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Design and development of a miniaturised flow-through measuring device for the in vivo monitoring of glucose and lactate

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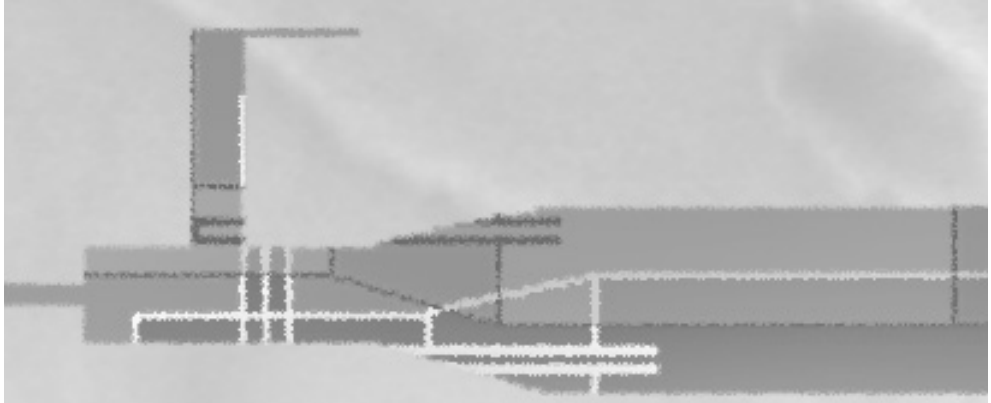


Chapter 7

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

Summary

A lightweight measuring device for continuous *in vivo* monitoring of glucose in biological compartments is presented. The measuring device consists of a miniaturised flow-through biosensor, connected to a microdialysis probe and a semi-vacuum pump. The biosensor is based on the amperometric detection of hydrogen peroxide after conversion of glucose by immobilised glucose oxidase. A portable potentiostat equipped with data logging is used for detection and registration. The device is validated for its accuracy, precision, linearity, selectivity and stability during ex vivo and *in vivo* experiments. The accuracy was found to be well in accordance with the criteria set for methods of Self Monitoring of Blood Glucose for patients with diabetes mellitus. *In vivo* monitoring of glucose in dialysate of subcutaneous sampled tissue during glucose tolerance tests in healthy volunteers demonstrates the potential of this measuring device.

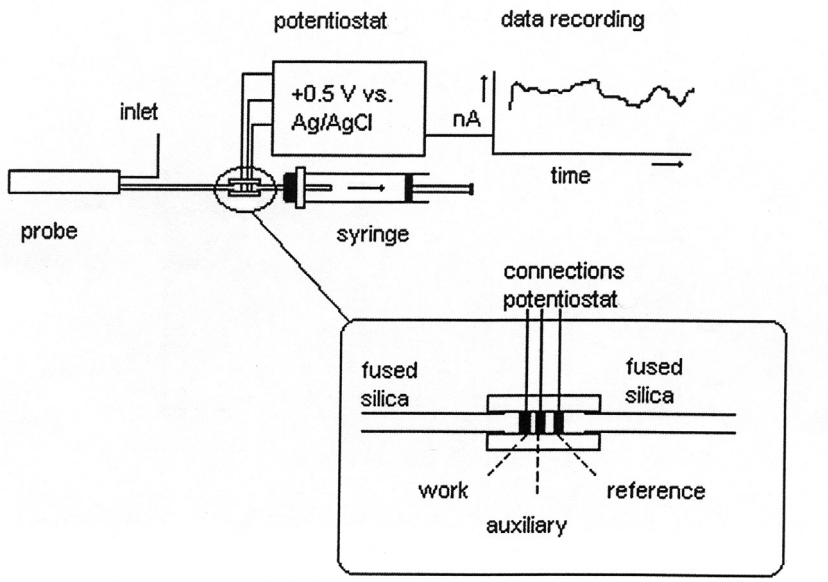
7.1 Introduction

Diabetes mellitus is a chronic disorder characterised by insulin deficiency, hyperglycaemia and a high risk of the development of irreversible damage to eyes, kidneys, peripheral nerves, heart and blood vessels[1,2]. A long-term study performed by the Diabetes Control and Complications Trial Research Group[3] has conclusively demonstrated that if glucose levels are tightly regulated diabetic complications are reduced. In addition, hypoglycaemic events can be prevented. In practice, blood glucose levels are controlled with the finger-prick method. The measurements thus obtained however are limited and inform only about the blood glucose levels at intermittent moments. Continuous *in vivo* glucose monitoring may therefore be a significant improvement. For this reason, throughout the years elaborate research efforts have been devoted to the development of a glucose sensor[4]. Biosensors may not only offer a viable route for continuous real-time glucose monitoring carried out by clinical laboratories at the patient's bedside or during operations but also for self monitoring at home[5]. An ideal biosensor has many advantages over conventional analysis such as reagentless analysis and independence of laboratory facilities. Several investigators reported the use of a peroxide based needle-type amperometric biosensor, that can be easily manufactured and miniaturised. To avoid thromboembolism and complications arising

from surface fouling of the electrode by proteins and coagulation composites (e.g. fibrinogen/fibrin, platelets, leukocytes and red cells), the needle-type glucose sensor was most often inserted in the subcutaneous tissue[6-12]. As an alternative to implanted needle glucose biosensors, microdialysis (MD) and/or ultrafiltration (UF)[13-24] as a sampling interface between the body and the biosensor have been proposed. These techniques are regarded as minimally invasive because the needles required for the insertion of the MD or UF probe are relatively small[25]. And although the effect of probe implantation on the glucose measurements is still a matter of concern, these sampling techniques have demonstrated an improvement of the stability and reliability of the biosensors tested thus far[26,27]. The sampling principle of MD is based on diffusion down the concentration gradient existing between the perfusion fluid (dialysate) and the outside environment of the semi-permeable membrane (interstitial fluid). Small analytes, such as glucose, are perfused whereas the semi-permeable membrane excludes cells and/or large molecules. A relatively clean matrix is obtained which is transported by pumping and analysed outside the body with a glucose biosensor. A major drawback of conventional MD is that the concentration of glucose is always lower in the dialysate than in the sampled interstitial fluid, and excessive (*in vivo*) calibration procedures are required for accurate monitoring[28]. The *in vivo* recovery, the dialysate/interstitial concentration ratio, depends on variables such as probe dimensions and perfusion flow rate as well as on tissue characteristics as tissue tortuosity, extracellular volume and metabolism[29-31]. A generally accepted method for the quantitative analysis of the interstitial concentration is based on the principle that diffusion of substances from the interstitial fluids tends to reach equilibrium between dialysate and interstitial concentrations at extremely low perfusion flow rates[32,33]. In our laboratory, (near) quantitative *in vivo* recoveries have been observed at flow rates of less than 300 $nl.min^{-1}$ (ultraslow MD)[28]. 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. Because, to our knowledge, no hardware is yet available to perform *in vivo* sampling by ultraslow MD followed by on-line continuous analysis, a portable lightweight measuring device was developed by us as demonstrated schematically in figure 1. The measuring device comprises a MD probe, a flow-through biosensor with an internal volume of 10 – 20 nanoliter and a semi-vacuum pump. The semi-vacuum pump is capable of producing a stable low flow rate for several days[34] and does not need (additional) batteries. A home-made portable potentiostat equipped with rechargeable battery and a data-logger is used for detection and registration of the analytical results during monitoring.

In this paper the construction and production of the lightweight portable measuring device

Figure 1:
Schematic demonstration of the instrumental set-up.



are presented. The performance characteristics (precision, selectivity, linearity, accuracy and stability) of the measuring device, which have been tested *in vitro* and ex vivo, will be described. The performance of the measuring device *in vivo* was assessed with glucose tolerance tests (OGTT).

7.2 Materials and methods

7.2.1 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). 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 aqueous solutions containing 0.1% (by volume) Kathon CG (Rhom and Haas, Croydon, UK)

to inhibit bacterial growth. The composition of the carrier solution during microdialysis is a Dulbecco's buffer phosphate-buffered saline (PBS) (mmol.l^{-1}): NaCl (136.9), KCl (2.7), KH_2PO_4 (1.5), $CaCl_2$ (0.9), $MgCl_2$ (0.5), Na_2PO_4 (8.1) and EDTA (2). The pH is adjusted to pH 7.4 and purged with helium before use. Standard solutions of glucose are prepared by diluting the stock solution of glucose (50 mmol.l^{-1}) in PBS and are allowed to reach mutarotational equilibrium before use (24 hr).

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). Connections with the flow-through biosensor are made by using 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).

During *in vitro* and *in vivo* measurements, sampling is performed by means of ultraslow microdialysis thereby using a CMA 60 microdialysis probe (Aurora Borealis Control, Assen, the Netherlands).

7.2.2 Production of the flow-through biosensor

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 close to each other into the 0.005 inch ID tygon tubing (see figure 1). By means of a multi-meter the correct position of the electrodes in the tubing is checked. Possible leakage formed in the tygon tubing is eliminated with cyanoacrylic glue (Henkel, Nieuwegein, the Netherlands). The flow-through cell thus prepared is washed prior to electropolymerisation by connected the cell with a 1 ml syringe (Becton Dickinson, Etten-Leur, the Netherlands) equipped with a 0.40 x 12 mm Luer Lock needle (B.Braun, Melsungen, Germany). The flow-through cell as well as the electrodes are washed by filling the syringe and pushing the following solutions consecutively through the flow-through cell: methanol (pro analysis, E. Merck, Amsterdam, the Netherlands), 10 %v/v hydrogen peroxide solution in water (pro analysis, E. Merck, Amsterdam, the Netherlands) and finally 0.1 M phosphate buffer pH 6.9. After washing the syringe is filled with 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. The syringe is placed in a model 22 (Harvard Apparatus, Kent, United

Kingdom) syringe pump. The flow-through cell is connected to the syringe and the wires outside the tubing of the flow-through cell are connected to a model DECADE electrochemical detector (Antec Leyden, Leiden, the Netherlands) by means of crocodile clips. Electropolymerisation is performed at +0.8 V vs Ag/AgCl for one hour at a flow rate of 0.5 µl.min^{-1} followed by electropolymerisation for an additional 30 minutes using the monomer solution without the enzyme. Before storage and/or use, the biosensors are rinsed with 0.1 M phosphate buffer pH 6.9 for 30 minutes at a flow rate of 0.5 µl.min^{-1}. In between, the flow-through biosensors thus produced are stored in the refrigerator at 4–8 °C.

7.2.3 Testing of the flow-through biosensor by means of Flow Injection Analysis

For the *in vitro* testing of the flow-through biosensor, measurements are carried out by means of Flow Injection Analysis (FIA). The flow-through biosensor is connected to a VICI Cheminert C4 valve (Valco Instruments, Houston, USA) and equipped with a 100 nanoliter internal loop for sample injection. With the model 22 (Harvard Apparatus, Kent, GB) syringe pump, carrier solution and sample is transported to the biosensor at a flow rate of 5 µl/min. The internal loop of the valve is continuously filled with dialysate by connecting the microdialysis probe and a home-made semi-vacuum syringe pump (1.2 ml monovette, Sarstedt, Nümbrecht, Germany) as previously described[34] each at a side of the VICI Cheminert C4 valve. Dialysis is carried out at a flow rate of 300 nl.min^{-1} by means of under-pressure. To avoid high dead volumes, connections between the various parts of the flow injection analysis system are made with 150 µm OD x 50 µm ID fused silica capillary (Aurora Borealis Control, Assen, the Netherlands). Injection and analysis of the sample is carried out every two minutes. Detection is carried out at +0.5 V vs. Ag/AgCl by connecting the wires outside the tubing of the flow-through cell to a model DECADE electrochemical detector (Antec Leyden, Leiden, the Netherlands) by crocodile clips. Data is recorded on a model BD 112 flatbed recorder (Kipp & Zonen, Delft, the Netherlands).

7.2.4 A lightweight measuring device for the continuous monitoring of glucose

For the continuous monitoring of glucose during *in vitro* and *in vivo* experiments, a lightweight measuring device (see figure 1) was constructed. One side of the flow-through

biosensor is connected with the microdialysis probe whereas the other side is connected with the semi-vacuum syringe pump (1.2 ml monovette, Sarstedt, Nümbrecht, Germany) as previously described[34]. Low dead volume connections between the different parts of the device are made with fused silica tubing (150 µm OD, 50 µm ID) (Aurora Borealis Control, Assen, the Netherlands). Dialysate is sampled by means of under-pressure and is continuously analysed by the flow-through biosensor at a flow rate of 300 nl.min^{-1}. Detection is carried out at +0.5 V vs. Ag/AgCl and data is collected with a model Dextralert^{TM} (Analytic Devices, Zeist, the Netherlands) portable potentiostat.

7.2.5 Oral glucose tolerance test (OGTT)

OGTTs were performed with healthy young 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 is placed in one side of the umbilicus in the subcutaneous fat tissue by means of a 16G catheter. At day 2, following an overnight fast, subjects are monitored from 8.30 a.m. until 1 p.m. At 8 a.m. the implanted probe is washed and de-aerated with sterile 0.9% NaCl solution. One end of the probe is connected to the lightweight measuring device as described, whereas the other end of the probe is connected to a buffer reservoir containing sterile PBS buffer (without Kathon CG). By pulling the plunger of the semi-vacuum syringe pump a vacuum is created and sampling is started. The dialysate thus obtained is continuously analysed for 5 hours at a flow rate of 300 nl.min^{-1}. After reaching a steady state (mostly within 30 minutes), the subjects received 100 g of glucose dissolved in 200 ml of water via an oral administration. During the OGTTs, blood samples were taken from a forearm vein cannula and analysed by the clinical laboratory with an ECA 180 glucoanalyser (Medingen GmbH, Dresden, Germany). To correct for the difference between glucose molarity (mol/l blood) of haemolysed whole blood and molality (mol/kg water), the data obtained from the blood samples are multiplied by 1.176 as prescribed by the manufacturer's report. In this way a correct comparison can be made between the content of glucose in blood versus dialysate. At the end of the study, the subject is disconnected and the flow-through biosensor is coupled to the microdialysis probe, which is placed in PBS buffer containing 5 mmol.l^{-1} of glucose for one-point calibration.

7.3 Results and discussion

7.3.1 Performance characteristics

The determination of glucose is based upon the conversion of glucose by the immobilised glucose oxidase (GOx) into the electrochemically detectable product, hydrogen peroxide. To enable the efficient immobilisation of the enzyme and to improve the performance characteristics of the final biosensor, we preferred a permselective membrane based upon the electropolymerisation of the monomer 1,3-phenylenediamine in the presence of the enzyme GOx. Accordingly, a substrate diffusion-limiting barrier is introduced which prevents the reaction from following the intrinsic enzyme kinetics and as a consequence extends the linear range. The linearity is investigated by analysing standard solutions of glucose in the FIA and the continuous mode. For both modes a linearity of up to 30 mmol.l^{-1} of glucose is found. The signal/noise (S/N = 3) ratio indicates a limit of detection of 0.5 mmol.l^{-1} in the FIA mode and 0.05 mmol.l^{-1} in the continuous mode. This means that the linear range as well as the sensitivity of the biosensor is sufficient for the determination of glucose levels in diabetic patients. The precision of several biosensors (n = 10) was tested by analysing a standard solution of 5 mmol.l^{-1} of glucose in six-fold in the FIA mode. The repeatability, expressed as the relative standard deviation in the peak height after six consecutive injections was found to be 2-4% depending on the biosensor tested. To investigate the selectivity of the biosensor regarding interference from electroactive species, standard solutions, containing 0.1 mmol.l^{-1} ascorbic acid or 0.25 mmol.l^{-1} uric acid in the presence of 0, 5 or 25 mmol.l^{-1} of glucose, are analysed in the continuous mode with several (fresh and one week old) biosensors. No significant contribution to the signal is observed and demonstrated the selectivity and stability of the permselective membrane.
Regarding the stability of the measuring device, it has to be emphasised that due to electrode fouling by small endogenous proteins and the hydrogen peroxide mediated enzyme inactivation, theoretically, *in vivo* measurements will not take longer than a couple of days without re-calibration of the device. For this reason, it was decided to test the stability of several flow-through biosensors by monitoring for only up to three days in the continuous mode respectively a standard solution of 5 mmol.l^{-1} glucose and a dialysate of serum sample containing approximately 6 mmol.l^{-1} glucose. It was observed that after an initial decrease in sensitivity from 100% to approximately 70% within several hours of practice, the biosensors remained their activity during the period tested. This is in accordance with data reported by others[35], who attributed this initial decline to leaching

out of not fully immobilised enzyme and/or polymer film. Although more studies are performed to evaluate the storage conditions and to increase the stability of the biosensors in use and during storage, the biosensors are sufficiently stable for the purpose of this study. To examine the accuracy of the measuring device, ex vivo studies have been conducted by analysing serum samples obtained from the clinical laboratory for their content of glucose in the FIA mode. By comparing the results with those obtained from the clinical laboratory which have been analysed with validated methods, the accuracy of the measuring device is determined. The results are presented as prescribed for methods of Self Monitoring of Blood Glucose for patients with diabetes mellitus. For this reason the method presented by Clarke et al[36] was used. They described the error grid analysis, where the x-axis represents the reference blood values and the y-axis the value generated by the measuring device tested. The diagonal represents perfect agreements whereas data points above and below the diagonal represent overestimates and underestimates. Based on the assumption that the target blood glucose level ranges from 70 – 180 $mg.dl^{-1}$, 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. As can be seen in figure 2, the results are well in line with those obtained by the clinical laboratory; a correlation of $Y = 0.98X + 0.19$ with a correlation coefficient of 0.99 for $n = 54$ was calculated. No results were found which deviated more than 20% from the reference value. Based on these results, it was decided to proceed with *in vivo* monitoring studies.

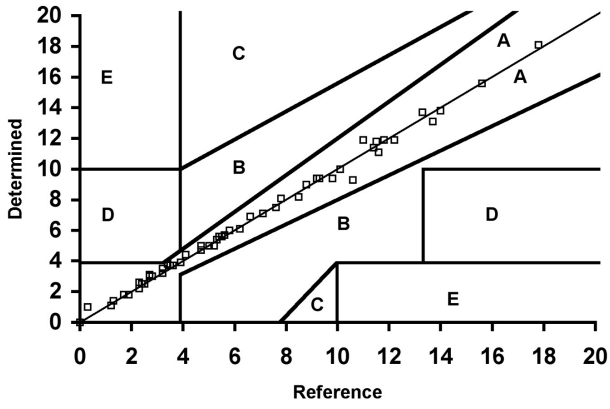


Figure 2:
Error grid analysis for the evaluation of clinical implications of patient-generated blood glucose values. The Y-axis represents the values determined in the serum samples by means of the biosensor; the X-axis represents the values determined in the serum samples by means of validated clinical methods.

7.3.2 The lightweight measuring device

Although numerous parameters determine the performance of the sampling by MD, in general (near) quantitative recoveries are obtained at very low flow rates (less than 0.5 $\mu l.min^{-1}$)[28]. To enable, however, real time on-line monitoring of the dialysate, the internal volume of the measuring device has to be extremely small. By using tygon tubing with a defined diameter, a flow-through cell with a defined volume can be easily constructed. With an internal diameter of 0.005 inch (0.127 mm), the tubing has a specified volume of 127 $nl.cm^{-1}$. If the electrodes are positioned within 1-2 mm of each other, a cell with a total internal volume of 10-20 nanoliter is obtained. By connecting the flow-through cell with, for instance, 4 cm of 50 µm ID x 150 µm OD fused silica tubing (with a specified volume of 20 $nl.cm^{-1}$), the total internal volume of the measuring device is 100 nanoliter or less. This means that at a flow rate of 300 $nl.min^{-1}$, the delay time between sampling and analysis is less than a minute. Because of the relatively large internal volume of the CMA 60 MD probe however, a total delay time of approximately 2-3 minutes was found. Although this delay time is one of the smallest reported for a MD based measuring device, *in vivo* correlation studies will have to prove whether or not this delay time will be sufficiently low for adequate and practical use of diabetic patients.

By using a semi-vacuum syringe pump, as described earlier in detail[34], a stable flow rate of 300 $nl.min^{-1}$ can be produced for up to several days[3] without the need of (additional) batteries. This means that the wet part of the measuring device outside the body (biosensor, pump, fused silica and electrical connections and housing) weighs less then 5 gram, which is negligible, compared to the insulin pumps worn by many diabetic patients.

For detection and data collection, a portable potentiostat provided with internal data-logging and rechargeable battery is used. The Dextralert™ collects continuously data and every minute the mean value of the sampled minute is filed in its internal memory. The results can directly be recorded by connecting the Dextralert™ to a flatbed recorder or via the Dextralert™ software program installed on a Windows95/98/NT PC. This first prototype of the Dextralert™, with a weight of approximately 250 gram can be easily worn by attaching the instrument to a belt with the provided clip.

7.3.3 Oral glucose tolerance test (OGTT)

To demonstrate the applicability of the lightweight measuring device described in this article, the first set of results of our ongoing *in vivo* studies are presented. On the first day,

a MD probe is placed in one side of the umbilicus in the subcutaneous fat tissue by means of a 16G catheter. On the second day a fresh biosensor is connected to the implanted MD probe after allowing to reach an equilibrium for 30 minutes. Dialysate is then continuously analysed at a flow rate of 300 nl.min^{-1} as schematically demonstrated in figure 1. During the OGTT blood samples are collected and analysed in the clinical laboratory on their content of glucose. In figure 3, some typical data obtained during the OGTT on healthy volunteers are demonstrated. The data presented are corrected for the delay time, which is found to be several minutes. As can be seen in figure 3a, in one case the content of glucose in the dialysate reasonably follows the blood glucose content. However, frequently we have observed a lower glucose content in the dialysate (see figure 3a) compared to blood and a relative low correlation was found ($Y = 0.82$, $R^2 = 0.56$) (see figure 4a). Based upon the *in vitro* and *ex vivo* performance characteristics of the measuring device, this phenomenon can not (only) be attributed to the measuring device. We believe, as already suggested by others[37,38], that the values found in the interstitial fluid are influenced by the characters of the tissue surrounding the probe. The present results are comparable with those obtained during similar studies[24] applying other (validated) analytical methods. For instance Wientjes[37] demonstrated increasing glucose recoveries in interstitial fluid until a plateau is reached 6-9 days after placing the probe. Especially the (individual) change in recovery during the first day was significant. This effect was explained by him that insertion of the probe can cause trauma to cells and capillaries. As a consequence, no (fully) functional layer of tissue surrounds the probe and limits diffusional glucose transport. The increase of glucose recoveries during the days after implantation follows from repair of the tissue microstructure around the probe. Tiessen et al[24] also observed relatively low interstitial glucose contents compared to blood glucose levels in fat tissue. He attributed this effect to that, depending on the location of the MD probe, the diffusion path of glucose as well as possible glucose uptake by the cells in the subcutaneous interstitium may differ. Although the amount of data are in this stage too limited to draw conclusions concerning this phenomenon, it seems worthwhile to investigate whether or not a better correlation is obtained if the measuring device is calibrated by means of the glucose levels found in blood. Especially during home-care, diabetic patients must be able to calibrate and control in a simple way their measuring device. It has to be emphasised however that, in order to improve the quality of life of diabetic patients excessive calibration should be avoided. For this reason, the data from the sampled interstitium are recalculated on the first blood glucose value only. The results are demonstrated in figure 3b and 4b, and as can be seen from both figures a better correlation is found between the values in blood and dialysate

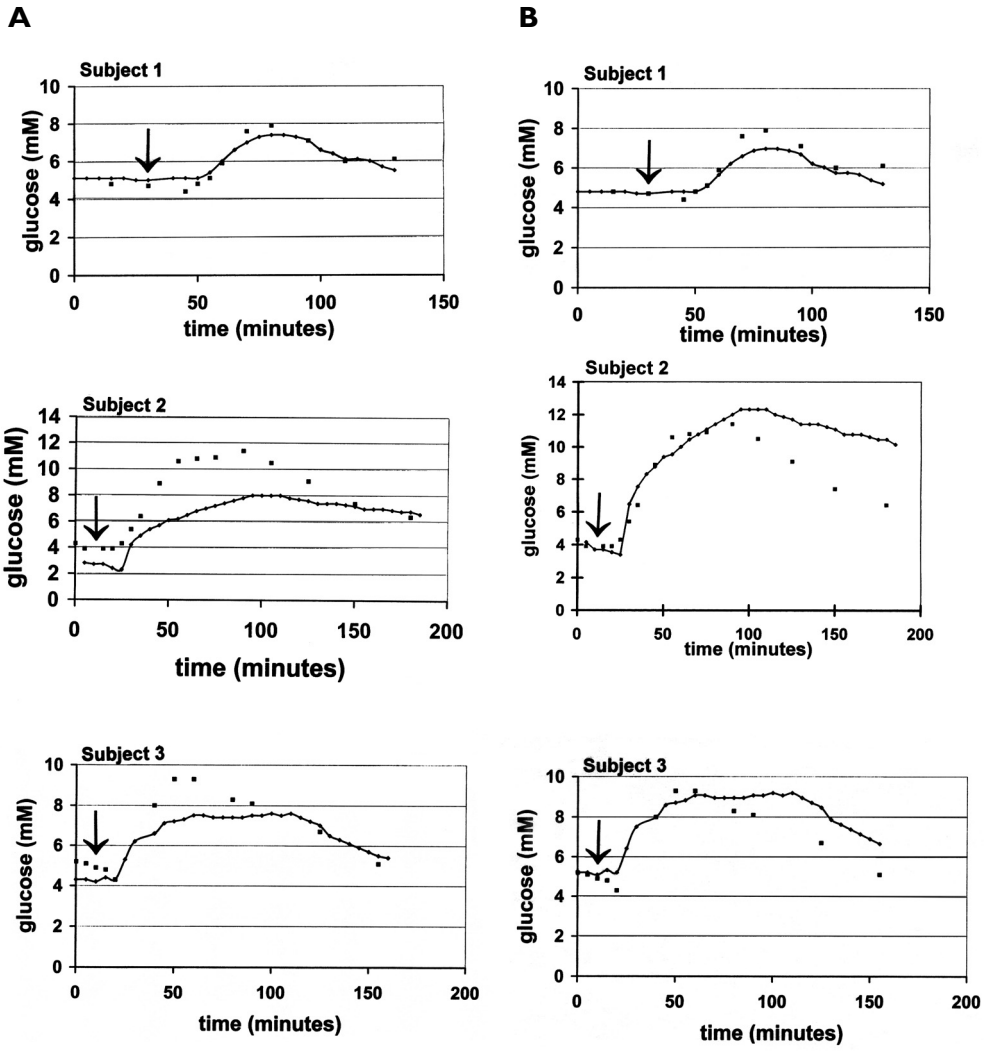


Figure 3a and 3b:

Typical graphs obtained for in vivo monitoring of glucose during glucose tolerance tests on respectively three healthy volunteers (a) calibration of biosensor in vitro and (b) calibration of biosensor on the first blood glucose value measured. Blood glucose concentrations in time (■), and subcutaneous glucose concentrations in time measured by the biosensor after ultraslow microdialysis (◆).The arrow in the figures indicates the oral administration of glucose.

(Y = 1.06, R^2 = 0.80). However, for several subjects a significant delay time is found in the decrease of the dialysate glucose content compared to the decrease in the blood glucose values (see figure 3b), and may lead to a potentially dangerous situation for the diabetic patient. Although these typical data have been observed in earlier studies[24], no explanation has been given so far.

In order to investigate this phenomenon more thoroughly, more clinical studies will therefore be necessary. For this reason and to investigate the possibilities of the measuring device for the monitoring of glucose for a longer period of time, more clinical studies will be carried out in the near future. These studies will be extended by the monitoring of both healthy volunteers and diabetic patients.

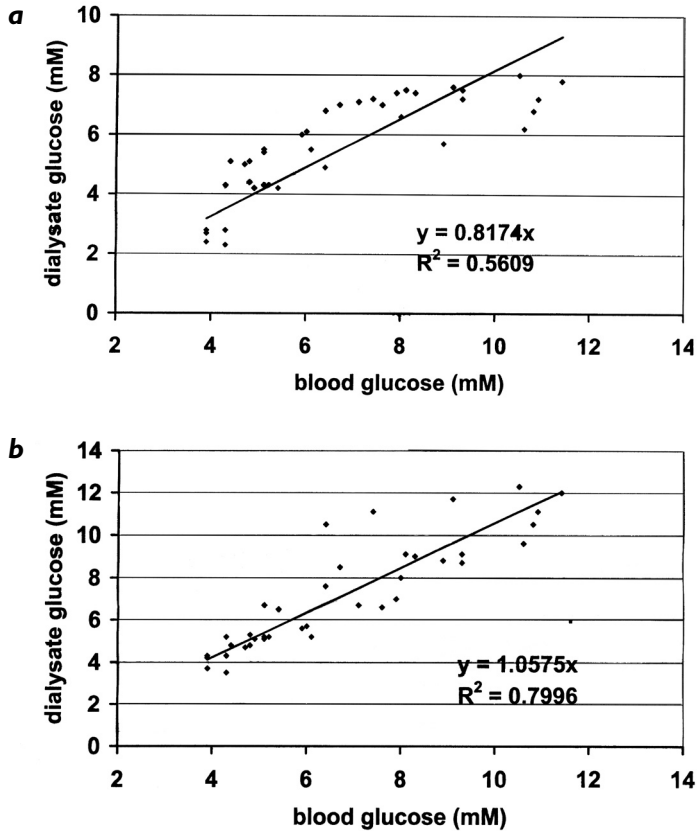


Figure 4a and 4b:
Correlation between glucose in dialysate measured with biosensor and blood glucose measured in the clinical laboratory: (a) calibration of biosensor in vitro, and (b) calibration of biosensor on the first blood glucose value measured.

7.4 Conclusions

Our ultimate goal is to develop a measuring device, which reliably measures glucose for at least several days without excessive and difficult calibration steps. In addition, if only (disposable) parts of the measuring device outside the body have to be replaced after defined moments without the need of hospital personnel, the quality of life of diabetic patients can thus be improved. Here, a portable lightweight measuring device is presented, which comprises a sampling unit (MD probe), a miniaturised flow-through biosensor and a semi-vacuum pump. Thanks to the low perfusion rate, (near) quantitative *in vivo* recoveries are established which circumvents excessive calibration normally used for microdialysis based measuring devices. The performance characteristics (linearity, precision, accuracy, selectivity and stability) which have been determined during *in vitro* and *ex vivo* studies, justifies the application of this measuring device for the *in vivo* monitoring of glucose in subcutaneous sampled interstitium of diabetic patients.

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