL⁻¹) in sterile 0.9% saline solution and are allowed to reach mutarotational equilibrium before use (24 hr). All other chemicals are of pro-analysis quality.
and are purchased from E. Merck (Amsterdam, The Netherlands). Double quartz distilled water is used for all other aqueous solutions.

For the construction of the flow-through biosensor, tygon tubing (ID 0.005 inch) is purchased from Skalar Analytical (Breda, the Netherlands). The auxiliary and work electrode is made from platinum wire (0.10 mm diameter) whereas the reference electrode is made from silver wire coated with AgCl (0.125 mm). All these materials are purchased from Drijfhout (Amsterdam, the Netherlands). Low dead volume connections are made with fused silica tubing (150 µm OD, 50 µm ID) (Aurora Borealis Control, Assen, the Netherlands). Connections are glued with cyanoacrylic glue (Henkel, Nieuwegein, the Netherlands).

Sampling is performed by means of ultraslow microdialysis thereby using a CMA 60 microdialysis probe (Aurora Borealis Control, Assen, the Netherlands).

### 8.2.3 Production of the measuring device

Production of the measuring device is essentially carried out as described in detail elsewhere. By pushing a 0.50 x 16 mm Luer Lock needle (B.Braun, Melsungen, Germany) perpendicularly through a 0.005 inch ID tygon tubing, consecutively two platinum wires and a Ag/AgCl wire are placed within 1-2 mm to each other into the 0.005 inch ID tygon tubing. A multi-meter was used to check the correct position of the electrodes in the tubing. Leakages in the tygon tubing are eliminated with cyanoacrylic glue (Henkel, Nieuwegein, the Netherlands) and the flow-through cell thus obtained is washed with respectively methanol (pro analysis, E. Merck, Amsterdam, The Netherlands), 10 %v/v hydrogen peroxide solution in water (pro analysis, E. Merck, Amsterdam, The Netherlands) and 0.1 M phosphate buffer pH 6.9. Electropolymerisation is performed at +0.8 V vs. Ag/AgCl for one hour at a flow rate of 0.5 µl.min⁻¹ using a solution containing 2 mg.ml⁻¹ of enzyme and 10 mg.ml⁻¹ of 1,3-phenylenediamine in 0.1 M phosphate buffer pH 6.9, followed by electropolymerisation for an additional 30 minutes using the monomer solution without the enzyme. Afterwards, the biosensors thus produced are rinsed with 0.1 M phosphate buffer pH 6.9 for 30 minutes at a flow rate of 0.5 µl.min⁻¹ and stored in the refrigerator at 4-8 °C. The measuring device was constructed by connecting one side of the flow-through biosensor with the microdialysis probe and the other side with the semi-vacuum syringe pump (1.2 ml monovette, Sarstedt, Nümbrecht, Germany) as previously described. Low dead volume connections between the different parts of the device are made with 4 cm fused silica tubing (150 µm OD, 50 µm ID) (Aurora Borealis Control, Assen, the Netherlands).
8.2.4 Performance characteristics of the measuring device

The device had been validated earlier in detail for its accuracy, precision, linearity, sensitivity, selectivity and stability during ex vivo experiments. The linearity was found to be up to 30 mmol·l⁻¹ with a detection limit of 0.05 mmol·l⁻¹. The precision was found to be 2-4%, whereas no contribution to the signal could be observed from electroactive species, such as ascorbic acid and uric acid. The accuracy of the device had been investigated by analysing 50 serum samples for their content of glucose. The results were compared with those obtained from the clinical laboratory, which have been analysed with validated methods, and demonstrated that the accuracy of the device was found to be well in accordance with the criteria set for methods of Self Monitoring of Blood Glucose. After an initial decrease from 100% to 70% in sensor response within several hours of practice, no further loss of the signal for three consecutive days was demonstrated during continuous monitoring of a dialysate of a standard solution of 5 mmol/l of glucose or a serum sample respectively. The semi-vacuum syringe pump, as reported earlier, produces a stable flow rate of 300 nl·min⁻¹ for almost a week and does not need additional batteries. The portable potentiostat, the Dextralert™ detects and collects data and files every minute the mean value of the sampled minute in its internal value.

8.2.5 Twenty-four hours profiling of glucose in subcutaneous tissue

In vivo tests were performed with healthy young male and female subjects. All subjects gave their informed consent and the study was approved by the Ethical Committee of the University of Groningen. On day 1, a microdialysis probe was placed in one side of the umbilicus in the subcutaneous loose connective tissue by means of a 16G catheter. At day 2, subjects were monitored from 9.00 a.m. up to 24 hours until the next day 9.00 a.m. At 8.00 a.m. the implanted probe was washed and de-aerated with sterile 0.9% NaCl solution. One end of the probe was connected to the lightweight measuring device, as described and provided with a fresh biosensor, whereas the other end to a 1 ml buffer reservoir containing sterile 0.9% saline solution. By pulling the plunger of the semi-vacuum syringe pump a vacuum was created and sampling was started. The dialysate thus obtained was continuously analysed for at least 24 hours at a flow rate of 300 nl·min⁻¹. After reaching a steady state (mosty within 30 minutes) the blood glucose content was determined every hour until 6.00 p.m. Blood samples were taken by finger pricking and the content of glucose was analysed with the Accutrend method (Boehringer Mannheim, Almere, The
Netherlands). After 6.00 p.m. the subject was free to go. At day 3, following an overnight monitoring, the last blood sample was taken at 9.00 a.m. and the subject was disconnected from the measuring device. After that, the measurements obtained were recorded via the Dextralert™ software program installed on a Windows 95/98/NT PC.

To stabilise the biosensor and to examine the performance, the measuring device was calibrated at day one and at day 3, prior after the study, by means of a standard solution containing 5 mmol.l⁻¹ of glucose.

8.2.6 Presentation of the data

The results are presented as prescribed for methods of Self Monitoring of Blood Glucose for patients with diabetes mellitus²¹. This method describes an error grid analysis where the x-axis represents the reference blood values and the y-axis the value generated by the measuring device tested, whereas the diagonal represents the perfect agreement. Based upon the assumption that the target blood glucose levels ranges from 70 – 180 mg/dl, the grid is divided into five regions of varying degrees of accuracy. In short, values found in zone A and B are clinically acceptable, whereas values in zone C, D and E are potentially dangerous for patients and are therefore clinically significant errors.

8.3 Results and discussion

To enable real time continuous on-line monitoring at very low flow rates (less than 0.5 µl/min), the internal volume of the measuring device has to be extremely small. In this case a device is presented which is equipped with a flow-through biosensor with an internal volume ≤ 20 nl. Connections between the microdialysis probe and biosensor are such that the internal volume of the measuring device is calculated to be less than 100 nl. In combination with the CMA 60 MD probe, which has a relative large internal volume, a total delay time of approximately 3 minutes was found at a dialysis flow rate of 300 nl/min. The device has been validated earlier in detail for its performance characteristics¹⁹. Based upon the results obtained during these ex vivo tests and some limited short-term in vivo tests, the potential of this lightweight portable measuring device was further investigated. Additionally, to avoid deviations between the blood and subcutaneous glucose levels due to glucose gradient differences in subcutaneous tissue, as mentioned by others¹⁶, it was decided to place the probe in the subcutaneous loose connective tissue of the subject. For
practical reasons and to comfort the volunteer, the MD probe was placed only one day before the measurements. The next day, continuous in vivo measurements were carried out up to twenty-four hours whereas during these measurements the volunteers were entirely free to move.

Some typical data obtained during this study are presented in Figure 2 (A till F). To mimic the practical use of this measuring device as much as possible, calibration of the biosensor was carried out on the first blood glucose value only. The data presented are corrected for the delay time, which was approximately 3 minutes. As can be seen in Figure 2, in most cases the content of in the dialysate reasonably follows the blood glucose content. However, probably due to bad connections between the electrical parts of the measuring device, a relatively large noise in the baseline was observed, whereas in some cases even a default value was given corresponding to an overload of the potentiostat.

**Figure 2:** Typical graphs obtained during the in vivo monitoring of glucose in subcutaneous tissue by ultraslow MD for twenty-four hours in freely moving healthy volunteers: (a) till (f).

Blood glucose concentrations in time (■), and subcutaneous glucose concentrations in time (◆). Calibration of the biosensor was performed on the first blood glucose value measured.
Nevertheless, if the results are presented as prescribed for methods of Self Monitoring of Blood Glucose for patients with diabetes mellitus, a good correlation was observed. As can be seen in figure 3, the results are well in line with the criteria set, and no results were found in zone C, D and E.

Regarding the performance of the measuring device after a period of 24 hours monitoring, a mean loss in of 3% in signal was found for all biosensors tested; this loss is in accordance with earlier observations and was found to be negligible and felt within the deviation of the output of the measuring device.

Based upon the present results, the studies will be extended by the monitoring of both healthy volunteers and diabetic patients for a longer period of time (a week). Before we are capable to do so, the robustness of the device needs to be improved. Additionally, extended stability studies will have to be performed to proof the performance of these biosensors necessary during these long-term monitoring studies. As an alternative or in addition, the connection between the measuring device and the MD probe needs to adjusted such, that the (disposable) parts of the measuring device (biosensor and pump) outside the body can be easily replaced by any person without the need of trained personnel.

![Error grid analysis for the evaluation of clinical implications of patient-generated blood glucose values. The Y-axis represents the values in the subcutaneous sampled interstitium by means of ultraslow MD; the X-axis represents the values determined in the blood samples by means of a validated method.](image-url)
8.4 Conclusions

A portable lightweight measuring device of less than 5 gram is presented, which comprises a sampling unit (MD probe), a miniaturised flow-through biosensor and a semi-vacuum pump. Owing to the low perfusion rate, (near) quantitative in vivo recoveries are established which circumvents excessive calibration normally used for microdialysis based measuring devices. Provided that the MD probe was placed in the subcutaneous loose connective tissue, a good correlation was found with blood glucose levels during a twenty-four hours profiling of glucose in the subcutaneous tissue of freely moving healthy volunteers. Our next goal is to test this measuring device for the monitoring of glucose for up to a week without excessive and difficult calibration steps in both volunteers and patients suffering from diabetes mellitus.

8.5 References

Chapter 8


