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

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

PROEFSCHRIFT

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door

Wilhelmina Anneriek Kaptein

geboren op 15 februari 1970
te Hardenberg

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Referenten:

Dr. J.F.C. Glatz

Dr. M.H.J. Ruiters

Voorwoord

Zo'n vier jaar geleden ben ik met een boot uitgevaren. De havens die ik onderweg moest aandoen waren onbekend, evenals welke goederen ik mee zou moeten nemen. Ook de bemanning moest nog grotendeels aangemonsterd worden. Hier voor jullie zien jullie mijn scheepsjournaal, dat ik heb geschreven over de afgelopen jaren. Ik heb regelmatig alle zeilen bij moeten zetten, en wel eens in het schip gezeten. Toch moest deze vaart gevaren worden, en heb ik na vele omzwervingen de behouden haven bereikt.

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aan mijn ouders

The studies described in this thesis were carried out at the Department of Biological Psychiatry and the Hong Kong University of Science and Technology.

Contents

Chapter 1

General introduction

Biosensoren zijn gewoon te moeilijk
(J. Koopal, Technisch Weekblad, 9 nov 1994)

Biomedical analysis

The first diagnosis of a disease by measurement of a constituent in a body fluid took place in the Middle Ages. Diabetes mellitus was diagnosed by tasting the sweetness of urine (Calbreath, 1992). Since then, knowledge of the composition of the body fluids increased, and by the end of the 19th century, many biochemical compounds were isolated and characterized (Calbreath, 1992). Nowadays, salts, sugars, proteins, hormones etc. are analysed to assist in diagnosis and to assess disease. The major metabolite measured today is glucose, mainly for diabetes care (Pickup, 1993). Other examples of the analysis of biochemical compounds can be found in cardiology (e.g. measurement of fatty acid-binding protein for diagnosis of an acute myocardial infarction) (Glatz et al., 1997), hormonal diseases (Cushing syndrome diagnosis by measuring cortisol) (Gosling et al., 1993) and in sports medicine (measuring lactate concentrations for fitness monitoring and to investigate tissue damage after training) (Kearney, 1996). The biochemical parameters for diagnosis of physiological abnormalities are normally measured batch-wise in discrete samples. The (bio)chemical analysis itself is usually performed in a laboratory. An important improvement for rapid clinical intervention or for a follow-up of the progression of a disease would be to apply analysis methods that measure constituents in body fluids without laboratory handling procedures. The ultimate goal would be the development of an on-line, bedside analysis system measuring the body fluid parameters continuously. Biosensors may anticipate in this area, for these can meet such requirements.

A biosensor is a self-contained integrated device which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biological receptor, selector) which is combined with a transducing (detecting) element.

This chapter concentrates on giving examples of optical and electrochemical techniques, which have been used, or have great potential, in biomedical applications. For more detailed description of fundamental aspects of biosensors and their applications, many excellent books and reviews are available, and are suggested for further reading (Canh, 1991; Aizawa, 1994; Wilkins and Atanasov, 1996; Hansen, 1996; Taylor and Schultz, 1996; Scheller et al., 1997a).

Biosensor concept

A biosensor consists of two elements (see Fig. 1.1):

1) (Bio)selector. The selector recognizes the analyte and reacts or binds to it.
2) Transducer. The transducer is the detector, monitoring the chemical or biochemical reaction initiated by the sample.

The two elements are methodologically closely linked to each other, preferably in a single device. In principle, no additional separation step should be applied and no extra reagents should be added to perform the measurement, though in some literature, devices

needing such additional procedures are also called biosensors (Aizawa, 1994). We use this broader understanding of the term “biosensors” in this thesis.

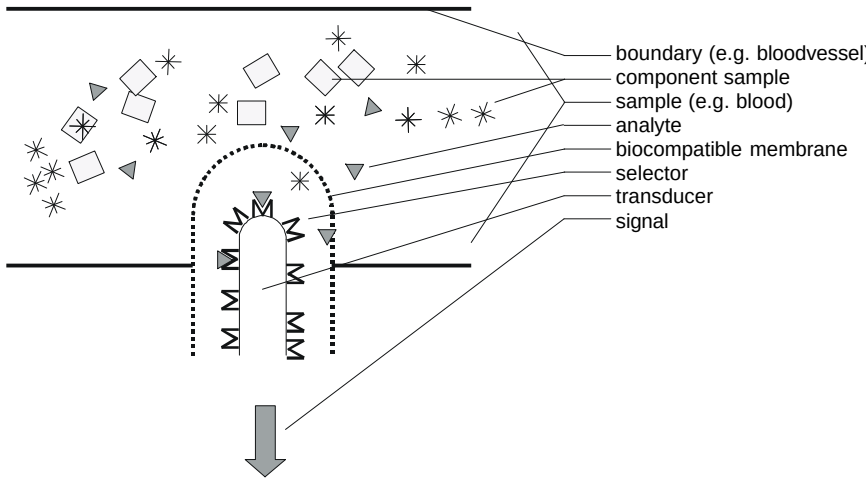

Fig. 1.1. Biosensor concept.

Table 1.1 Types of biosensors
(modified from Lambrechts and Sansen, 1992a; Scheller et al., 1997b)

Bioaffinity sensors			Biocatalytic sensors		
Device name	**Receptor**	**Analyte**	**Device name**	**Receptor**	**Analyte**
	dye	protein			
	lectin	saccharide glycoprotein			
	enzyme	substrate inhibitor	enzyme sensor	enzyme	substrate cofactor inhibitor activator enzyme activity
	apoenzyme	prosthetic group	organelle sensor	organelle	
immunosensors	antibody	antigen	microbial sensor	microbe	
	receptor	hormone	tissue sensor	tissue slice	
ion selective electrode	ionophore	ions			
	transport system	substrate analogue			

Selectors

The selectors of biosensors consist of biomolecules, biorecognition elements or analogues thereof (Table 1.1). The selectors determine the selectivity, so that only the compound which has to be measured leads to a signal. The selection can be based on bioaffinity, in which the bioelement does not change the chemical structure of the analyte

(e.g. an antibody), or biocatalysis, in which the bioelement catalyses a biochemical reaction of the analyte (e.g. an enzyme) (see Fig. 1.2).

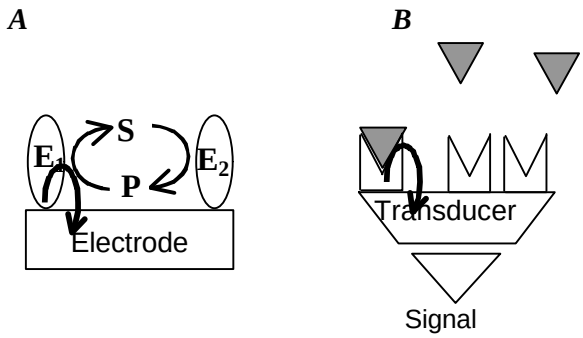

Fig. 1.2. Biocatalysis (A) and bioaffinity (B). E_1, E_2 enzymes; S: substrate; P: product.

Most biosensors described in literature use an enzyme as selecting element (Table 1.2). Purification of enzymes is relatively cheap, because most enzymes can be isolated from micro-organisms, such as fungi or bacteria, which produce these enzymes in excess, naturally or after genetic manipulation (Kopetzki et al., 1994). The enzyme catalyses specifically a conversion of the analyte. In most cases, this reaction only uses the analyte, but sometimes also other compounds (co-substrates). Such a reaction has usually a high specificity and can be followed by measuring the increase of the formed product or the decrease of a co-substrate, which is consumed during the enzyme reaction. Glucose, oxidized by the enzyme glucose oxidase, is an example of an enzymatically detectable metabolite.

When selective enzymes are not available for the detection of an analyte, antibodies can serve as selecting element. Antibodies can bind to analytes very selectively. Biosensors based on monitoring antibody-antigen interaction, are termed immunosensors (e.g. see review Morgan et al. (1996)). A genuine immunosensor essentially comprises an antibody-bearing sensing probe that relies on direct (on-line) measurement of antigen binding by a detecting system (Ekins, 1994). Immunosensors are often more complex than enzyme biosensors, because there are hardly any (sensitive) detection methods that can measure the interaction between antibody and antigen directly. Mass sensitive or optical immunosensors (see below) are based on measuring the change of bound material onto a surface. Antigens (the analyte of interest) or antigen-analogues present in (or added to) the sample associate to surface immobilized antibodies, thus forming a complex which is larger than the antibody alone. Other immunosensors operate following the ELISA-principle, i.e. one immuno compound is labelled, e.g. with a fluorophore or enzyme. This labelled compound can then compete with an unlabelled immuno compound (the analyte) or form a sandwich complex. If an enzyme is used, the signal can be amplified by increasing the incubation time.

Table 1.2. Enzyme biosensors
(modified from Lambrechts and Sansen, 1992; Pfeiffer et al., 1996)

Determinant	Substrate	Enzyme	Indicator
saccharides	glucose	glucose oxidase	O_2 H_2O_2
	lactate	lactate oxidase	O_2 H_2O_2
alcohols	ethanol	ethanol oxidase	O_2
aminoacids	glutamate	glutamate dehydrogenase	NH_4^+
acids	acetic acid	alcohol oxidase	O_2
	uric acid	uricase	H_2O_2
lipids	cholesterol	cholesterol oxidase	H_2O_2
antibiotics	penicillin	penicillinase	pH electrode
other substrates	urea	urease	NH_4^+
	nitrate	nitrate reductase	
	nitrite	nitrite reductase	
	creatine	Creatinase	NH_4^+
	inorganic phosphorus	Alkaline phosphatase	H_2O_2

Besides the problem that the binding of an antigen to an antibody can rarely be detected itself, there is another reason why creating immunosensors is more difficult than creating enzyme biosensors. The analytes measured by immunosensors, e.g. proteins and hormones, are often present in the body fluids in a much lower concentration than the analytes detected with an enzyme biosensor. For instance, glucose, a typical analyte for enzyme biosensors, has physiological concentrations in the millimolar range. Analytes that are usually detected with immunosensors, such as proteins and hormones, normally have to be detected in nano- and picomolar concentrations or even below that. This requires a more specific, more sensitive detection system.

As illustrated in Table 1.1, other selective elements can be applied in biosensors. However, these are not applied as frequently as enzymes and antibodies, especially in the medical field. Therefore, they will not be discussed in this chapter.

Transducers

The transducer (detector) translates the recognition of the selector into a digital or analogue (preferably quantitative) signal. Possible transducer technologies are optical, electrochemical and acoustical/mechanical or calorimetrical (see Table 1.3).

The choice of the detection method is not only determined by the sensitivity of the detection method, but also by the contaminants present in the matrix. Most biosensors used so far apply optical or electrochemical detection. The detection of an analyte (e.g. as a result of an enzymatic reaction of the analyte) by measuring the change of the light absorption at a certain wavelength is normally not very sensitive. For analytes present at sufficiently high concentrations, this is not a problem. An advantage of this optical detection system is that there are not many compounds, which might interfere in measurement, therefore the selectivity is high.

Table 1.3. Transducer devices
(modified from Schultz and Taylor, 1996; Wang, 1996; Hock, 1997)

Device	Output change	Examples
Electronic		
amperiometric	applied current	immunosensor
potentiometric	voltage (potential)	ISFET
capacitance/impedance	impedance (modulation)	conductiometers
Optical /Photometric		
light adsorption or scattering; refractive index	light intensity. colour, emission	ellipsometry, internal reflectometry, laser light scattering
fluorescence or luminiscence activation, quenching, polarization	fluorescence or chemiluminiscence	surface plasmon resonance, fiber optic wave guides, fluorescence polarization
Acoustical/mechanical		
acoustical	amplitude, phase or frequency (acoustic wave)	surface Acoustic Wave Devices
mass/density	weight	piezoelectrometric devices
Calometric		
thermistor	temperature	enzyme sensors

Electrochemical detection can be amperometric (measuring changes of current) or potentiometric (measuring a difference in potential) or conductiometric (measuring changes in conductance on the electrode) (Wang, 1996). An example of an electrochemical sensor is an ISFET (ion-sensitive field-effect transistor). These ISFET systems opened possibilities for measuring ions and miniaturization of the transduction system, and have been used for example for the detection of heparin in blood samples (Van Kerkhof et al., 1995). An advantage of electrochemical detection (ECD) devices is that the sensitivity is high, compared to most optical detection methods (Pfeiffer et al., 1996). Furthermore, the materials that are required are cheap, compared to fluorimetric (or the older radiochemical) detection devices. Disadvantages of ECD-based sensors are that their signal may drift and that unspecific reduction or oxidation of compounds present in the sample may occur. Thus, ECD devices have high sensitivity but relatively low specificity.

An alternative optical detection method is measuring fluorimetric changes (Hemmila, 1985; Morgan et al., 1996). Fluorescent signals can be created by probes carrying fluorescent dyes, or by probes containing enzymes, catalysing reactions forming fluorescent products. The sensitivity of electrochemical and fluorescent detection is often comparable, but fluorescent measurements encounter less interference. However, the apparatus required is large and both the equipment and the fluorescent dyes or substrates are expensive. Application of fiber optics may improve this technique considerably (Abel et al., 1996).

During the last decades some new detection methods for immunosensors were developed. Examples of these methods are sensors based on measurement of SPR (surface plasmon resonance) (Jonsson et al., 1991; Brecht and Gauglitz, 1997) and piezo immunosensors (Kricka, 1994; Joracek and Skaladal, 1997), detecting antigen-antibody

interactions without additional labels and in real time. However, the antigens should have high molecular weight, and only increases of antigen amounts can normally be detected.

Clinical biosensors

In 1962, Clark et al. (1962) introduced the first biosensor for glucose. This enzyme biosensor was based on detecting the decrease of oxygen, which was the co-substrate for the conversion of glucose by the enzyme glucose oxidase. Later, the oxidation of glucose was also followed by the increase of hydrogen peroxide (McNeil et al., 1997).

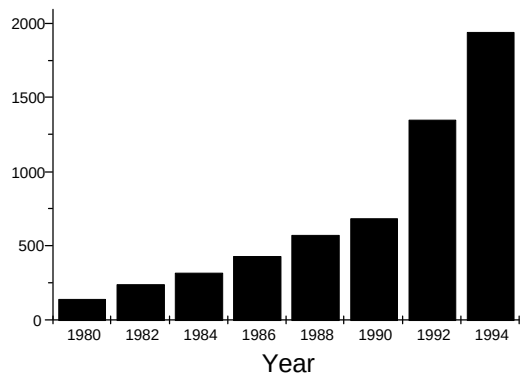

Fig. 1.3. Number of publications on glucose electrodes and glucose sensors from 1980 to 1994 (adapted from Lundi, 1997).

Of all biosensors, the glucose biosensor has been studied most. In 1994, almost 2000 articles describing glucose electrodes and glucose sensors have been published (Fig. 1.3) (Lundi, 1997). Glucose dipsticks (e.g. the Glucocard or Medisense Glucopen) became available in the eighties. Since then, diabetic patients are able to monitor their blood glucose themselves, pricking a blood sample and using dry strip chemistry analysis (see Table 1.4) (Pfeiffer et al., 1996). However, the patients experience this as troublesome and painful. Furthermore, the measurement only gives the glucose concentration at the time the fingerprick was performed and the measurement is still rather expensive (Pfeiffer, 1997).

Biosensors, continuously monitoring the glucose concentration of blood, would enable the patient to keep the glucose concentration in the blood more constant, and avoid the painful fingerprick. Research to develop such a sensor has started a long time ago. When such a sensor is linked to an insulin pump, being a kind of “artificial pancreas”, discomfort and complications will be reduced (Reach and Wilson, 1992). In the first glucose biosensors, the enzyme glucose oxidase was immobilized on electrodes. The glucose sensors were improved over the years, for example by the utilization of mediators. Mediators, electroactive chemical compounds which can reoxidize the enzyme and itself reoxidizes at the electrode, create a better link between the selection and the detection part

and turn the measurements independent on oxygen (Boutelle et al., 1996; Parellada et al., 1997). Prototypes of glucose sensors are reported to measure up to ten days now (Wilkins et al., 1995). Needle-type biosensors, in which the sensor system is perfused with an exogenous fluid have also been tested in volunteers (Poitout et al., 1993; Hashiguchi et al., 1994). Despite all research, *in vivo* glucose sensors have not yet been introduced into clinical practice (Wilkins and Atanasov, 1996).

Table 1.4 Enzyme-electrode-based portable devices (adapted from Lundi, 1997)

Model	Company	Analyte	Measuring range (mM)	Functional stability
ExactTech	MediSence (USA)	glucose	1.1-33.3	disposables
Satillite G		glucose	2.0-33.3	disposables
Glucometer Elite	Bayer Diagnostics (Germany)	glucose		disposables
Glucocard	Kyoto Daiichi Kagaku Co (Japan)	glucose	2.2-27.8	disposables
i-STAT PCA	i-STAT Corp. Princeton (USA)	glucose	2.9-23.6	disposables
		urea	1.0-43.0	disposables
BSE 5500	Orion Anal. Technol. Inc. (USA)/Dosivit (France)	glucose	1.6-16.0	disposables
		sucrose	1.6-16.0	disposables
		lactose	1.6-16.0	disposables
Biosen 6020 G	EKF Industrial Electronics (Germany)	glucose	0.5-20.0	24 days
Biosen 5020 L		lactate	0.5-20.0	10 days

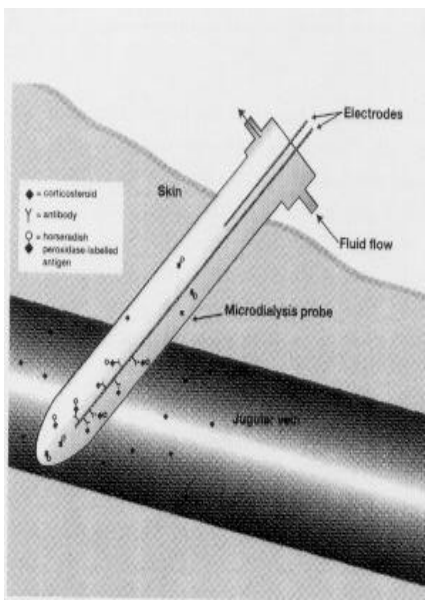

Fig. 1.4. In vivo *immunosensor Cook (adapted from Turner, 1997).*

Although many model systems have been described for (electrochemical) immunosensors, there are no commercial sensor devices available yet (McNeil et al., 1997). A first *in vivo* immunosensor device has been recently described (Cook, 1997). He demonstrated an electrochemical immunosensor for cortisol, placing an electrochemical sensor in a needle type microdialysis probe (see Fig. 1.4). With this, he detected cortisol and corticosterone in a competition immunoassay. This approach was posed as the next generation of immunosensors (Turner, 1997). However, the implementation of this method may harm the surrounding tissue, e.g. because HCl-solutions are perfused through the probe and may therefore penetrate into the tissue. Furthermore, the applied cortisol —horseradish-peroxidase — complex may (partially) leak into the tissue because of small irregularities in the membrane. Above all, the proposed device still requires complex pumping and valve connections.

Main chemical and physiological characteristics studied compounds

Glucose

Glucose is a sugar that is an important source of energy in the body and the sole source of energy for the brain. In clinical medicine, its determination is a routine clinical test to measure glucose plasma levels in several diseases or during hospitalization. One of the main reasons to monitor the glucose concentrations is to achieve adequate metabolic control of diabetics. For this, frequent analysis is required (several times a day). Besides diabetology, there is a strong demand for miniaturized integrated glucose biosensors for *in vitro*, *in vivo* and *ex vivo* applications in the intensive care, the operation theatre and in the field of bedside analysis (Urban and Jobst, 1997).

Lactate

Lactate is an intermediate metabolite of the anaerobic glycolysis. It has been of interest in physiology, for example because of its relation to anaerobic metabolism during muscle contraction (Lamont, 1987; Pilardeau et al., 1988; Scheller et al., 1997). In clinical medicine, several pathological conditions cause increased lactate production, for example vascular occlusion and lacto-acidosis caused by shock or mitochondrial enzyme deficiencies (De Boer et al., 1991).

Cortisol

Cortisol (hydrocortisone) is a steroid hormone of the adrenal cortex. The cortisol concentration in blood is strongly influenced by stress. It has relatively high concentrations in blood and it exhibits a strong circadian rhythm (see Fig. 1.5).

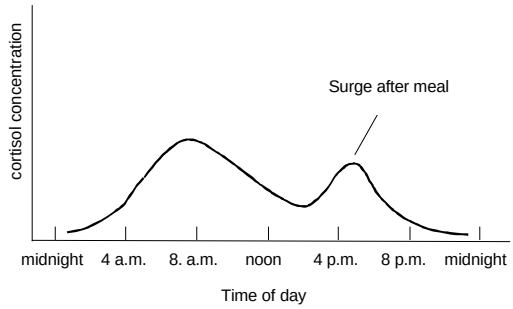

Fig. 1.5. Cortisol circadian rhythm.

Fatty acid-binding protein

Fatty acid-binding protein (FABP) is a 15 kD protein. It is a novel plasma marker protein for acute myocardial infarction (AMI), which has a high sensitivity and specificity for early detection of AMI (see Fig. 1.6) (Glatz et al., 1997).

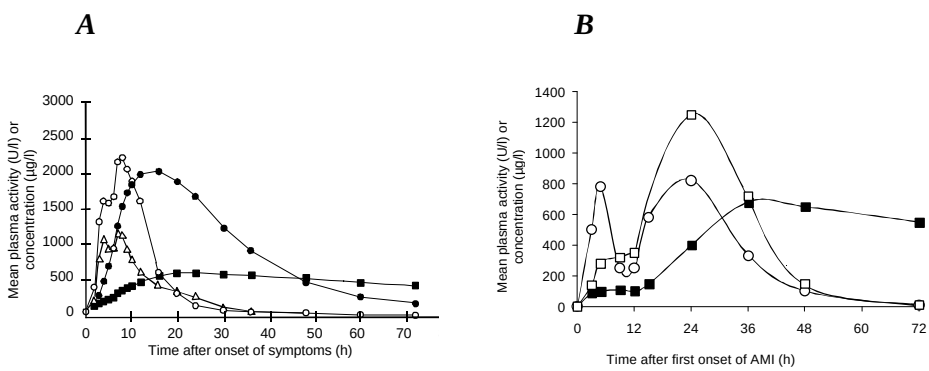

Fig 1.6. FABP after infarction (A) and reinfarction (B). FABP (#); myoglobin ( )creatine kinase (!); hydroxybutyrate dehydrogenase (+); lactate dehydrogenase (▲) (adapted from Wodzig et al., 1997a, Glatz et al., 1997).

Other so-called biochemical markers for myocardial tissue injury are released later and/or have lower specificity (Apple, 1992). Because of the small size of FABP, its concentration is returned to normal values within 12 to 24 hours (Tsuji et al., 1993b; Van Nieuwenhoven et al., 1995; Wodzig et al., 1997b). Because of this fast raise and fall, the infarct size and recurrent infarctions can be monitored when FABP is determined on a regular base (Kleine et al., 1992a; Glatz et al., 1994a; Wodzig et al., 1997a; Ishii et al., 1997a).

Development of *in vivo* or *ex vivo* biosensors

To develop an on-line clinical biosensor device for *in vivo* or *ex vivo* applications, there are physiological and technical aspects to be considered. The physiological aspects are merely concerned with the choice of analyte and, subsequently, the site in the body where this analyte has to be measured. The technical aspects of an on-line biosensor can be divided into three parts: Firstly, contact with the body fluid has to be made. Secondly, the analyte in the (complex) matrix has to be recognized specifically. Thirdly, the recognition should be translated into a quantitative signal, e.g. a number or an analogue signal.

The occurrence and "behaviour" of the analyte in a certain body fluid dictate the place of sampling. Most analytes have to be monitored in blood, but sometimes subcutaneous (under the skin) or transcutaneous (on top of the skin) measurements can be performed, even to estimate analyte concentrations in blood.

The configuration of a biosensor has to be adjusted to the sampling site. When intravenous measurements are necessary, a small contact area between the device and the body is essential, because it has to be inserted into a blood vessel. For transcutaneous analysis, a large contact surface may be preferred to establish a larger signal.

For the first requirement, continuous contact with the matrix is needed. To enable this, there are two options: measurement directly inside the body or a combination of a sampling and an analysis system. The direct measurement inside the body with biosensors often leads to problems, such as instability of the sensor signal and biocompatibility. Problems that might occur in these sensors are calibration (because the response *in vivo* can be rather different compared to the *in vitro* response) and the stability of the sensor (Arner and Bolinder, 1991; Fischer et al., 1994, 1995; Fischer, 1995).

An alternative for measuring parameters *in vivo* can be found in combining an analysis system with a continuous sampling system, such as microdialysis or ultrafiltration sampling (Fig. 1.7) (Ungerstedt, 1991; Moscone et al., 1996; Ballerstadt and Schultz, 1996; Elmquist and Sawchuk, 1997).

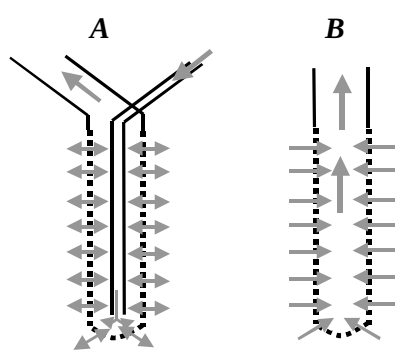

Fig. 1.7. Schematic representation of microdialysis (A) and ultrafiltration (B).

Microdialysis (MD) is a dynamic sampling method based on analyte diffusion across a semi-permeable membrane, due to a concentration gradient (Palmisano et al., 1997). The membrane forms the contact area with the body fluid, thus determining the biocompatibility. Furthermore, the membrane may serve as a selector, because it prevents diffusion of large molecules or cells to the analysis system. Accordingly, the sensing surface remains rather clean and, when membranes with the right characteristics are chosen, interference of many substances is reduced. MD probes are not only useful in intravenous sampling, they also provide the possibility for subcutaneous sampling. This is because the invasive part, the probe, can be very small and the sampling does not withdraw the scarce extracellular fluid (Arner and Bolinder, 1991). A problem encountered when using MD is that the absolute amounts of analyte are difficult to estimate (Justice, Jr. 1993). Moreover, MD can cause depletion of the analyte at the sampling site (Rosdahl et al., 1993).

Recently, ultrafiltration (UF) has been proposed as alternative for on-line sampling (Moscone et al., 1996). The UF technique consists of a sampling probe withdrawing fluid from the surrounding tissue by means of an underpressure. This technique has been tested in rats and humans. It may become (under certain circumstances) a suitable alternative for MD, because depletion is prevented and absolute concentrations can be measured. When the sampling is performed at very low flow rates (e.g. 100 nl min^{-1}, as proposed by Moscone et al. (1996)), tissue damage as a result of the withdrawal of fluid is expected to be minimal.

For the second requirement of the biosensor (the selection), a continuous selection method is necessary. For metabolites like glucose, lactate and glutamate, specific enzymes (oxidases) are used to produce electrochemically active species. As these species are continuously produced by the enzymes, they can also be continuously monitored. For immuno detection, e.g. of hormones or proteins, one of the immuno compounds (the antigen or antibody) is labelled. However, the conventional competitive or sandwich immunoassays do not allow continuous detection. The only immunological selection system allowing an on-line measurement is called displacement (see Fig. 1.8).

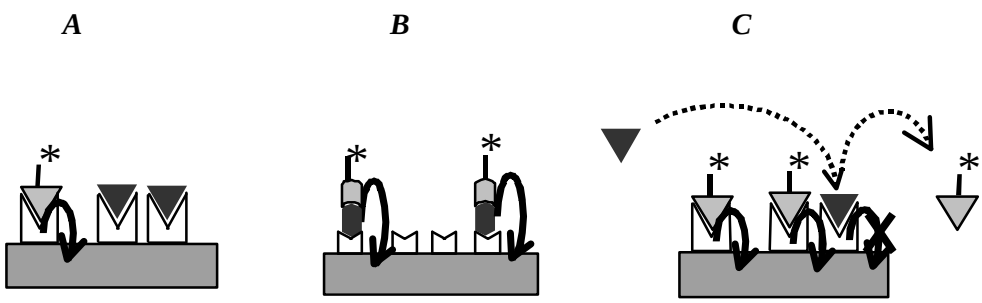

Fig. 1.8. Immunoassays. A. Competition; B. sandwich; C. displacement.

Selection based on displacement utilizes the replacement of a previously associated antigen (or its analogue) from the antibody binding site of an antibody by an antigen of the sample. In displacement detection either antibodies or antigens are immobilized, and the respective interacting antigen (analogue) or antibody is labelled. After the antibody binding sites are saturated with antigens, the actual displacement consists of the release of labelled molecules from the immobilized ones as a result of the binding of the antigen from the sample to the antibody binding site. When these antigen-antibody complexes are placed in a flow system, the amount of displaced molecules will be dependent on the concentration of the analyte. When the complexes are immobilized in excess, the displaced fraction after a certain time interval can be neglected, and continuous samples can be analysed in a constant displacement system.

Scope of the thesis

The aim of the studies described in this thesis is to develop sampling and bioselective techniques, which can be used in continuous *in vivo* or *ex vivo* clinical biosensors. There are three elements required for the on-line clinical biosensor devices: contact with the analysis site, specific recognition of the analyte of interest, and translation of this recognition into a signal. The present thesis emphasizes on the creation of a contact with the analysis site (the body fluids) and a selection of the analyte, rather than on signal translation (see Fig. 1.9).

Chapter 2 introduces the techniques for continuous *in vivo* sampling. The techniques available are, apart from direct, undiluted blood sampling, microdialysis (MD) and ultrafiltration (UF). These techniques, their merits and their disadvantages are discussed. Furthermore, sampling in various body fluids is reviewed, and the physiological implications of the analysis of samples from these body fluids, are shortly addressed. Special attention is given to measurements performed to estimate concentrations of an analyte in blood, which can not only be done by sampling in blood itself, but also in other body compartments, e.g. in subcutaneous tissue, because all body compartments are connected to each other.

Chapter 3 describes UF sampling experiments in subcutaneous tissue as well as in the jugular vein. Very low flow rates have been obtained with an underpressure driven, homemade pump. The homemade UF probe consists of a semi-permeable dialysis tube. In this study the performance of the sampling system was analysed. Furthermore, the subcutaneous and intravenous glucose concentrations were compared.

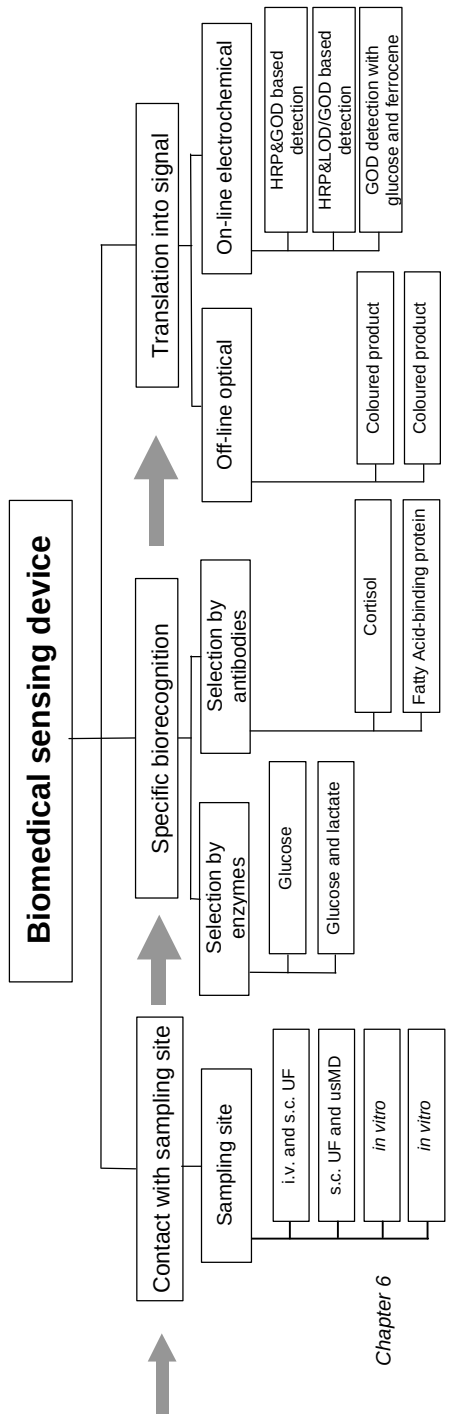

Fig. 1.9. Overview research articles thesis.

Chapter 4 describes on-line sampling experiments in the subcutaneous tissue of the rat. The sampling was performed with UF and with a new ultraslow microdialysis (usMD) design. The aim of this study was to investigate the differences between these two sampling techniques at different flow rates. To test both sampling systems, the glucose and lactate concentrations of the samples were analysed simultaneously, using a dual bienzymatic reactor system (for selection) and electrochemical detection (for transduction).

Chapter 5 describes the chosen continuous immuno selective method: displacement. Displacement in non-flow and flow systems, and for discrete or continuous sample is reviewed. Also, examples of displacement of labelled antigen and labelled antibodies are given. The displacement is addressed both from a theoretical and a practical point of view.

Chapter 6 describes the displacement of cortisol labelled with horseradish peroxidase from immobilized antibodies initiated by the cortisol hormone present in the matrix. The aim of the study is to investigate whether displacement occurs when the displacement unit is continuously perfused with buffer containing cortisol at flow rates of a microliter scale. The selectivity and sensitivity of the displacement reaction was tested under different conditions for two different monoclonal antibodies.

Chapter 7 investigates the displacement for fatty acid-binding protein (FABP), a 15 kD protein. FABP is a novel plasma marker protein for acute myocardial infarction (AMI), which has a high sensitivity and specificity for early detection of AMI. FABP is detected by the displacement of labelled antibodies from immobilized FABP. Unique in this experiment is the displacement in a flow system of labelled antibodies, whereas this is normally done with labelled antigens. Also, displacement has never been demonstrated for proteins before. In this chapter, the sensitivity and specificity of the displacement have been investigated, as well as different FABP-immobilization methods. The relative displacement signal for different FABP concentration has been studied.

The final chapter, *chapter 8* will summarize, discuss and interpret the main results of the previous chapters and discuss the merits of the research for future biosensor development.

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Chapter 2

Methods of in vivo *continuous sampling for clinical applications*

Get your facts first,
and then you can distort them
as much as you please (Mark Twain)

Introduction

Since the diagnosis of diabetes mellitus in the Middle Ages was performed by tasting the sweetness of the urine, our knowledge of biochemical parameters in health and illness has expanded enormously (Calbreath, 1992). In the 19th Century, the first biochemical techniques for characterizing and analysing compounds in the body fluids became available (Calbreath, 1992). Presently, it is possible to measure sugars or other enzymatically convertible, relatively easy-to-detect body metabolites, and, with the introduction of immunoassays, many other compounds, e.g. hormones and proteins (Gosling, 1990). Measuring analytes in body fluids is routinely used to assist in the medical diagnosis; many clinical decisions are made based on laboratory analysis (Gilbert and Vender, 1996). However, some improvements can still be made. One major improvement would be to have techniques available that would provide concentrations of body fluid constituents continuously and instantaneously (Anderson et al., 1997). To date, samples are often taken batch-wise, and subsequently analysed in a laboratory. The results of such measurements are usually only after several hours available (Gilbert and Vender, 1996), and these analyses are generally rather expensive (Linhares and Kissinger, 1992b). Continuous analysis offers the advantage of providing instantly information of the analyte concentration and facilitating the creation of time-profiles. Methods of continuous analysis avoid the need for frequent sampling, which would be necessary when the concentration of the analyte has to stay within a certain range or when a concentration profile of the analyte over the day is required (Trajanoski et al., 1996). Therefore, one can expect that the quality of patient care would be improved if batch-wise sampling and analysis were replaced by on-line, continuous analysis.

Requirements for accurate on-line analysis

For continuous (bio)chemical monitoring, two methodologically different invasive approaches can be applied (Fischer et al., 1995). The first approach, direct *in vivo* measurement at the sampling site inside the body (as utilized in many (bio)sensors) has several technical limitations. Among these problems are *in vivo* calibration and stability (Reach and Wilson, 1992; Carlsson et al., 1996). In addition, the probes are often rather large, thus creating large artefacts in the surrounding tissue (Reach and Wilson, 1992).

The second approach consists in continuous (on-line) sampling, preferably directly coupled to an analysis system. In this overview, we will primarily focus on the current state of the latter approach.

For pathophysiologically relevant continuous measurements following an approach of this kind, two issues have to be addressed: 1) Which sampling technique should be used? and 2) In which tissue or body fluid should the sampling be performed?

This overview will outline two techniques suitable for on-line sampling for clinical practice: microdialysis and ultrafiltration. The merits and limitations of both sampling

methods are evaluated. Literature on the application of the microdialysis and ultrafiltration to various sampling sites will be reviewed. Special attention will be given on approaches to estimate the concentration of analytes in blood.

Methods for continuous on-line sampling

At present, most biochemical parameters are measured in (discrete) blood and urine samples. Among these, blood sampling is predominant, because many times changes in the levels of an analyte in blood often occur rapidly after a clinical event, as all tissues are in close contact with the blood circulation (Linhares and Kissinger, 1993b). Furthermore, compared to other body compartments, blood samples are relatively easy to sample and the disturbance of the physiological state of the subject is minimal. Continuous sampling performed rapidly and free of compounds interfering in the detection of the analyte would be ideal for patient monitoring (Linhares and Kissinger, 1992a). Blood is the only body compartment that can be used for continuous, undiluted sampling. However, on-line blood sampling is not performed routinely, because it has some serious drawbacks:

1) Infection hazards (Reach and Wilson, 1992). Placement of a catheter in the blood stream has to be done and controlled by professionals and special care must be taken to prevent infections. Normally this is only possible in a hospital setting.
2) The subject has to be heparinized to prevent blood clotting both intracorporally as well as in the sampling system. Heparinization also holds certain risks, for example internal bleedings.
3) The internal diameters of the tubings have to be rather large to prevent blood clots blocking the tube (Linhares and Kissinger, 1993b). For this same reason, the flow rate has to be rather large. This means that much blood of the donor is lost in the process of continuous sampling.
4) Continuous sampling and subsequent biochemical analysis is only useful when the result of the assay is not changed by endogenous physiological processes. Blood cells and enzymes often metabolize compounds and this can only be prevented when special reagents are added (such as citrate).
5) The instruments (such as the Biostator) for continuous sampling of blood, e.g. for analysing the glucose, are rare and very expensive (Ash and Janle-Swain, 1988).

To overcome these drawbacks, alternative body fluids for sampling have been considered. However, most tissues and body fluids do not have enough fluid to be sampled directly, except in case of urine and saliva. As the variations of volume influence the concentration profiles of analytes in urine and saliva, on-line measurements do not reveal relevant information.

Alternative sampling techniques for more general applications and sampling of other body fluids have been developed some decades ago. The first device was a push-pull canulla (Gaddum, 1961; Delgado and Rubinstein, 1964; Gliessman et al., 1986), developed specifically for sampling at places where the amount of fluid is limited. This semi-continuous device was based on adding and subsequent withdrawing of a buffer. The

probe (the part which is in direct contact with the sampling site) consisted of a double tubular system (Gardner et al., 1993). One tube (push) infuses a physiological buffer into the sampling site. The other tube (pull) withdraws, with the same flow rate, fluid for analysis (see Fig. 2.1A). Although the technique seems rather straightforward, its biochemical application is difficult. At too low flow rates, blockage of the pull cannulla is risked, whereas high flow rates can damage the surrounding tissue. Additionally, even small differences in flow rates of the two pumps will cause massive tissue damage (Gardner et al., 1993). The push-pull sampling technique has been used for subcutaneous and brain fluid sampling, but it is not suitable for blood sampling because blood cells and blood platelets block the tubes (Rada et al., 1993). The technique was soon replaced by a more sophisticated technique: microdialysis.

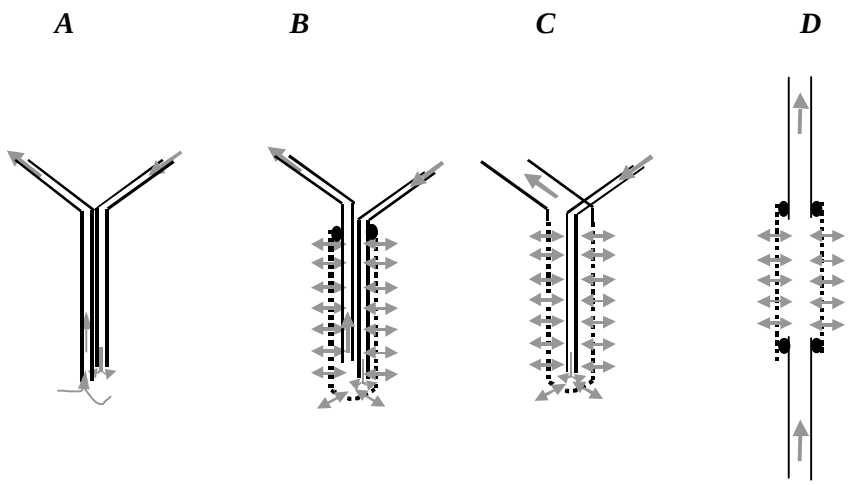


Fig. 2.1. Sampling devices. A Push-pull canulla; B-D. Microdialysis canullas.

Microdialysis

Microdialysis (MD) is the first universally working continuous sampling technique, a dynamic sampling method based on analyte diffusion across a semi-permeable membrane due to a concentration gradient (Delgado and Rubinstein, 1964; Palmisano et al., 1997). A fluid is pumped with a single pump through a dialysis probe, the semi-permeable membrane. The incoming fluid, the perfusate, is a buffer balanced in pH and ion content with the extracellular fluid in the surrounding tissue. The outgoing fluid, the dialysate, contains body fluid constituents, which have been diffused into the fluid by passive diffusion through the membrane of the probe. The membrane forms the contact area with the body tissue.

MD has many advantages over other sampling techniques. MD allows sampling in the extracellular space of virtually all tissues (Robinson, 1995). Due to the semi-permeable membrane the influx and the outflux of fluid are balanced, preventing tissue damage as in

the case of the push-pull canullas (Gardner et al., 1993, Robinson, 1995). Due to the cut-off of the semi-permeable membrane of the MD probe, the sample is relatively clean (Deterding et al., 1992; Paez and Hernandez, 1997). Therefore, not only is the chemical analysis less complex (deproteinisation is unnecessary), but the analyte in the dialysate can not be degraded by enzymes. It is an inexpensive, readily applicable, and relatively non-invasive method, causing minor tissue trauma and allowing to define the sampling site clearly (Linhares and Kissinger, 1993b). The introduction of a MD probe creates only moderate pain, comparable to an intramuscular or subcutaneous injection (Müller et al., 1995). It is also possible to infuse compounds *into* the tissue (Robinson, 1995). Because the focus of this review is sampling, this possibility will not be discussed further in this chapter. Although there are several dialysis probes available, in general they can be schematically represented as in Figs 2.1B-D.

Analyte recovery and its estimation

When MD sampling is performed, the relative recovery of the analyte has to be determined. The relative recovery is defined as the percentage of the concentration found in the dialysate and of that of the original body fluid concentration (Gardner et al., 1993). Sometimes, also the absolute recovery is used to characterize the concentration in the dialysate. The absolute recovery is the total amount of analyte in the dialysate per time interval. In general, the relative recovery is independent of the concentration of the analyte, but depends on:

1) The flow rate and composition of the MD fluid. The higher the flow, the lower the recovery (Linhares and Kissinger, 1993b). In contrast, the absolute recovery will increase at higher flow rates.
2) The features of the probe. For example, the surface area of the probe is directly proportional to the recovery (Robinson, 1995).
3) The chemical nature of the MD probe (Hsiao et al., 1990; Linhares and Kissinger, 1993). There are numerous membrane materials, each with different physical and chemical properties. Depending on the analyte, a selection of membrane material has to be made. The membranes normally used are organic polymers (e.g. cellulose, polycarbonate, polysulphone, polyacrylonitrile) which have a cut-off value of 10-30 kDa and hydrophylic properties. The chemical characteristics also determine the biocompatibility (Gaddum, 1961).
4) The nature of the sample. The higher the fluid/tissue ratio of the sampling site, the higher the recovery. When the fluid is in motion, like in blood vessels, the recovery is also higher (Linhares and Kissinger, 1992b).
5) The temperature (Linhares and Kissinger, 1992b). At higher temperature, the diffusion through the membrane will increase, the recovery will therefore be higher.

When the absolute concentration of the analyte in the body compartment is required, the recovery has to be calculated. There are several methods for the quantification of the recovery, enabling calculation of the absolute concentration in the tissue (see also Fig. 2.2).

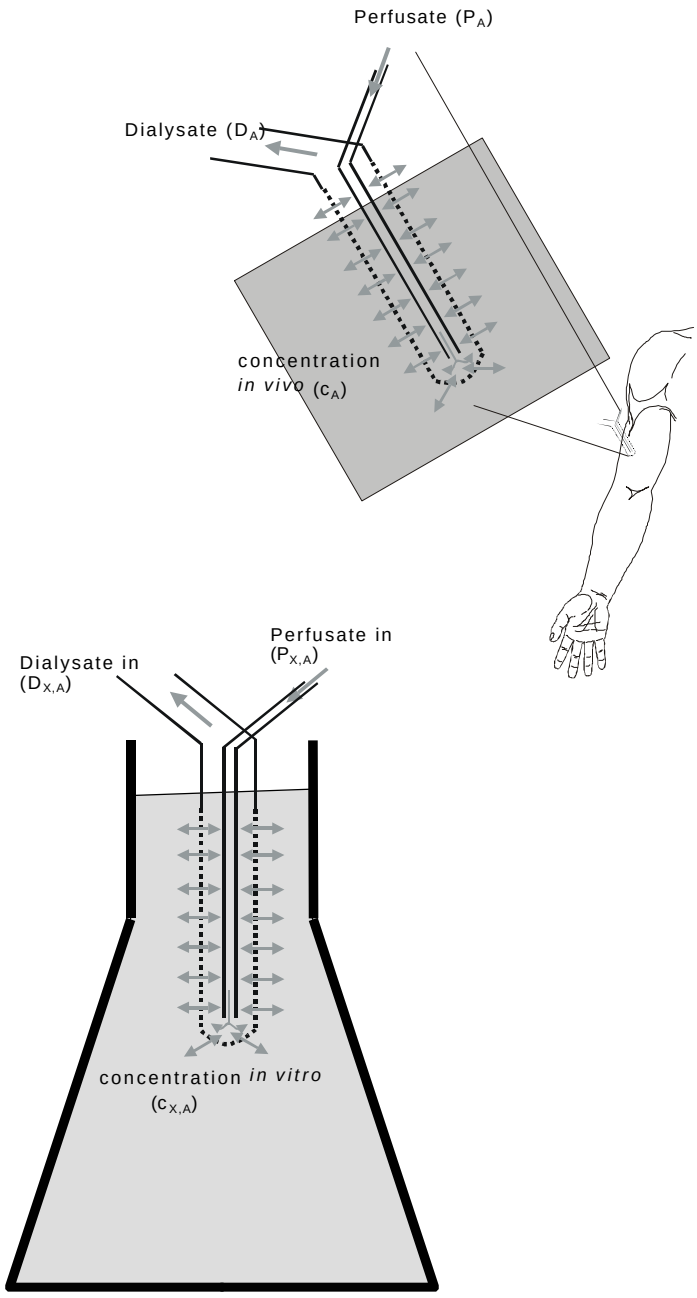


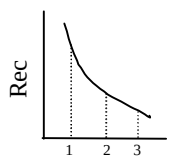
Fig. 2.2. Recovery estimation approaches. A. Cartoon.

$$Rec_A = \frac{D_A}{c_A} * 100\% \Leftrightarrow c_A = \frac{D_A}{Rec_A / 100\%}$$

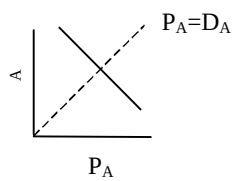
1. $Rec_A = Rec_{X,A} \Leftrightarrow \frac{D_{X,A}}{c_{X,A}} = \frac{D_A}{c_A} \Leftrightarrow c_A = \frac{c_{X,A} D_A}{D_{X,A}}$

2. $D_A = c_A$ if $t \to \infty$

3. $[\frac{D_A}{c_A}]_{flow=1} = Rec_1 ; [\frac{D_A}{c_A}]_{flow=2} = Rec_2 ; [\frac{D_A}{c_A}]_{flow=3} = Rec_3 \Rightarrow c_A = [D_A]_{flow \to 0}$



4. if $P_A = D_A$, then $P_A = c_A$



5. $Rec_A = (1 - (\frac{D_A}{P_A})_{before}) * 100\%$

Rec_A:	recovery *in vivo* of component A
$Rec_{X,A}$:	recovery *in vitro* of component A
c_A:	concentration of component A *in vivo*
$c_{X,A}$:	concentration of component A *in vitro*
D_A:	concentration of component A in dialysate after *in vivo* sampling
$D_{X,A}$:	concentration of component A in dialysate after *in vitro* sampling
P_A:	concentration of component A in perfusate after *in vivo* sampling
$P_{X,A}$:	concentration of component A in perfusate after *in vitro* sampling

6. $1 - \frac{D_B}{P_B} = \frac{D_A}{c_A} \Leftrightarrow c_A = \frac{D_A}{1 - (D_B / P_B)}$

7. c_A known $\rightarrow Rec = \frac{D_A}{c_A} * 100\%$ known

Fig. 2.2. Recovery (Rec) estimation approaches. B. Formulas. See Fig. 2.2A and text for details.. 1. In vivo *recovery equals to* in vitro *recovery; 2. Low flow method and stop-flow method; 3. Changing flow rate method; 4. No-net-flux method or equilibrium technique; 5. Retrodialysis method; 6. Internal reference technique; 7. Calibration with an independent method.*

Methods for estimation of the *in vivo* recovery Rec_A and the subsequent estimation of the *in vivo* concentration c_A:

1) *In vivo* recovery equals to *in vitro* recovery (Zetterstrom and Ungerstedt, 1984).
   Originally, the *in vivo* recovery Rec_A was estimated by direct usage of the *in vitro* recovery $Rec_{X,A}$. However, they can not be corrected for the small extracellular volume, and variations in viscosity and tortuosity of the tissue (Sarre et al., 1995).
2) Low flow method (Menacherry et al., 1992; Justice, Jr. 1993; Kaptein et al., 1997) and stop-flow method (Gardner et al., 1993).
   These methods are based upon the concept that when dialysis fluid D_A and extracellular fluid c_A are in contact with each other for a long period, the recovery will approximate to 100%. When the flow rate is chosen low enough (<0.5 µl min^{-1}) or alternatively, when the flow is stopped long enough, a nearly 100% recovery may be expected. This assumption can be correct, but can not easily be checked.
3) Changing flow rate method (Hegemann et al., 1995).
   The recovery Rec_A is calculated by measuring the concentrations at changing flow rates, whereupon the flow rate is extrapolated to zero. Disadvantages of this method are that it is rather time consuming and that it requires a steady-state condition.
4) No-net-flux method (Gardner et al., 1993) or equilibrium technique (Arner and Bolinder, 1991).
   Perfusates contain a range of pre-set concentrations of the analyte P_A. When the concentration of the analyte in the extracellular compartment c_A is higher than in the perfusate, the analyte concentration of the dialysate will increase as compared to the perfusate. At higher concentrations in the perfusate, the analyte concentration in the dialysate will be lower. Interpolation will give the exact extracellular concentration. This method is quite labour intensive and works only for analytes which do not vary during the calibration. Additionally, the method may create an artefact, because the analyte in the perfusate may disturb the physiological extracellular concentration.
5) The retrodialysis method (Müller et al., 1995).
   This calibration technique is based on the assumption that the diffusion of the analyte through the membrane is equal in both directions. Before the measurement, concentrations around 100 times above normal *in vivo* concentrations are perfused. The recovery can be calculated by measuring the percentage of the fraction of the concentration of this analyte in the perfusate D_A and the perfusate P_A and subtracting this percentage from 100%. This method has two drawbacks. The first is that the local analyte concentration around the probe may increase as a result of this perfusion. The second is that, if the analyte concentration is chosen too low, the endogenous concentration may influence the measured recovery.
6) Internal reference technique (Rosdahl et al., 1993; Sarre et al., 1995; Lonnroth and Strindberg, 1995).
   In this calibration technique, it is assumed that the ratio of the recovery of the analyte and the loss of an internal standard (a kind of an inverse recovery) *in vivo* and *in vitro* is the same. The internal standard (also called *in vivo* marker) is a compound with a "behaviour" similar to the analyte. The advantage of this method is that it can also be applied in non steady-state conditions and that it detects recovery changes during

sampling. Major problems of this technique are finding a good marker and the need for a dual detection system, detecting the analyte as well as the *in vivo* marker.

7) Calibration with an independent method (Chen and Steger, 1993).
   When the MD sample can be calibrated with an independent technique, the data will be most reliable. For example, discrete blood samples can be analysed and compared with the MD data. However, many MD sampling sites (e.g. subcutaneous tissue) had no alternative methods for sampling.

Of all the afore-mentioned methods, calibration with another technique seems to be the most reliable, but, in practice, only the blood compartment has an optional sampling possibility. An important deficiency of all mentioned methods, with the exception of the internal reference technique, is that they do not account for changes in recovery during the sampling (Sarre et al., 1995). These changes occur often, even within a short period of time, as a result of blockage of the membrane by proteins (e.g. fibrin) or cell structures, or due to pathophysiological changes in the tissue structure (Ash et al., 1992).

In addition to the technical problems of recovery determination, MD may also cause artefacts in the tissue. This is not necessarily due to tissue trauma as a result of insertion of the probe, but rather to drainage of the analyte or of other compounds present in the extracellular fluid (Rosdahl et al., 1993). Drainage is the depletion of analytes from the tissue around the probe as a result of the sampling process, in which the analyte diffuses into the dialysis fluid. Balancing the pH and ion content of the perfusion medium with that of the living tissue is required but is not easily accomplished (Osborne et al., 1991). A MD buffer lacking certain compounds, for example ions present in the endogenous fluid, can influence the physiology at the sampling site. Moreover, drainage of the analyte may not only influence the tissue physiology, but also affects the actual measurement. Rosdahl et al. (1993) calculated that in their experimental set-up the drainage of glucose was in the same order of magnitude as the physiological supply of the analyte in the subcutaneous and muscular interstitial fluid sampling they performed. The best way to counter this phenomenon is to select a flow rate as low as possible and to emulate the extracellular fluid as well as possible (Osborne et al., 1991). The minimal flow rate depends on the recovery of the analyte, the sensitivity of the analysis method and the time resolution required.

In 1987, the first report was published on a sampling technique alternative to MD (Janle-Swain et al., 1987): Ultrafiltration. This alternative not only avoids the difficult and time consuming recovery calculations of MD, but prevents drainage as well.

Ultrafiltration

Ultrafiltration (UF) is a technique in which endogenous fluid is withdrawn from the sampling site driven by underpressure (Janle-Swain et al., 1987). Lust like MD, the UF probe consists of a semi-permeable membrane that excludes large molecules (e.g. proteins), whereas small analytes (e.g. glucose) enter the probe together with the water and salts

(Fig. 2.3). UF was first described by Janle-Swain et.al. (1987) for batch-wise sampling in subcutaneous tissue. Approximately 40-50 µl h^{-1} (0.01 µl mm^{-1} probe min^{-1}) (Ash and Janle-Swain, 1988; Ash et al., 1992) can be sampled with this technique. In 20-minute time intervals, they did not observe any significant difference in the acetaminophen concentration measured with two probes placed contralaterally in a single subject (Linhares and Kissinger, 1993a).

Recently, Moscone et al. (1996) and Kaptein et al. (1997) have used UF for on-line sampling (see also *chapter 3* and *4*). The underpressure was created with a small, disposable syringe and the flow rate was regulated with a flow restriction tube, a tube with a very small internal diameter. Accordingly, a constant flow rate of 100-300 nl min^{-1} for a 4 cm probe was obtained, and sampling was performed up to 24 hours. Sampling was performed in subcutaneous tissue as well as intravenously in the rat, and the sample was analysed on the glucose and lactate concentration.

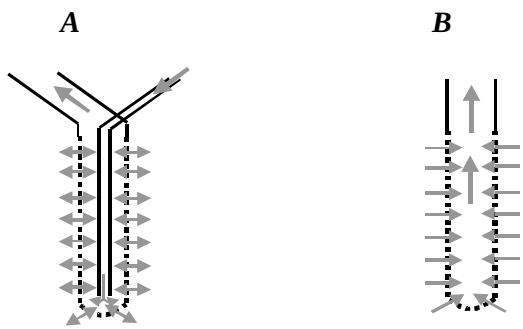


Fig. 2.3. Schematic representation of microdialysis (A) and ultrafiltration (B).

UF provides “recoveries” above 95 % for low molecular weight molecules (Linhares and Kissinger, 1993a), because there is no dilution factor. A small correction factor, by which the tissue concentration appears to be smaller than the measured concentration, must be included for the osmotic pressure due to the exclusion of restricted compounds (e.g. proteins) (Linhares and Kissinger, 1992b). Another correction factor should be included, because in particular larger molecules are hindered to pass the membrane of the UF probe, leading to an underestimation of the analyte concentration (Linhares and Kissinger, 1992b; Scheiderheinze and Hogan, 1996). In theory, both correction factors can be determined *in vitro* (Linhares and Kissinger, 1993a) and it is hypothesized that it will not be changed by partial blocking of the probe surface.

Reflection upon MD and UF

MD and UF have many similarities: Both techniques can use disposable material, and create relatively clean, on-line samples. The partial purification of the sample allows direct

analysis for example with HPLC-systems. They also share the drawback that hydrophobic compounds (such as steroids) can not be measured accurately, because they do not diffuse through the membrane easily and/or adsorb to the tubing material. Moreover, these compounds are *in vivo* mainly bound to (serum) proteins.

Larger molecules are barely or not at all diffusing through the membrane in case of MD (Ungerstedt, 1991). In contrast, for UF, large molecules can enter, though the "recovery" is below 100% (Scheiderheinze and Hogan, 1996).

The necessity of an additional fluid in MD sampling may lead to changes in the surrounding tissue. Sterility is more difficult to achieve and to maintain, because of the addition of extra fluid. Furthermore, the probe configuration is more complex. However, little is known about the effect of having a probe implemented and the withdrawal of fluid when UF sampling is performed. Janle-Swain et al. (1987) have demonstrated a zone of fibrin tissue around the probe after three months of sampling with an UF probe. In case of MD, such a fibrin layer would not allow to recover the analyte. The influence of this on UF sampling with low flow rates has not yet been investigated in detail, though it might be expected that it may change some of the analyte concentrations because of metabolic activities of the tissue (Ash et al., 1992). Examination using electron microscopy should also be performed in order to gain insight into the direct effects of the probe on the tissue.

A problem that was encountered when on-line UF was performed in abdominal (subcutaneous) tissue in man was that the probe also held a resistance (Tiessen et al., 1997), thereby decreasing the flow rate. When analysis is based on absolute amounts of an analyte per time interval, the concentration is then difficult to estimate.

In conclusion, a general decision as to whether MD or UF should be used can not be made. All parameters involved in the planned analysis have to be considered before choosing the sampling technique. Therefore, not only the sampling site, but also the (expected) recovery, the sensitivity of the analysis method and the conditions in which the measurements will be accomplished (e.g. in a hospital or at home) have to be taken into account. When, for example, a 24-hour profile of an analyte of an ambulant patient is to be made, UF holds a lower infection risk, and no additional fluid has to be carried, so if the applied flow rate does not cause hindering for passing the membrane of the probe, this is the method to be chosen. However, when the sampling site has a limited fluid production, MD is a better alternative.

To prevent drainage and improve recovery in MD, studies have been made with MD with a low flow rate (100-300 nl min^{-1}) in subcutaneous tissue of rats. In comparison with UF it showed a recovery for MD at these flow rates of 100% (Kaptein et al., 1998). Also, there was no flow restriction over the probe for UF sampling. This means that MD at low flow rates might be an alternative for UF when the sampling sites do not have sufficient fluid, whereas in other cases, if enough fluid is available, UF may be the best choice.

Places for sampling

A very important issue in obtaining useful data is the decision in which tissue or which body fluid the sampling should be performed. The outcome of this decision is influenced by:

1) Safety considerations. This includes risk evaluation of infections, inflammation and blood clotting.
2) Occurrence of the analyte at the sampling site and fast change of the analyte after the event of interest. For example, protein markers for acute myocardial infarction are not expected to diffuse to subcutaneous tissue, whereas small molecules like glucose do. On the other hand, monitoring of these protein markers might well be performed in heart muscle.
3) Knowledge of the concentration of the analyte at the sampling site. Clinical decisions of biochemical parameters can only be made when the "behaviour" of the analyte at the particular sampling site is known.

As stated in the introduction, at present most clinically relevant analytes are measured in discrete blood samples and estimation of the concentration of an analyte in blood is preferable. For such measurements there are four places of sampling, as described in literature: intravenous, subcutaneous, transcutaneous and in the peritonial cavity (Reach and Wilson, 1992). Of these four, the peritoneal cavity is not an attractive option. It is difficult to reach, carries a large risk of damaging the probe due to the peristaltic movement of the intestines, and the probe will be covered by, among other things, fibroblasts (Woodward, 1982). The intravenous, subcutaneous and transcutaneous samplings, however, all have useful applications and will be discussed below. Also, some examples of sampling in other organs, e.g. the brain, liver or kidney, are discussed, as such applications reveal important information for specific cases.

Intravenous

Both MD and UF can be used for continuous sampling of the blood compartment. The properties of the membrane prevent blood cells and large compounds (e.g. proteins) to enter the sampling tube (Paez and Hernandez, 1997).

MD sampling in the blood compartment has been used to study pharmacokinetics (Telting-Diaz et al., 1992; Ekstrom et al., 1995) and endogenous compounds such as glucose (Rada et al., 1993; Chen and Steger, 1993; Paez et al., 1996). The apparently controversial findings in the recovery with the intravenous (i.v.) probes is quite remarkable. Chen and Steger (1993) found that the recovery of glucose in heparinized rats remained stable, whereas in unheparinized rats the recovery dropped from 28% to 1% within 24 hours. On the other hand, Rada et al. (1993) and Stenken et al. (1993) stated that for the analytes they measured (glucose, epinephrine), the *in vivo* recovery was equivalent to the *in vitro* recovery, and remained stable during the seven days of measurements. However, most current reports show that the *in vivo* and *in vitro* recovery differ (Sarre et al., 1995). In

humans, glucose measurements with i.v. MD have been performed in the cubical vein of healthy volunteers (Paez and Hernandez, 1997). Until present, intravenous UF sampling has only been described once. Kaptein et al. (*Chapter 3*) measured glucose in the jugular vein of rats with a sample flow rate of 100 nl min^{-1}.

Despite their physiological advantages, i.v. MD and UF are not yet used routinely, obviously due to the risks of having an artificial object in the blood stream and the relative unfamiliarity of the technique. The technique is still rather invasive. Subcutaneous sampling is, therefore, suggested as an alternative for i.v. sampling.

Subcutaneous

Subcutaneous (s.c.) monitoring has been performed for two reasons: to study local effects and to estimate analyte concentrations in blood. Subcutaneous tissue fluid is believed to reflect the blood characteristics of many analytes relatively well (Poitout et al., 1993; Thome Duret et al., 1996). The implantation of a sampling or measurement device in sc. tissue is relatively easy, and the risks for infection and body-reactions are mild when compared to full-blood contact (Arner and Bolinder, 1991). Subcutaneous implantation of an UF or MD probe is quite 'patient-friendly', because in most cases it can be performed without anaesthetic or complicated surgical procedures (Ash et al., 1993). The recovery in s.c. tissue is generally lower than in blood (Bolinder et al., 1983; 1989), and larger molecules, such as proteins, rarely penetrate into subcutaneous tissue. However, long term effects such as blocking of the sampling probe by proteins or encapsulation by collagen may occur (Reach and Wilson, 1992).

Most research with MD sampling in humans has been performed in sc tissue. In particular, glucose has been studied intensively, for the monitoring of diabetic patients and to set up an insuline administration schedule. Literature on subcutaneous glucose is all but conclusive as to whether it represents an accurate reflection of blood concentrations. Clinical decisions on the outcome of sc tissue concentrations are therefore controversial. Several reports suggest that the kinetics of glucose in blood and s.c. tissue are similar (e.g. Bolinder et al., 1989; Reach and Wilson, 1992), because blood capillaries are rarely more than 20 µm away from any single functioning cell (Linhares and Kissinger, 1993b). However, after close inspection of the available data (Pickup et al., 1989; Ertefai and Gough, 1989; Lonnroth, 1996; Sternberg et al., 1996) and the preliminary results of our studies (Tiessen et al., 1997; Kaptein et al., 1997) we are prompted to believe that the subcutaneous tissue fluids form a distinct body compartment, and that analyte concentrations exhibit different time profiles than in case of blood. From a physiological viewpoint, this can be explained by the fact that the cells in the subcutaneous tissue might change the concentration of glucose, e.g. metabolism of glucose by subcutaneous fibroblasts (Ash et al., 1992). Obviously, the relation of s.c. tissue concentrations and plasma water concentrations are different for every analyte studied and may vary from subject to subject.

Besides estimation of the blood compound concentration, it is also possible to perform measurements in subcutaneous tissue to study local metabolism and kinetics (Arner and Bolinder, 1991). For example, chemotherapy and local drug deliveries require low systemic and high local concentrations. If the chemicals are perfused in subcutaneous tissue, such measurement in this tissue may provide very useful information. Hegemann et al. (1995) studied local effects of the nicotine distribution in the dermis of healthy volunteers. They observed an increase in blood flow around the probe for up to one hour, and noticed that the maximum levels may depend on the location of the probe. When the probe is in close contact to blood vessels, it might (partially) sample blood compounds directly, either because of damage to blood vessels or because of the short diffusion distance (Linhares and Kissinger, 1993b).

Transcutaneous

An even more controversial way of estimating blood concentrations is measuring transcutaneously. For example, sweat can be collected continuously. Non-invasive sampling of sweat or analyte on the surface of the skin can be performed with MD (Korf et al., 1993; De Boer et al., 1994). However, the recovery is low (approx. 2%) and the concentration of analytes is strongly influenced by the volume of sweat produced. In addition, many analytes do not, or only in very small amounts, diffuse through the skin. In particular, large molecules (e.g.proteins), hardly penetrate the skin.

An interesting application of transcutaneous sampling has been described by de Boer et al. (1994) They measured glucose in neonates with a transcutaneous microdialysis probe. Transcutaneous measurements seem to be restricted to cases when the skin is very thin, for example, the transcutaneous samples of the neonates resembled the glucose concentration in blood almost perfectly.

Sampling in other organs

Besides the previously mentioned body compartments, there are other organs which have been the subject of sampling, as described in many (animal) studies. Most of these organs are not expected to become routinely available for sampling for clinical diagnosis in humans, because of ethical and experimental considerations (Hamberger et al., 1991). However, in some particular cases, there might be a need to perform sampling in other tissues.

Brain

MD has predominantly been applied in the study of brain chemistry of experimental animals *in vivo* (Justice, Jr. 1993). MD is the first method allowing continuous sampling of the hitherto relatively inaccessible neural compartment *in vivo* (Robinson, 1995).

One of the first reports describing microdialysis was written by Ungerstedt and Pycock (1974). They implanted a hollow dialysis fiber in the brain of a rat and applied a constant perfusion. The MD technique has been further optimised and characterized over the years. Over 2000 studies have been performed since then, studying neurotransmitters (Rosdahl et al., 1993), drugs (Stahle, 1992), metabolites (Stjernstrom et al., 1993), allergic mechanisms (Petersen et al., 1992) etc. in animals *in vivo*.

An example of intracerebral microdialysis in human subjects was given by Kanthan et al. (1995). They reported a study about the glutamate release during ischemia. Because of ethical and experimental reasons, this type of application is not likely to develop significantly. The same can be said about sampling in lumbar subarchnoid space in humans (Persson et al., 1993). Such methods are only applicable under very special circumstances, e.g. after surgery, when important medical information can be obtained by this technique.

Muscle

Müller et al. (1995) demonstrated time profiles of paracetamol and gentamicin from MD probes in muscle, comparing the data to serum samples. They did not find significant differences in case of simultaneously measuring in the muscle on the left and right side of the body. This was especially true for gentamicin, a compound that responses differently in the blood and the muscle compartment, so that differences at the sampling site might be expected, because in this case, the distance of the probe from a blood vessel is very important. However, possible differences may have gone unnoticed, because of the poor time resolution (20 minutes).

Langeman et al. (1996) inserted a probe into the interventricular septum of the heart during an aorta-coronary bypass operation. They claim that the MD in this tissue is feasible, although this would require an additional effort from the surgeon.

Other organs

In case of specific organ malfunctions or physiology, sampling in other organs can provide important information. For example, chemotherapy and local drug deliveries can be checked locally instead of systemically. This enables one to adjust the concentrations so that they are high locally and low in blood. Ekstrom et al. (1995) have reported successful MD in muscle, kidney and liver. These organs are very different in structure, morphology, enzymatic activity and uniformity. Additionally, sampling has been performed in the uterus of the rat, the ovary, the pineal, the pituitary gland (Robinson, 1995), the adrenal gland (Jarry et al., 1989) and the corpus luteum (Maas et al., 1992).

Concluding remarks

The MD sampling technique holds the potential to become a powerful new technique, for both research and clinical applications. On-line, continuous *in vivo* sampling can be performed, not only in blood, but also in other body compartments. For some analytes, monitoring the concentration in the subcutaneous extracellular space, or even with transcutaneous sampling, can estimate the blood concentration. Insertion of sampling probes in these areas is less invasive than in blood vessels, and reduces the risks. However, in many cases it is not possible to predict concentrations in blood by these measurements. For example, in some cases the analyte profile might be significantly delayed, and in other cases it is impossible for the analyte to diffuse to the s.c. tissue because of its size or polarity, and thus to be present in the s.c. fluid. Therefore, a decision on the best sampling site has to be made after careful consideration of all parameters involved in that particular application.

A definite general choice between MD or UF sampling in all conditions can not be made. The amount of fluid present at the sampling site, the risks of infection and the sensitivity and required sample size of the subsequent analysis method, are the factors which determine the choice between these two methods.

This overview clearly demonstrates the potential of on-line sampling with UF or MD. Implementation of on-line sampling in clinical practice for diagnosis of illness and studiest in the pathophysiology, will lead to new insights and new findings.

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Chapter 3

Methodological aspects of glucose monitoring with a slow continuous subcutaneous and intravenous ultrafiltration system in rats[1]

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).

[1] Kaptein, W.A., Kemper, R.H.A., Ruiters, M.H.J., Venema, K., Tiessen, R.G. and Korf, J. In: *Biosensors and Bioelectronics* **12** (9&10): 967-76 (1997)

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^{-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 *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 *in vivo* monitoring of patients -bedside or ambulant- are very limited. Fur 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 *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^{-1}, approximately 1 ml day^{-1} (0.01 µl min^{-1} mm $probe^{-1}$) 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^{-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 cannulla 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.

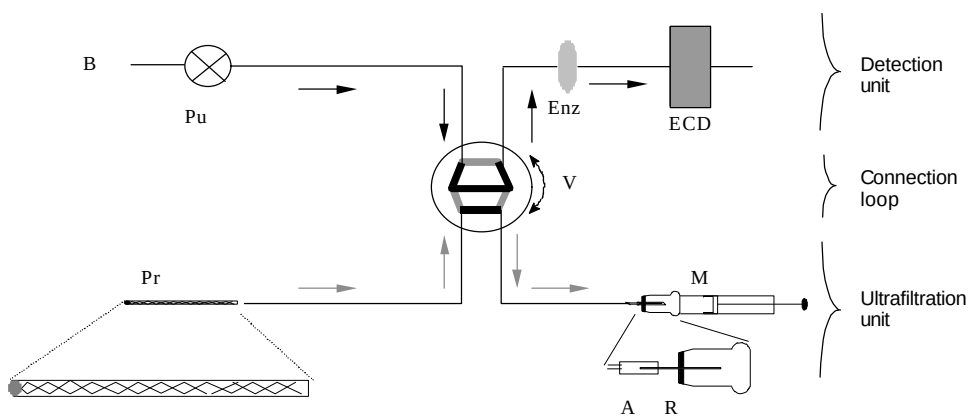

Fig. 3.1. Set-up of the ultrafiltration sampling and analysis. ® direction of the flow; A. air chamber; B. buffer. stock; ECD: electrochemical detector; Enz: enzyme reactor; M: monovette; Pr: probe; Pu: pump; R: restriction; V: valve

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 intraperitonial (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 Autoanalyser, 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 Na_2HPO_4, 2.5 mM KH_2PO_4 (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 intraperitonial (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.*

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.

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.

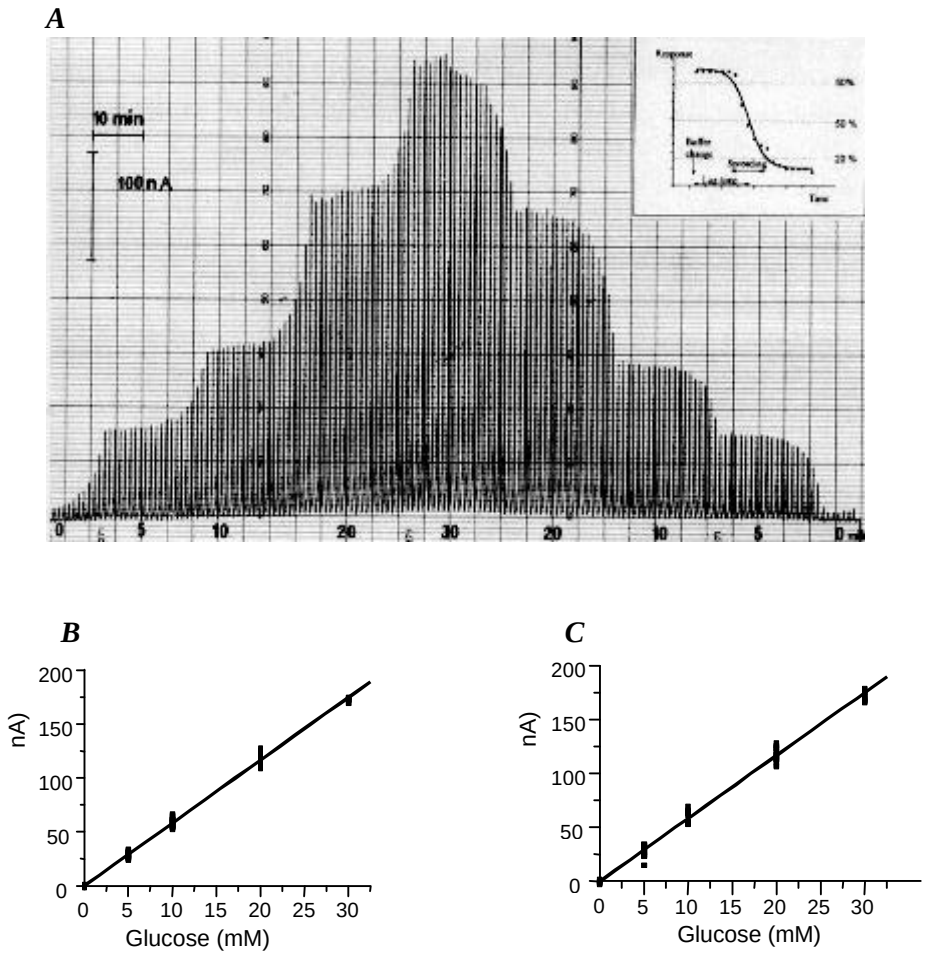

Fig. 3.2. In vitro *experiment A. Recorder output of standard curve (without probe). In window: Explanation of terms lag-time and spreading. B.* In vitro *regression curve the ultrafiltration system with probe for standard concentrations of glucose. C.* In vitro *regression curve the ultrafiltration 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

Glucose steps (in mM)	**With probe Delay (spreading) in minutes**		**Without probe Delay (spreading) in minutes**	
	=>	<=	=>	<=
0 <=> 5	9.7 (3.5)	9.4 (4.1)	8.4 (2.3)	8.9 (3.3)
5 <=> 10	9.1 (4.5)	9.6 (4.7)	8.9 (3.0)	9.1 (2.6)
10 <=> 20	9.7 (4.3)	9.5 (5.1)	9.3 (3.4)	9.0 (3.7)
20 <=> 30	9.2 (3.3)	9.3 (3.2)	9.5 (3.7)	9.0 (3.6)

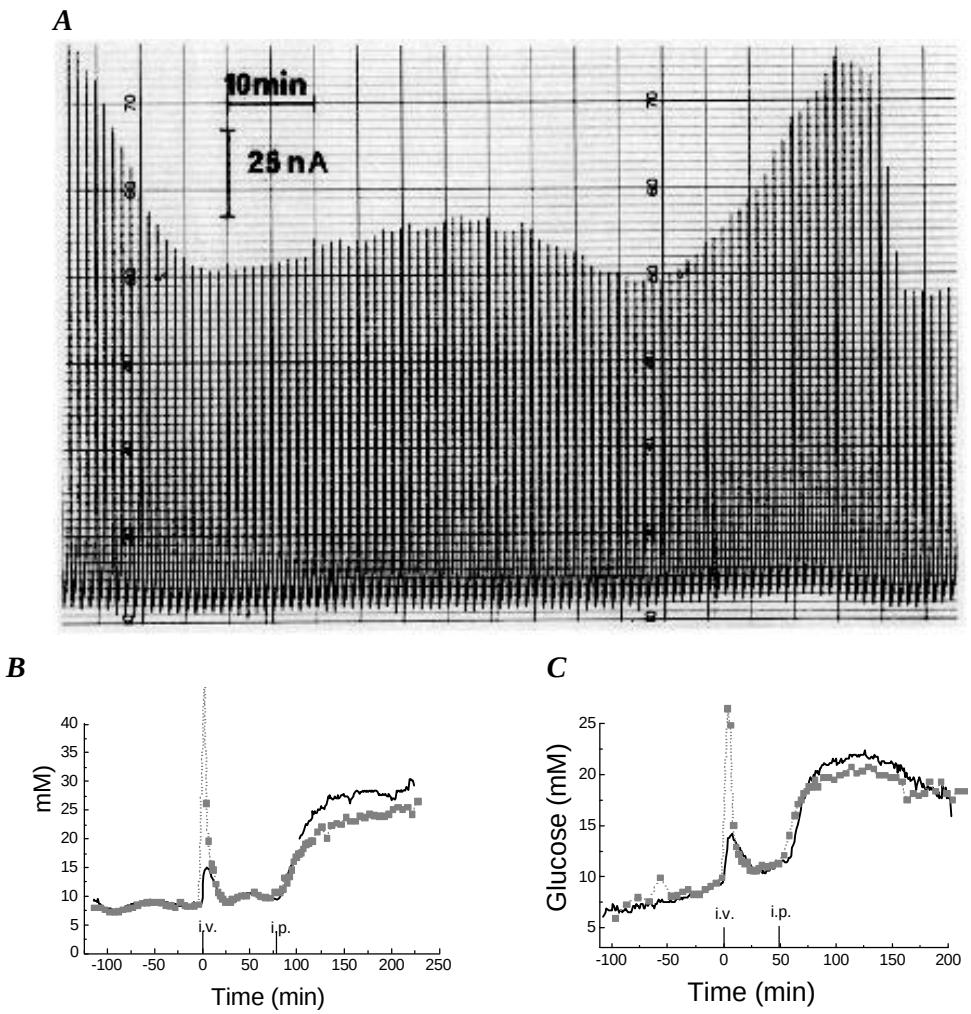

Fig. 3.3. Intravenous ultrafiltration A. Recorder output of intravenous ultrafiltrate glucose monitoring. B, C. Examples of UF (──) and blood sample (-- --) glucose concentration with i.v. and i.p. glucose injections.

In vivo *system evaluation*

Intravenous: Flow changes from in vitro => 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%.

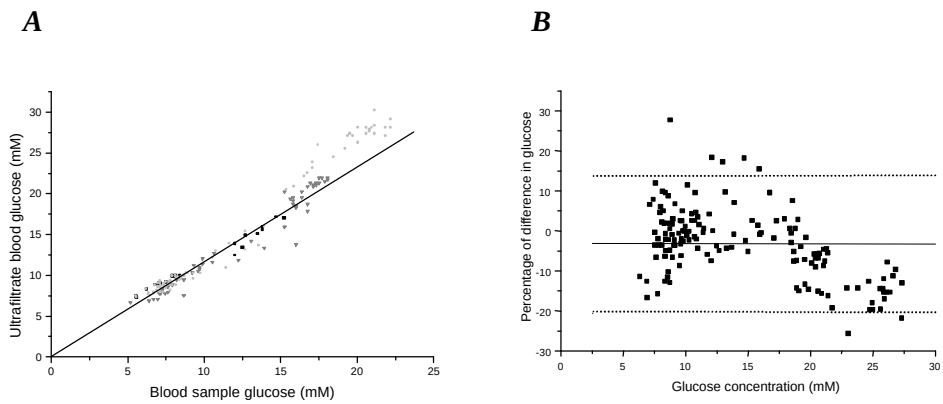

Fig. 3.4. Mathematical analysis A. Linear regression on ultrafiltrate measurements versus blood sample glucose for three rats (indicated with different symbols) B. Bland-Altman plot of proportional differences versus the average of blood samples and UF. The proportional differences are the ratio between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

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.

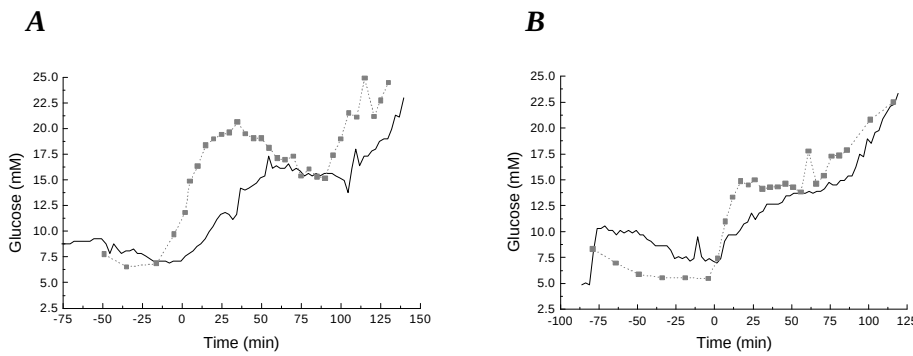

Fig 3.5. A,B. Examples of glucose concentrations of interstitium UF (──) and blood (--■--).

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 subcuteneous 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

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.

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Chapter 4

Continuous ultraslow microdialysis and ultrafiltration for subcutaneous sampling as demonstrated by glucose and lactate measurements in rats[1]

Voor een succesvolle technologie moet de realiteit voorrang krijgen boven public relations, want de natuur kan niet voor de gek gehouden worden (Richard Feyman, Nobelprijswinnaar 1965).

[1] Kaptein, W.A., Zwaagstra, J.J., Venema, K. and Korf, J. Submitted

Classical microdialysis has some drawbacks. Two main issues in this respect are the time consuming calculations (due to partial recovery of an analyte) and depletion near the sampling site. In this paper we describe a new sampling method, called ultraslow microdialysis (usMD), and compare this with ultrafiltration (UF) at flow rates between 100-300 nl min^{-1}. For the usMD, the recovery at the applied flow rates was 100%. For UF, the flow rates *in vitro* and *in vivo* were the same. As an example of an application of this method, we conducted in a dual enzyme analysis for simultaneous measuring of the glucose and lactate concentration using these sampling techniques in the subcutaneous (sc) tissue of the rat. Both sampling techniques have the potential of on-line *in vivo* monitoring, as well as the measurement of time profiles of analytes by continuous collection and off-line analysis afterwards.

Introduction

Biochemical constituents in blood can serve as parameters for illness, drug metabolism and regulation of homeostasis. Blood sampling and subsequent laboratory analysis is labour intensive, patient unfriendly and often expensive. *In vivo* applied biosensors may overcome many of these problems (Gilbert and Vender, 1996; Anderson et al., 1997). However, until now, there are only few examples of such devices used (batch-wise) in clinical practice. A problem of the biosensors placed in direct contact with the body is that they often evoke a physical reaction, such as covering of the sensor with collagen (Reach and Wilson, 1992) or, when placed in the blood stream, with fibrin (Ash et al., 1992). This creates an additional diffusion barrier, which results in lower measured analyte concentrations (Fischer et al., 1994). In addition, calibration of the sensor in-situ in man is often difficult. Furthermore, a sensor placed *in vivo* often becomes unstable. Measurements with the sensor *in vivo* are, therefore, often inaccurate (Pickup, 1993; Johne et al., 1995).

With microdialysis (MD), relatively clean samples can be obtained. MD is a dynamic sampling method based on analyte diffusion across a semi-permeable membrane driven by a concentration gradient. (Palmisano et al., 1997). MD probes have successfully been applied in both laboratory animals and in man, for example in the brain (Kanthan et al., 1995; Paez et al., 1996), subcutaneous tissue (Lonnroth et al., 1987; Arner et al., 1988) and blood (Telting-Diaz et al., 1992; Stjernstrom et al., 1993; Chen and Steger, 1993; Paez and Hernandez, 1997). A drawback of the MD technique is that calculation of the recovery *in vivo* is difficult, labour intensive and often rather imprecise (Justice, Jr. 1993; Johne et al., 1995). In addition, the sampling can lead to a local depletion of the analytes, because of a removal of the analytes near the MD probe, which leads to an underestimation of the concentrations (Petyovka et al., 1995). To reduce the drainage and to obtain a (near) 100% recovery, the flow of the dialysis fluid has to be very low (less than 0.5 µl min^{-1} (Petyovka et al., 1995)).

Recently (Moscone et al., 1996a; Kaptein et al., 1997a), we have introduced an on-line ultrafiltration (UF) method as an alternative for continuous sampling. UF is a sampling technique that withdraws fluid from the tissue through a semi-permeable membrane. Others (Linhares and Kissinger, 1992; Linhares and Kissinger, 1993; Scheiderheinze and Hogan, 1996) have previously described UF for off-line analysis. The driving force in this process is underpressure in the sampling tube, which creates an influx of extracellular fluid through

the semi-permeable membrane of the probe. We have applied UF for subcutaneous and intravenous sampling in rats with an ultra-slow continuous flow (100 nl min^{-1}) in combination with direct analysis of the glucose content (Moscone et al., 1996). Also, we analysed a concentration profile in a continuously collected 24 hours sample (Kaptein et al., 1997a). The underpressure was created with a disposable syringe, a monovette, with a fluid restriction to ensure a constant flow over several hours.

When we tested the UF in sc tissue of healthy human volunteers (Tiessen et al., 1997), we occasionally observed an additional resistance over the probe, leading to a decrease in the flow rate. Even at flow rates as low as 100 nl min^{-1}, there was insufficient formation of the extracellular fluid. Therefore, in this study we investigated ultraslow microdialysis (usMD) sampling in the subcutaneous tissue of the rat and compare the sampling with ultrafiltration (UF). At flow rates between 100-300 nl min^{-1}, we study the recovery of the usMD, and for UF we evaluate resistance over the probe. We compared the sampling techniques using a dual enzymatic analysis technique for glucose and lactate.

Material and methods

General description of the system

The experimental set-up of a measurement is shown schematically in Fig. 4.1. The probe (for sampling) was coupled to a dual detection system, consisting of an HPLC pump (HPu), a splitter (S; 50-50% splitting ratio) and two enzyme reactors (L and G), both upstream of an electrochemical detector (ECD1 and ECD2). One of the enzyme reactors contained horseradish peroxidase, HRP and glucose oxidase, GOD, whereas the other contained HRP and lactate oxidase, LOD.

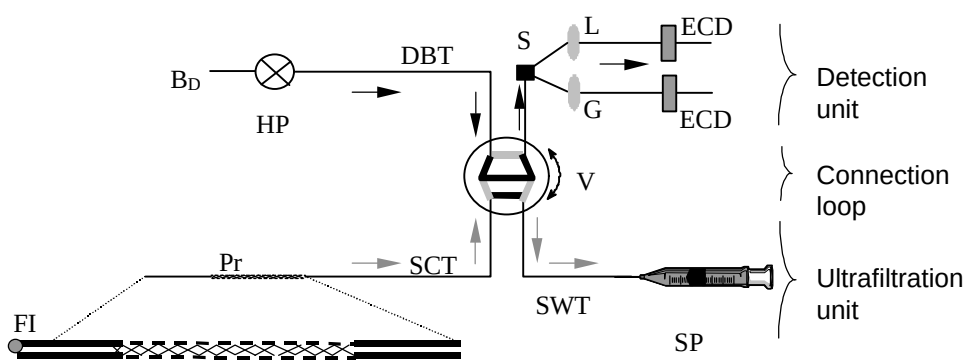

Fig. 4.1. Schematic set-up of the dual enzyme UF/usMD system. BD: buffer detection unit; HPu: HPLC pump detection unit; DBT: detection buffer tube; S: splitter; L: LOD/HRP enzyme reactor; G: GOD/HRP enzyme reactor; ECD1,2: electrochemical detector; V: valve; SCT: sample connection tube; SWT: sample waste tube; BMD: buffer for usMD; FI: fluid inlet for usMD/UF transition; Pr: probe; SP: syringe pump sample unit; =>: flow direction.

Instrumental set-up

Our probe was a modified design of a previously described ultrafiltration probe (Moscone et al., 1996; Kaptein et al., 1997). We used a four centimetre long probe from semi-permeable membrane (fibers of an artificial kidney, AN69HF Filtral 16; Hospal Ind., Meyzieu, France, outer diameter = 290 µm, inner diameter = 240 µm) with a hand-made spring inside (stainless steel wire; Vogelsang, Hagen, Germany; 60 µm diameter, axial length 12 windings cm^{-1}) to prevent collapsing of the fibre. This probe was connected on both ends to a 20-30 cm long fused silica tube (inner diameter 50 µm, outer diameter = 150 µm, Applied Science Group, Emmen, The Netherlands). The spring was glued to the fused silica tubes, and the connection of this with the fibre was closed with cyano-acrylic glue (Henkel, Nieuwegein, The Netherlands). One end was screwed into the valve of the Trident (Spark Holland, The Netherlands), the other end was closed (UF) or held in a PBS-buffer (usMD). The UF or usMD flow (100-300 nl min^{-1}) was driven by the underpressure of a Hamilton syringe (0.5 ml) by pulling with the Harvard apparatus 22 syringe pump. The valve, switched pneumatically by the Trident (load/inject 15/90 seconds), had a loop of 20 µl, which was only partially filled with the sample.

Glucose and lactate were detected electrochemically using bi-enzyme reactors as described by Elekes et al. for glucose (Elekes et al., 1995). The flow injection system has been described before by our group (Kaptein et al., 1997a). In brief, the buffer used for the detection system is a ferrocene-PBS buffer, containing 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 2.5 mM KH_2PO_4. All the chemicals were of pro-analysis quality and were purchased from Merck, Darmstadt, Germany. To this, 0.5 mM Ferrocenemonocarboxylic acid, (Sigma Chemical Co, St. Louis, MO, USA) and 0.1 volume% Kathon CG (Rhom and Haas, Croydon, UK) was added in double quarts distilled water. An HPLC pump (LKB 2150, Pharmacia Bromma, Sweden) pumped the ferrocene-PBS buffer (bubbled with helium to remove air), with a flow of 0.25 ml min^{-1}. This flow was divided in two separate streams after the injection valve and each was pumped through the one enzyme reactor and electrochemical cell. In the enzyme reactor for glucose, 250 U GOD (EC 1.1.3.4, grade I) and 250 U HRP (EC 1.11.1.7), obtained from Boehringer Mannheim (Germany), was immobilized between 0.01 µm cellulose nitrate filters (thickness 100 µm; pore size 0.01 µm, cut-off 50 kDa, Sartorius, Göttingen, Germany). The enzyme reactor for lactate comprised of 25 U LOD and 250 U HRP. The electrochemical cells were thin layer-type cells, with a glassy carbon working electrode hold at 0.00 mV relative to an Ag/AgCl reference electrode and a Teflon/carbon counter electrode (Amor, Spark Holland, Emmen, The Netherlands).

Theoretical considerations for the interpretation of the measurements

To explain the different interventions in the system and the effects from these on the signal, we show a schematic representation of a recorder output for one analyte with these changes (Fig. 4.2).

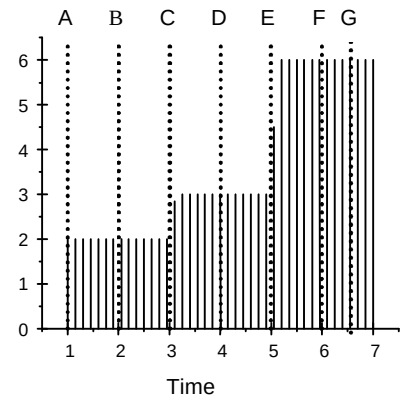

Fig. 4.2. Schematic representation of the different interventions of an in vivo *experiment. A. probe* in vitro*; B. probe* in vivo*; C. sc sample in detector; D. UF => usMD; E. usMD sample in detector; F. flow increase 2-fold; G. high flow sample in detector See text for details.*

At t=1, the measurement starts with analysing a buffer containing a certain concentration of the analyte (glucose or lactate) with the detection system. In this hypothetical case, we assume a start with UF. At t=2, the probe (Pr in Fig. 4.1) is transferred from *in vitro* to subcutaneous tissue. The sample connection tube (SCT) is then still filled with sample from the (*in vitro*) buffer. In case of any change in flow (e.g. as a result of a restriction over the probe membrane), the signal will immediately change. At t=3 the sample of the subcutaneous tissue reaches the detector. In this case, the concentration of the analyte is higher than in the previous buffer. At t=4, the fluid inlet (FI) for usMD is opened, thus starting dialysis. Again, if this would influence the flow rate because of restriction on the probe during UF, this would result in a change in signal. At t=5, the usMD sample reaches the valve (V), thus injected and analysed. If the recovery of the microdialysis is lower than 100%, the signal will decrease. At t=6, the flow is increased two-fold. This leads to a two-fold increase of signal, if the signal increase has a linear relationship with the sample amount. At t=6.5, the first sample from the higher flow reaches V. Changes in recovery at the higher flow, will be shown at this time.

In vitro *experiments*

The calibration of the measurement was performed by placing a probe in the ferrocene buffer with glucose concentrations of 0, 2.5, 7.5 and 10 mM, or lactate 0, 1, 2 and 4 mM, was changed incrementally. The load/inject time was 15/90, therefore the sample was measured every 105 seconds with the ECD and the currents (in nA) were recorded. The calibration curve was made with UF and usMD, which determined the system delay (the lag-time) that was caused by the volume of the connecting tube from the probe to the analysis system and the sensitivity for the analytes.

In vivo *experiments*

Male Wistar rats (250-350 g, Harlan, Zeist, The Netherlands) were housed groupwise on a 12-12h light/dark regime. Their food and drink were provided ad libitum. The rats were anaesthetized by an intraperitonial (i.p.) injection of 6 mg kg^{-1} body wt pentobarbital sodium (Sanofi, Maassluis, The Netherlands) and remained anaesthetized with these drugs. Using a rectal temperature probe and a heat pad, the body temperature was maintained between 36.5 and 37.5 °C.

Before starting an *in vivo* experiment, the sensitivity of the system was determined *in vitro*, using a buffer with 2.5 mM glucose and 1 mM lactate. The probe was placed in sc tissue on the back of the rat with a 16G catheter needle. The flow of the sampling system varied from 100-300 nl min^{-1} by changing the flow rate of the syringe pump (SP). The probe was alternated between UF and usMD by blocking or unblocking the fluid inlet (FI) with a stopper.

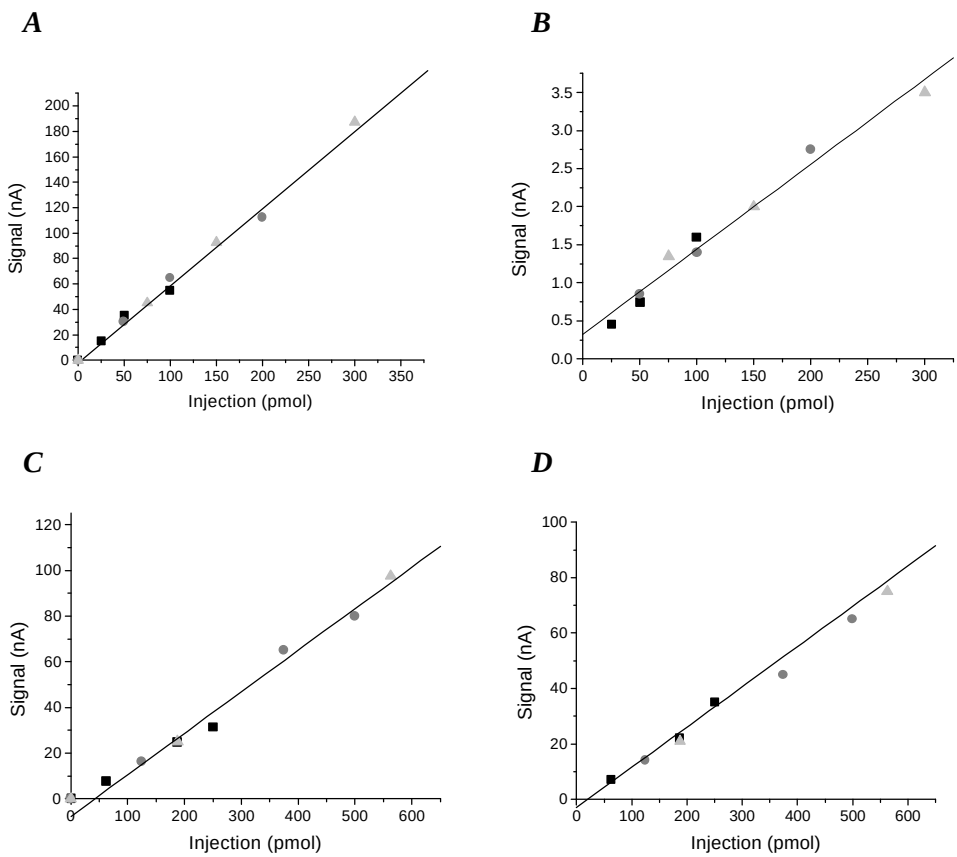

Fig. 4.3. Calibration curves A. lactate, UF (r>0.99); B. lactate, usMD (r>0.98); C. glucose, UF (r>0.99); D. glucose, usMD (r>0.99) ■ *:100 nl* min^{-1}; ● *: 200 nl* min^{-1};▲*: 300 nl* min^{-1}; ——*: regression.*

Results

In vitro *experiments*

Fig. 4.3 shows the effect of a different flow rate on the electrochemical signal for lactate (A&B) and glucose (C&D). For UF (Figs A&C) as well as usMD (Figs B&D) the current was linearly correlated to the concentration of the analyte. The sensitivity for lactate was 0.6 A mmol $^{-1}$ for UF ($r>0.99$; $p<0.000$) and 0.01 A mmol $^{-1}$ for usMD ($r>0.98$; $p<0.000$). The difference in sensitivity for lactate with UF or usMD was caused by the different enzyme activity. The enzyme activity for LOD, and therefore its electrochemical signal, decreases considerably with time. However, the decrease during an (*in vivo*) experiment was negligible, as checked with standards before and after the experiment. For glucose, the sensitivities were respectively 0.18 A mmol $^{-1}$ ($r>0.99$; $p<0.000$) and 0.12 A mmol^{-1} ($r>0.99$; $p<0.000$). The curve remained linear for all curves for the applied amounts from 0-350 pmol for lactate and 0-750 pmol for glucose.

In vivo *experiments*

Fig. 4.4 shows representative data of a measurement in the sc tissue of a rat. As was indicated, the flow was continuously changing and the sampling method (usMD or UF) was alternating. The transition from UF to usMD and vice versa did not change a signal either directly or after the delay caused by the volume of the SCT. This implies that the UF sampling does not have a significant restriction over the probe and that the recovery for this *in vivo* usMD at the applied flow rates is 100%. There was some slight fluctuation in the flow, which can be seen when both analytes fluctuate simultaneously, e.g. at time interval II. This is often seen as a result of irregularities of the pumping system under these extremely low flow conditions. Sometimes, a decrease in flow at UF occurred at the highest flow (300 nl min^{-1}) as a result of the resistance over the membrane of the probe (data not shown).

When the signals for different flows (*in vitro*) were calculated, it appeared that a certain amount of fluid leaked, probably in the valve. This can be seen in Fig. 4.5, when the average of three measurements of glucose before and after a change of flow rate is calculated. The leakage is equivalent to 60 nl min^{-1}, thus with the 15 seconds load this would mean 15 nl per injection. The standard curves shown in Fig. 4.3 did not show this, because at that time the valves did not leak. Correction for this leakage did not influence the results. The concentration of glucose and lactate in the rat during the experiment is shown in Fig. 4.5. The glucose concentration is quite constant, whereas the lactate concentration fluctuates with a trend to lower values.

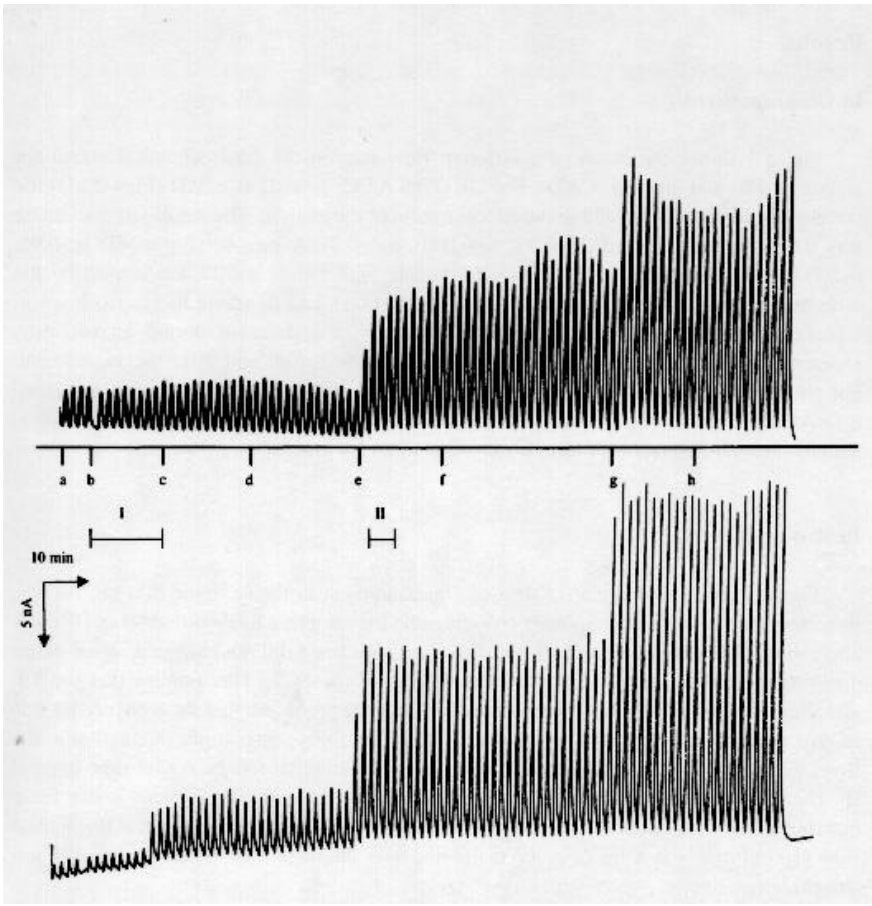

Fig. 4.4. Example of dual enzyme UF/usMD measurement in vivo *for lactate (upper) and glucose (lower). Indicated are: a: probe in UF sampling placed in buffer solution with 2.5 mM glucose and 1 mM lactate; b: probe placed in sc tissue of rat; c: UF sample in detection unit; d: transition UF => usMD; e: flow increase from 100 nl* min^{-1} *=> 200 nl* min^{-1}*; f: transition usMD => UF; g: flow increase from 200 nl* min^{-1} *=> 300 nl* min^{-1}*; h: transition UF => usMD; I: lag-time; II: see text.*

Discussion

In the present study, we demonstrated a system for UF and usMD with different flow rates. To illustrate its application, we coupled the sampling system to a dual enzyme detection system, analysing glucose and lactate simultaneously in one continuous sample in a flow injection analysis system with a splitter. The splitter volume ratio can be changed, and the amount of enzyme in the reactors and the sample volumes injected can also be adjusted. This allows for simultaneous analysis of other constituents, even if different

substrates are added or if sample sizes and sensitivities are different. Leakage in the valve connection must be reduced for these low flows. In combination with a collection tube, as described earlier, profiles can collected and analysed afterwards in laboratory conditions for any analyte, as long as there is an assay with enough sensitivity.

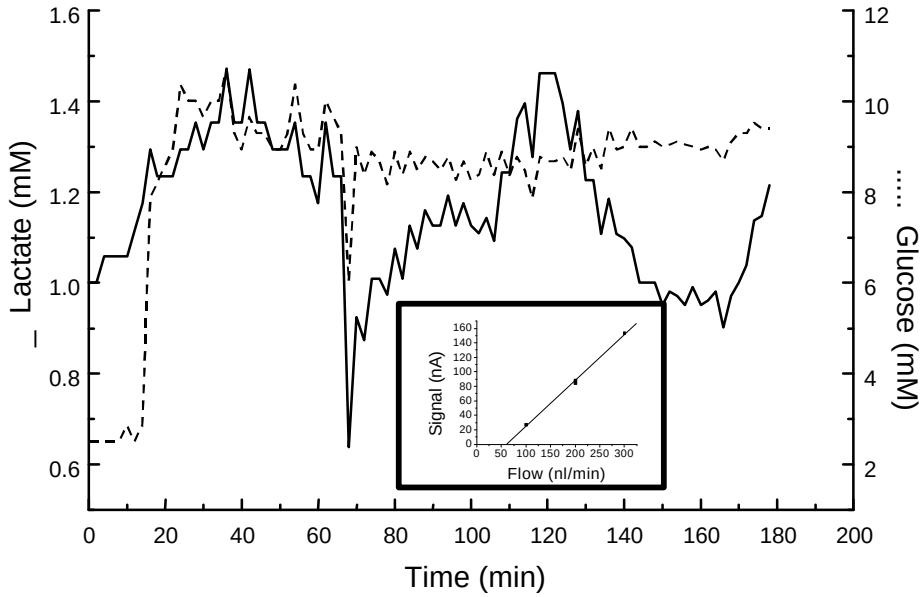

Fig. 4.5. Calculated lactate and glucose concentrations for recorder output of Fig. 4.4. Window: response at different flow rates for glucose in vivo.

The main study object of this research was the comparison of our new continuous sampling system (the ultraslow microdialysis, usMD) to ultrafiltration. By repetitive switches from usMD and ultrafiltration and vice versa, we were able to demonstrate a 100% recovery for this usMD under the applied conditions. We also showed that the UF sampling in the interstitial fluid in the back of the rats can be realized with a flow of 200 nl min^{-1}, and in most cases, such as in the given example, this flow was realized up to 300 nl min^{-1}. As mentioned in the introduction, these findings may be different when the UF technique is used in abdominal tissue in human subjects. When the flow is not measured, correction is difficult.

In the present study, we used a syringe pump, allowing flow changes during the experiment. Unfortunately, at these low flow rates this pump showed some flow disturbances as a result of pulsation caused by the moving parts in the pump. Previously (Moscone et al., 1996; Kaptein et al., 1997), we described a more precise, inexpensive pumping system for fixed ultraslow flow rates: a monovette who's driving force comes from an underpressure and a flow regulation by a fused silica restriction. For the present

experiment this was not utilized because changes of the flow rate are difficult to achieve with this pump.

The low flows for both UF as well as usMD have several advantages. The intervention of the tissue (fluid) around the probe is reduced, because of the small amount of the fluid or analytes removed. In addition, we demonstrated that for usMD the recovery of glucose and lactate is 100% at the applied probe size and flow rates. Labour-intensive, inaccurate recovery calculations are therefore not necessary.

Whether usMD or UF is the best option depends on the *in vivo* application. Both techniques can use disposable material, and both can create the relatively clean samples with (for small analytes) a 100 % recovery. UsMD is the best choice when the amount of fluid at the sampling site is limited. We noticed that in the brain of a rat and in subcutaneous tissue of humans, an *in vitro* flow rate of 100 nl min^{-1} is lower *in vivo* because of a restriction over the membrane of the probe. The exact concentration is then difficult to estimate. The drawback of the usMD technique is the necessity of an additional fluid, making it more difficult to keep the sampling system sterile (especially because the flow is created by the underpressure of the monovette "pump"). When, for example, a 24 hour profile of an analyte of an ambulant patient has to be made, UF will give less risks on infection, so if a constant flow can be created, this is the method of choice. However, when the sampling site is very limited in fluid production, usMD is preferable.

The described usMD and UF techniques can be integrated in many selection and detection systems. Selection can be performed in flow injection systems. The undiluted samples can then be separated with microtechniques, such as capillary electrophoresis (Hadwiger et al., 1997; Dawson et al., 1997) and microbore HPLC (Steele and Lunte, 1995). The absence of large molecules enables direct analysis. Selection can also be performed with enzymes creating products from the analyte which are detectable. An example for this is the enzymatic conversion of glucose into (among other) hydrogen peroxide, an electrochemical detectable compound (Fang et al., 1997; Anderson et al., 1997). This enzymatic reaction is often used in biosensors. Other biosensor selection devices are based on immunological reactions (Morgan et al., 1996b; Kaptein et al., 1997b). Biosensors might better function in UF/usMD sampling devices than in direct contact with the body, because of the relatively "clean" sample, whereas calibration (because of the 100% recovery) is easier than in MD sampling with µl min^{-1} flow rates. The total integration of the sampling, selection and detection in on-line biosensors, as demonstrated by Rigby et al. (1996), using a cheap, pulse-free pumping system as we described earlier (Moscone et al., 1996a; Kaptein et al., 1997a) enables on-line bedside measurements.

In summary, we described a sampling probe for ultraslow microdialysis or ultrafiltration. With simultaneous glucose and lactate analysis, we demonstrated a 100% recovery for microdialysis *in vivo* in subcutaneous tissue of the rat at a flow rate of 100-300 nl min^{-1}. No restriction over the probe was encountered at UF sampling up to at least 200 nl min^{-1}. Both sampling techniques enable on-line *in vivo* measurements as well as sample collection to study time profiles of various compounds by subsequent analysis of the collected sample and can be combined with several microanalysis systems.

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Chapter 5

Displacement: An incubation-step-free, quantitative immunological method for applications in flow systems

Discovery consists of seeing what everybody has seen and thinking what nobody has thought (A. Szent-Gyögyi)

Introduction

For the continuous on-line measurements of analytes, there are hardly any analysis techniques available. Except for specific cases, where the analyte has characteristics different from those of compounds in the matrix (such as particular electrochemical or optical properties) the techniques available are in the best case suitable for semi-continuous analysis. HPLC in combination with a detection system such as electrochemical detection (Sharp et al., 1987; Cheng et al., 1994) is one example of such semi-continuous analysis. Another example is capillary electrophoresis (Kissinger, 1996; Jardemark et al., 1997). These methods give, depending on the analyte and sensitivity of the detection a time resolution of 5-10 minutes. Besides this, these methods require expensive apparatus as well as highly trained personnel. They are, therefore, normally not suitable for bedside monitoring.

An alternative for this complex, expensive methodology might be found in sensor technology. A sensor is a device where selection and detection are integrated in one apparatus (Taylor and Schultz, 1996). Sensors for measuring pH or ion content are now used routinely (Hafeman et al., 1988; Van der Schoot and Bergveld, 1990). ISFETS, ion-selective field effect transistors, enable measurements of ions in complex matrices (Van Kerkhof et al., 1993). When the analytes are more complex, biosensors might be considered. A biological element, such as an enzyme or antibody, recognizes the analyte. This recognition eventually leads to a signal that is dependent on analyte concentration. For endogenous compounds such as glucose, this is well documented (Wilkins and Atanasov, 1996). On-line continuous biosensors are only described for the detection of metabolite molecules that can be specifically converted by enzymes. So for many other important analytes, techniques for on-line continuous measurements are not available. A technique studied recently that may offer on-line selection for other analytes is displacement, an immuno-based analysis method.

In the following, the new technique will be described. A theoretical model will try to explain the different parameters and to convey the interaction between them. A qualitative analysis will show how displacement can be manipulated to set up an analysis system. Finally, displacement systems will be compared to other techniques, to emphasize their possibilities.

Defining displacement

Displacement is the event that the complementary binding between an antigen binding site (ABS) of the antibody (Ab) and the corresponding antigen (Ag) or antigen analogue is actively broken after free antigen, the analyte, is added (Kusterbeck et al., 1990). This phenomenon may be explained assuming that the complex between the Ag and the Ab is reversible, and that the analyte in the sample block the ABS of the Ab, thus impair reassociation of the system Ag. An other explanation might be that the analyte induces dissociation, by active pushing out system Ag. Which explanation is true, can not be

explained scientifically. Other displacement descriptions, as e.g. used by Middlebrooks et al. (1991) in their "ligand displacement assay" is in reality a competition reaction. In case of competition, labelled antigen (Ag*) and Ag, present in comparable amounts in the solution, compete simultaneously for a limited amount of ABS. The two main differences between competition and displacement as defined here are therefore the time when the compounds are mixed and the molar proportions of the antibodies, antigens and labelled antigens. In case of displacement, the ABS of the antibody are (nearly) pre-saturated, and the binding will be broken after exposure to free analyte antigen in the matrix. In case of competition, the binding sites are empty before the simultaneous addition of the sample and labelled analyte analogue. These compounds are then competing for the (limited) sites. Furthermore, the binding process in competition assays is normally performed in (near-)equilibrium conditions, whereas displacement is performed in non-equilibrium.

Displacement principles

There are two methodologically different displacement detection systems: One with immobilized antibodies, Ab_{imm} and one with immobilized antigens, Ag_{imm}. In the first case labelled antigens (Ag*), in the latter case labelled antibodies (Ab*) are displaced (see Fig. 5.1).

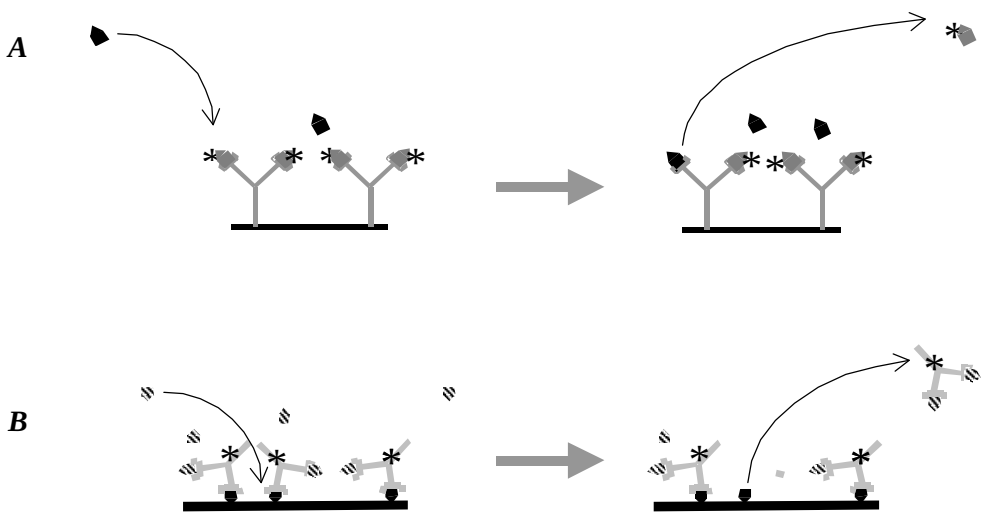

Fig. 5.1. Displacement principles A. Displacement of labelled antigens; B. Displacement of labelled antibodies.

Displacement of labelled antigen analogues

The displacement of labelled antigen (analogues) Ag*, is most commonly applied (Fig. 5.1A). To prepare the system, Ab are immobilized. Subsequently, they are saturated with Ag*. Displacement is achieved by adding the sample to these complexes. Some of the

Ag (the analyte of interest) from the sample then bind to the ABS, previously occupied by Ag*. Thus, these Ag* are displaced and therefore released into the solution. This process can be detected after separation of the immobilized and unbound molecules. This results in an increase of Ag* in the solution and a decrease of Ag* that are immobilized to the ABS. When the system has to be used repetitively or continuously over a long period of time, the conditions are chosen in such a way, that this decrease of Ag* bound to the ABS is negligible. This can be achieved by using a relative large amount of Ag*-saturated Ab.

Displacement of labelled antibodies

In the displacement of labelled antibodies (Fig. 5.1B) or antibody fragments (Ab*), Ab* is associated to immobilized Ag. The Ag may be conformationally changed by the binding to the carrier. At displacement, Ag (the analytes) from the sample may occupy some of the ABS, thus causing release of Ab* from the matrix. The Ag-Ab* complex can then be detected in the solution by its label.

For the readers convenience, all processes are described and explained for the displacement of Ag* from Ab_{imm} only (unless stated otherwise), to prevent confusion and repeats. In most cases, changing Ag* into Ab* and Ab_{imm} into Ag_{imm} restated all into the displacement of Ab*. Also, when stated Ag, this refers to antigens as well as antigen analogues.

Displacement detection

The detection methods, and therefore the labels used in displacement may vary. They can be detected directly or indirectly. “Direct” labels can be detected themselves, whereas “indirect” labels, such as enzymes or cofactors, should create reaction products that are detectable. These can be detected with a colourimetrical (Warden et al., 1987), fluorometrical (Yu et al., 1996) or electrochemical (Tiefenauer et al., 1997) detection methods. An advantage of indirect labels is the amplification of the signal, for example, a huge amount of electrochemically active molecules can be formed by a single enzyme molecule. A disadvantage is that in case of enzymes, the stability and activity of the label may fluctuate. An advantage of direct labels is that the label can be chosen with more freedom, therefore the label and detection method may be better tuned to each other.

The layout of the displacement detection unit can vary as well. The displacement experiment can be performed in ELISA plates. In this non-flow system, the solution and immobilized fraction have to be separated manually. At displacement, a decrease of the amount of label can be observed on the plate, and an increase in the solution. This can for example be detected when the label is an enzyme, such as horseradish peroxidase. Normally, this displacement should be not very effective due to the chance for recombination of the displaced labelled compound with the immobilized partner.

In flow displacement immunoassays, the displaced Ab* or Ag* will be taken downstream of the displacement unit. Depending on the chosen label, the detection can be performed with an on-line detector such as a fluorimeter or electrochemical detector.

Examples of displacement in literature

Displacement in non-flow systems

Displacement in non-flow systems has been done several times (see below). However, there can hardly be seen any advantage in using displacement instead of a sandwich or competition assay, except for the reduction of pippetting procedures and (in contrast to competition assays) it has a linear response. A disadvantage is that displacement assays are less sensitive than competition or sandwich assays because only a small percentage of the analyte leads to a release of the labelled molecule. Most of the literature on displacement in non-flow systems is not aimed at practical assays, but at kinetic studies, comparing the example association/dissociation of IgG molecules (with two antigen binding sites) and Fab-fragments (with only one binding site per molecule).

Interactions in microtiter plates

Nygren and Stenberg and colleages (Nygren et al. 1987; 1988; Stenberg and Nygren, 1988) studied the kinetics of antibody dissociation of surface immobilized antigen at non-flow conditions. They immobilized antigen (TNP) in a well of a microtitre plate and associated labelled polyclonal antibodies to the TNP. Subsequently, they attempted to displace with different concentrations of antigen. They observed no significant dissociation or displacement of the polyclonal antibody even after 3 days of incubation. When they performed the same experiment with Fab-fragments, they observed a decrease of signal on the surface, depending on the amount of antigen and the incubation time (Hinds et al., 1985). This was explained by the bivalence of the antibody, but also by assuming that the antibody has a higher affinity for the antigen compared to the Fab-fragment. The latter explanation is supported by their finding (Nygren et al., 1988) that when a comparable experiment with monoclonal antibodies is performed, a decrease of the antibodies associated to the antigens on the surface is observed in the presence of free antigen in the buffer. Polyclonals have in general higher affinities than monoclonals, and therefore a lower dissociation rate.

Displacement on beads

Hinds et al. (1984) showed an assay for theophylline which he called LIDIA, LIgand Displacement Immuno Assay. They used beads for the immobilization of antibodies. The antibodies were saturated with Ag*, and after removal of the unbound Ag*, the complexes

were mixed with the sample. The sample was incubated for five minutes, and the beads were separated from the solution. A linear correlation between analyte concentration and released Ag* was found in the liquid. Surprisingly, they also found a linear relationship with polyclonal Ab (Hinds et al., 1985). Because of the differences in affinities of the antibodies, a decrease in displacement per Ag molecule would be expected at higher concentrations of the Ag theophylline. These results suggest that the displacement in these experiments is merely controlled by diffusion rather by than association/dissociation kinetics.

Displacement in flow systems

Displacement in stop-flow systems

Warden et al. (1987; 1990) described displacement, denoted as “repetitive hit-and-run immunoassay” for T-2 toxin. In this assay, they bound labelled Fab-fragments of monoclonal antibodies (Ab*) to antigen immobilized onto column material. The displacement experiment was performed by injection of a sample onto the column and a five minutes stop of the flow for incubation. After each analysis, the column was reloaded by adding fresh Ab*. They found linear relationships between the concentration of T-2 toxin and the released Ab*, in which 29% of the Ag molecules actually displaced Ab* (Warden et al., 1987). Wortberg et al. (1994) showed a similar displacement approach for triazine herbicides. In their system, the flow was stopped 15 minutes during loading with Ab* and 20-40 minutes during the sample incubation.

Displacement in flow injection analysis systems

Kronkvist et al. (1997) published a cortisol assay based on displacement. A column contained immobilized cortisol monoclonal antibodies complexed with cortisol-alkaline-phosphatase. To avoid a lower efficiency of the displacement they resaturated the column by injecting labelled cortisol after each measurement. They compared their displacement (as called here competitive flow injection enzyme immunoassay) with a normal competition assay, in which they preincubated the sample with a known amount of labelled cortisol using a limited amount of antibodies. The advantage of the displacement here was that no preincubation was necessary and labour intensive pipetting could be avoided. However, the sample throughput, the signal per molecule and the precision of the measurement was lower than in classical competition assays.

Ligler and colleagues (e.g. Kusterbeck (1990), Rabbany et al (1994;1995)), were the first group who applied displacement for the detection of low-molecular analytes in a real flow injection system. Their first system, published in 1990 by Kusterbeck (1990), describes the detection of DNP, 2,4-dinitrophenol. After that, they described in a sequence

of articles this approach, also for the analytes cocaine (Ligler et al., 1992; Ogert et al., 1992; Wemhoff et al., 1992) and TNT (trinitrotoluene) (Whelan et al., 1993). Generally, they immobilize a large quantity of Ab on a Sepharose column, and saturate this with a fluororescence dye-labelled antigen. After this, they perfuse the column until the spontaneous dissociation of the label is stable. The actual displacement experiment is performed with discrete samples that are batch-wise perfused through the column at a flow rate of 0.1-3 ml min^{-1} (see Fig. 5.2) (Ligler et al., 1992). In their articles, they demonstrate qualitative detection of displacement, using developed formula for the non-equilibrium flow system they applied. They introduced several correction factors and variables of displacement.

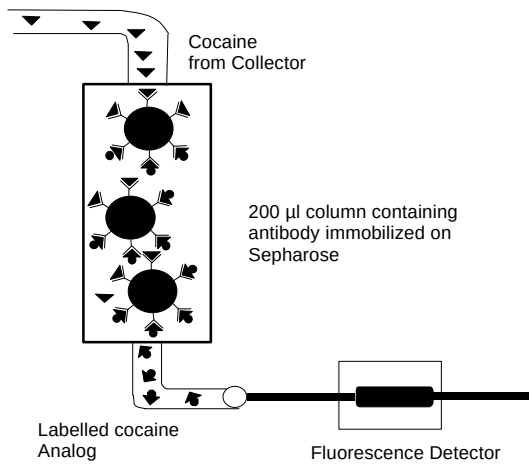

Fig. 5.2. Displacement setup Ligler and collegues.

Displacement in continuous flow systems

Schramm and Paek described in 1992 a displacement method for continuous monitoring of analyte concentrations (Schramm and Paek, 1992). They applied a shuttle system, based on displacement of the low MW analyte progesterone (see Fig. 5.3). Two different Ab were immobilized; one recognizing the analyte (Ab_A) and one recognizing the enzyme (Ab_E). The Ab were immobilized on spatially separated areas, and trapped with a membrane, so that large molecules could not enter or leave. The used enzyme-antigen conjugate (Ag-E) was assumed to have a higher affinity for Ab_A. In the absence of Ag, Ag-E was bound to Ab_A. When Ag was introduced, part of Ag displaced Ag-E and subsequently, Ag-E was bound to the other antibody, because it can not leave the Ab-compartment. In theory, the reverse reaction was possible (when the analyte is dissociated from the Ab_A and Ab_A and Ab_E are in close proximity to each other), but it was not observed. With this system, small analytes could be monitored continuously, but the response was slow (hours) and it was in principle only likely to work at increasing concentrations of the antigen.

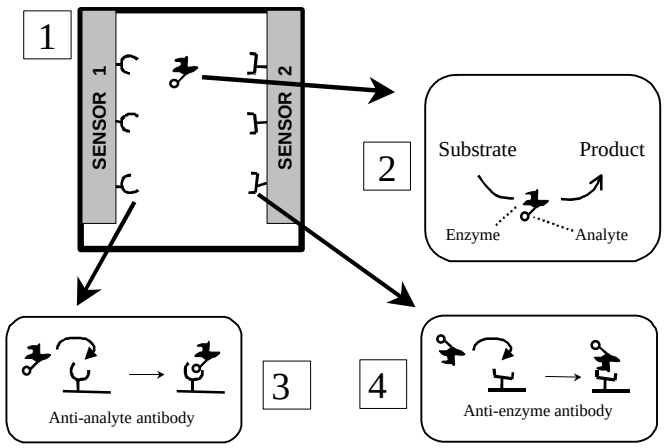

Fig. 5.3. Displacement setup Schramm and Paek.

In *chapter 6* and *7* of this thesis, two approaches of displacement for continuous analysis are demonstrated. The first one, for the low molecular hormone cortisol (*chapter 6*), was similar to the system of Ligler and colleagues, as described before. In this chapter, cortisol is detected by displacement of a conjugate of cortisol and horseradish peroxidase.

Another approach was made in *chapter 7* for the detection of a protein, fatty acid-binding protein (FABP). FABP was immobilized to column material, and Ab* were associated to this. The displacement released these Ab* from the matrix, and were detected downstream. There is only one other example in which antibodies or antibody fragments are displaced. Freytag (1984) published a patent describing a displacement system, where labelled antibodies were displaced. He described the displacement of monovalent and divalent digoxin Ab from oubain. This oubain, to which the antibodies had a lower affinity, was immobilized onto a column. Detection of ng ml^{-1} digoxin was possible. No displacement flow system for proteins has been described in literature.

Theoretical model of displacement

Mathematical models for antibody-antigen interactions are described for many systems. However, neither the fluid phase system for equilibrium nor the solid phase systems for static ELISA systems can be used for a quantitative description of flow displacement systems, for they are working at a solid-liquid interface under non-equilibrium conditions (Rabbany et al., 1992). For example, using the formulas of Nygren et al. (1988) for solid-phase systems under non-flow conditions, there would not be any displacement signal. Using the dissociation constants obtained from Ab in solution, more than 99% of the antigen analogue would theoretically be washed away after five minutes.

Therefore, a new model was introduced (Kusterbeck et al., 1990; Rabbany et al., 1995; Yu et al., 1996; Rabbany et al., 1997). The theoretical model describes displacement as a first order function under non-equilibrium conditions.

The displacement reaction can be written as:

$$Ag + Ag^{*}\ Ab \underset{\leftarrow}{\overset{\rightarrow}{}} AgAb + Ag^{*}$$

Kusterbeck et al. (1990) and Rabbany et al. (1995) formulated q as the undissociated fraction; the amount of label still present on the column.

$$q = \frac{[bound.Ag^{*}]_{t=0} - [displaced Ag^{*}]_{total}}{[bound.Ag^{*}]_{t=0}} \qquad (-)$$

whereas:

Ag*: amount of labelled antigen (mol)

Ab_{imm} : immobilized antibody

t=0: time starting the experiment

q = undissociated fraction: the proportion of Ag* still bound to Ab_{imm} in the column compared to the amount present at t=0 (-)

$[bound.Ag^{*}]_{t=0}$: releasable labelled antigen (mol): the amount of labelled antigen present at t=0

$[displaced.Ag^{*}]_{total}$: total of released antigen (mol): the amount of labelled antigen already been displaced

This undissociated fraction q is used to define a displacement efficiency D_E .

$$D_E = \frac{[displaced.Ag^{*}]}{[loaded.Ag]} * \frac{1}{q} \qquad (-)$$

whereas:

D_E : displacement efficiency (-)

: the amount of Ag in the sample (mol)

The displacement efficiency is therefore a size for the amount of Ag* that is actually displaced by Ag from the sample. Ideally, D_E would be constant in one system.

Qualitative analysis of displacement

The quantitative framework of displacement as given above describes characteristics of a displacement system. However, for setting up a displacement assay, it is essential to obtain insight into the way changes in the system influence the displacement signal. A qualitative approach of the displacement reaction, or, more specifically, for the displacement signal in a displacement detection system, may be helpful to optimise the assay systematically. Therefore, the most important parameters are explained below.

Affinity: association and dissociation rate

As can be expected, the equilibrium constant of the Ab to the Ag is largely influencing the displacement signal. High affinities lead to lower displacement signals, but also to lower spontaneous dissociation. Interestingly, it appeared that sometimes Ab with low affinity for high concentrations of Ag show a signal that was not depending on Ag concentration (Addendum *Chapter 6*), whereas the high-affinity Ab do. An explanation for this phenomenon is not easily been given. In contrast, low concentrations of Ag are only detected with low-affinity Ab, because there is no visible change of the spontaneous dissociation after perfusion of low Ag concentrations when the high-affinity Ab was used. Therefore, it appeared that, depending on, for example, the concentration interval and the time the displacement system has to be used, a choice of affinity has to be made. Important in this aspect is also that polyclonal Ab may help in semi-quantitative or qualitative measurement. They will detect low concentrations (as there are Ab with low affinity), but also at high Ag concentrations, they will (in theory) show an Ag-dependent signal. However, this can only be used for batch-wise analysis, because the system changes over time.

Immobilization procedure

The immobilization procedure is influencing the displacement signal in two ways. The first way is that material chosen for immobilization determines the diffusion characteristics for the Ag towards Ab. Ligler and collegues found that for their system immobilization on the inner walls of glass capillaries decreased the detection limit, compared to immobilization of Sepharose beads (Narang et al., 1997). The displacement efficiency was higher as well as the signal-noise ratio.

Immobilization can also influence the displacement signal by changing the binding affinity. For example, chemical linking can alternate the binding site. This effect is heterogeneous and difficult to address, especially for larger analytes (such as proteins). Also, when molecules are immobilized with another Ab (in a sandwich configuration), these Ab may hinder binding of a second Ab to it, thus influencing the displacement system.

Antibody density

One of the factors strongly affecting the displacement reaction is the Ab density per (column) volume (Dufaux et al., 1983; Ogert et al., 1992). At high antibody densities, the first perfusions of sample have lower displacement efficiencies than the following perfusions. This effect is less strong for lower antibody densities. An explanation for this is that after preparation of the column there are still empty sites (Wemhoff et al., 1992). Alternatively, this might be caused because of steric hindrance of the Ag* when the Ab are closely together (Kaptein et al., 1998). The higher Ab density enables analysis of more samples without reloading (Ogert et al., 1992). The fact that more complexes in the system are available might explain this, rather than the density. When the effect is caused by the density itself, this may be an effect of (re)association (Dufaux et al., 1983). No data are available in which the same absolute amount of Ab are loaded in different densities.

As expected, a higher antibody density increases the displacement rate, but decreases the displacement efficiency (Rabbany et al., 1994). This can be explained by the interaction effects of the Ab. At high flow rates (1 ml min^{-1}) high and low density of the antibody show the same displacement efficiency (Rabbany et al., 1995). This means that, when the flow is high enough, the distribution of the antibodies is not influencing the displacement signal. The interaction between the antibodies is negligible. Low flows and high Ab density, thus a high concentration of the labelled Ag throughout the experiment resemble the theoretical constant value for the displacement efficiency the best (Rabbany et al., 1995).

Antigen or antigen analogue

The choice of an antigen or antigen analogue to create displacement may be essential. Some researchers claim out of theoretical reasons that the displacement can only occur when the binding between Ag* and Ab is weaker than the binding of Ag to Ab (Freytag, 1984). In general, there is no experimental proof for this statement. In most cases, an Ag (not Ag analogue) is labelled, but it may well be that these Ag* do have a lower affinity to the ABS. No research has been investigating this in detail. The only report, where deliberately the formatted complex was weaker than the complex that would be formed after displacement, was in the displacement of Ab* from antigens of a different species (*chapter 7*). Here, the dissociation was too large to create a constant dissociation signal and displacement at Ag perfusions. Unpublished reports (Ligler, personal communication) found that even if the Ab has a higher affinity for Ag* than for Ag, displacement may occur. That Ab have higher affinity for the complexes occurs especially when the analyte in small (e.g. a hormone) and the label is large (e.g. an enzyme). This is caused by the immunization method for small analytes.

The relative size of the Ag to Ag* may also influence the displacement signal, because this influences the diffusion of the molecule. It may be expected that an optimal signal can be obtained when Ag/Ag* is as small as possible, because the spontaneous dissociation of Ag* from the Ab is relatively small (reassociation might occur regularly), whereas displacement is large (Ag can easily reach the sampling site).

Type of label

The type of label influences the detection of a displacement reaction at different levels. The first level is on the displacement reaction itself. When a large label (e.g. an enzyme) is chosen, this might decrease the displacement efficiency. Diffusion will be slower, and therefore reassociation of the Ab-Ag* complex may occur more often (see above). Also, the label might sterically hinder the analyte to approach the antibody binding site, resulting in a lower efficiency. Another decrease in efficiency can be caused by poor solubility of the labelled analogue (Ogert et al., 1992). The second level of influence of the displacement detection concerns the sensitivity of the measurement of the label itself. For example, a fluorescent label will raise above background signal earlier than a label detection, based of light absorption at a certain wavelength. An exception on this might be measurements in urine. Many urine samples fluoresce at the same wavelength as fluoresceine, a commonly used fluorescent label. Another risk with fluorescent labels is that they bind to the column material, because they are hydrophobic.

Higher sensitivity is to be expected from enzymatic labels, for these multiply the detectable products, therefore raising the sensitivity. A disadvantage of these labels is not only that they are bulkier (resulting in effects described above), but they might also be unstable themselves (*chapter 6*).

Saturation grade of antibody with labelled antigen

Although the amount of Ag* should only be enough to saturate the column, there can be a difference between a 100-times higher amount and a 10 times higher amount. This can be caused by primary unspecific binding of the Ag* (Ogert et al., 1992). The problem of always using high amounts of Ag* to set up the system, is not so much the price involved, but merely the long time the column has to be perfused with buffer before reaching a stable baseline.

Valency of the label/antigen

When an antigen has more than one labelled molecule per antigen molecule, the sensitivity of the displacement detection increases. However, the solubility might decrease, as well as the binding properties to the antibody may. Also, when, for example, a fluorescent label is chosen, the labelled antigen might cluster together because the (apolar) labels are attracted to each other.

What might occur when large labels, for example enzymes, are used, is that more than one antigen binds to one enzyme. This might decrease the displacement efficiency, because reassociation might occur more often, or because a labelled antigen analogue is even bound to more than one antibody binding site.

Perfusion fluid and matrix effects

Addition of ethanol or propanol in the flow buffer increased the sensitivity, because of higher solubility and/or a higher quantum yield of the fluorescent probe (Whelan et al., 1993). However, the binding affinity of the Ab may decrease (as a result of unfolding of the protein), so displacement of Ab* or protein-Ag* may be influenced by this change negatively. Proteases and non-physiological pH of the sample may deactivate the antibody binding sites (Ligler et al., 1992)

Flow

As expected, the applied flow rate has large impact on the displacement efficiency. The lower the flow, the more displacement will occur (e.g. (Whelan et al., 1993)). The sensitivity increases because of an increase of displacement without a change in background signal. However, when the flow decreases, the peak in which the displaced label will be found after a (discontinuous, injected) sample will also broaden. The signal/noise (injection with analyte compared injection without analyte) will worsen. Also, the time between samples has to be longer, because the time to get the signal back to base level will be longer (Kusterbeck et al., 1990), and the column lifetime (in which the column give satisfying signals) decreases (Whelan et al., 1993). The maximum displacement rate occurs at the highest flow rate. This phenomenon is explained with the hypothesis that the reassociation is reduced at the fastest flow. To describe the system with corrections for the above factors, the apparent dissociation rate constant k_d has been introduced, a unit decreasing at lower flow rates (Wemhoff et al., 1992). For high density antibody system and low flow, the displacement efficiency stays rather constant, in contrast to low density, high flow systems (Rabbany et al., 1994). The displacement efficiency of low density columns with low flow rates is the highest, although it decreases fast. When a high sensitive system for only a few analysis is necessary, this is the system of choice. If a rather constant, long-lasting, unsensitive system is required, a high density column and a high rate are the option of choice (Rabbany et al., 1994).

Concluding remarks

Although several groups have performed displacement assays, they are still not commonly used. The explanation for this may be found in more than one reason. Firstly, as shown in the theoretical description, it may only be possible to estimate the absolute concentration of an analyte after labour intensive, complicated calculations. However, when the characteristics of the displacement system are chosen in a way, that the amount of displaced Ag* or Ab* is negligible to the amount of Ag* or Ab* present in the displacement system, these calculations may be omitted. It may well be, that in that case, the displacement signal is linear. Secondly, the preciseness of the assay is less than competition or sandwich assays. Although this may be critical, for many applications an

indication of concentration or even only an indication of changes in the concentration is sufficient. Thirdly, the system may be less sensitive than classical immunoassays, because only part of the analyte leads to a displacement signal. Here, it is also important to consider the requirements of the monitoring. For many analytes, displacement can measure (patho)physiological concentrations. In that case, the sensitivity should only be sufficient to measure these concentrations.

The advantages of displacement are numerous. Firstly, when applied in flow injection immuno systems, it prevents labour intensive pipetting and incubation steps, when compared to e.g. competition-based systems. Secondly, when applied in continuous sampling analysis, no alternative is available so far. In general it can be concluded that, depending on the requirements of the assay, the set-up has to be chosen considering linearity or sensitivity and lifetime extension.

Incorporation of the displacement technique in on-line immunosensors primary allows early detection of myocardial damage and successful reperfusion. For this, a low, constant flow of the system is required, which can be established with slow flow on-line sampling techniques such as microdialysis or ultrafiltration (Ungerstedt, 1991; Moscone et al., 1996; Kaptein et al., 1997). Especially for this application, the continuous signal of the flow displacement immunoassay is used in its full potential, and to our best knowledge, no other assays based on immunological detection have been described for the creation of an on-line signal.

Glossary

Affinity constant, K is the ratio of the association divided by the dissociation

Antibody binding site (ABS): The site of an antibody with the binding capacity. This site defines mainly which epitope is recognised and with which affinity. For IgG, there are two sites per Ab

Continuous: without interruption, such as an injection into a detection system, batch-wise samples.

Equilibrium (affinity, association, binding) constant ($l.mol^{-1}$): The state in which the association rate is the same as the dissociation rate.

Affinity: the attractive force between the complementary conformations of the antigenic determinant and the antibody combining site. The affinity of the antibody (Ab) to the antigen (Ag) is an effect of different molecular forces, e.g. electrostatic bonds, hydrogen bonding, hydrophobic interactions and van der Waals forces.

Dissociated fraction: The labelled Ag already been released from the immobilized Ab

Undissociated fraction: The labelled Ag bound to the immobilized Ab

Fab-fragment: a specific part of the antibody with only one ABS

Monoclonal antibodies: Antibodies with all the same molecular structure, therefore the same ABS

On-line: directly linked to the sampling site

Polyclonal antibodies: Antibodies isolated from serum of an immunized animal. These antibodies are mainly recognising the antigen, but all have different ABS. They can recognize different epitopes of an antigen, with different affinities

Sensitivity: determines the detection limit

Specificity is the property of Ab to recognize only one antigen, or a few closely related antigens

Spontaneous dissociation: the process of unbinding of the Ag and the ABS

(Re)association: the process of binding of an Ag to an ABS

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Chapter 6

Analysis of cortisol with a flow displacement immunoassay[1]

Results! Why, man,
I have gotten a lot of results.
I know several thousands things
that won't work (Thomas E. Edison).

[1] Kaptein, W.A., Zwaagstra, J.J., Venema, K., Ruiters, M.H.J. and Korf, J. In: *Sensors and Actuators* **B45** (1): 63-9 (1997)

This study investigates a flow immunoassay for cortisol based on displacement of labelled antigen analogues from immobilized antibodies. On a displacement column, two antibodies with different affinities were immobilized on protein-A Sepharose, and a construct of cortisol and horseradish peroxidase (cort-HRP) was associated to the antibodies. Perfusion of the column with cortisol displaced a proportional amount of cort-HRP. On-line detection was performed with an electrochemical detector measuring cort-HRP directly in a flow injection system. For investigating some characteristics of the displacement system, cort-HRP was also detected off-line, with a substrate for HRP, which changes OD_{492}. We tested our system in buffer as well in serum for two days at a flow of 4-6 µl min^{-1}. Analysis of the data show a detectable displacement signal at physiological cortisol concentrations. The specificity, stability of the columns and the influence of the antibody affinity were studied.

Introduction

Total serum cortisol concentrations are 20-200 µg l^{-1} (55-550 nM) of which over 90 % is bound to proteins and has daily fluctuations. To get accurate measurements of the amount of cortisol or of the circadian profile, one needs to measure blood several times a day. Besides this, for some clinical diagnosis, it is necessary to know the exact cortisol fluctuations during the day (Sachar et al. 1973). To comply with this, it is necessary to develop a technique, which enables analysis of continuous samples.

There are several methods to measure cortisol in body fluids. The conventional method, analysis of sera with a HPLC is very labour intensive (Oka et al. 1987). Radio immunoassays have been used for some time now (Huang and Zweig, 1989), but they have the disadvantage of using radioactive compounds.

New techniques, using antibodies and labelled cortisol have been developed during the last decade (Gosling et al. 1993). Nowadays, there are some fully-automated analysers available for the analysis of cortisol, such as Enzymun-Test Assay (Boehringer), ACS:180 (Ciba Corning Diagnostics) (Yatscoff et al. 1996) and Stratus (Baxter) (Rogers et al. 1986). Most of these are based on competitive antibody-antigen assays, where immobilized antibodies are incubated simultaneously with a sample and a known amount of enzyme-labelled cortisol. The cortisol concentration is, therefore, inversely proportional to the enzyme activity. Also, a piezoelectric immunosensor (Attili and Suleiman, 1995) and capillary electrophoresis method (Schmalzing et al. 1995) for cortisol have been studied recently. However, none of these methods allow the continuous monitoring of cortisol *in vivo.* For this, a homogeneous assay is essential. One suitable technique for on-line analysis is a displacement system, described by Kusterbeck, Rabbany et al., e.g. for cocaine and dinitrophenol (Kusterbeck et al. 1990; Wemhoff et al. 1992; Rabbany et al. 1994; 1995; Yu et al. 1996). Here, antibodies are covalently immobilized to column material or fused silica (Narang et al. 1997b), and saturated with fluorophore or radioactive labelled analyte-analogues. Discontinuous sample analysis is achieved after perfusion of the samples through the column and measuring the released label. Measurements with these columns are performed up to 15 times (Yu et al. 1996). Recently, the displacement

technique has also been described for the off-line analysis of cortisol. Kronkvist et al. (1997) have immobilized antibodies and an alkaline phosphatase labelled cortisol-analogue on a column. Between every measurement the column is regenerated and the flow applied (0.2 ml min^{-1}) is not suitable for continuous sampling.

Here, we describe a technique, which can be used for both discontinuous and continuous analysis. We immobilize cortisol antibodies to Protein-A Sepharose and associate horseradish peroxidase labelled cortisol (cort-HRP) to these antibodies. The saturated immobilized antibodies are placed in a column in a buffer flow. When a sample containing cortisol is introduced, a proportional amount of cort-HRP is displaced from the binding sites of the immobilized antibodies and subsequently detected downstream. In principle, the detection of the enzyme is performed with an electrochemical detection of cort-HRP. For this, glucose oxidase, placed downstream of the column, produces hydrogen peroxide, the substrate of the released cort-HRP. The electrochemical detection (ECD) can be applied in the future in small, relatively cheap and low energy consuming devices (McNeil et al. 1997). Moreover, the enzymatic labelling of the antigen with HRP increases the sensitivity. This enables lower perfusion rates of the column, thus enhancing the stability of the system and possible incorporation in body fluid sampling devices such as microdialysis (De Boer et al. 1991) or ultrafiltration (Moscone et al. 1996; Kaptein et al. 1997). Characteristics of the displacement system are studied analysing cort-HRP off-line, measuring the enzyme activity photometrically. We analysed cortisol in different (physiological) concentrations in buffers. The specificity and sensitivity of the displacement reaction is investigated for the antibodies with different affinities by measuring the cort-HRP concentration after perfusions with glucose and cholesterol. We also investigate the system with ultrafiltrated sera.

Materials and methods

Materials

Chemicals and antibodies

Protein-A Sepharose was obtained from Pharmacia, Biotech, Uppsala, Sweden. The Mαcort-H, a high affinity monoclonal antibody ($K=1.2\ 10^{9}$ M l^{-1}) and Mαcort-L, a low affinity monoclonal antibody ($K=2.1\ 10^{7}$ M l^{-1}) against cortisol were obtained from Fitzgerald Industries Internationals Inc., Concord MA (clone M94144); cortisol-HRP (cortisol conjugated at the 3-position to horseradish peroxidase) from Eurogenetics, Tessenderlo, Belgium. Glucose oxidase (EC 1.1.3.4, grade I) was obtained from Boehringer Mannheim, Germany, ureum peroxide tablets from Organon, Weesp, The Netherlands and Kathon CG from Rhom and Haas, Croydon UK. Ferrocenemonocarboxylic acid (FcA), Tween 20 and o-phenylene diamine dihydrochloride (OPD) tablets were obtained from Sigma, St. Louis, MO, whereas other chemicals were of pro-analysis quality and purchased from Merck, Darmstadt, Germany. Sera were obtained from the University Hospital of

Groningen, The Netherlands. They were ultrafiltrated with fibres of dialysis membrane AN69HF (Filtral 16; Hospital Ind., Meyzien, France) as described by Moscone et al. (1996).

Buffers

Special buffer (SB) consisted of 0.35 M NaCl, 2.5 mM KCl, 8 mM Na_2HPO_4, 2.5 mM KH_2PO_4, 0.03 vol.% Tween-20 and 0.1 vol.% Kathon CG. For the glucose buffer (GB), 5 mM glucose was added to SB. The FcA buffer (the buffer for the flow injection analysis in the detection system) contained 0.5 mM FcA, in GB. The substrate for the off-line quantification of the HRP in the eluent was made as follows: a 4 mg OPD tablet was dissolved in 10 ml distilled quarts water with 100 µl ureum peroxide (in 10 ml). The reaction was stopped with 50 µl 1.5 M H_2SO_4.

Equipment

For the displacement unit, a Minipuls 3 peristaltic pump (Gilson Medical Electronics, WI) was used with clear standard pump tubes (Skalar, Breda, The Netherlands). The columns were made in 25 µl transferpettor-cap (Brand, Germany). The OD_{492} of the sampled fractions was measured with a Lucy1 spectophotometer (Anthos, Salzburg, Austria).

For the flow injection analysis, a HPLC pump (LKB 2150, Pharmacia) was connected to a Marathon HPLC Autosampler (Spark Holland, Emmen, The Netherlands). The sampler was equipped with a Rheodyne 7010 Valve (Cotati, CA) with a loop (homemade, 1.25 µl). Glucose oxidase was immobilized with a 0.01 µm cellulose nitrate filter obtained from Sartorius (Gottingen, Germany). The electrochemical cell was a thin layer-type cell (AMOR, purchased from Spark Holland, Emmen, The Netherlands), 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 potentiostate (Decade, Antec Leyden B.V., Leiden, The Netherlands).

General description of the displacement system

Our cortisol analysis system was based on a displacement detection. Antibodies were immobilized on column material, followed by perfusion with cort-HRP to bind to the antigen binding sites (ABS) of the antibodies. The experimental set-up is shown in Fig. 6.1. The column (displacement unit) was perfused continuously with varying amounts of cortisol or other contents. Cort-HRP in the eluent of the column was analysed either off-line or on-line.

In off-line analysis, the continuous sample was divided into 10 minutes-fractions (see Fig. 6.1A) and analysed with a colour substrate. On-line analysis was performed in a flow

injection analysis system. In this, cort-HRP was detected electrochemically. The displacement unit was coupled to a second flow system with a valve to a connection loop, which enables injections of the samples into a relatively higher flow system. The buffer in this system was the same as used for perfusion of the displacement column but also contained FcA. Immobilized glucose oxidase in this system produced H_2O_2 from glucose. The HRP reduced the H_2O_2, oxidizing ferrocene(II) to ferrocene(III). At the electrode ferrocene(III) was then reduced back to ferrocene(II). This set-up enables incorporation of the detection system in an on-line ECD system without an additional fluid system, for in body derived fluids glucose is often present in excess, and may serve as substrate-producing substance.

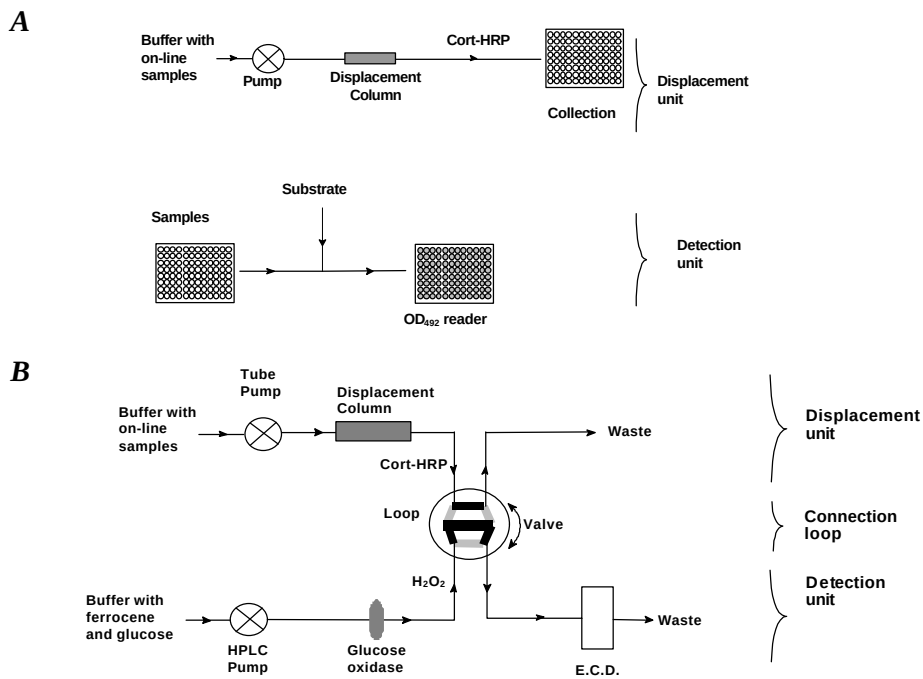

Fig. 6.1. Schematic representation of the displacement detection. A. off-line; B. on-line.

Preparation and operation of the displacement unit

Glass wool was placed at the bottom of a part of a 25 µl transferpettor-cap (10- 50 µl internal volume). Tubes, fitted in a Gilson Minipuls 3 pump, were connected to the bottom of the cap. The cap was filled with Protein-A Sepharose. The standard flow of the displacement unit for loading and running was 4-6 µl min^{-1}.

100 µl (330 ng μl^{-1} in GB) Mαcort-H antibody was immobilized on the protein-A. After this, the column was washed with at least two column volumes, followed by an incubation with 50 µl (64 µg ml^{-1}) cort-HRP in GB. A tube at the top of the column

connected the column to the pump and the system was washed for at least 16 hours with GB. The column in the displacement unit was continuously perfused maximally two days with GB or with various concentrations of cortisol. The column was also perfused with SB and 50 µM cholesterol in SB (as a compound present in body fluids with similar chemical properties), or with 5 mM glucose in SB (as a compound with physiologically high concentrations) to determine the specificity of the displacement. Non-protein bound cortisol in filtrated serum was also analysed. Extra cortisol (275 nM) in GB was added before and after filtration.

Description of the off-line detection system

From the displacement unit described previously, the 10 min fractions of the on-line sample were analysed by adding 100 µl substrate to 5 µl of these fractions (see Fig. 6.1). The reaction was stopped with H_2SO_4 when $OD_{492,max} \approx 1$.

Description of the on-line detection unit

As described in above, the on-line detection was performed as a flow injection analysis. Injections of the perfused fluid into the detection unit was performed every 2 min. The flow of the displacement unit was 6 µl min^{-1}, the flow in the detection unit was 100 µl min^{-1}.

Presentation of the results

Several examples of our experiments are given to illustrate the significance of the various parameters studied. In other cases linear regression between data was calculated. In several experiments the efficiency of the displacement was expressed as the percentage of the ration of the cort-HRP (in mol) eluted and the cortisol perfused (in mol) analogue to the definition of Rabbany et al. (1995).

Results and discussion

Analysis with the off-line detection unit

Specificity of the displacement

The specificity of the displacement reaction was tested in two ways. Firstly, physiological concentrations of cholesterol and glucose were perfused; these did not displace cort-HRP (Fig. 6.2A). Secondly, ultrafiltrates of sera (with or without extra cortisol) were tested, showing no more increase of cort-HRP in the eluates than was

expected on the unbound cortisol concentrations in the sera (see Fig. 6.2B). In the serum ultrafiltrates, little cortisol was present because only free cortisol (approximately 0.5 µg l^{-1}) passed the membrane. When 10 µg l^{-1} cortisol was added to the ultrafiltrate the displacement was similar to the displacement after perfusion with 10 µg l^{-1} cortisol (about 1.9 10^{-14} mol) in buffer. In some serum filtrates samples unknown compounds disturbed the HRP-activity. In general, these were not present or physically separated from the displaced cort-HRP (see Fig. 6.2B).

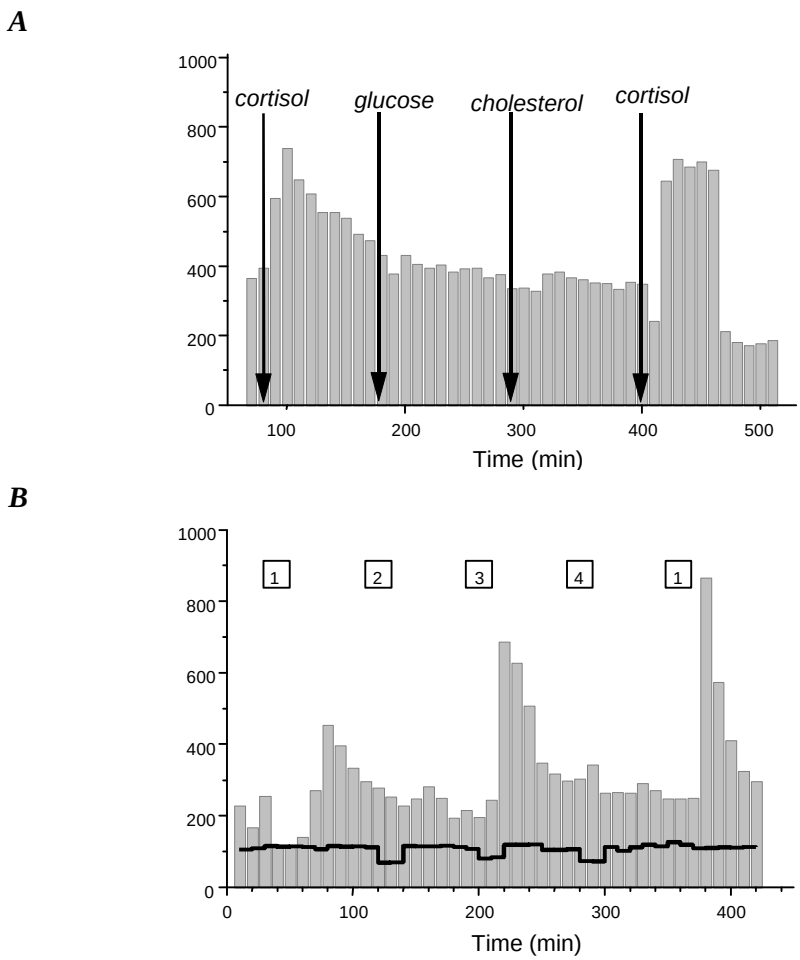

Fig. 6.2. Cort-HRP release and activity in fractions after perfusion. A. Specificity of the displacement. Bars indicate the cort-HRP activity present in the fraction. Arrows indicate the perfusion starts: 275 nM cortisol; 5 mM glucose; 50 µM cholesterol. B. Displacement and enzyme activity after serum perfusions. Bars: Column loaded with cort-HRP-saturated antibodies; Line: Unloaded column perfusate analysed with extra cort-HRP (OD devided by 3); Perfusions with samples were performed for 20 minutes as indicated. 1. 275 nM cortisol buffer; 2. dialysed sera and buffer; 3. sera and 275 nM cortisol buffer, dialysed; 4. dialysed sera and 275 nM cortisol buffer.

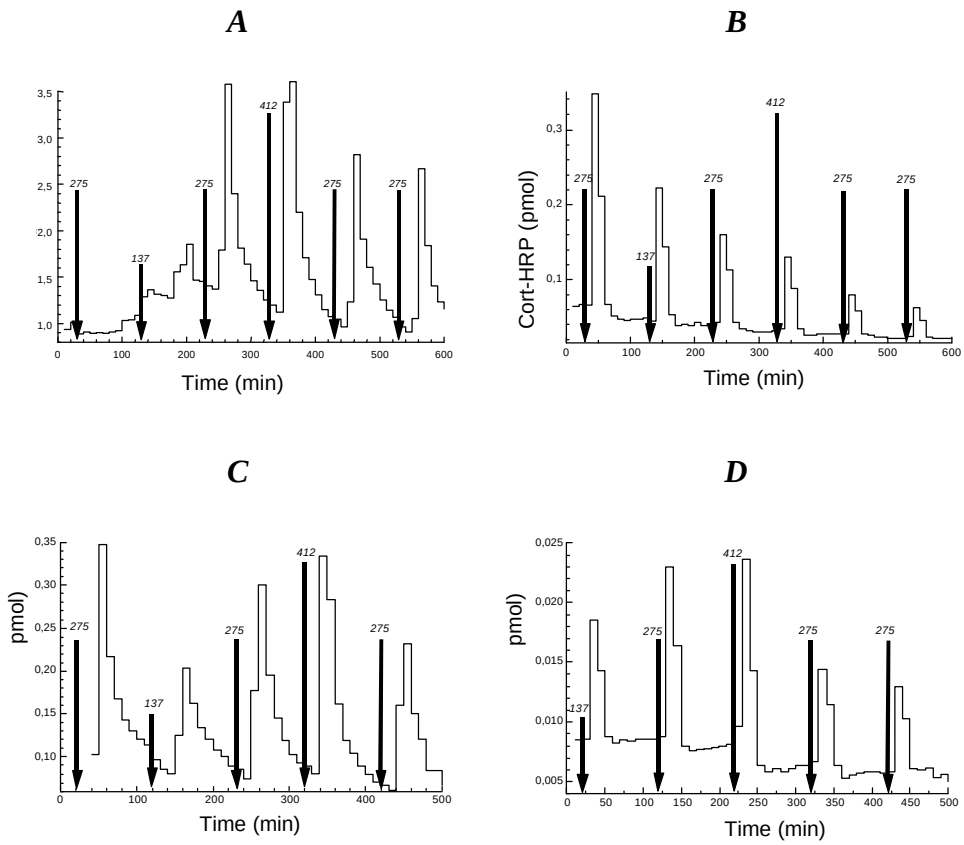

Fig. 6.3. Displacement experiments. Off-line detection of cort-HRP in 10 minute fractions from continuously perfused columns. Arrows indicate the perfusion starts of 20-min cortisol perfusion in the concentrations indicated (nM). A. column with Macort-H one day after column preparation; B. column with Macort-L one day after column preparation; C. column with Macort-H four days after column preparation; D. column with Macort-L two days after column preparation..

Influence of the antibody affinity on the signal

Fig. 6.3 shows the elution profiles of two columns loaded with high affinity or low affinity antibodies. The first cortisol perfusion of the column with Mαcort-H showed a lower displacement signal as perfusion of the same amount of cortisol later that day.

The ratio of the amount of ABS and of cort-HRP bound to this and of the amount of cortisol on the column when 275 nM cortisol is perfused 20 minutes equals to ABS:cort-HRP:cortisol=12:3:1. Thus, the ABS were not saturated with cort-HRP when the displacement experiments started. This might explain why the first cortisol perfusion did not displace cort-HRP as much as later cortisol perfusions, presumably because the first

cortisol became partially trapped at free antibody binding sites. However, perfusion for 20 minutes at a concentration of 275 nM cortisol only added 3.3 10^{-11} mol cortisol to the column and did not theoretically fill all the free sites, suggesting that some ABS were not available for antigen binding. Wemhoff *et al*. reported a lower release at the first injection of antigen even after loading the antibodies with a 100-fold excess of antigen homologues (Wemhoff et al. 1992). They explained this by assuming that there are empty ABS after the preparation of the column. We hypothesize that some ABS can not bind to relatively large cort-HRP due to steric hindrance, but are still accessible for the small unconjugated cortisol.

The initial decrease of displacement in the first cortisol sample for the Mαcort-H column was not seen after the first day, although the absolute signal decreased. There are several explanations for this. One is that the amount of cort-HRP on the columns decreases as a result of the perfusion. However, the spontaneous release of cort-HRP was less than 1 10^{-16} mol min^{-1}. The release of cort-HRP caused by displacement from 275 nM cortisol was at most 5 10^{-16} mol min^{-1} (calculated from 10 min fractions). Compared to the amount of cort-HRP present on the column (8 10^{-10} mol), the absolute amount of cort-HRP displaced was not changed significantly. Another explanation for the decrease of signal is that the apparent binding constants differ for the antibodies. Wemhoff et al. (1992) have described a similar phenomenon. It can be explained by the steric hindrance described previously, but it may also be (partly) an effect of inhomogeneity of the cort-HRP. For example, the number of cortisol molecules per HRP molecule may influence the affinity as described for a similar process: the binding of antibodies (with two ABS) compared to that of the Fab fragment of the antibody (with one ABS) (Nygren et al. 1987). A last explanation is that the cort-HRP enzyme activity decreases during the perfusion period. In this case, the calculated displacement rates will be lower than experimentally found.

The Mαcort-L column did not have a lower displacement for the first injection of cortisol, but here the displacement on the first day was not dependent on the concentration of cortisol but only on how long after the preparation of the column the cortisol was perfused. However, the signal seemed to be more concentration dependent on the second day than on the first. The reason for this is unknown, although it might be explained by assuming that there are more unstable complexes on the first day, for example because of steric hindrance for the large cort-HRP onto the column material. These can be easier released in their dissociation phase in the presence of cortisol. On the second day, these unstable complexes might have dissociated already.

For analysis of lower concentrations of cortisol-HRP, the antibody with the low affinity was preferred. The displacement from the high affinity antibody was too small to distinguish from the spontaneous dissociation, whereas the column with Mαcort-L showed concentration dependent signals (data not shown).

Quantification of the signal

Repeated perfusions of 3.3 10^{-11} mol cortisol in 20 minutes (275 nM) showed a decrease from 0.05 % to 0.02 % of this displacement efficiency for on the second day for the columns with Mαcort-H. Measurements on the same day did not show significant differences (see Fig. 6.4). The displacement efficiency for Mαcort-L was not calculated, for this was highly influenced by the perfusion time, i.e. the amount of cort-HRP dissociated from the column.

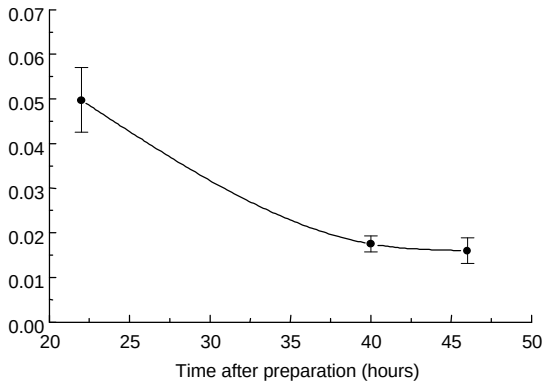

*Fig. 6.4. Displacement rate ± SEM (percentage release of cort-HRP/cortisol) for cortisol perfusion of 3.3 *10^{-11} mol.*

Using Mαcort-H, the displacement rate was not significantly different at the applied concentrations of cortisol used for perfusion. The cort-HRP present in the eluate showed, therefore, a linear relation (r=0.99; p=0.006) with the perfused cortisol concentration (Fig. 6.5). The intercept of the y-axis showed the spontaneous dissociation (0.16 fmol per sample of 10 minutes).

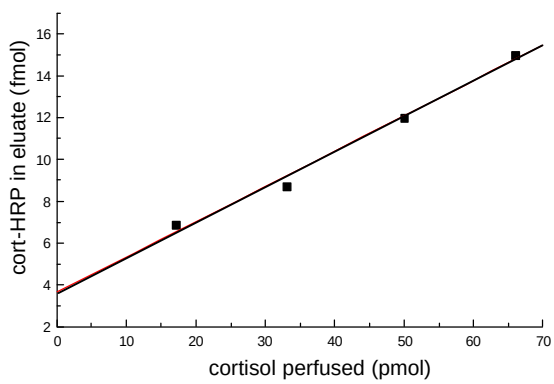

Fig. 6.5. Amount of cort-HRP in fraction for perfusion with different cortisol concentrations.

Analysis with the on-line detection unit

Fig. 6.6 shows a recording of *in vitro* experiments of our FIIA system. Above the baseline, where no column fractions were injected gave the displacement column eluates a basal background signal, even when no cortisol was perfused (the spontaneous dissociation). Upon perfusion with cortisol, this signal increased. We were able to measure physiological cortisol concentrations every two minutes. Cholesterol did not enhance the signal (data not shown).

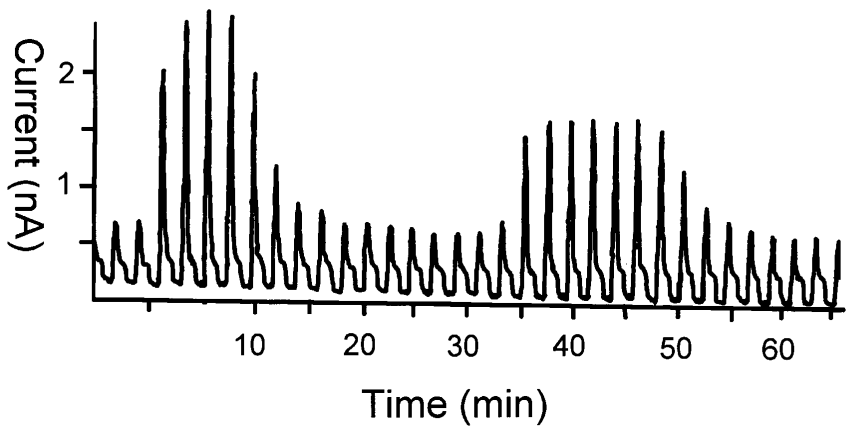

Fig. 6.6. On-line FIIA detection of cort-HRP at different physiological cortisol concentrations. Shown is a profile in which buffer is run continuously, with a 10-min 77 nM cortisol and a 15-min 39 nM cortisol perfusion.

Conclusion

This study demonstrates the potential of the displacement technique. Here, we applied this approach for continuous and batch-wise analysis of cortisol. Others (see Kusterbeck et al. 1990; Wemhoff et al. 1992;Whelan et al. 1993; Rabbany et al. 1995; Yu et al. 1996) have also described batch-wise displacement analysis, although our method reveals some differences. Firstly, we use another analyte in another sample matrix; secondly we perfuse at a much lower flow (6 µl min^{-1} instead of one ml min^{-1}); thirdly, we use another (indirect) label and detection system. And most important, we perfuse the displacement column continuously with on-line samples. Our results show a 100-fold lower displacement efficiency than Rabbany et al. (1994; 1995). This may have to be attributed to the antibody, having a high equilibrium constant of 1.2 10^9 M l^{-1}, resulting in a low displacement efficiency. The unoccupied binding sites, catching cortisol in the sample, do not offer an explanation (except for the first injection), because then the release of cort-HRP would have been increased during the experiment. When the detection of the label is sensitive

enough, a high affine antibody with a low displacement efficiency and low spontaneous dissociation and perfusion with a low flow rate might be preferable, because of the good stability and the longer lifetime of the column. This is clearly demonstrated with the Mαcort-L, where the sensitivity is largely reduced during extensive usage. Especially using high flow rates, the displacement efficiency is high, but the sensitivity decreases faster. However, low affinity antibodies are preferable for the detection of low concentrations of the analyte, because of the lower detection limit. Further research has to show whether this principle is more general, or whether this is only valid for the antibodies used here.

With the off-line detection of cort-HRP, it is possible to study many parameters simultaneously. Multichanel pumps enable serial running of columns, using simple apparatus (a pump and an ELISA reader) only. Because of its simplicity, the off-line analysis is a powerful research tool for further development of the on-line system. The on-line system itself provides a useful tool in research as well as in clinical applications, because of its minimal labour requirement and possibility of automation (Hitzmann et al. 1995).

In on-line body fluid analysis, interference of other compounds on the detection systems might lead to false measurements, as sometimes is seen in the detection of HRP. Therefore, the labelling method has to be chosen with great care.

In summary, the present report shows a novel application of the immunological technologies in the development for the fast and specific detection of a wide variety of analytes for biomedical research. The principle of this approach is to displace enzyme labelled antigens from an antibody complex by an analyte in a flow system, allowing sensitive electrochemical detection. We characterize some properties of the system, especially the difference between the displacement from antibodies with a high or low affinity.

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Addendum chapter 6

After publishing the above chapter of "analysis of cortisol with a flow displacement immonoassay", there have been performed some experiments, which results are worth mentioning here.

Firstly, we tested the displacement in a continuous flow system with electrochemical detection. The general set-up was comparable to the flow injection system as described, with some changes. The first is that there are no pulses, but a continuous perfusion with cortisol-containing buffer. The second is the size of the column (smaller: 1.5 µl) and flow rate (3 µl min^{-1}). With this, we were able to detect 1.25 µl of 22 µg l^{-1} cortisol. The peak width is 10 min at half height and shows some tailing, but this is caused by the size of the column and the flow used. For analysing continuous samples, the peak width may become a problem. The continuous samples enable us to monitor the concentration of the cortisol during the day, and will give valuable data. Miniaturizing the column as well as the electrochemical detection unit will increases the time resolution at low flow and. result in a portable device.

Secondly, we investigated the influence of the ultrafiltrate on the electrochemical detection. As described before, the results of the experiments with sera show inhibition in off-line (coloured-product-based measurements) of the cort-HRP enzyme activity by serum components. Recent studies with fresh sera showed that this interference was due to conservatives or drugs in the sample used in this report. However, in the electrochemical detection systems, there is also an electrochemical disturbance of signal in the cort-HRP displacement fractions. To overcome this problem, an extra size selecting column in the detection unit, separating serum compounds (due to the dialysis step smaller than 20.000 Da) and cort-HRP (42.000 Da) might solve this problem. However, this will make it impossible to create a continuous signal. Therefore, a solution should be found on the detection level itself, either by the application of special (coated) electrodes, or by finding another suitable detection system.

An interesting phenomenon has been observed in the off-line analysis system when cortisol concentrations were decreased further. As in "high" concentrations (around 10 µg l^{-1}), the antibody with the high affinity for cortisol gives the best, quantitative reactions, for lower concentrations (around 0.05 µg l^{-1}), the low affine antibody has to be preferred. The displacement from the high affinity antibody is too little to distinguish from the spontaneous dissociation, whereas the column with Mαcort-L shows cortisol-concentration dependent signals.

Chapter 7

On-line flow displacement immunoassay for fatty acid-binding protein[1]

It would be
possible to describe
everything scientifically,
but it would make no sense; it would be
without meaning, as if you described a Beethoven
symphony as a variation of wave pressure (Albert Einstein).

[1] Kaptein, W.A., Korf, J., Cheng, W., Yang, M., Glatz, J.F.C. and Renneberg, R. In: *Journal of Immunological Methods* Accepted.

In standard displacement flow immunoassays analyte in the sample creates an active dissociation of labelled antigens (or antigen homologues) from an antigen binding site of an immobilized antibody, whereafter the labelled substance is measured downstream. Such systems have been described for molecules up to 1 kD. In this study, we demonstrate displacement in a flow system for the detection of a small protein, cytoplasmic heart-type fatty acid-binding protein (15 kD), a plasma marker for myocardial injury. The displacement system applies an inverse set-up: enzyme labelled monoclonal antibodies are associated to immobilized antigen, displaced by analyte in the sample. It allows detection of both physiological (2-12 μg l^{-1}) and pathological concentrations (12-2000 μg l^{-1}) of fatty acid-binding protein in an on-line flow system.

Introduction

Since the technique for preparation of monoclonal antibodies has been developed by Köhler and Milstein (1975), antigen-antibody interactions are widely used for detection of antigens in complex matrices, such as body fluids (Gosling, 1990). Following solid phase detection procedures, such as ELISA and RIA (Anderson et al., 1997), more complex systems like flow injection immunosystems (Gubitz and Shellum, 1993) and immunosensors (Aizawa, 1994 ; Morgan et al., 1996; Schreiber et al., 1997) have been developed. One of the approaches used in immunosensors is displacement, as described by our group (Kaptein et al., 1997b) and by others (Kusterbeck et al., 1990; Rabbany et al., 1994; Kronkvist et al., 1997; Narang et al., 1997). Displacement is a principle in which either antibodies or antigens are immobilized, whereas the respective interacting antigen (analogue) or antibody is labelled. After the antibody binding sites are saturated with antigens, the actual displacement consists of perfusing the sample along these antibody-antigen complexes, releasing labelled molecules from the immobilized site.

Flow displacement immunoassays can be divided into two different categories, one with immobilized antibodies and the other with immobilized antigens. In the most frequently used set-up, labelled antigens (or antigen analogues), associated to immobilized antibodies, are placed in a flow system. Some of the antigens in the sample, running through this flow system, will displace labelled analogues from the antibodies, thereby raising the concentration of the label downstream. This label is then analysed, directly or indirectly, by a colourimetrical (Warden et al., 1987), fluorometrical (Yu et al., 1996) or electrochemical (McNeil et al., 1997) detection. Such a flow displacement of antigen analogues has been described for small analytes like cortisol (Aizawa, 1994; Kronkvist et al., 1997), cocaine (Ogert et al., 1992) and trinitrotoluene (Whelan et al., 1993).

The second displacement set-up is performed by immobilizing an analyte, and displacing a labelled antibody, which was previously bound to the immobilized analyte. Freytag (1984) published a patent describing such a displacement system. Warden et al. (1987; 1990) described a displacement, denoted “repetitive hit-and-run immunoassay”, for T-2 toxin. In this assay, a 5-minute incubation was applied, and after analysis the column was regenerated.

So far, the application of both displacement set-ups has been demonstrated only for molecules with a molecular weight of less than 1000 Da. To explore the usefulness of the displacement technique for small proteins in an on-line flow system, we studied the displacement system for cytoplasmic heart-type fatty acid-binding protein (FABP, 15 kD) (Offner et al., 1988; Veerkamp et al., 1991; Glatz and van der Vusse, 1996). FABP is a novel plasma marker protein for acute myocardial infarction (AMI), which has a high sensitivity and specificity for early detection of AMI (Yoshimoto et al., 1995; Glatz et al., 1997). With an immobilized FABP and displacement of a labelled antibody, we were able to apply the displacement technique of labelled antibodies for the detection of physiological and pathological concentrations of FABP (2-2000 µg l^{-1}).

Materials and Methods

Materials

Protein-A Sepharose and N-hydroxysuccinimide (NHS) activated Sepharose 4 fast flow were obtained from Pharmacia, Biotech, Uppsala, Sweden. Anti-human heart-type FABP monoclonal antibodies 53E9, 67D3 and 66E2 were obtained as earlier described (Roos et al., 1995). Antibodies for both immobilization methods and for the catcher as well as the detection antibody were selected on their performance in ELISA's and BIAcore experiments. The monoclonals 66E2 and 53E9 were labelled with horseradish peroxidase (with Sigma P8375, Sigma Chemical Co., St. Louis, MO). Recombinant human FABP (Schreiber et al., 1998) and recombinant rat FABP (Schaap et al., 1996) were obtained from T Börchers, Münster, Germany and F. Schaap, Maastricht, The Netherlands, respectively. Horse myoglobin, lysozyme and o-phenylene diamine dihydrochloride (OPD) for the detection of the horseradish peroxidase label were obtained from Sigma, St. Louis, MO.

Buffers and substrates

The perfusion buffer is a phosphate based buffer of pH 7.4, consisting of 5 mM glucose, 0.35 M NaCl, 2.5 mM KCl, 8 mM Na_2HPO_4, 2.5 mM KH_2PO_4 and 0.03 vol.% Tween-20. The substrate for the quantification of the HRP in the eluent contained 4 mg OPD dissolved in 10 ml distilled quarts water with 30 µl hydrogen peroxide. The enzyme reaction was stopped with 1.5 M H_2SO_4.

Equipment

For the displacement, a multichannel peristaltic pump (Ismatec, IPC, Switzerland) was used with clear standard pump tubes (Skalar, Breda, The Netherlands). The columns were made in 25 µl Transferpettor-caps (Brand, Germany). Sampled fractions were measured

spectrophotometrically at 492 nm using a Dias ELISA reader and the BioLinx software (Dynatech Laboratories).

Preparation of the displacement column

Immobilization of FABP by chemical linking

For the chemical immobilization, the transferpettor-cap column was filled with NHS activated Sepharose. After washing it with at least 3 column volumes of propanol, the column was washed with 1 mM HCl. Coupling of rat or human FABP was performed by addition of an FABP-solution (0.3 mg ml^{-1} FABP in 0.2 M $NaCO_3$, 0.5 M NaCl pH 8.3) to the column and incubation for 1 hour. Thereafter, the column was washed at least three times each with a sequence of 0.5 M ethanolamine, 0.5 M NaCl (pH 8.3) and 0.1 M acetate, 0.5 M NaCl (pH 5) for one hour. Then the column was equilibrated with buffer and 2 µg of the detection antibody was loaded. The column was subsequently washed for 16 hours to remove unbound and unspecifically bound label.

Immobilization of FABP with antibodies

For the immobilization of FABP with antibodies, 50 µl (0.2 mg ml^{-1} in buffer) catcher antibody was immobilized on the packed protein-A column material by perfusion. After this, the column was washed with at least two column volumes of buffer, followed by a perfusion of 30 µl (0.1 mg ml^{-1}) rat or human FABP in buffer, followed by another buffer wash of at least two column volumes. The detection antibody was subsequently bound to the FABP as described above.

Description of the displacement experiments

Filling of the column with modified Sepharose
⇓ *washing*
Loading of the column
⇓ *washing for 16 hours*
Perfusing of the column with buffer, with or without FABP
Collection of the fractions
⇓
Analysis of the 10 minutes fraction

After the preparation of the column, the actual experiment started. During the displacement experiments, the columns were perfused continuously. Eluates were collected

as 10-minute fractions. The column was continuously perfused with buffer or with buffer containing various concentrations of FABP. As a test for specificity, myoglobin (MW 18 kD), and lysozyme (MW 14.3 kD) were perfused. Displacement experiments were performed up to three days with the same columns. The flow rate was 4 µl min^{-1}.

Analysis of the displacement column eluates

From the displacement column described above, 5-20 µl aliquots of the 10-min fractions were taken and analysed by adding 100 µl substrate, and compared with a standard curve of the detection antibody. The colour reaction was stopped when $OD_{490,max} \approx 0.5$.

Presentation and interpretation of the results

For the interpretation of the semi-quantitative data, we compared columns with different characteristics. The concentrations of the labelled (detection) antibodies present in the 10-minute fractions are plotted against the time. Perfusions of different compounds are indicated in the graphs. The scale on the y-axis is arbitrary.

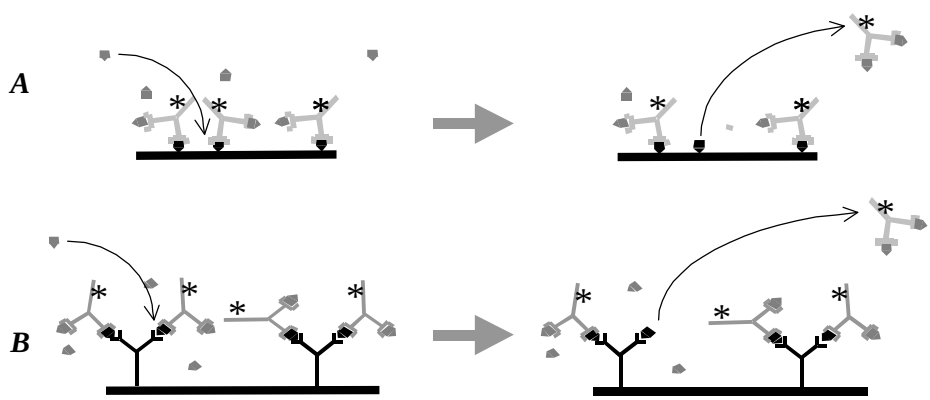

Fig. 7.1. Two approaches for displacement of labelled antibodies. A. Displacement from chemically immobilized FABP; B. Displacement from antibody-based immobilized FABP.

We applied a set-up displacing a labelled antibody from immobilized FABP. Two immobilization methods for the antigen FABP -- a direct chemical coupling and the use of a catcher antibody -- were evaluated. The actual displacement experiment (schematically represented in Fig. 7.1) is performed when these immobilized antibody-antigen complexes are placed in a flow system and exposed to FABP, which will partially displace the

antibodies. Evaluated are the two different immobilization procedures and antigens (human FABP and rat FABP), different antibodies as well as the sensitivity and specificity of the different systems.

Results

The sensitivity for FABP in displacement experiments for different catcher antibodies varied (Figs. 7.2A&B). The displacement after perfusion with 2000 ng ml^{-1} FABP was not above the spontaneous dissociation for the 67D3 — FABP — 66E2-HRP complex, whereas the released amount of 66E2-HRP for the 53E9 — FABP — 66E2-HRP complex was clearly higher.

Influence of different detection antibodies

Displacement experiments with 53E9-HRP as detection antibody resulted in a constant decrease of the dissociation signal. Also, the displacement was more time-dependent than FABP-dependent for both the antibody-based and the chemical immobilization (data not shown). The dissociation was also large when compared to the displacement signal.

Influence of method of FABP immobilization

Two methods of immobilization – one antibody-based and the other chemical – were studied. For high concentrations of FABP, the antibody-based immobilization gave a concentration-related signal (Fig. 7.2A). However, the sandwich-system was not sensitive enough to detect physiological concentrations of FABP (2-5 ng ml^{-1} (Wodzig et al., 1997)). The displacement signal for these FABP concentrations was not identifiable from the spontaneous dissociation signal. The chemically immobilized FABP displacement, where human FABP is coupled to Sepharose with an irreversible chemical reaction, enabled detection of sub-pathological (physiological) FABP concentrations (here 2-20 ng ml^{-1}; Fig. 7.2D).

To explore the specificity of the system, two proteins were used. Myoglobin (15 kD), a protein released at the same time after infarction (van Nieuwenhoven et al., 1995; Ishii et al., 1997) and roughly the same size, seemed to be the ideal test. However, using myoglobin (20 µg ml^{-1}) in a displacement experiment, we observed an increase of signal (Fig. 7.2C). When the perfused FABP concentrations were decreased to 2-20 ng ml^{-1}, the relative signal for myoglobin (20 µg ml^{-1}) was large (data not shown). This is most likely to be related to contamination of the (horse) myoglobin with (horse) FABP, because when myoglobin was tested with an ELISA for FABP (Wodzig et al., 1997), binding signals were observed. Similarly, human myoglobin (Fitzgerald Industries Int. Inc., Concord MA, USA), gave an even higher binding signal (around 0.1 percent), because of more contamination with FABP and/or higher binding affinities (data not shown). Lysozyme perfusion showed

hardly any increase of enzyme activity for the chemically immobilized FABP (Fig. 7.2D). The immobilization of FABP with antibodies showed more unspecific displacement, and chemical immobilization was therefore used for subsequent experiments (data not shown).

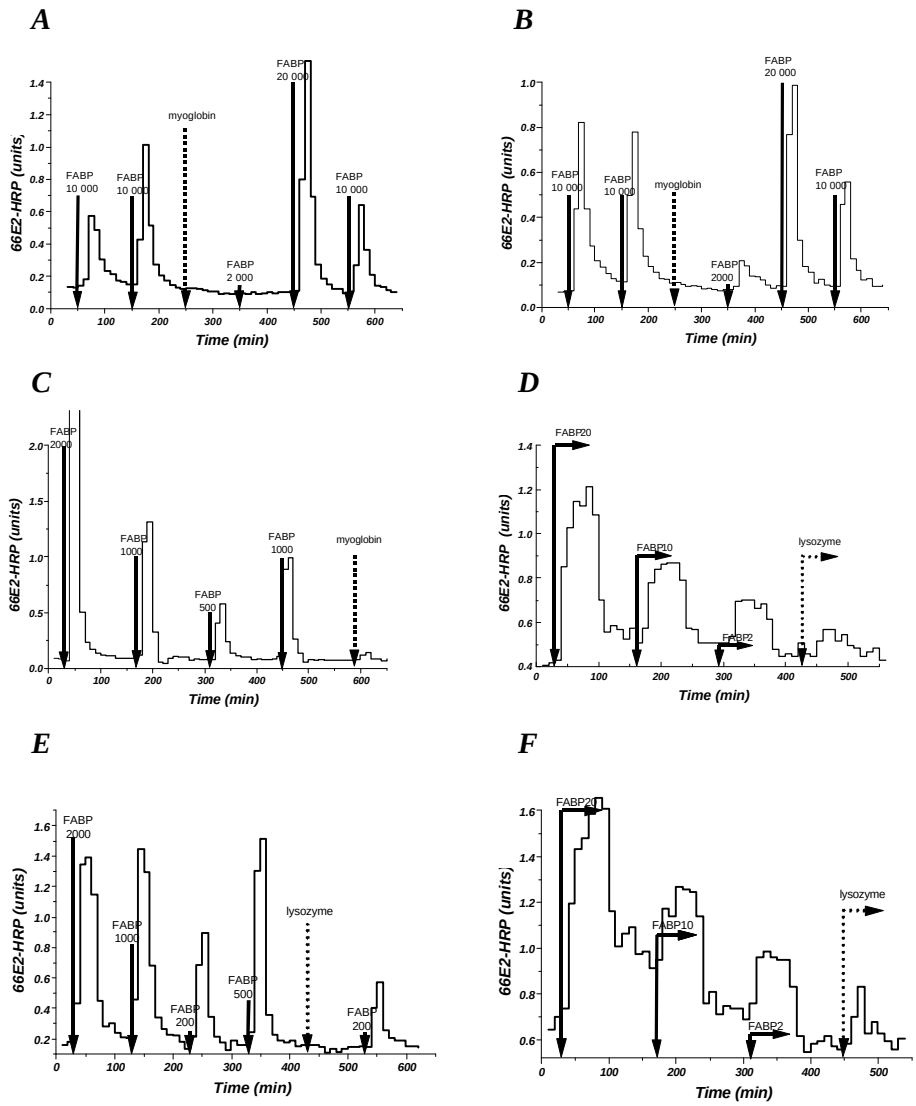

Fig. 7.2. Displacement experiments in continuous flow system after various FABP perfusions with different immobilization procedures. A. antibody-based immobilization; catcher antibody 67D3; B. antibody-based immobilization; catcher antibody 53E9; C, D. chemically immobilized FABP; E, F. chemically immobilized rat FABP. The drawn line indicates the amount of label detected, whereas the start of the FABP perfused are indicated with arrows, the length reflecting the concentration (in ng ml^{-1}) Myoglobin (in a concentration 20 µg ml^{-1}), and lysozyme (20 µg ml^{-1}) are indicated with the dotted arrows. For profile C and F the FABP perfusions are performed for 60 minutes, for the other 20 minutes. The 66E2-HRP units in the graphs are not equivalent.

Influence of species (rat or human) of immobilized FABP

We also studied immobilized rat FABP in an attempt to lower the affinity of some antibody-binding site - antigen complexes. It is known that the homology of rat FABP and human FABP is large (Londraville and Sidell, 1995). Cross-reaction of rat FABP with the human FABP monoclonal antibodies is therefore expected. Immobilization with catcher antibodies and rat FABP resulted in a high, fastly decreasing dissociation signal, of which no significant increase at FABP perfusions occurred (data not shown). The results for chemically immobilized rat FABP with 53E9-HRP were comparable (data not shown). For 66E2-HRP, the spontaneous dissociation was less. FABP concentrations of 200-2000 ng ml^{-1} showed concentration dependent displacement signals (Fig. 7.2E). However, for 2-20 ng ml^{-1} FABP, the spontaneous dissociation is more interfering than for immobilized human FABP (Fig. 7.2D compared to Fig. 7.2F). Interpretation of the signal is hampered because of simultaneous influence of dissociation, displacement and time effects on the amount of label released. Therefore, chemical immobilization of human FABP is preferred and used in subsequent experiments.

Influence of measurements on subsequent analysis

As described, the columns were used for up to 3 days. This enabled perfusions of different sequences through the column. For columns with chemically immobilized human FABP, the signals of subsequent days became lower than previous days (data not shown). However, because the dissociation signal decreased as much as the displacement signal, this decrease did not influence the relative amounts. For unstable, fast dissociation, such as when 53E9-HRP was used, the 53E9-HRP was washed out after one analysis day, leading to no release of label during perfusion after that (data not shown).

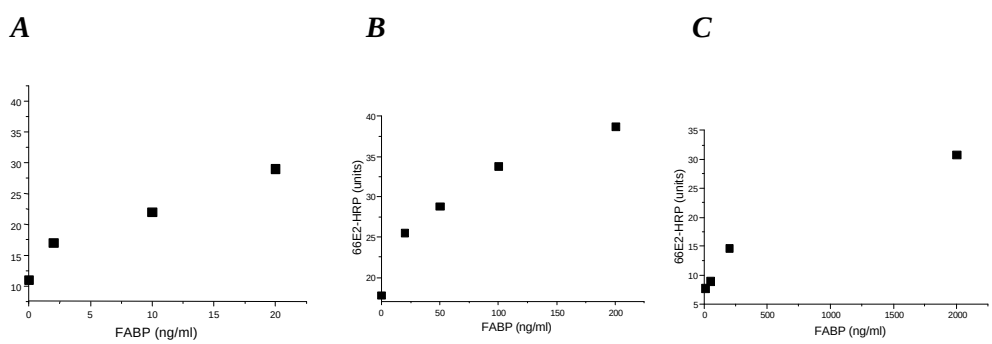

Fig. 7.3. Cumulative fractions per FABP perfusion with following buffer perfusate fractions. A. Low concentrations of FABP in perfusate; B. Medium concentrations of FABP in perfusate; C. High concentrations of FABP in perfusate For Fig. A, the top values of Fig. 7.2H are used. For Fig. B&C, the surface under the curve of a response curve of 20-minute FABP perfusions is summed up. The 66E2-HRP units in the graphs are not equivalent.

Quantification of the signal

To explore the quantitative characteristics of the displacement for the chemically immobilized FABP, we compared the amount of label released at different FABP concentration perfusions. When the displacement signals per FABP perfusion are added, the displacement increases non-linear for the intervals 0-20, 0-200 and 0-2000 ng ml^{-1} FABP. The total amount of released detection antibodies increases when the FABP concentration increases, whereas the amount of detection antibodies per FABP molecule decreases on the complete FABP concentration tested (Figs. 7.3A-C).

Evaluation of the results

As illustrated above, displacement of antibodies can be used to detect an antigen in a flow system. The method of immobilization and the choice of antibody influence the sensitivity and selectivity. Because all monoclonal antibodies had approximately the same equilibrium constant, as well as the same association rate (Roos et al., 1995), the different sensitivity and selectivity can not simply be explained by differences in binding characteristics.

For the antibody-based immobilization, more blocking of the antibody-binding site of catcher antibody 53E9 in combination with 66E2-HRP compared to the 67D3 — FABP — 66E2-HRP might explain the difference, because FABP can reach the site more easily. Experiments with the detection antibody 53E9-HRP were also less successful than with 66E2-HRP in both antibody-based and chemically immobilized FABP. It is unclear whether this is a result of the labelling procedure or of the different epitope recognition.

The reason for a higher sensitivity for chemically immobilized FABP compared to antibody-based immobilization might be that less steric hinderance at the antibody binding site leads to an increase of the displacement. The explanation of a lower affinity of the antibody for the chemically immobilized FABP is less likely, because that would also increase the spontaneous dissociation signal and decrease the specificity.

The displacement signal was non-linearly related to the amount of FABP perfused. At higher FABP concentrations, the release of labelled antibody per molecule FABP was lower. This phenomenon has been described before for trinitrophenol in a displacement system where a labelled antigen homologue was displaced (Whelan et al., 1993a; Narang et al., 1997a). Displacement became linear at higher flow rates. This seems to indicate that at higher flow rates the displacement is diffusion-rate limited, whereas at lower flow rates, as used for the FABP detection, other effects, such as association-dissociation kinetics have an influence on the signal. It is unknown, for example, whether displacement is due to active pushing or preventing re-association with the immobilized antigen. However, the semi-quantitative data are not precise enough to set up an accurate model for the displacement phenomenon.

Discussion

The aim of this study is to investigate whether displacement can be used for the detection of FABP in a continuous sample and, if so, what influence some parameters have. A displacement selection system for fatty acid-binding protein is investigated, where labelled antibodies, associated to immobilized FABP, are displaced by FABP in the sample. These studies were successful, for both chemical and antibody-based immobilization of the FABP. Because the displacement detection is based on immunological recognition, this principle might be useful for more analytes, as previously shown, for the displacement of small, labelled analytes by analyte in the sample. Further research has to be performed to support this hypothesis.

The chemical immobilization of FABP is preferred because of the higher sensitivity and specificity. Different antibodies and antibody-combinations are studied, and they yield different results despite their similar binding characteristics. Semi-quantitative analysis of the displacement signal for the preferred set-up (chemically immobilized human FABP with 66E2-HRP associated) showed a non-linear FABP concentration-dependent displacement over the whole interval measured (0-2000 ng ml^{-1} FABP).

The data of this research demonstrate a non-linear displacement signal, which created a high sensitivity (concentrations of 2 ng ml^{-1} FABP give a signal above base level) and a large detection range (0-2000 ng ml^{-1} gave changes in signal). However, the quantification is rather complex. Whelan et al., showed that raising the flow creates a linear displacement efficiency (Whelan et al., 1993). However, increasing the flow rates decreases the sensitivity and lifespan of the system. A higher flow might be preferable for applications, where measurements are performed for a short time and the amounts of FABP are important, for example in the estimation of the size of an infarction after an AMI. For an alarm system, that detects an infarct after a major heart operation, a relatively stable system, which can be used for more than 24 hours, might be preferred. In respect to our findings, an exact configuration can not yet be described. The displacement column and the detection have to be made more homogeneous from one system to another. For example, using immobilization on fused silica in stead of Sepharose column material for trinitrotoluene, as described by Narang et al., (1997a), might lead to higher sensitivity and reproducibility. In general it can be concluded that, depending on the requirements of the assay, the set-up has to be chosen with consideration of linearity or sensitivity and lifespan extension.

The results described here demonstrate the displacement principle for FABP. However, additional experiments have to be performed to create reproducibility and quantitative measurements before clinical applications can be considered as an option. Additionally, a suitable on-line detection method has be to found, because the 10-minute fraction collection method is labour intensive, imprecise and impeding direct information about the concentration of the analyte. The development of a good detection method, especially in complex matrices such as body fluids, is a major issue in research for further quantification

and following clinical evaluation. One of the methods presently under study is electrochemical detection, which is quite simple to use, relatively inexpensive and needs only small apparatus. Electrochemical detection enables the incorporation of the displacement technique with a small-size disposable immunosensor. This immunosensor can be applied for early detection of myocardial damage and reperfusion. For this, a low, constant flow of the system is required, which can be established with slow flow on-line sampling techniques such as microdialysis (Stjernstrom et al., 1993; Paez and Hernandez, 1997) or ultrafiltration (Moscone et al., 1996; Kaptein et al., 1997). For a combination with this type of sampling, the continuous signal of the flow displacement immunoassay is used to its full potential. To the best of our knowledge, no other assays based on immunological detection have been described for the creation of an on-line signal.

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Chapter 8

Summary and concluding remarks

Some are wise, some otherwise.

This thesis describes the development of techniques designed for continuous on-line bedside and ambulant biosensor devices. Two aspects for continuous on-line analysis are studied: the creation of contact with the analysis site and the development of bioselective methods. The contact with the analysis site was acquired with an on-line sampling device. The selection was created with an enzymatic detection for two metabolites, glucose and lactate and an immuno displacement method for the detection of the hormone cortisol and the early infarction marker fatty acid-binding protein (FABP).

These two aspects are described in subsequent sections in this thesis. The two sections are introduced by an overview of research in this area. A general introduction is given to contextualize the research in the broader perspective of clinical biosensor development.

Chapter 1 presents a general introduction of biosensors that are currently available for *in vivo*, *ex vivo* and *in vitro* (bio)chemical monitoring in clinical medicine. At present, most biosensors are not able to perform *in vivo*, but are utilized with discrete (blood) samples. The original goal to implant biosensors in ways similar to the implantation of pacemakers is risky because of stability, reliability and biocompatibility problems. In addition, it is difficult to calibrate an implanted sensor. Realistic on-line monitoring might be achieved by combining biosensors with an on-line microdialysis or ultrafiltration sampling device. Techniques which might be implemented in such a device, are also introduced.

The first section (*chapter 2*, *chapter 3* and *chapter 4*) of the thesis focuses on on-line sampling techniques and the bioselective detection of two metabolites, glucose and lactate. *Chapter 2* surveys the microdialysis (MD) and ultrafiltration (UF) literature. MD is a dynamic sampling method based on analyte diffusion across a semi-permeable membrane due to a concentration gradient. UF is a technique during which endogenous fluid is withdrawn from the sampling site by means of an underpressure. These are the only two on-line sampling techniques available besides undiluted blood sampling. Our group (in Groningen) recently introduced on-line UF. It relies on the constant withdrawal of fluid with a sampling probe, using underpressure as the driving force. Different sources of sampling are described. Special attention is given to subcutaneous and transcutaneous sampling for estimation of blood (glucose) concentrations. In our opinion, the blood compartment and subcutaneous compartment are definitely two clearly distinguished sections of the body, whereas the differences between the blood and the transcutaneous compartment are even more distinct. Even for small molecules like glucose, changes in the blood compartment can be observed in the subcutaneous tissue only with a large delay. The sampling of body fluids is therefore not only a technical challenge, but also requires a detailed knowledge of the pathophysiology.

Chapter 3 describes ultrafiltration (UF) experiments in anaesthesized rats. Contrary to MD, UF does not cause drainage of analytes (depletion of the analyte being removed by the dialysis fluid), and may therefore give better insight into the real “undisturbed tissue” physiology. Probes were implemented into the subcutaneous (s.c.) tissue and into the jugular vein (i.v.). Sampling was performed with an ultraslow, continuous flow of 100 nl min^{-1}, created with a disposable syringe and controlled by a fluid restriction in the

outlet of this syringe. This syringe (a "monovette") enabled pulsefree pumping of these ultraslow flow rates, using very inexpensive material. In both s.c. and i.v., the samples were analysed for the glucose concentration with a bioselective enzyme reactor, using electrochemical detection. These concentrations were compared with discrete blood samples. Reported for the first time here, the small hollow fiber probes enabled i.v. sampling, and therefore created a new field of UF sampling. Batch-wise sampling in s.c. tissue with large probes has been described before. However, continuous UF sampling experiments for on-line monitoring of analytes in the s.c. compartment has never been described before. The outcomes of the experiments of *chapter 3* demonstrate that UF sampling is a successful continuous sampling system for both the s.c. and i.v. compartments. The correlation between the glucose concentrations found with the UF i.v. samples and the discrete samples was very high ($r>0.995$; $p<0.001$). After a mathematic correction for the difference between whole blood and UF (for the presence or absence of cells), the concentrations of glucose in the discrete blood samples and the UF were nearly the same, indicating that the barrier (if any) of the membrane for glucose molecules is the same as for water molecules. Obviously, because both samples are taken from the same body compartment, there are no physiological differences. With s.c. sampling, a clear difference in the glucose concentrations between subcutaneous fluid and blood was observed. This was not only a delay in changes of the glucose concentrations, but also the shape of the curve of the glucose concentration over time after a glucose load was different. This supports our statement that blood glucose control performed on subcutaneous analysis may create large artefacts. The development of probes for intravenous sampling is a good way to prevent these complicated problems.

A new ultraslow sampling technique is described in *chapter 4*. Whereas normally MD is performed with flow rates of 1-20 µl min^{-1}, we describe sampling with an ultraslow MD technique, usMD, with flow rates as low as 100-300 nl min^{-1}. By this, artefacts caused by drainage are reduced. We compared this usMD with UF by alternating between UF and usMD and changing flow rates. A dual bi-enzymatic system combined with electrochemical detection for glucose and lactate was applied to study both sampling techniques. The probe was inserted into the s.c. tissue of the rat. Under the conditions of the experiments, there was no difference in the sampled fluid (dialysate or ultrafiltrate). In contrast to state-of-the-art MD, with at least one order of magnitude higher flow rates (µl min^{-1}), the recovery of this usMD was 100 percent. This means that the actual concentrations in the dialysate are the same as in the extracellular s.c. tissue fluid, and therefore, complicated calculations for absolute concentrations can be avoided. For UF, the fluid influx from the tissue to the probe through the membrane was not hindered in the applied flows. This is in contrast to findings in our group concerning UF sampling in s.c. tissue in humans, where obstruction of the fluid to enter the probe was observed. This means that UF is a suitable sampling technique for on-line sampling in s.c. tissue in the rat for flow rates up to (at least) 300 nl min^{-1}.

A careful evaluation of the sampling site, the (expected) recovery, the sensitivity of the analysis method and the conditions in which the measurements are taken all have to be made before choosing between the UF or MD sampling method. In the described

application, there was no preference for one sampling method above the other. However, in general, the UF sampling may be preferable, because the probe configuration is less complex and sterilization can be achieved more easily. Also, when the probe will be blocked during the experiment, this can be checked immediately with UF (especially when, in the future, flow meters are accessible to measure flow rates this low), whereas the recovery of the usMD might decrease without awareness of the experimentalist.

Both MD and UF have the potential to be used in *in vivo* or *ex vivo* monitoring systems. Also, they can be used for analysis of time profiles by continuous collection and off-line analysis afterwards. Biosensors might function better in combination with UF/usMD sampling devices than in direct contact with the body, because of the relatively "clean" sample. Due to the approximate 100% recovery, calibration is easier than in conventional, fast-flow MD sampling.

The second section (*chapter 5*, *chapter 6* and *chapter 7*) investigates the immuno displacement method as a bioselective step for continuous sampling to monitor the hormone cortisol and the early heart infarction marker FABP. Displacement happens when the complementary binding between an antigen binding site (ABS) of the antibody (Ab) and the corresponding antigen (Ag) or antigen analogue is actively broken after free antigen, the analyte, is added. Displacement immunoassays have been used for the analysis of discrete samples. *Chapter 5* discusses the literature on displacement. On the one hand, compared to conventional competition and sandwich immunoassays, displacement assays have a lower sensitivity. On the other hand, displacement is the only way to analyze samples continuously.

Chapter 6 describes the experiments on displacement for cortisol in a continuous flow system. Cortisol-recognising antibodies were immobilized on sepharose protein-A columns; cortisol labelled with horseradish peroxidase is associated with this. When the columns are subsequently perfused with cortisol, displacement can be obtained. To detect concentrations of cortisol above 100 nM, high-affinity antibodies were found to be best suited, because the spontaneous dissociation was low, and the displacement was dependent on the cortisol concentration. When low-affinity antibodies were used, the spontaneous dissociation was too high, and the displacement was dependent only on the time of perfusion and not on the cortisol amount. For lower concentrations of cortisol (10-100 nM), the displacement signal of the high-affinity antibody did not exceed the background dissociation signal. Here, the low-affinity antibodies were a better option, because they released a concentration-dependent signal.

Chapter 7 describes the displacement for the protein cytoplasmic heart fatty acid-binding protein (FABP). The approach of these displacement experiments was different from the experiments with cortisol: the displacement assay applied to a high-molecular protein instead of a low-molecular hormone, and instead of displacement of a labelled antigen (analogue), the antibody was labelled and released in solution at displacement. Displacement of labelled antibodies has been observed in non-flow systems only after hours or even days of incubation. All the literature on displacement in flow

systems so far has described displacement for small analytes up to 500 Da. No experiments to date have reported a displacement signal for proteins. In our experiments, we have shown that for physiological (2-6 µg l^{-1}) and pathophysiological (6-2000 µg l^{-1}) concentrations of FABP, the displacement is detectable from the background signal of spontaneous dissociation. We immobilized the FABP on Sepharose beads in a column following two different methods: by direct chemical linkage to the carrier and by binding it with another antibody ("sandwich" format). Of these methods, the chemical linkage gave by far the best results, both for sensitivity as well as selectivity. It showed a displacement signal up to 2 µg l^{-1}, whereas 20 µg ml^{-1} lysozyme (used as an unspecific control) did not show any increase of displacement signal.

A very important advantage of the application of labelled antibodies instead of labelled antigens is that labelling of antibodies can be done following a standard method, leaving the binding sites unaffected. Labelling of the antigen itself has to be adapted to every single analyte, and may harm the binding site.

The on-line sampling and bioselecting system developed for FABP may be the first step for a future bedside alarm system that detects a reinfarction as well as an infarction after a major heart operation. It might be useful for more analytes as well.

Future outlook: on-line clinical biosensors.

Future biosensors combining the ultraslow microdialysis or ultrafiltration with enzymatic metabolite conversion or displacement immunoselection will enable on-line biomedical monitoring systems for major metabolites and risk factors (e.g. a sensor as schematically represented in fig. 8.1). Harmful fluids to the tissue do not contact the body fluid, and potential toxic elements can be kept outside the body. Moreover, the proposed underpressure pump is not only pulse-free, but also very cheap (below 1 US$).

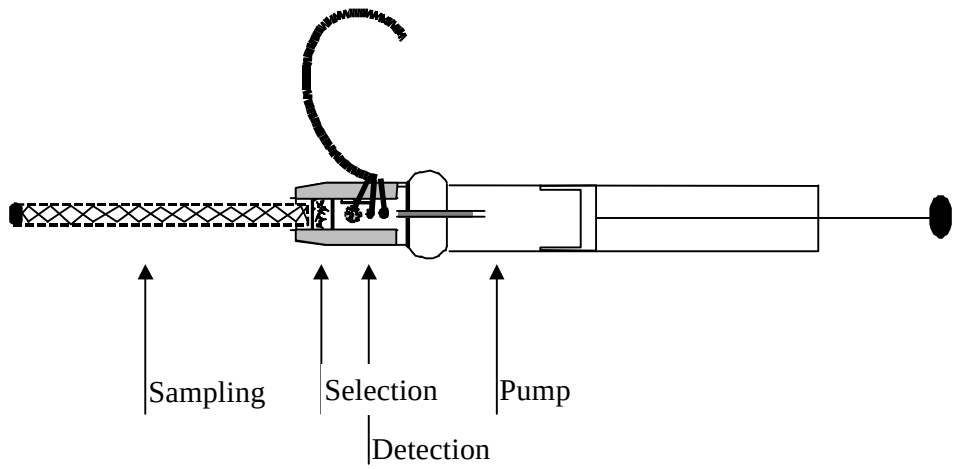

Fig. 8.1. Future on-line clinical biosensor device.

To establish the proposed devices, some technical challenges have to be overcome. The major next task that lies ahead, consists in developing a suitable on-line detection system. The creation of a detection system is an underestimated problem. Detection in physiological samples such as blood is extremely complex. The problem is merely not the detection limit, but the interference from other compounds in the matrix with the signal. We have not been able to solve the problem concerning the interference-free measurement of the displacement signal within the framework of this thesis.

After establishing the detection system, the different parts for sampling, bioselection and detection have to be combined. Flow rates, sensitivities and fluid handling all have to be carefully considered. As for the choice between an usMD or UF device, the effects of removing the fluid from the tissue have to be studied more in detail to make a good decision.

Clinically relevant applications of biosensors can be found across the entire field of medicine. On-line monitoring in combination with a feed-back mechanism would be an especially important new application for biosensors. At present, the research on this specific type of biosensor aims at controlling blood glucose for diabetics. However, other applications can be found in the monitoring and controlling of drugs. In particular, biosensors will be useful in the control of drugs with small therapeutic intervals, such as digoxine and cytostatics. The proposed UF/MD displacement immunosensor system may create devices for this type of application. Other application fields can be found in on-line monitoring of cardial protein markers, not only for detection of (recurrent) infarctions, but also for visualising reperfusion of the blockage and estimation of the infarct size.

The ideal biosensor should be small, fast, safe, easy to handle and cheap. Furthermore, the reproducibility of the biosensor should be high and the shelf life long. It should create a maximum of information with minimal human intervention. The possibilities of biosensors seem to be unlimited. Research all over the world focuses on such devices. After the release of the first successful biosensors, future release will cascade because of the similarities of many designs. In an enzyme sensor, for example, an enzyme can be replaced by a similarly acting enzyme (e.g., glucose oxidase by lactate oxidase or glutamate oxidase) and the sensor design can be maintained. Analogously, replacing an antibody recognising one analyte by an antibody selective for another analyte will increase the diversity of immunosensors and reduce the development time.

Essential for this future work is good interaction between researchers in (bio) electronics, chemists and biologists on one hand and medical doctors, the future end-users, on the other hand. When this can be achieved, on-line clinical biosensors will have a major impact on the future of medical care.

Chapter 9

Samenvatting

Het leven is een feest
en jij bent uitgenodigd (Loesje, kalender 1997)

In dit proefschrift worden technieken beschreven die kunnen worden geïmplementeerd in *in vivo* (in het lichaam geplaatste) toepasbare biosensoren. Biosensoren zijn ontwerpen die snel en specifiek een signaal creëren als reactie op de aanwezigheid van een stof. Deze stof wordt selectief herkend door een biologisch molecuul, zoals een enzym of een antilichaam. Dit signaal ontstaat zonder toevoeging van een extra substraat of scheidingsstap. Biosensoren, die tot nu toe beschreven zijn in de literatuur, hebben nog een aantal technische tekortkomingen. Vaak treedt er een afweerreactie van het lichaam op en is het signaal van de sensor niet constant. Bovendien zijn tot nu toe de technieken beperkt gebleven tot de detectie van stoffen, die met behulp van een enzymreactie uiteenvallen in detecteerbare produkten, zoals dat bijvoorbeeld gebeurt bij glucose en lactaat. In een algemene inleiding wordt de toepassing van biosensoren binnen de geneeskunde van dit moment beschreven. Deze toepassing ligt vooral in de detectie van glucoseconcentraties in bloedmonsters. Een belangrijke stap voorwaarts zou liggen in het gebruik van biosensoren om continu stoffen in lichaamsvloeistoffen te meten. Het doel van het onderzoek zoals beschreven in dit proefschrift, dat in dit hoofdstuk wordt toegelicht, is hierin te vinden. In het proefschrift worden technieken geïntroduceerd, waarmee continu contact wordt gecreëerd met lichaamsvloeistoffen. Bovendien wordt er een nieuwe, op antilichaam-herkenning gebaseerde techniek beschreven waarmee ook niet-enzymatisch detecteerbare stoffen kunnen worden gemeten. Na deze algemene inleiding is de tekst globaal te verdelen in twee gedeelten.

In het eerste gedeelte van het proefschrift wordt aandacht besteed aan een nieuw soort “monstername”, een samplingsysteem. Deze techniek wordt ultrafiltratie genoemd. Met ultrafiltratie kunnen continu zeer kleine hoeveelheden vloeistof aan het lichaam worden onttrokken, die dan buiten het lichaam geanalyseerd kunnen worden. Een voordeel van het analyseren buiten het lichaam is, dat een daaraan gekoppeld sensorsysteem, geen afweerreactie van het lichaam geeft. Deze afweerreactie treedt vaak op wanneer de sensor rechtstreeks in contact wordt gebracht met het lichaam. Ook kan de sensor eventueel wat groter uitgevoerd worden, en kan de sensor gemakkelijker vervangen worden.

In een overzichtsartikel in het proefschrift wordt de literatuur over deze techniek beschreven en vergeleken met een andere techniek: microdialyse. Bij microdialyse wordt geen vloeistof onttrokken, maar worden lichaamsvloeistoffen in contact gebracht met een vloeistof die door een zeer kleine, half-doorlaatbare buis door het weefsel stroomt. Er treedt uitwisseling van stoffen op tussen de lichaamsvloeistof en deze vloeistof. De hoeveelheid van een stof in dit “monster” geeft dan een afspiegeling van de hoeveelheden van die stof in de lichaamsvloeistof waarmee dit in contact is geweest.

Na het overzichtsartikel zijn twee artikelen opgenomen waarin experimenten met ratten worden beschreven. Het eerste artikel beschrijft de toepassing van ultrafiltratie voor monstername in een bloedvat en subcutaan weefsel. Hierin zijn duidelijk verschillen te zien tussen de glucosehoeveelheden in het subcutane weefsel en die in het bloed. Het instellen en onderhouden van een insuline-toedieningsschema op grond van deze subcutane glucoseconcentraties, zoals vaak in de literatuur wordt voorgesteld, lijkt door deze waarneming zeer discutabel. Het tweede artikel beschrijft experimenten waarin de verschillen van microdialyse en ultrafiltratie worden onderzocht aan de hand van

gelijktijdige glucose- en lactaatmetingen. Bij deze proeven werden slechts zeer kleine monsters per tijdseenheid (100-300 nl/min) genomen. Het bleek dat bij deze perfusiesnelheden de concentraties van glucose en lactaat in het microdialysemonster in evenwicht was met de lichaamsvloeistof en dat ook monstername met behulp van ultrafiltratie mogelijk was.

Het tweede gedeelte van het proefschrift beschrijft een nieuwe selectiemethode: de verdringing (displacement). Bij verdringing wordt de binding tussen een antilichaam (een molecuul dat specifiek een bepaalde stof herkent) en een antigeen (de stof die het antilichaam specifiek herkent) verbroken doordat een andere stof aan de specifieke bindingsplek van het antilichaam gaat binden. Wanneer verdringing wordt gebruikt voor de specifieke herkenning van stoffen, is het antigeen een stof die gelijk is of sterk lijkt op de stof die gemeten moet worden. Deze methode kan gebruikt worden voor stoffen die niet rechtstreeks met behulp van een enzymreactie detecteerbaar zijn. Hiervoor worden bijvoorbeeld antilichamen vastgezet en de bindingsplaatsen van deze antilichamen verzadigd met een gemerkte stof. Tijdens de verdringing wordt het eerder gevormde complex tussen het antilichaam en de gemerkte stof verbroken, doordat de stof uit het monster aan het antilichaam gaat binden. De ongebonden gemerkte stoffen kunnen vervolgens apart worden gemeten. Deze verdringing is in theorie voor bijna alle biologische moleculen mogelijk, en voor de vele moleculen zijn er antilichamen te koop. Deze techniek heeft daarom de potentie om voor analyse van vele stoffen inzetbaar te zijn.

Een algemene inleiding geeft een overzicht over de mogelijkheden en toepassingen van deze verdringing. Hierin wordt de tot nu toe verschenen literatuur beschreven. Daarnaast worden de mogelijkheden besproken waarmee de verdringingsreactie gebruikt kan worden om een detectie van stoffen op te zetten, en hoe zo'n systeem kan worden aangepast. De hierop volgende twee hoofdstukken geven illustraties van de verdringing als detectiemethode. De verdringingsdetectie van cortisol, een stresshormoon, demonstreert de techniek voor de meting van de concentratie van een klein molecuul. Het blijkt dat het mogelijk is om cortisol te meten in hoeveelheden zoals ze in het menselijk lichaam voorkomen.

In het tweede hoofdstuk wordt een eiwit, het fatty acid-binding protein (FABP) met behulp van deze verdringingsdetectie gemeten. FABP is een eiwit dat vrijkomt bij beschadiging van het hart. De verdringingsexperimenten voor dit FABP laten zien dat verdringing ook gebruikt kan worden voor grote moleculen. Ook is de methode waarin de verdringing hier is opgezet nieuw. In plaats van een verdringing van de gemerkte stof wordt het antilichaam zelf gemerkt en door de aanwezigheid van het FABP losgemaakt van vastgezet "antigeen". Met deze twee toepassingen wordt niet alleen de eerder geclaimde universele toepasbaarheid ondersteund, maar ook een nieuwe methode voor het gebruik van de verdringing gedemonstreerd. Deze nieuwe methode, waarbij antilichamen worden gemerkt, is in praktijk voor meer toepassingen geschikt dan het merken van de stof zelf.

Tot slot wordt het proefschrift afgerond met een evaluatie van de hierin besproken technieken, en een terugkoppeling naar het oorspronkelijke doel: implementatie in *in vivo* biosensoren.