Slow microfiltration or slow microdialysis to versatile biomonitoring

Huinink, Kirsten D.

Slow microfiltration or slow microdialysis to versatile biomonitoring

Studies presented in this thesis were performed at the University Center Psychiatry of Groningen, Animal Sciences Group of Wageningen and Biomonitoring & Sensoring Group of Groningen.

Publication of this thesis was financially supported by the University of Groningen, University Medical Center Groningen and Dutch Technology Foundation STW, applied science division of NWO and the Technology Program of the Ministry of Economic Affairs, grant number GPG 06038.

Cover design: Sjors Sevenhuijsen

Printed by: Het Grafisch Huis

RIJKSUNIVERSITEIT GRONINGEN

Slow microfiltration or slow microdialysis to versatile biomonitoring

Proefschrift

ter verkrijging van het doctoraat in de

Medische wetenschappen

aan de Rijksuniversiteit Groningen

op gezag van de

Rector Magnificus, dr. F. Zwarts,

in het openbaar te verdedigen op

maandag 8 juni 2009

om 13.15 uur

door

Debby Huinink

geboren op 30 maart 1976

te Meppel

Slow microfiltration or slow microdialysis to versatile biosampling

Promotor:	Prof. dr. J. Korf
Copromotores:	Dr. A. Lambooij Dr. T. I. F. H. Cremers
Beoordelingscommissie:	Prof. dr. J. Strubbe Prof. dr. R. Vonk Prof. dr. B. H. C. Westerink

Contents

Chapter 1
General introduction

Important in human and animal research is the methodology to monitor stress without interference with the normal stress physiology. Related to this issue is or how to collect biological samples from an intact organism without stress, and how to store and analyse biochemical and pharmaceutical compounds. The present thesis is focused on the development of continuous monitoring devices in the freely moving subject that can be applied to a wide variety of compounds, including the glucocorticoids corticosterone and/or cortisol. The present methodologies to monitor pharmaceutical compounds and various brain energy metabolites illustrate their versatility and their usefulness. To explore the temporal patterns of peptides, drugs, metabolites or hormones, conventionally multiple samples have to be collected manually which is often stressful and invasive.

Stress is often an unwanted source of experimental error, because stress affects both the physiology and metabolism, thus interfering with or even impairing subtle experimental designs. This may add to between-animal variations and eventually leading to inconsistencies of experimental procedures and results. To reduce stress and release of stress-related hormones, there is a need to refine animal experimental procedures, thus avoiding handling, restraining and bio-sampling. To reduce experimental stress in animals we propose and explored two sampling techniques: microfiltration and MetaQuant microdialysis.

1.1 Stress response in short

The term stress refers to the physiological reaction caused by the perception of aversive or threatening situation and will renders an organism ready for the fight-or-flight response (Cannon). Once the threat is over, the physiological condition can return to "normal" again. Important is the distinction between an essentially non-conditionable stress response, usually indicating a vital mechanism for survival, and that of a cognitively perceived response that is primarily a subjective interpretation of a challenge [101]. A human example of a vital response is the threat of an attacking lion, leading to an immediate action, as compared to the impact of a loss of a loved person that may cause a longer lasting emotional state. The latter rather than the firstly mentioned challenge may eventually evoke a psychiatric condition, such as depression. Exposure to lasting stress, rather than incidental and transient stress, may lead to a long-lasting response, eventually facilitating cardiovascular diseases, damage to muscle tissue, steroid diabetes, infertility, inhibition of growth, inhibition of the inflammatory response, and suppression of the immune-system making an individual more vulnerable to infections.

The hypothalamic-pituitary-adrenal axis (HPA) is the central control area of the stress response and subsequent hormonal fluctuations **(figure 1; left)**. In response to non-conditionable stress among other the amygdala is activated leading to the release of corticotropin-releasing hormone (CRH) in the paraventricular nucleus (PVN) of the brain. CRH acts on the pituitary gland, causing the release of adrenocorticotrophic hormone (ACTH), which in turn causes the adrenal gland to release cortisol (in the human and the cow), or corticosterone (in rodents) in the blood circulation. Is the stress conditionable than the external signal is firstly processed and interpreted in or by the cerebral cortex, the amygdala and the hippocampus, before activating hypothalamic structures and so the release of CRH. Positive and negative feedback occurs at various sites in the brain to ensure that cortisol production stays within certain boundaries both in time and in magnitude. The stress hormones cortisol and corticosterone are glucorticoids and have profound effects on

glucose metabolism. Accordingly, proteins are broken down and converted into glucose, fats are made available for energy production, blood flow is increased and behavioural responsiveness is enhanced by these stress hormones [243].

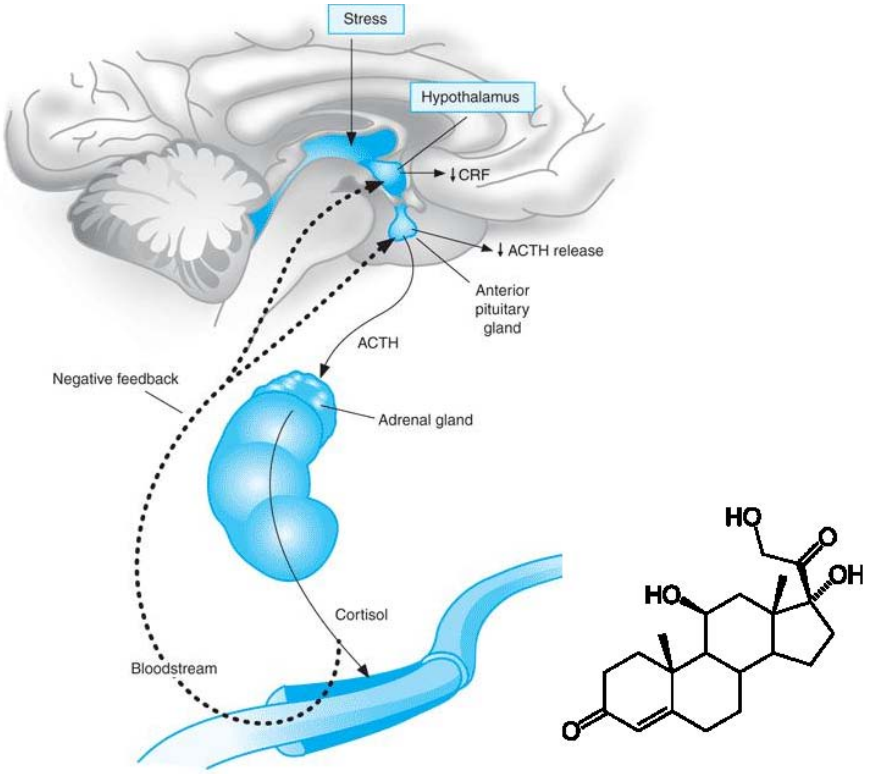

Figure 1 Left: Schematic drawing hypothalamic-pituitary-adrenal (HPA) axis. In response to non-conditionable stress among other the amygdala is activated leading to the release of corticotropin-releasing hormone (CRH) in the paraventricular nucleus (PVN) of the brain. CRH acts on the pituitary gland, causing the release of adrenocorticotrophic hormone (ACTH), which in turn causes the adrenal gland to release cortisol (in the human and the cow), or corticosterone (in rodents) in the blood circulation (Molina PE: Endocrine Physiology, 2nd edition). **Right**: structure formula of cortisol $C_{21}H_{30}O_5$.

1.2 Glucocorticoid cortisol

Cortisol **(figure 1; right)** is a lipophylic hormone and is in the human circulation bound to proteins, such as the cortisol-carrier globulin (CBG, transcortin, for 75%) and albumin (for 15%), whereas a small percentage remains unbound (10%). Only the unbound or free fraction cortisol is biologically active. CBG is produced in the liver with a molecular weight of 48 kDa binding cortisol with high affinity. Albumin has a greater binding capacity but its binding affinity is lower. The plasma half-life of cortisol is 60-90 minutes and is determined by both protein binding and metabolism. Under basal (= non-stressed) conditions the human urinary excretion of cortisol ranges from 8-25 $mg \cdot day^{-1}$ (22-69 $\mu mol \cdot day^{-1}$), with a mean of about 9.2 $mg \cdot day^{-1}$ (25 $\mu mol \cdot day^{-1}$).

All species known from amphibian to humans have stress hormones corticosterone and/or cortisol. Birds, mice and rats secrete almost exclusively corticosterone; dogs secrete approximately equal amounts of corticosterone and cortisol; and cats, cows, sheep, monkeys, and humans secrete predominantly cortisol. In humans the ratio of secreted cortisol to corticosterone is approximately 7:1. In pharmacology the synthetic form of cortisol is referred to as hydrocortisone and is used to treat inflammations and allergies.

1.3 Cortisol rhythm

During normal, non-stressed situations, blood cortisol level is subject to circadian rhythm; high in the early morning and low around midnight,

3-5 hours after the onset of sleep [171, 200]. This rhythm is not present at birth (estimates of when it starts vary from two weeks to 9 months) [65]. Although cortisol rhythm is generally consistent, there is considerable intra- and inter-individual variability, and the circadian rhythm may be altered by changes in sleep pattern, light-dark exposure, and feeding times. The rhythm is also changed by (1) physical stresses such as major illness, surgery, trauma, or starvation; (2) psychological stress, including severe anxiety, endogenous depression, and the manic phase of manic-depressive psychosis [19]; (3) central nervous system and pituitary disorders; (4) Cushing's syndrome; (5) liver disease and other conditions that affect cortisol metabolism; (6) chronic renal failure; and (7) alcoholism [111, 171, 200, 243].

1.3.1 Monitoring cortisol rhythms

In the clinic blood, urine and saliva are the most sampled body fluids to investigate the concentration of the analyte of interest for e.g. hormones, metabolites, and ions. Blood samples are relatively easily achieved in humans and animals, but it is an invasive procedure that becomes in particular stressful when multiple samples have to be collected. In rodents blood is usually sampled via a cannula placed in the jugular vein, via a cut in the tail, or trunk blood is collected after decapitation. The disadvantages of jugular sampling is that the number of blood samples being collected is limiting and to keeping the cannula open for several days is quite a challenge. Tail bleeding causes pain and discomfort, thus influencing stress hormone levels, and only a limited number of samples to be collected. Only one single sample of trunk blood can be collected, and thus increasing the number of animals to be included when studying the time course of stress hormones. It should also be questioned if the blood values represent tissue values, as their concentrations may differ because of differences of tissue distribution, protein binding and metabolism. Urine and saliva can easily and non-invasively be collected. However these body fluids have limitations as well, as not all analytes may end up in this body fluid or may be representative for tissue levels.

To circumvent the many of the above mentioned issues, sampling techniques like microdialysis and microfiltration have been introduced,

that are still mostly applied in pre-clinical settings [207], but both techniques have clinical potential. The basic principle is shown in **figure 2** and **table 1** shows the most important differences between microdialysis and microfiltration.

1.3.2 Microdialysis technique

The basic principle of microdialysis is to mimic the function of capillary blood vessels by implanting a hollow fiber membrane into tissue, perfusing the tube with a physiological fluid and subsequently collecting the dialysate. The dialysate reflects the composition of the extracellular fluid containing substances that diffuse through the pores of the hollow fiber membrane. The driving force of microdialysis is the concentration gradients of the various diffusible constituents between the extracellular fluid and the perfusion fluid over the probe membrane. Ideally no fluid is withdrawn from the sampling site with microdialysis.

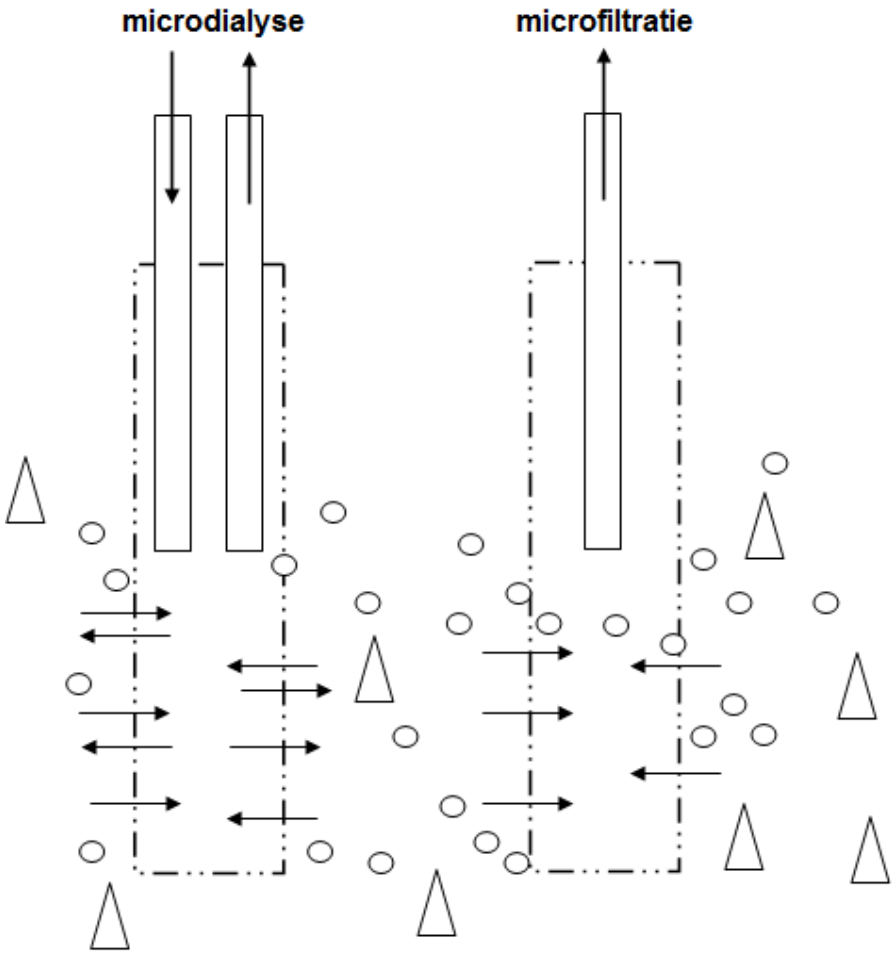

Figure 2 The principle of conventional microdialysis **(left)** and microfiltration **(right)**. Microfiltration uses under pressure to collect a filtrate of body fluid; Microdialysis is based on the use of a carrier solution, a concentration gradient and diffusion of analytes across the semi-permeable membrane. The cut-off value of the membrane determines the size of molecules that pass the membrane. In contrast to conventional microdialysis, in which the recovery has to be calculated, the microfiltration recovery is 100% as the microfiltrate directly reflects the tissue concentration.

Table 1 Comparison between microdialysis and microfiltration.

method	microdialysis	microfiltration
driving force	pump	under-pressure
method of analyte retrieval	diffusion	Filtration
retrieved fluid	protein free or with proteins*1	protein free or with proteins*1
MWCO hollow fiber	10-3000 kDa	10-3000 kDa
in vivo sampling	all tissues including the brain	all tissues except the brain
sampling rate ($\mu l \cdot min^{-1}$)	0.1-10	0.05-10
highest sampling	every minute	Continuous
calculation after retrieval	yes, except when using ultraslow flow rate	100% recovery
drug delivery	yes	No
used in clinic	yes	No

There are several variables to consider when applying microdialysis. The most important variable is the flow rate. In conventional microdialysis studies, perfusion rates of 1 $\mu l \cdot min^{-1}$ and higher have been used for in vivo sampling, resulting in relative recoveries far from 100%. The term relative recovery describes the ratio between the concentrations of the substance in the dialysate and (peripheral) tissue. Complicated procedures and computations are required to calculate (or -perhaps better- to estimate) the real concentration of the analyte in the tissue compartment. These calculations are nicely reviewed by Plock and Kloft [250]. Quantitative microdialysis, without calibrating the microdialysis device, is realized by applying an ultraslow perfusion rate (<0.3 $\mu l \cdot min^{-1}$), which results in similar concentrations of the analyte in the dialysate and the tissue compartment. Kaptein et al. [160], Ekberg et al. [76], Rosdahl et al. [262], and Cremers [52] proved that using ultraslow-microdialysis the recovery is near 100% in contrast to the lower recoveries obtained with conventional microdialysis. An inconvenience of ultraslow flow rates is the long lag times between sampling and

subsequent chemical analysis. This disadvantage is circumvented by using the MQ-microdialysis **(figure 3)** sampling technique (patented by Brainlink B.V.), which combines the advantage of high and quantitative microdialysis at ultraslow perfusion rates and the fast transport of dialysate of conventional microdialysis. In this type of microdialysis probe an extra fluid line is added, which rapidly transports the dialysate sample to the detection apparatus [52].

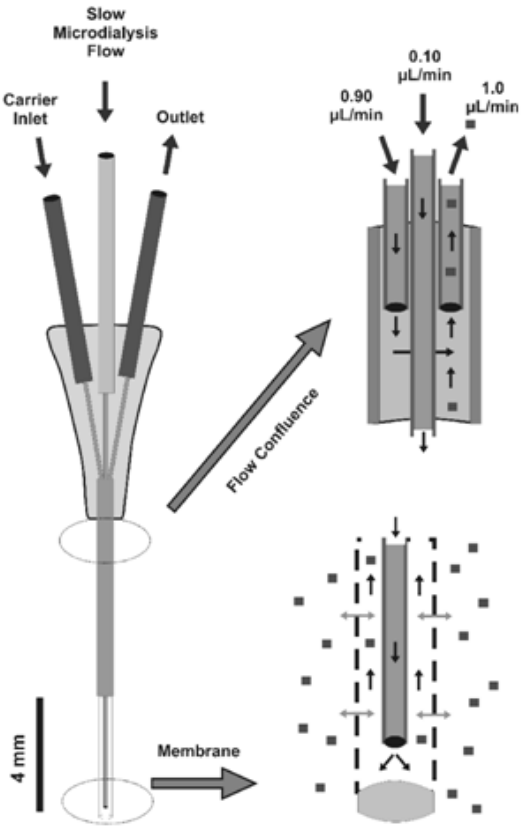

Figure 3 Schematic drawing of the MetaQuant microdialysis probe.

1.3.3 Microfiltration technique

The driving force of the flow rate in microfiltration is a pressure gradient over the probe membrane, pulling sample from the in vivo environment. Microfiltration samples are undiluted (100% recovery),

small (µl or nl size) and sterile. The microfiltration probe can be built into a continuous collection device, enabling continuous sampling for a selected period of time and simultaneous storing the samples in the collection capillary coil. This integrated device is called the ultra/micro-filtration collection device (MCD/UCD; **figure 4**). With this device time-profiles can be created without manual intervention and the profile can be stored in de collection device until further analysis up to several days. The amount of fluid withdrawn from the tissue compartment is usually relatively small, so the chemical balances of the extracellular fluid (ECF) is not or minimally disturbed and in most tissues the body fluid is easily replenished. Because microfiltration concentrations are (nearly) identical to those in the ECF concentrations, the measured levels of the analyte reflect rather accurately the metabolic changes in ECF [232]. It appeared, however, that the volume of the ECF may differ between animal species, condition of the organism and the investigated organ. For instance, Kissinger et al. mentioned that an anesthetized rat produces almost no microfiltration fluid, whereas a rat, that has free access to water and food and is moving around, produced plenty of microfiltration fluid [172]. Microfiltration sampling is not possible in the rat brain striatum at flow rates of 50-100 nl·min^{-1} [160]. Apparently the ECF production and/or supply in the rat striatum is too low. Whether this limitation applies to the human brain is unlikely, considering the high percentage – as compared to the rat brain – of extracellular volume of more than 30% of the total brain volume.

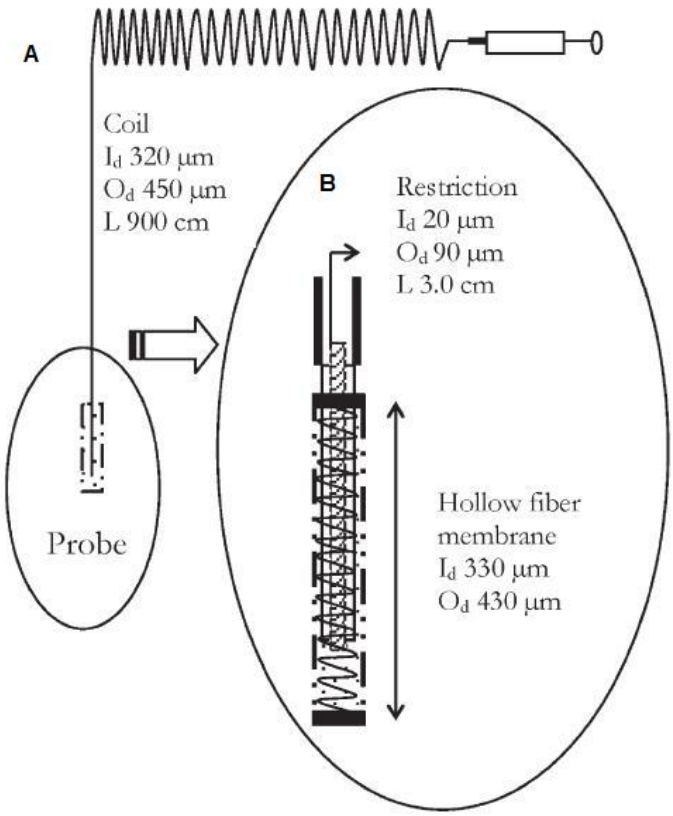

Figure 4 Schematic design of the microfiltration collection device (MCD). (**A**) The hollow fiber membrane (probe) is connected to the fused silica tubing storage coil. The coil is on his turn connected to the 9-ml S-Monovette the under-pressure generator. The probe could be placed into a sample solution or in vivo subcutaneously or intravascular. (**B**) Schematic blow-up of microfiltration probe consisting of the hollow fiber membrane, spring and flow restriction. The restriction specification gives in combination with the under-pressure of 34800 Pa, a certain flow rate. This flow rate can be calculated according with Poiseuilles law.

1.4 Analytes sampled with microdialysis and microfiltration

1.4.1 Low molecular analytes

Most studies describing microdialysis and microfiltration sample low molecular weight (LMW) analytes. The most sampled analytes with microdialysis are neurotransmitters as the microdialysis method was originally developed for sampling the brain, but the energy substrates glucose and lactate make a second best. Microdialysis has been applied also in the human and among the most promising application is the monitoring of glucose in diabetes and in intensive care medicine. Microfiltration has little if any medical applications beyond the experimental setting. In microfiltration sampling, glucose [146, 152, 196, 197, 271, 307, 309] and lactate [196, 197, 271, 307, 309] are the most sampled analytes. But other analytes such as electrolytes [145, 153, 205, 291] and drugs [28, 58, 204, 206] are sampled as well in both techniques.

To sample LMW analytes with microdialysis and microfiltration, hollow fiber membranes with a MWCO <30 kDa are used, in this manner large particles like proteins and cellular elements are excluded.

1.4.2 High molecular analytes

As in the beginning of microdialysis, the emphasis was on monitoring LMW, thus ignoring the possibilities to collect and analyse high molecular weight (HMW) analytes. More recently HMW sampling is gaining interest [120, 129, 130, 132, 133, 138, 208, 346 274]. Microfiltration has only occasionally been applied to collect HMW and the present thesis is one of the first attempts to explore this application systematically.

1.5 Aims and scope of this thesis

Microdialysis and microfiltration sampling is most often used for the collection of hydrophilic compounds and it appears difficult to sample hydrophobic analytes. The glucocorticoids cortisol and corticosterone are rather lipophilic hormones, exhibiting high protein binding, so the free (unbound) concentrations in the blood and extracellular fluid are relatively low. Not only do lipophilic hormones bind to proteins they

also bind non-specifically to microdialysis and microfiltration probe-materials and tubing. To monitor total cortisol levels the proteins which bind cortisol must be sampled as well. To realise this hollow fiber membranes with relatively large pores must be used.

Both microdialysis and microfiltration use ultraslow flow rates. Handling of such small flow rates is difficult as the sample might concentrate influencing the concentration, resulting in long lag times, and sampling small volumes necessitates sensitive analytical methods.

Chapter 2 gives a general overview about ultraslow-microfiltration since its invention in 1987 [154]. In addition various aspects of ultraslow-microdialysis are summarized.

Experiments described in **chapter 3** were aimed to find the fiber material with the least non-specific binding of proteins and to explore the potential of a storage capillary. Adsorption of hydrophobic and hydrophilic peptides to different hollow fiber membranes was tested in vitro and in a complex bio-fluid. Part of the experiments utilizes mass-spectrometry to analyse the various peptides and proteins. Next we tested the capillary device for long-term storage of microfiltration-samples. To preserve a time-profile little or no diffusion should occur of peptides and proteins during storage in the capillary coil.

Chapter 4 describes in vitro testing of various hollow fiber membranes for optimising MQ-microdialysis sampling of corticosteron and a number of psychoactive pharmaceuticals. We tested how quantitative MQ-microdialysis as a sampling technique was: i.e. whether the recovery is precise or near 100%.

In **chapter 5** sampling was tested in vitro and in vivo to assess quantitative sampling with different hollow fiber membranes and the drugs, used also in the studies described in **chapter 4**.

The microfiltration probes found optimal in the previous studies were applied in vivo to sample biofluid with continuous microfiltration **(chapter 6)**. In the rat microfiltration-probes were applied subcutaneously, intraperitoneally, and intravenously. In the cow microfiltration probes were placed in an udder-vein to sample

microfiltrates for storage in a capillary for assessing cortisol time profiles.

An extensive overview on the biomedical applications of ultraslow-microdialysis and microfiltration is provided in **chapter 7**. Not only some of the studies in the present thesis are discussed, but also applications and interpretations of various experiments on brain energy metabolism are considered.

The thesis is concluded **(chapter 8)** with a general discussion and English and Dutch summaries **(chapter 9)** of the present studies and overviews.

Chapter 2

Ultraslow-microfiltration and microdialysis for in vivo sampling: principle, techniques, and applications

This chapter is published in Handbook of microdialysis-Methods, Applications and Clinical Aspects, Edited by: Ben H.C. Westerink and Thomas I.F.H. Cremers, Chapter 2.6, page 217-230.

Abstract

Microfiltration often referred as ultrafiltration (UF) is a sampling method next to microdialysis which is underexposed, and it should not be. In this review we tried to be as complete as possible as far for microfiltration. Ultraslow-microdialysis is far from complete as many microdialysis studies are performed but not always in the light of an ultraslow-flow, we report a few. First, we explain the principles of microfiltration and outline the similarities and differences between microdialysis and microfiltration. After this we will go over the topic biocompatibility of the hollow fiber probes placed in vivo briefly. Then we will summarize what has been done in the field of microfiltration the last 5 years. In addition a few articles about ultraslow-microdialysis will be mentioned briefly and the analytical (detection) devices will be highlighted only shortly as this topic deserves a report on its own.

Keywords

microfiltration, ultrafiltration, ultraslow flow, microdialysis, in vivo, sampling techniques, in vitro

2.1 Introduction and scope

The first reports on microdialysis appeared in the late seventies and early eighties of the last century, more than 5 years before methods and applications of microfiltration were reported. We preferred to speak about microfiltration instead of UF as this is more conform microdialysis terminology. Pioneering studies on microdialysis were described by Delgado [66], Brodin [33], Ungerstedt [320] and their co-workers. Microdialysis now a well established technique to monitor metabolites, energy substrates, neurotransmitters and hormones of experimental animals from a wide variety of sites, with an early emphasis on cerebral applications. The clinical potential of microdialysis was recognised from the early onset and in particular its application to monitor glucose in diabetes, have been pursued ever since. In 1987 the potential of in vivo UF or microfiltration as a clinical device was recognized for the first time. It was suggested by Janle-Swain [154] that microfiltration could be used in hospitalised patients in whom frequent biochemical parameters have to be monitored, without the necessity of repeated blood sampling. In vivo microfiltration has been performed in human subjects [13, 45, 307] and in animals including dogs [154], cat [146], rats [150, 159, 160, 172, 195-197, 204-206, 232, 274], mice [148, 152], chicken [271], sheep [139, 145, 153, 289], pigs [309], and horses [291]. Most in vivo experiments with microfiltration are performed subcutaneously, but intravenous application of microfiltration [159, 195-197, 271, 309] of saliva [172, 204, 275, 276], bone [145] and muscle [291] have also been described.

On microdialysis more than 5,000 experimental studies and numerous reviews have been published since 1982. In contrast, since 1987 only about 40 scientific papers on microfiltration have been published **(table 1)**, together with 2 review articles [96, 193]. In far the most studies on microfiltration and microdialysis, perfusion rates of over 1 µl·minute^{-1} have been used for in vivo sampling. For microfiltration estimation of the real recovery of the analyte from tissue is not a real issue, because essentially undiluted body fluid is collected. In contrast, from microdialysis performed at relatively high perfusion rates complicated procedures are required to calculate (or estimate) the real

concentrations of the analyte in the tissue compartment. One option to overcome this issue is to apply such slow rates of perfusion that the concentration of the analyte in the dialysate and in the tissue compartment, become the same. The main focus of the present review is on articles on ultraslow-microfiltration that were published between 2000 and 2009.

2.2 Microfiltration sampling techniques

Two different types of microfiltration probes have been developed of which one is commercially available (Bioanalytical Systems, Lafayette, Ind., USA). Basically a microfiltration device consists of a hollow fiber membrane which is connected via tubing to a syringe pump. The flow rate/sampling rate is induced by applying a negative pressure over the hollow fiber membrane; this can conveniently be accomplished by a syringe pump or Monovette/Vacutainer. The maximum size of particles that are transported over the hollow fiber membrane is limited not only by the molecular weight cut off (MWCO) value of the hollow fiber membrane, but also by the configuration and the charge of the analyte. Microfiltration samples are undiluted, small (µl or nl size) and sterile.

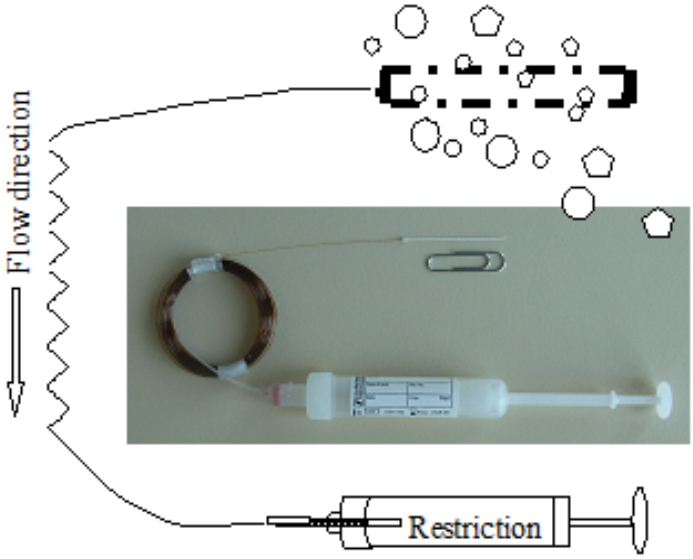

Figure 1 Schematic design and photograph ultrafiltration collection device (UCD).

Table 1 An overview of microfiltration sample analysis.

analyte	type	length (cm)	hollow fiber membrane				flow ($\mu l \cdot min^{-1}$)	site	animal	analysis method	sampling time (hours)	ref.	Year
			polymer	o.d. (µm)	i.d. (µm)	mwco (kDa)							
glucose	3 l	20	-	200		30	1 – 2	sub	dogs	glucose oxidase strips accucheck II reflactance meter spectrophotometric	7 – 63 d	154	1987
theophylline acetaminophen	3 l	7 12 16	polyacrylonitrile	310	220	30	2 – 5	saliva sub	humans rats	HPLC-UV HPLC-ECD	4 8 - 12	204	1992
glucose	3 l	-	-	-	-	-	-	sub	cat	-	30 d	146	1992
glucose	3 l 1 l	2 2	-	-	-	-	0.13 – 0.16 0.02 – 0.03	sub	mice	lc-FIA-enzymereactor	21 – 48 d	152	1992
theophylline acetaminophen	3 l 3 l	12 16	polyacrylonitrile	310	220	30	2 – 3	sub	rats	HPLC-UV HPLC-ECD	4.5 2	206	1993
sodium potassium calcium phosphorus	3 l	16	-	310	220	30	1 – 3	sub	rats	ion-selective electrode spectrophotometric	-	205	1993
glucose	3 l	-	-	-	-	30	0.7 – 1	sub	humans	glucose-oxidase	30 d	13	1993

analyte	type	length (cm)	hollow fiber membrane				flow (µl·min^{-1})	site	animal	analysis method	sampling time (hours)	ref.	Year
			polymer	o.d. (µm)	i.d. (µm)	mwco (kDa)							
glucose cefazolin	3 l	12	polyacrylonitrile	-	-	-	-	sub	mice	-	15, 30, and 50 d 5.5	148	1993
glucose	3 l	12	-	-	-	-	-	sub	rats	LC-FIA-enzyme reactor	24	149	1995
insulin	con	1 – 1.5	polyacrylonitrile	340	240	40	-	in vitro sub	rats	capillary electrophoresis SDS-page	<1 5	274	1996
myoglobulin		1 – 1.5	polyacrylonitrile	340	240	40							
albumin		0.5 – 1	polysulfone	1100	-	100							
proteins		1.5	polyacrylonitrile	340	240	40							
sodium potassium chloride glucose lactate	3 l	12	-		-	-	5	in vitro	na	Cardi compact ion meter spectrophotometric BAS LC method glucose oxidase	1	147	1996

analyte	type	length (cm)	hollow fiber membrane				flow ($\mu l \cdot min^{-1}$)	site	animal	analysis method	sampling time (hours)	ref.	Year
			polymer	o.d. (µm)	i.d. (µm)	mwco (kDa)							
acebutolol cephalothin chloroamphenicol salicylic acid acetaminophen isoniazid	3 l	2	polyacrylonitrile	-	-	-	-	in vitro	na	HPLC-UV HPLC-ECD	-	188	1996
glucose	con	4	acrylonitril-sodium methallyl sulfonate	290	240	-	0.2	sub	rats	ECD	24	232	1996
glucose	con	4	acrylonitril-sodium methallyl sulfonate	290	240	-	0.1	iv sub	rats	FIA-enzymereactor-ECD	5 3.5	159	1997
glucose lactate	con	4	acrylonitril-sodium methallyl sulfonate	290	240	-	0.1 – 0.3	sub	rats	FIA-enzymereactor-ECD	3	160	1998

analyte	type	length (cm)	hollow fiber membrane				flow (µl·min^{-1})	site	animal	analysis method	sampling time (hours)	ref.	Year
			polymer	o.d. (µm)	i.d. (µm)	mwco (kDa)							
sodium Potassium chloride lactate glucose	3 l	12	-	220	320	-	5	sub	rats	ion selective electrode Spectrophotometric BAS LC methode glucose oxidase	25 d 15 d - 16 d -	151	1998
sodium potassium calcium	-	-	-	-	-	-	1	sub musc	horses	ion selective electrode Spectrophotometric	- - 8	291	1998
glucose lactate	con	2.5	acrylonitril-sodium methallyl sulfonate	340	240	50	0.05	sub	humans	FIA-enzymereactor-ECD	2.5	307	1999
glucose	con	2	acrylonitril-sodium methallyl sulfonate	340	240	-	0.2	in vitro	na	FIA-enzymereactor-ECD	12	258	1999

analyte	type	length (cm)	hollow fiber membrane				flow (µl·min^{-1})	site	animal	analysis method	sampling time (hours)	ref.	Year
			polymer	o.d. (µm)	i.d. (µm)	mwco (kDa)							
lactate	con	4	acrylonitril-sodium methallyl sulfonate	290	240	-	0.1 – 0.35	In vitro	na	FIA-enzymereactor-ECD	5.5	45	2000
calcium magnesium phosphorus	3 l	12	-	-	-	40	-	sub musc bone	sheeps	spectrophotometric ionselective electrode	6.5	153	2000
inflammation study	con	2	-	-	-	30	na	musc	sheeps	-	na	139	2000
glucose	con	2	-	-	-	-	0.1	in vitro	na	FIA-enzymereactor-ECD	-	255	2001
calcium magnesium phosphorus	3 l	12	-	-	-	40	-	sub musc bone	sheeps	spectrophotometric ionselective electrode	-	145	2001
glucose lactate	con	-	acrylonitrilsodium methallyl sulfonate	340	240	20	-	iv	pigs	FIA-enzymereactor-ECD	27	309	2001

analyte	type	length (cm)	hollow fiber membrane				flow ($\mu l \cdot min^{-1}$)	site	animal	analysis method	sampling time (hours)	ref.	Year
			polymer	o.d. (µm)	i.d. (µm)	mwco (kDa)							
glucose lactate	con	4	acrylonitril-sodium methallyl sulfonate	290	240	-	0.05	iv sub	chickens	FIA-enzymereactor-ECD	8	271	2003
carbamazepine	3 l	2	polyacrylonitrile	-	-	-	-	sub	rats	-	48	172	2003
cimetidine	con	7	-	300	150	11.5	5	in vitro	na	chemiluminescense	-	333	2003
na	con	4	-	-	-	5	-	skin flap	rats	na	8	235	2003
glucose lactate	con	-	acrylonitril-sodium methallyl sulfonate	290	240	20	0.1	iv	rats	FIA-enzymereactor-ECD	3 - 4	197 196 195	2001 2003 2004
na	con	5	-	-	-	-	-	skin flap	rats	na	8	236	2004
poly-l-lysine poly-l-tryptophan	con	4	polyethylene coated with ethylenevinyl-alcohol polysulfone	430 450	330 320	0.3 µm 0.2 µm	0.5 3.0	In vitro	na	spectrophotometric	-	135	2005

analyte	type	length (cm)	hollow fiber membrane				flow ($\mu l \cdot min^{-1}$)	site	animal	analysis method	sampling time (hours)	ref.	Year
			polymer	o.d. (µm)	i.d. (µm)	mwco (kDa)							
marbofloxacin enrofloxacin ciprofloxacin	3 l	12	-	-	-	30	-	sub	dogs	HPLC-UV	24, 60	[28]	2005
cephalexin	3 l	12	-	-	-	-	-	sub	horses	HPLC-UV	12	[58]	2005
cofilin-1 futuin-A complement C3 gelsolin apoliprotein C-1	c	-	polysulfone	700	500	400	0.83 $\mu l \cdot h^{-1}$	sub	mice	MALDI-TOF-MS/MS Q-TOF-MS/MS	6	[133]	2005
thymosin	c	-	polyacrylonitrile	-	-	50	0,33 $\mu l \cdot h^{-1}$	sub	mice	MALDI-TOF-MS Q-TOF-MS/MS	3	[132]	2006
peptides/proteins (n=10)	c	-	polysulfone	700	500	400	1.67 $\mu l \cdot h^{-1}$	sub	mice	MALDI-TOF-MS Q-TOF-MS/MS	6	[130]	2006
thymosin	c	-	-	-	-	50	0.33 $\mu l \cdot h^{-1}$	sub	mice	MALDI-TOF-MS Q-TOF-MS/MS	3	[208]	2007

The commercially available microfiltration-probe (Bioanalytical systems) consists of one or more hollow fiber loops with various hollow fiber lengths. The smallest loop has a length of 2 cm and the biggest is 12 cm. The sampling rate is relatively high, in the range of 0.5-2 $\mu l \cdot h^{-1} \cdot cm^{-1}$ hollow fiber membrane. By using a relatively high negative pressure, fluid and dissolved metabolites are directly pulled from the blood capillaries to the ultrafiltrate membrane and thus, the ultrafiltrate closely resembles the plasma water composition of blood [154]. We refer to this type of microfiltration fast microfiltration as for e.g. the UF-3-12 (Bioanalytical Systems) posses a total length of 36 cm of hollow fiber so at e a high negative pressure the typical filtration rate is between 0.3-1.2 $\mu l \cdot min^{-1}$. Disadvantages of fast microfiltration are the relatively large size of the hollow fiber that has to be implanted through a 14 gauge cannule with (local) anasthesia, the large hollow fiber can cause tissue damage and the microfiltration sampling construction may not suited for intravenous microfiltration sampling, possibly except in the larger blood vessels. Normal rate microfiltration allows the collection of discrete samples of µl size in small commercial tubes that can conveniently be processed manually for subsequent analysis.

A far smaller microfiltration probe was introduced by Moscone et al. which allows to sample continuously for at least 30 hours at flow rates of 200 $nl \cdot min^{-1}$ and less [232]. Such samples are too small to handle manually. This microfiltration probe has been combined with a storage device, so time-profiles of analytes in e.g. the subcutaneous or blood compartment can be collected and stored before further analysis, without manual intervention. This integrated device is called the ultrafiltration collection device (UCD), see **figure 1**. This device consisted of a hollow fiber probe, a long coil for sampling storage of fused silica or coated glass; as with other materials evaporation of the collected fluid disturbed flow patterns and a flow creator by means of negative pressure (Monovette). Because of the small size of the probe, the probe can be introduced subcutaneously via adapted needles with minimal tissue damage and without anasthesia. The amount of fluid withdrawn from the tissue compartment is usually small relative to the size of that compartment, so the chemical balances of the extracellular fluid (ECF) are not or minimally disturbed and the body fluid is easily

being replenished. Because microfiltration concentrations are (nearly) identical to those in the ECF concentrations, the measured levels of the analyte reflect rather accurately the metabolic changes in the intercellular spaces [232]. It appeared, however, that the volume of the ECF may differ between animal species, condition of the organism and investigated organ. For instance, Kissinger and coworkers mention that an anesthesized rat produces almost no ultrafiltrate, whereas a rat, that has free access to water and food and is moving around, produced plenty of ultrafiltrate [172].

The microfiltration sampling technique is comparable to microdialysis likewise also separating chemicals by moving them across a semi-permeable membrane, but there are differences. The most important differences are that in microdialysis, the separation is exclusively due to a concentration gradient and diffusion of the analytes, whereas in microfiltration, analytes are transported over the membrane together with the body fluids in which they are dissolved. Due to the diffusion gradient in microdialysis technique one has to calculate the recovery of the analyte, which is time-consuming and complicated as equilibrium is almost never reached. A possible solution to this problem is lowering the perfusion rate in order to reach diffusion equilibrium, so that calibration becomes redundant. This can be accomplished by applying ultraslow-microdialysis, a technique developed by our laboratory and firstly described by Kaptein et al. [160]. Normal and ultraslow-microdialysis can not be performed in the brain as microdialysis and ultraslow-microdialysis can. Indeed, Kaptein et al. [160] concluded that at flow rates between 50-100 nl·min^{-1} microfiltration (the usual range of ultraslow-microfiltration) could not be performed in the striatum of the conscious rat. Their probe length was only 0.5 mm, the flow rate decreased during measurement and often stopped completely. Of course, larger probes could be used, but then the sampling area exceeds the site of interest of the rat brain. Apparently, the fluid production and/or supply in the rat brain is to low. They also showed that in contrast to the normal rate microfiltration, sampling with ultraslow-microfiltration up to 300 nl·min^{-1} was well possible in the subcutaneous interstitial fluid in the back of the rats. In healthy volunteers subcutaneous ultraslow-microfiltration sampling was also possible, e.g.

for monitoring glucose on-line [307]. From a physical point of view the flow rate should be as low as possible in ultraslow-microdialysis. Whether ultraslow-microdialysis or microfiltration is the best option depends on the in vivo application. Both techniques can use disposable material and relatively clean samples can be created. When the amount of extracellular tissue fluid is limited, ultraslow-microdialysis is preferable. A drawback of ultraslow-microdialysis technique is the necessity of an additional fluid, making it more difficult to keep the sampling system sterile in contrast to microfiltration. On the other hand local application of drug near the probe is easier with microdialysis.

The microdialysis and microfiltration sampling technique is also applied as biosensor interface between the body and the sensor. Leegsma-Vogt [194] reviewed glucose and lactate biosensors and explained how the two biosensors are working and reviewed clinical and experimental results. More differences and/or in common characteristics between microfiltration and microdialysis were described by Leegsma-Vogt [193] and Garrison [96].

2.2.1 Pumps for ultraslow-microfiltration and ultraslow-microdialysis

For the collection of nl samples by ultraslow-microfiltration and ultraslow-microdialysis, flow rates as low as 10 nl·min^{-1} are preferred. In particular when storage in capillary coils of the UCD, is desired, the pumps should generate a pulse-less flow that is constant over several hours. Such low flow rates can not easily be achieved with mechanical pumps. One option is the use of pumps based on the use of under-pressure, combined with a fluid resistance. Such stable flow rates can be realised by using Poiseuilles law combined with a capillary restriction placed in the UCD.

Poiseuilles law is:

$$v = \frac{\Delta P \cdot \pi \cdot r^4}{8 \cdot l \cdot \eta}$$

Where ν = flow ($m3 \cdot s^{-1}$); l = length (m) and r = radius (m) of the restriction tube, ΔP = pressure (Pa) is the difference in pressure between the tubing ends; and η = viscosity ($Pa \cdot s^{-1}$) of the sampled fluid. Different flow rates can be realised by changing either the applied vacuum or the restriction length or diameter. We tested the usefulness of various under-pressure devices, in particular cheep disposable injection syringes. Monovettes were tested (1.2, 5.5 and 9.0 ml) with different under-pressure values and different restriction specifications. Flow rates could be maintained for several hours or even days, values ranging from 10-500 $nl \cdot min^{-1}$ can thus be obtained. Advantages of such pumping devices are that they are very cheep, energy friendly and light weight, so they can be used in freely moving animals and humans. For instance, we have applied them for freely moving chickens [271], where the devices were installed under the wing for subcutaneous and intravenous sampling.

Recently alternative pulse-less pumps have been described. In particular pumps based on electro-osmotic fluid movements are a promising alternative for fluid collection. The principle is that electrical fields in thin laminar channels induce the movement of electrolytes and concomitant water. Electro-osmotic pumping is based on the drag force exerted on an electrolyte by double layer charge moving in an electric field. Electro-osmotic flow can be switched on/off easily through switching of the voltages that establish the electric field. Similarly, switching of flows from and to different liquid lines is possible, without (mechanical) valves, allowing complex sample and reagent manipulation. Like the under-pressure systems developed by us, electro-osmotic pumps can be constructed with small (nl) dead volumes. Various designs of electro-osmotic pumps have been described and most of the earlier problems that were associated with these devices have been solved. For example, gas bubbles generated at the electrodes that provide the electric field are prevented from entering and thereby obstructing the main flow channel by decoupling the electrode compartments from the flow channel via porous frits or (nano-) channels [192, 230], or by preventing electrolysis completely using asymmetric electrode patterns [233] or an asymmetric AC-voltage [281]. A drawback of the conventional electro-osmotic pumps is that high

voltages are required to achieve reasonable flows. Particularly for the application of systems to be carried on or close to the human body, the accessible voltages should not exceed c.a. 50 Volts, and preferably stay below 10 Volts. Low voltage electro-osmotic pumps have been described Takamura et al. [298] and a theoretical analysis of the experimental results obtained by the latter groups was provided by Brask et al. [31].

2.3 Membrane biofouling and tissue changes

The hollow fiber membranes, the sampling part of microfiltration and microdialysis, placed in vivo can elicit a material-tissue/blood interaction. Several articles appeared regarding this topic in the context of microfiltration, microdialysis sampling and/or biosensors [47, 139, 337-341] and some articles described blood-hollow fiber interaction in haemodialysis studies, hollow fibers are than being tested extracorporeal [141, 280, 330].

In the first article of Janle-Swain on microfiltration [154] the subcutaneously implanted hollow fiber in rats was evaluated for tissue reaction. However the material of the hollow fiber was not mentioned. Minimal tissue reaction was noticed and only a small sheath of fibrous connective tissue was formed. According to Burhop et al. [36] and Hakim and Lowrie [107] polyacrylonitrile implanted as dialyzer caused little complement activation and was classified as low complement activity material. However the period of sampling in both studies lasted only 3-4 hours. Microfiltration (UF-3-2 and UF-1-2, BAS) implanted subcutaneously in rats by Janle and Kissinger [148] showed no macroscopically apparent inflammatory response 6 weeks after implantation. Also no local oedema was seen within 2 days after intramuscular implantation of RUF-3-12 in horses [291]. The histopathological effect of microfiltration probe UF-3-2 made from polyacrylonitrile was tested in sheep in combination without and with intramuscular injections procaine and benzathine salts of penicillin G [139]. This group found that implantation of the hollow fiber alone caused greater inflammatory response than the injections of procaine or procaine plus benzathine penicillin G at remote intramuscular sites.

Implantation of the microfiltration probe caused thus inflammation and degeneration of the surrounding muscle, microscopically and macroscopically. The intensity of the inflammation differ depending on the time elapsed after implantation of the hollow fiber probe (8-11 days). The longer the implantation the magnitude of the inflammation was greater. Microfiltration probes made from polyacrylonitrile may not be suitable for intramuscular UF sampling in sheep [139]. Clark et al. [47] investigated a range of commercial hollow fibers for subcutaneously implanted applications. The tested hollow fibers were made of mixed esters of cellulose (pore size = 0.2 µm; inner diameter = 350 µm; outer diameter = 500 µm), polysulfone (pore size = 0.2 µm; inner diameter = 1000 µm; outer diamter = 1800 µm, pore size = 0.65 µm; inner diameter = 750 µm; outer diameter = 1200 µm, MWCO = 10 kDa; inner diameter = 500 µm; outer diameter = 820 µm), regenerated cellulose (MWCO = 13 kDa; inner diameter = 200 µm; outer diameter = 216 µm) and cellulose-diacetate (MWCO = 68 kDa; inner diameter = 195 µm; outer diameter = 255 µm). The hollow fibers were implanted subcutaneously in rats and 3, 6 and 12 weeks the implants with surrounded tissue were harvested. The samples were evaluated histologically, on thickness of the fibrotic capsule and for membrane integrity. As early as 3 weeks the hollow fiber membranes made of polysulfone with a pore size of 0.2 µm and the cellulose diacetate were extensively degradated and were considered as unsuitable. The mixed ester cellulose and regenerated retained there integrity even after 12 weeks. All membranes exhibited typical foreign body response with fibrotic capsule formation. The regenerated cellulose (MWCO = 13 kDa) and polysulfone membranes (MWCO = 10 kDa) had the thinnest fibrotic capsule formation. Not only is the implanted material important for material/tissue-interaction, also the MWCO value and the size of the implanted hollow fiber may have additional effect.

The advantage of sampling subcutaneously is that it avoids the complement cascade. In microdialysis a substances can be added to the perfusate en to reduce or lower the complement cascade. In microfiltration this can't be done. Sefton et al. discussed the topic: What really is blood compatibility? [280]. In haemodialysis studies most of the time continuous administration of an anticoagulant is necessary.

Heparin is applied most often, forming a complex with ATIII and thrombin the enzyme that converts fibrinogen into fibrin. Von Baeyer et al. [330] tested the following membrane types extracorporeal: cuprophan, cellulose diacetate, symmetric and asymmetric polyacrylonitrile, polyamide, polysulfone and polymethylmethacrylate. The cuprophan, cellulose diacetate, and symmetric polyacrylonitrile showed continuous fibrin precipitation including white blood cells. Polysulfone, asymmetric polyacrilonitrile and polyamide remained clear except of a few white blood cells and platelets. The haemodialyis devices filled with polymethylmethacrylate the surface was covered with a mural white thrombus. It was proposed that the more rigid surface of symmetric membranes hinders sterically the action of heparin near the membrane wall. Micro-elasticity of asymmetric membranes avoids such inhibition [330].

Our group applied heparin to enable intravenous blood sampling with microfiltration in rats [159] and pigs [289]. Heparin was not necessary for the intravenous sampling of chickens [271]. Biomedical and clinical application of microfiltration and ultraslow-microdialysis

Blood and urine are the most sampled body fluids to investigate the concentration of the analyte of interest for e.g. hormones, metabolites, and ions. Blood samples are relatively easily achieved and clinicians increasingly relying on fast biochemical analysis urge on continuous monitoring. However, continuous blood sampling poses infectious hazards and risks of internal bleedings, because the patient is often heparinized. It also remains the question if the blood values represent tissue values after all concentrations may differ because of different distribution, protein binding or metabolism. Microfiltration is an ideal sampling technique to investigate analyte values in the different tissues and it can be performed continuously. Most of the studies describing microfiltration, sample low molecular weight (LMW) analytes. For example: glucose and lactate are the most sampled analytes, but minerals like magnesium, calcium, phosphorus, chloride are sampled as well. To sample small analytes, hollow fiber membranes with a MWCO <50 kDa are used, in this way large particles like proteins and cellular elements are excluded. Macro-analytes such as proteins and biopharmaceutical agents which pharmacokinetic and metabolism are

of interest both systemically and in local tissue are also interesting to sample. One might consider cytokines and peptide hormones, these are high molecular weight (HMW) analytes in the order of 20-90 kDa. In this section the LMW analytes sampled with microfiltration are discussed with at the end two articles discussing HWM analyte sampling.

2.4 Microfiltration

2.4.1 Minerals

Janle and Soika [153] investigated calcium and magnesium distribution in bone, muscle, and subcutaneously in sheep, by infusing calcium gluconate over a period of 1 hour. Peak concentration occurred for all studied minerals after 90 minutes in bone, muscle, and subcutaneous tissue, but the magnitude of the peak varied between the tissues. The mineral concentration in muscle and subcutaneously were similar, but the concentration in bone was lower. This group studied also mineral metabolism in sheep also in bone, muscle and subcutaneous tissue but now without infusing calcium gluconate [145]. The calcium plasma concentration from the ultrafiltrate was higher in plasma comparing the value of calcium to ultrafiltrated samples taken from bone, muscle and subcutaneous tissue. The concentration of magnesium was the opposit, this value was lower in plasma.

This study shows differences between plasma and interstitial bone mineral concentrations for calcium, magnesium and phosphorus. Variation in concentration from one tissue to another also differed for the various bone minerals. For e.g. magnesium bone interstitial value was greater than subcutaneously interstitial magnesium, calcium bone interstitial value was lower compared to other tissues. Part of such difference is likely to be due calcium protein binding.

2.4.2 Drugs

Carbamazepine is extensively used for the management of epilepsy and psychiatric diseases. It is almost completely metabolized in the body, so only small traces are excreted unchanged in the urine [38]. Kissinger et al. [172] sampled this drug and its epoxide metabolite in subcutaneous tissue in rats. They infused carbamezepine intraveneously and withdraw

ultrafiltrate and blood concomitantly. It appeared that the mean concentration in the ultrafiltrates was less than half of the plasma levels.

2.4.3 Glucose and lactate

Lactate is an important metabolite in sport medicine, but also an analyte of interest in the intensive care medicine for e.g. lactate is released from the relation to the extent of ischemic heart [143, 261]. High blood lactate is associated with poor prognosis in septic patients [59]. Cheng et al. [45] sampled lactate with continuous microfiltration and stored the sample simultaneously in a collection coil made from PEEK (l = 6 m; inner diameter = 125 μm). The sample was collected with a Monovette syringe disposable pump in combination with a capillary restriction for 6 hours. The coil was analysed afterwards with a lactate sensor. Such set up is ideal at it does not require the direct connection of the sampling system to analytical detection system. It could thus be applied to monitor athletes actively performing sports.

Glucose is an important metabolite in, for example, in diabetes and stroke management. Tiessen et al. [307] explored the on-line microfiltration technique subcutaneously in human volunteers, using the same analysis set-up as Kaptein et al. [159] but now glucose and lactate were determined simultaneously. It was confirmed that subcutaneous glucose concentration is not clearly exclusively linked to blood levels [307]. Continuous but intravenous microfiltration of lactate and glucose was extended in pigs for several hours in pigs, during which ischemia of the heart was induced for various periods of time. A significant increase in the myocardial lactate efflux was detected within minutes after the cardial arterial flow was restored [309]. Balloon inflation experiment to induce ischemic brain damage was also performed in the rat [197], also monitoring arterial and venous glucose and lactate simultaneously so two microfiltration probes were inserted in a single animal allowing the measurement of arterio-venous differences related to brain energy metabolism. Glucose and lactate concentrations were measured in the jugelar vein and the aorta. These analytes were measured under control conditions and the experimental condition, during and after inflation of an embolectomy balloon for 2 minutes. Under control condition a small net cerebral lactate efflux and glucose uptake was observed, whereas

during brain injury the release of lactate and glucose uptake were reduced and there was an increased lactate influx at high arterial lactate levels.

Savenije et al. sampled ultraslow (50 nl·min^{-1}) glucose and lactate ultrafiltrate continuously in vivo in Broiler chickens in subcutaneous tissue and intravascular, simultaneously the ultrafiltrate was stored in a collection coil made from fused silica tubing for 6 hours. The intravenous glucose and lactate concentration were 8.1 ± 0.4 and 2.4 ± 0.3 mM and subcutaneous 6.7 ± 0.2 and 2.6 ± 0.2 mM, respectively, before feeding. Glucose levels rose significant after food was provided to 11.4 ± 0.5 intravenously and 10.6 ± 0.7 subcutaneously. In the plasma samples the concentration of glucose and lactate did not different significantly from the ultrafiltrate values [271].

2.4.4 Therapeutical UF

Therapeutic UF was described by Odland et al., this group used microfiltration as a method to remove excess intersitial tissue fluid thereby reducing oedema. The hypothesis was that reduction of tissue oedema improved tissue viability and according to their report, it did [235, 236].

2.4.5 High molecular weight analytes

Microfiltration allows the sampling of high molecular weight analytes (HMW) [135, 274]. Schneiderheinze et al. investigated microfiltration sampling in vitro and in vivo subcutaneously in rats and they a showed that proteins with a MW up to 68 kDa could be recovered for >90% in vitro. In vivo in the extracellular space proteins of 9.8, 13.0 and 26.8 kDa were measured [274]. In an in vitro study [135] the UCD was tested for adsorption to the hollow fiber membrane and for diffusion of stored proteins in the collection coil. Two model proteins were tested: poly-l-lysine a hydrophilic protein and poly-l-tryptophan a hydrophobic protein with a MW of 227-354 kDa and 1-5 kDa, respectively. The hollow fiber made from polyethylene coated with ethylenevinylalcohol (Plasmaflo OP-05W(L), Asahi Medical Co., Japan) gave near 100% recoveries for both proteins. Filling the collection coil with various poly-l-lysine concentrations gave also good recoveries and insignificant

diffusion even after storage for 6 days at 37°C. Huinink sampled with a rather fast flow rate of 500 nl·min^{-1}. This approach is convenient for blood sampling and reconstructing time profiles.

2.5 Ultraslow-microdialysis

Kaptein et al. was the first describing ultraslow-microdialysis [160], this group compared ultraslow-microdialysis with microfiltration sampling technique. Two sampling rates of 100 and 300 nl·min^{-1} were subcutaneously investigated in anesthesized rats. The measured levels of glucose measured in the effluent of ultraslow-microdialysis obtained from the rat subcutis were exactly the same to the ultrafiltrate, so the recovery was 100% for glucose and lactate sampled with ultraslow-microdialysis method. Ultraslow-microdialysis was used sample in healthy humans at flow rates of 300 nl·min^{-1} and 100 nl·min^{-1}, respectively [257, 308]. Tiessen et al. measured glucose levels during an oral glucose tolerance test (100 g glucose) simultaneously in blood, adipose tissue and loose connective tissue of the abdominal subcitus. Fasting glucose levels were 2.52 ± 0.77 mM in adipose tissue and 4.67 ± 0.17 mM in blood. This discrepancy increased further to 6.40 ± 1.57 and 11.59 ± 1.52 at maximum glucose concentration. Unlike in connective tissue insignificant different glucose values were found compared to blood levels [308]. Ekberg et al. [77] also measured glucose in humans ingesting glucose (75 g glucose) using four flow rates: 0.3 (= ultraslow-microdialysis), 1, 2, and 5 µl·min^{-1} in interstitial fluid of subcutaneous adipose tissue. Lactate, pyruvate and glycerol were also measured. This group mentions that in healthy volunteers the concentrations of substances in interstitial fluid is not know but they assume that concentrations in interstitial fluid are equal to concentration in blood [340], however this may be questioned considering other results [308]. The mean maximum blood glucose level was 7.8 ± 0.4 mM and the mean maximum glucose level in interstitial adipose tissue using a flow rate of 0.3 µl·min^{-1} was 8.9 ± 0.5 mM. In both studies the mean maximum concentration in the adipose tissue were observed 80 minutes after glucose consumption, but there is contradiction between the levels observed in blood and adipose tissue. This could be due to different kinetics and/or different flow rates and/or the measurements in the

connective tissue. The common conclusion was that the ultraslow-microdialysis flow rate was preferred in studies of local tissue metabolism measuring true interstitial glucose concentrations. Another study [211] examined the correlation obtained from ultraslow-microdialysis (flow rate = 0.3 μl·min^{-1}) of subcutaneous adipose tissue in patients with severe brain injury. The correlation between glucose in blood and subcutaneous adipose tissue was not as good during intensive care as in normal healthy humans. An important factor could be differences in local blood flow.

Rosdahl and coworkers identified a perfusion flow rate at which the interstitial fluid completely equilibrates with the microdialysis perfusion fluid in human skeletal muscle and adipose tissue [262]. Complete equilibrium was reached in both tissues at a flow rate of 0.16 μl·min^{-1} and the measured concentrations of glucose, glycerol, and urea were in good agreement with the expected tissue-specific levels. The glucose concentration in adipose tissue (4.98 ± 0.14 mM) was equal to plasma (5.07 ± 0.07 mM), whereas the concentration in muscle (4.41 ± 0.11 mM) was lower than in plasma and adipose tissue. The concentration of lactate was higher in muscle (2.39 ± 0.22 mM) than in adipose tissue (1.30 ± 0.12 mM). The concentration of glycerol was higher in adipose tissue (233 ± 19.7 μM) than in muscle (40.8 ± 3.0 μM) and in plasma (38.7 ± 3.97 μM). The urea concentration was equal in both tissues.

Ultraslow-microdialysis is also used to monitor cerebral metabolism in patients. A methodological study was performed by Hutchinson et al. [136] in patients with head injury. A number of issues were studied, like the effect of hollow fiber length, and the relative recovery using different flow rates (0.3 and 1.0 μl·min^{-1}). The relative recovery for glucose, lactate, pyruvate and glutamate were 65%, 67%, 72, and 69% using a flow rate of 0.3 μl·min^{-1} and for 1.0 μl·min^{-1} these values were 21, 27, 34, and 30%, respectively. Stahl et al. [292] monitored cerebral biochemical alterations, that precede and accompany increases in intracranial pressure. Approaching a complete cessation of cerebral blood flow showed that glucose and pyruvate values decreased, and that lactate, glycerol, glutamate and lactate/pyruvate ratio (characterized cerebral

ischaemia) were increased. Values of the healthy brain were obtained with microdialysis [253].

Trafficking of substrates between cells is considered to meet energy demand of the brain. Accordingly, it has been hypothesized that neuronal energy consumption depends, at least in part, on the release of glucose and lactate of astroglia cells and on subsequent diffusion to neurons via the brain ECS. The importance of an estimation of the concentration of glucose and lactate in the ECS has been recognized by several authors [67, 82, 83, 90, 212, 215, 225, 283]. Concentrations ranging from 0.35-3.3 mM glucose [225] and 0.35-1.1 mM lactate [67, 283] have been reported. There are, however, no reports on either estimates of the turnover rate of glucose and lactate of the ECS or of the proportion of energy substrates that is transferred via this compartment.

Our group proposed using ultraslow-microdialysis as a simple approach to estimate these turnover rates based on the loss of-for example-glucose or lactate added to the perfusate at steady state levels (see chapter 7). In practice, the decrease of glucose or lactate in the influx and efflux were measure at a constant flow rate of 100 nl·min^{-1}. This approach is justified, because it has been shown that acute hyperglycemia does not affect glucose cerebral conscious in rats [73, 239]. During low rate perfusion rapidly new steady state levels are achieved and the difference between the amounts infused and collected per time unit reflects the sum of diffusion and consumption of the substrates in the ECS surrounding the microdialysis-probe. Diffusion appeared to be a minor component. The normal ECS compartment of the brain is 18% [192, 215]. Steady state levels of glucose and lactate 2-4 days after probe implantation were 0.23 ± 0.12 and 0.66 ± 0.36 mM, respectively. The turnover rate of lactate was 0.13 ± 0.03 µM·g^{-1}·min^{-1} and that of glucose 0.03 ± 0.01 µM·g^{-1}·min^{-1}. These figures show that less than 10% of the rat striatal energy substrates are transferred via the extracellular space and was mostly attributed lactate trafficking. The values were close to those of the glucose-lactate shuttle estimated from a total energy balance [201].

2.6 Analytical detection

Samples collected with microdialysis and microfiltration can be collected with conventional manual or automated collection devices. Particular attention deserves the analysis of nanoliter samples, as obtained with ultraslow-microfiltration and ultraslow-microdialysis. Only few attempts have been described to combine these micro-perfusion techniques with analytical devices. Promising applications van be expected from a wide variety of analytical techniques such as HPLC [22, 313], Microbore HPLC [198], MS [9, 22, 144, 335], capillary electrophoresis [165, 216, 234, 245], biosensors [46, 75, 182, 194, 255, 257, 347], microfluidics [41, 126], and/or the coupling of several systems to each other. For an overview of microfiltration sample analysis see **table 1**.

2.7 Conclusion

Microfiltration is a good alternative sampling technique as compared to microdialysis. We emphasized the versatility of ultraslow-microdialysis and microfiltration. In **table 1**, half of the overviewed reports are about sampling glucose and/or lactate with microfiltration. Whilst other analytes could be of interest to sample as well. Herewith you could think of proteins (small and big), nucleic-acids, hormones (bound versus unbound) or cytokines. Different tissues can be sampled, however not the brain, simultaneously and compared and microfiltration can also be utilized intravenously. Prospective research would be optimising this technique, with the final goal of using ultraslow-microfiltration and ultraslow-microdialysis as a sampling technique in hospital setting with the current trend to increase detector sensitivity and decrease sample size, application of low rate microdialysis and microfiltration for biomedical research in now becoming a realistic option. The current overview may serve as a reference for low perfusion technologies.

2.8 Acknowledgement

This research is supported by the Dutch Technology Foundation STW, applied science division of NWO and the Technology Program of the Ministry of Economic Affairs, grant number GPG 06038.

Chapter 3
In vitro sampling and storage of proteins with an ultrafiltration collection device (UCD) and analysis with absorbance spectrometry and SELDI-TOF-MS

This chapter is published in The Analyst, volume 130, numer 8, August 2005, page 1168-1174.

Abstract

Frequent in-vivo sampling of blood proteins is most often stressful and only a few samples can be obtained, so limited time-profiles can be made. We have developed an ultrafiltration collection device (UCD) for continuous sampling. The UCD consists of hollow fiber, coil and flow creator. Hollow fiber membranes are often hydrophobic and this may result in adsorption of protein and/or peptides and finally clogging. Adsorption was tested with a hydrophobic and hydrophilic peptide and two biocompatible hollow fibers made from different materials. The hollow fiber made from polyethylene coated with ethylenevinyl alcohol gave for both peptides near 100% recoveries. This was in contrast with the polysulfone hollow fiber sampling hydrophobic peptide. Filling the coil with various peptide concentrations gave good recoveries and insignificant diffusion even after storage for six days at 37°C. Continuous pulse-free sampling was tested by vacuum. An average flow rate of 423 ± 50 nl·min^{-1} over a period of 4 days was created using S-Monovette. The flow rate gradually declined during this period with <5% every consecutive day. In addition we also sampled a complex sample; serum with the polyethylene hollow fiber. Serum and ultrafiltrate were spotted onto a protein chip and analysed on surface enhanced laser desorption/ionization-time of flight-mass spectrometry (SELDI-TOF-MS). Six proteins out of 64 proteins were found to be significantly different between serum and ultrafiltrate ($p < 0.05$). The UCD has the potential being used for in vivo real time monitoring assessing adsorption, diffusion and flow parameters.

Keywords

hydrophobic peptides, hydrophilic peptides, mass spectrometry, ultrafiltration sampling, in vitro, continuous sampling, hollow fiber membranes

3.1 Introduction

In 1987 the potential of in vivo UF for a clinical device and research tool was recognized for the first time. UF samples are undiluted, small (µl), sterile and UF sampling is most often less invasive compared to blood sampling [342]. UF can be used in hospitalized patients in whom parameters have to be monitored frequently, without the necessity of repeated blood sampling [154]. UF can be applied in blood, saliva, and in subcutaneous tissue. UF may also be useful for in vitro experiments for example in bioreactors. There is a general tendency to use smaller samples for analysis and UF can provide them. Several detection techniques can manage small samples, like microbore or capillary HPLC, capillary electrophoresis, mass spectrometry, and (bio)-sensors.

UF sampling is based on the transition of fluid, containing the analytes, over a semipermeable membrane driven by negative pressure. The maximum size of particles that pass the UF membrane is determined by the molecular weight cut-off value (MWCO) of the hollow fiber, but also by the configuration and the charge of the analyte. The amount of negative pressure determines the fluid sampling rate, but the membrane surface area and the hydraulic resistance, protein layer on the membrane surface and probe environment have additional effects on the flow rate. Hollow fiber membranes with a MWCO between 20 and 50 kDa have been used in UF, by thus excluding large particles like proteins and cellular elements. UF samples like this can often be analysed directly [193].

Despite the well-recognized potential of UF for monitoring analytes, only a few biomedical and clinical applications have been published so far. Subcutaneaously UF e.g. ion dynamics [205, 291], mineral and drug kinetics [145, 153, 206], and glucose and lactate monitoring [150]. Blood UF was performed in rats [159, 197, 309] pigs[309] and in Broiler chickens [271]. All these studies described sampling of low molecular weight analytes (LMW), but UF is also very suitable for sampling high molecular weight (HMW) analytes.

We describe an ultrafiltration collection device (UCD) to sample hydrophilic and hydrophobic proteins and subsequent storage. The UCD

was previously described by Savenije et al. [271] for glucose and lactate. The advantage from a collection coil connected to the UF probe is that sampling is performed continuously, stress-free, and samples can be stored in time.

Current materials for the hollow fiber membrane include polysulfone, polyacrylonitrile, polyethylene, polypropylene, and cellulose. Hollow fibers like polyethylene and polysulfone are hydrophobic. The hydrophobic character of the membrane may cause non-selective adsorption of proteins, resulting in plugging the pores and finally clogging of the UF-probe. Non-selective adsorption of proteins is reduced by hydrophylization of the hydrophobic hollow fiber membrane. This is achieved by adding alcoholic hydroxyl group to the membrane polymer [342]. Ethylenevinylalcohol, consisting of hydrophobic ethylene and hydrophilic vinyl alcohol segment, is water insoluble and blood compatible [314]. The plasmaflo hollow fiber used in the present study is covered with ethylenevinylalcohol copolymer. But also other surface modifications have been described such as adding carboxyl, amine, sulfonate or phosphate groups reducing adsorption [142]. Hasegawa et al. made a hemodialysis membrane with a protein-adsorption resistant surface using polysulfone as the base material by incorporating phospholipids [110, 141]. Higuchi et al. modified polysulfone hollow fiber by covalently conjugating polyvinylpyrrolidone on the surface [114].

In literature only a few studies compare two or more haemodialysis hollow fiber membranes for adsorption and/or biocompatibility [114, 140, 319, 324, 330, 341, 342]. The hollow fibers described here were chosen because they are biocompatible, commercially available and possess suitable diameter and pore size dimensions.

The goal of this study was to investigate as a proof of principle that both hydrophilic and hydrophobic peptides can be sampled with UF, stored in a coil at 37°C without significant diffusion. To illustrate the potential of UF for protein analysis in a biomedical setting we applied mass spectrometry of serum samples. Serum was ultrafiltrated and analysed on surface enhanced laser desorption/ionization-time of flight-mass spectrometry (SELDI-TOF-MS). The advantage of this analysis method is

it can handle small volumes, in low concentrations and multiple analytes can be monitored simultaneously.

3.2 Experimental

3.2.1 Probe and UCD construction

The UF probe is composed of an UF hollow fiber that is connected via fused silica tubing to a syringe pump (Harvard Apparatus 22). The hollow fibers from 2 manufacturers were employed: plasmaflo (Asahi Medical Co., Ltd., Tokyo, Japan) and plasmacure (Kuraray Co., Ltd., Osaka, Japan) plasma separators. The specifications for both hemodialyzers are shown in **table 1**. The hollow fiber was cut into a 4-cm piece and a spiral spring (stainless steel wire, 60 µm wire diameter, 290-320 µm spiral diameter, 7-10 windings·cm^{-1}; Vogelsang, Hagen, Germany) was inserted into the hollow fiber to prevent collapsing during sampling. The spring also reduces the dead volume of the probe. Fused silica tubing (fused silica, outer diameter = 170 µm, inner diameter = 100 µm, Polymicro Technologies Inc., Phoenix, AZ) approximately 40 cm long was inserted into the probe up to 2 mm from the tip. Membrane, spiral, and fused silica tubing were fixated with cyanoacrylic glue (Ruplo B.V., Ten Boer, the Netherlands). The UF probe is via a 0.2 µl flow cell located in Spectroflow 757 absorbance detector (ABI Analytical Kratos Division) connected to a syringe pump (Harvard Apparatus 22).

Table 1 Specification hollow fibers given by manufacturer.

plasma separator	Plasmaflo OP-05W(L)	Plasmacure PS-06
material	polyethylene	polysulfone
coating	ethylenevinylalcohol	-
outer diameter (µm)	430	450
inner diameter (µm)	330	320
wall thickness (µm)	50	65
maximum pore size (µm)	0.3	0.2

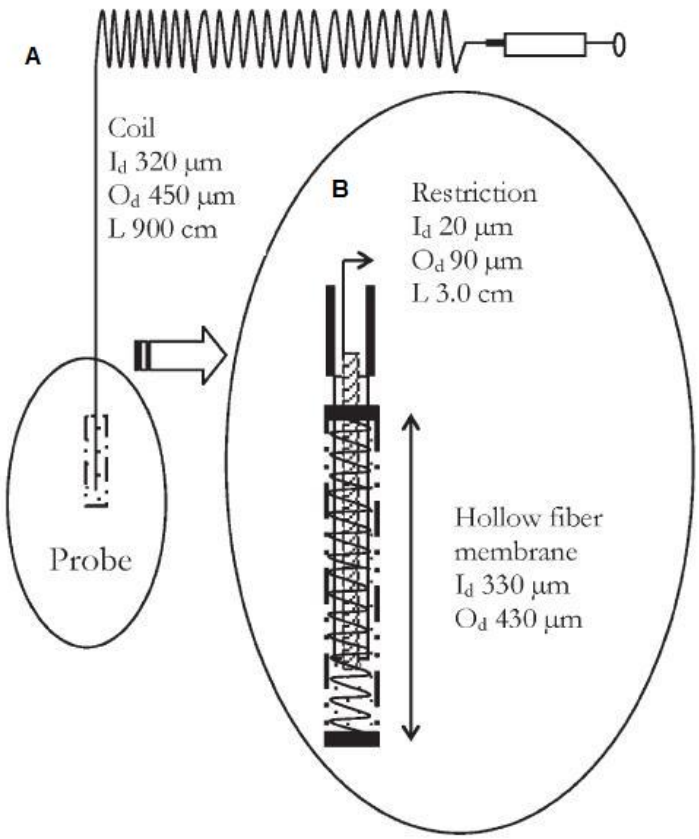

Figure 1 Schematic design of the ultrafiltration collection device (UCD. (**A)** The hollow fiber membrane (probe) is connected to the fused silica tubing storage coil. The coil is on his turn connected to the 9-ml S-Monovette the under-pressure generator. The probe could be placed into a sample solution or in vivo: subcutaneously or intravascular. (**B)** Schematic blow-up of the UF probe consisting of hollow fiber membrane, spring and flow restriction. The restriction specifications give in combination with the under-pressure of 34800 Pa, a certain flow rate. This flow rate can be calculated according with Poiseuilles law.

The UCD consisted of a plasmaflo hollow fiber probe connected to a fused silica tubing coil with a length of 900 cm. The outer diameter of the coil was 435 µm and inner diameter 320 µm. A volume of 724 µl will be collected in 24 hours time, sampling with a flow rate of 0.5 µl·min^{-1}. The schematic representation of the UCD is given in **figure 1**.

3.2.2 In vitro absorption of aqueous peptide solutions and analysis

The hollow fiber was placed in two peptide solutions: poly-l-lysine (Sigma Chemicals), a hydrophilic peptide, and the hydrophobic peptide poly-l-tryptophan (Sigma Chemicals) with molecular weight between 227.000-354.000 Da and 1.000-5.000 Da, respectively. The stock solution of poly-l-lysine contained 1.0 mg·ml^{-1} phosphate buffer solution (0.1M PBS, pH 7.4) + 0.1% Triton X-100 and diluted to 500, 250, 125, 63 and 31 µg·ml^{-1}. Poly-l-tryptophan, 2.0 mg·ml^{-1}, was dissolved in N-dimethylformamide (DMF) and diluted with PBS (0.1M) + 0.1% Triton X-100 into the following concentrations: 40, 20, 8, 4, and 2 µg·ml^{-1}, the end concentrations DMF were respectively 2.0, 1.0, 0.5, 0.3, and 0.1%. Poly-l-lysine was measured at wavelength 200 nm and poly-l-tryptophan at 280 nm. The established flow rate for the UF absorbance experiments was 3.0 µl·min^{-1} using a 1 ml syringe pump (Harvard Apparatus) at room temperature.

Serum was prepared from whole blood that was coagulated for 10 min at room temperature and centrifuged at 3000 rpm for 10 min. Serum was stored at –20°C until UF. The plasmaflo (Asahi Medical Co., Ltd., Tokyo, Japan) UF probe was used for serum sampling with an established flow rate of 3.0 µl·min^{-1} using a 1 ml syringe pump (Harvard Apparatus 22) at room-temperature.

3.2.3 Storage effects

For studying the storage effects of proteins in the coil, poly-l-lysine solutions as described above were used. Each concentration was sampled for 30 minutes using an auto-sampler (Promis, Spark Holland) and in between the standard blanks was sampled. Before usage the coil was filled with destilled water. The storage effect was studied by filling one coil at room temperature and analyzing the coil immediately. To mimic in vivo applications we stored also a coil at body temperature (37°C).

After filling the coil, with a flow rate of 0.5 µl·min^{-1} (syringe pump), the UCD was checked for damage and absence of air bubbles. For storage of the coil, the UF probe was removed from the UCD and disconnected

from the syringe pump, the ends of the coil were sealed with cyano-acrylic glue (Ruplo B.V., Ten Boer, The Netherlands) and stored at 37°C for 6 days. The absorbance was measured at 200 nm with the Spectroflow 757 absorbance detector (ABI Analytical Kratos Division) at a flow rate of 0.5 µl·min^{-1}. The signal output was registered analogously with a flatbed recorder and/or digitally using the Chromeleon software package.

3.2.4 Flow experiments

When using this device in the future for in vivo experiments, it is not possible to use a syringe pump as described above for the adsorption experiments. For this purpose a disposable 9-ml S-Monovette "pump" (Sarstedt B.V.), capable of applying a vacuum of 34800 Pa was tested. The restriction of 3.0 cm inside the plasmaflo hollow fiber probe (fused silica, outer diameter = 90 µm, inner diameter = 20 µm; Polymicro Technologies Inc., Phoenix, AZ) in combination with the under-pressure of the S-Monovette determines the flow rate (Poiseuilles law).

The restriction is glued in a bigger outer diameter fused silica tubing (fused silica, outer diameter = 245 µm, inner diameter = 100 µm, Polymicro Technologies Inc., Phoenix, AZ), thereby reducing the dead volume in the hollow fiber probe. Also around the restriction a spiral spring (stainless steel wire, 60 µm wire diameter, 290-320 µm spiral diameter, 7-10 windings·cm^{-1}; Vogelsang, Hagen, Germany) inside prevent collapsing of the hollow fiber, but it also reduces the hollow fiber dead volume.

3.2.5 Analysis of serum and UF by SELDI-TOF-MS

Serum and ultrafiltrate (20 µl each) were sampled one day before analysis and kept at 4°C. The samples were applied to weak cation exchange (CM10) protein chip with hydrophobic barrier coating and analysed on SELDI-TOF-MS (Ciphergen Biosystems, Fremont CA, USA). The CM10 proteinchip was inserted into a bioprocessor (Ciphergen). The surface of the CM10 protein chip was prepared by adding 200 µl 50 mM ammoniumacetate + 0.01% Triton X-100 washing buffer and allowed to incubate for 5 min at room temperature with vigorous shaking (ex 250 rpm). This was repeated once for a total of 2 washes.

The washing buffer was removed and 180 µl binding buffer, 50 mM ammoniumacetate + 0,01% Triton X-100, plus 20 µl serum/ultrafiltrate was added to each chip surface and allowed to incubate for 30 min. Each protein chip was washed 3x with 200 µl washing buffer and 1x with deionized H_2O for 5 min at room temperature with agitation. After drying 0.5 µl of saturated solution of the energy absorbing molecule for the high molecular range, sinapinic acid (SPA) in 50% v/v acetonitrile, 0.5% trifluoroacetic acid was applied to each spot on the protein array 2x, allowing the solution to dry between the applications.

The protein chips were read in a PBS-II mass spectrometer (Ciphergen Biosystems). Sample spectra were collected using the following spot protocol settings: detector set at 1800 V and focus mass optimized from 30.000 to 100.000 Da. The high mass limit was 200.000 Da, the sensitivity gain was set to 10 and the laser intensity was 300. The laser was set for 2 shots to be taken per position and the number of positions ranged from 20 to 80, incrementing at every 5 positions. A chip protocol was created which used the spot protocol to process automatically each sample identically.

3.2.6 Presentation of data and statistical analysis

Diffusion was calculated by measuring the peak width on half the peak height. A one- way ANOVA was used to calculate significant diffusion differences between immediate analysis and storage after 6°C at 37°C. For the flow experiments, the mean values over day 1, 2, 3, 4, 5, and 6 were calculated ± SD. Day 2, 3, 4, 5, and 6 flow rates were compared with the flow rate over day 1. For the SELDI-TOF-MS analysis, the Mann-Whitney U-test ($p < 0.05$) was used to assess significant profile differences between ultrafiltrate and serum. Cluster analysis was performed to compare data using biomarker wizard software that is part of the Ciphergen protein software version 3.1. First pass signal to noise ratio was 10 and the minimal threshold was 10%. Variation in mass was 0.3% and the second pas signal to noise ratio was 2. The SELDI-TOF-MS analysis was performed in quadruple for UF and serum on one chip to avoid inter-assay variability.

3.3 Results

3.3.1 Performance hollow fiber membranes

For each experiment a fresh hollow fiber was used and the recovery measured for 10 minutes with a 3.0 µl·min^{-1} flow rate, starting with the lowest standard concentration. The lowest standard concentrations were also the lowest measurable concentrations for absorbance spectroscopy. **Figures 2A** and **2B** illustrate the recovery of poly-l-lysine and poly-l-tryptophan without probes (only fused silica tubing), plasmacure and plasmaflo UF probe (polyethylene coated by ethylenevinylalcohol). The recovery of poly-l-lysine sampling with the plasmaflo and plasmacure UF probe, shown in **figure 2A**, is of similarly altitude. This appeared not to be the case for the poly-l-tryptophan solution sampled with the plasmacure UF probe, shown in **figure 2B**. Since the tested hollow fibers pore sizes were several orders of magnitude larger that the sampled peptides, the low recovery from the plasmacure UF probe was presumably the result of adsorption rather than membrane sieving. On the basis of this finding we continued with the plasmaflo UF hollow fiber probe.

These results imply that the more hydrophilic plasmaflo hollow fiber is less susceptible for protein adsorption than the plasmacure hollow fiber. This was in line with the study performed by Higuchi et al. [114]. Hollow fibers from polysulfone modified with polyvinylpyrrolidone, making the hollow fiber more hydrophilic, resulted in less plasma protein adsorption to the hollow fiber compared with the unmodified polysulfone hollow fiber. Not only protein adsorption was reduced but also the number of adhering platelets, thus enhancing blood biocompatibility [274].

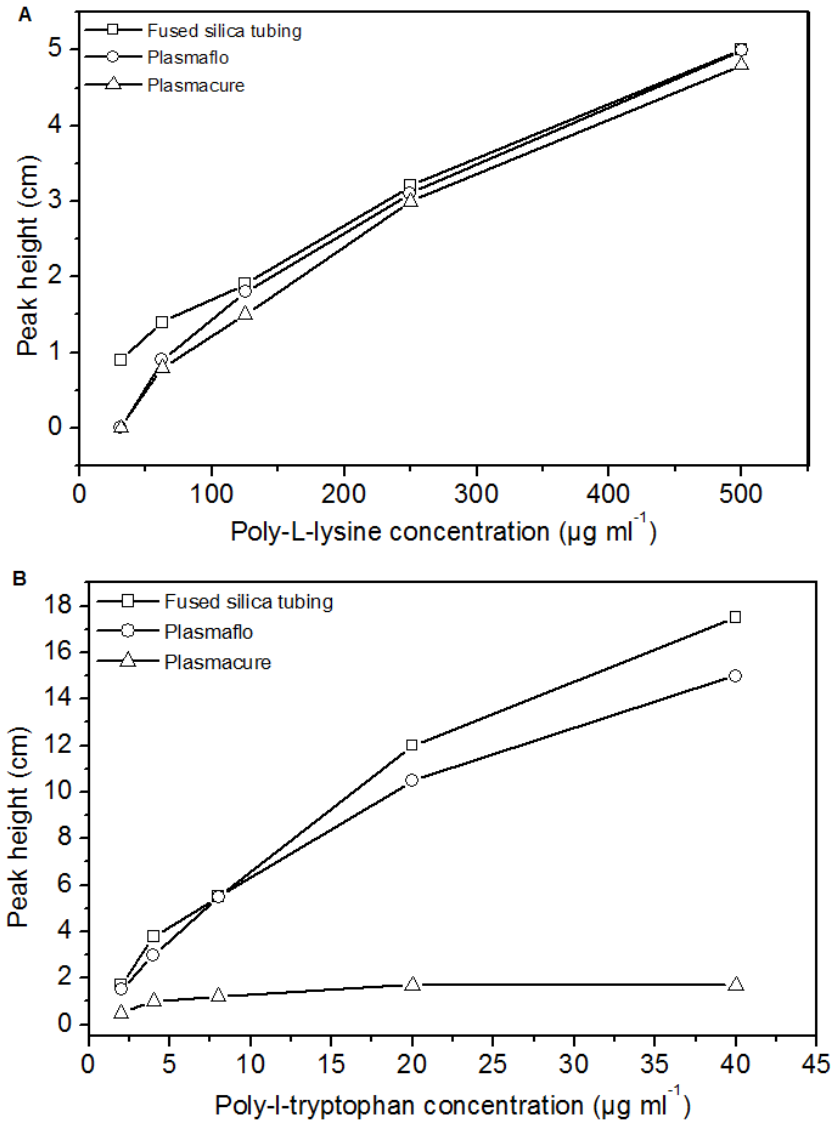

Figure 2 Poly-l-lysine sampled without hollow fiber (•), with plasmaflo hollow fiber (o) and with plasmacure hollow fiber (•) (**A**). Poly-l-tryptophan sampled without hollow fiber (•), with plasmaflo hollow fiber (o) and with plasmacure hollow fiber (•) (**B**).

To control membrane adsorption and thus fouling it is necessary to understand its causes and nature. Protein aggregation is the non-covalent bonding between proteins, such as van der Waals or hydrophobic interaction-the latter of these forces being more dominant. Poly-l-tryptophan did interact with the plasmacure probe but not with plasmaflo probe. It could be that poly-l-tryptophan peptides spherical aggregates are formed and not able to pass the 0.2 μm pore size of the plasmacure hollow fiber. The other possibility is that the more hydrophobic plasmacure hollow fiber adsorbs poly-l-tryptophan through hydrophobic interactions.

Protein behaves as a positive or negative ion at pH lower or higher than its isoelectric point (pI). Generally if a protein molecule or aggregate is of opposite charge to a membrane surface, electrostatic attraction will ensue and adsorption will take place. If the protein and membrane have the same charge sign then a high-energy barrier must be overcome before adsorption can occur. Like proteins, membranes also have isoelectric points and therefore the net overall surface charge is also pH dependent. All the above mentioned interactions influence adsorption. The suitability of a hollow fiber depends on the performance as reflected by the recovery of the analyte of interest and should be tested in advance.

3.3.2 Storage effects of peptides in coil

Profiles for direct sampling of poly-l-lysine solution series with probe is shown in **figure 3A**. Direct sampling profile of poly-l-lysine solution without probe was also performed (data not shown), the peaks are more rectangular. Ultrafiltrate stability during storage was checked by comparing profiles analysed immediately after sampling, with profiles analysed after 6 days of storage at 37°C **(figure 3B)**. The coils had storage capacities of 24 hours, but in **figure 3A** the data is shown for 10.5 hours.

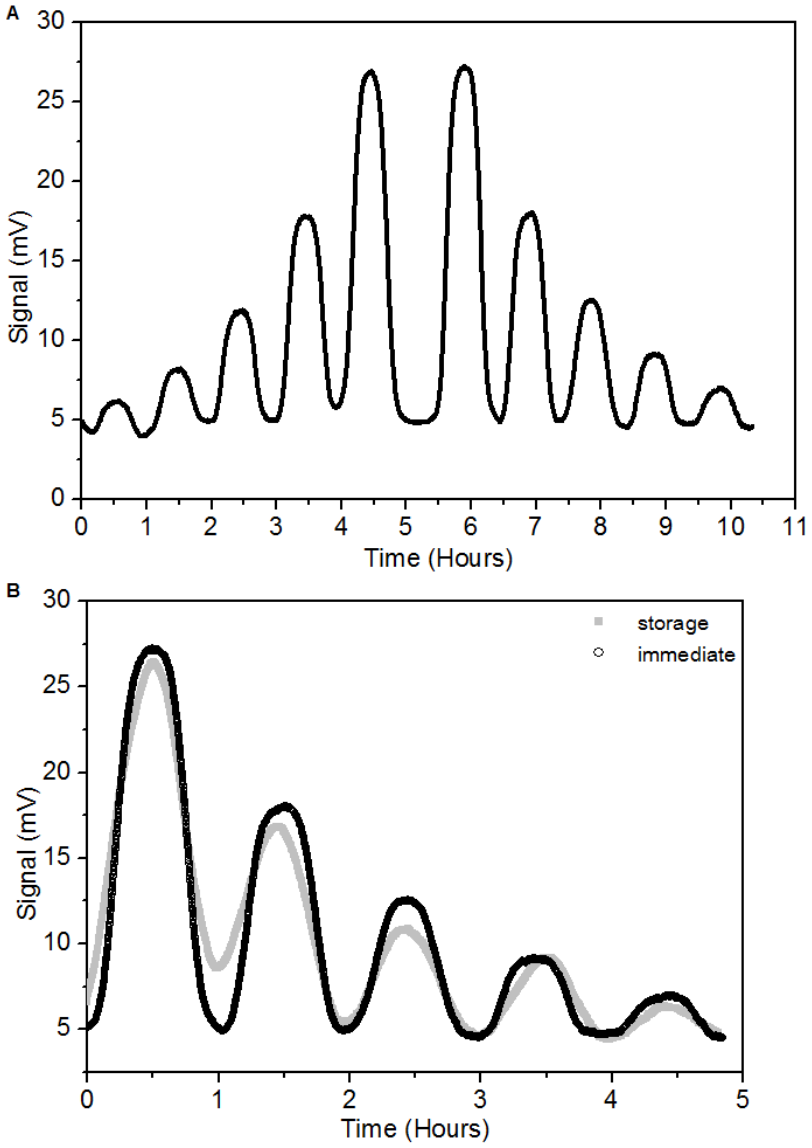

Figure 3 Poly-l-lysine profile sampled in storage coil analysed immediately at room temperature. Poly-l-lysine concentration 0/31-0/62-0/125-0/250-0/500-0-0/500-0/250-0/125-0/62-0/31-0 µg·ml^{-1} (**A**). Poly-l-lysine analysed immediately (open circles) and stored in a coil at 37°C for 6 days (shaded squares). Used poly-l-lysine concentrations 0/500-0/250-0/125-0/62-0/31-0 µg·ml^{-1} (**B**).

Figure 3B shows immediate analysis versus storing poly-l-lysine at 37°C for 6 days, analyzing the coil on day 6 peak width as measured at half peak to assess diffusion showed no significant difference in variation for the different protein concentrations (ANOVA) in the coil. During continuous sampling of poly-l-lysine in vitro, sampling is performed at room temperature. When sampling in vivo the temperature will gradually increase to a maximum of 37°C, in the coil. No significant diffusion was found comparing the coil stored at 37°C for 6 days with immediate analysis. We concluded that the coil could be used for storing peptides/proteins during in vivo sampling, in future, and that analysis can be postponed for at least 6 days.

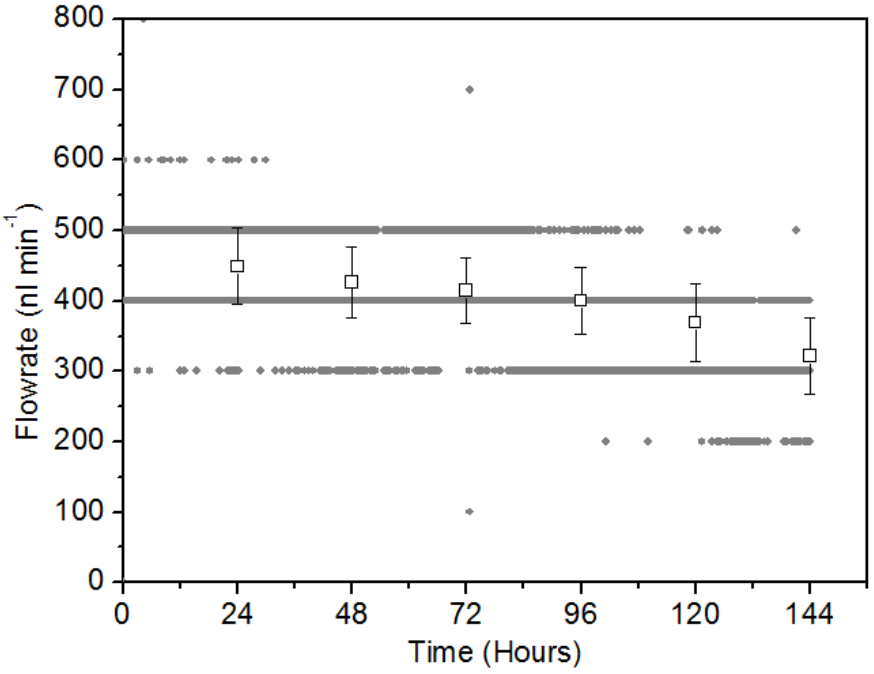

Figure 4 S-Monovette flow experiments nl/min (grey) and average flow rate nl/min per day ± SD (•).

3.3.3 S-Monovette flow experiments

Water was sampled with Monovette-restriction device and weight gain was measured each minute. The calculated flows are given in **figure 4**.

Fused silica restriction (outer diameter = 90 µm, inner diameter = 20 µm; Polymicro Technologies Inc., Phoenix, AZ) with a length of 3.0 cm was glued, via a 30 cm long piece fused silica tubing fused silica (outer diameter = 170 µm, inner diameter = 100 µm; Polymicro Technologies Inc., Phoenix, AZ) glued into the S-Monovette. According to the law of Poiseuille (air pressure 101500 Pa and internal diameter 20 µm) a flow of 523 nl·min^{-1} would be generated. However the internal diameter specifications of the fused silica tubing TSP020090 is 20 µm ± 0.3 µm; so calculating the flow for an internal diameter of 19 µm the theoretical flow would be 427 nl·min^{-1}.

The 9 ml S-Monovette calculated flows over day 1, 2, 3, 4, 5 and 6 mean values were 449 ± 54, 427 ± 50, 415 ± 46, 400 ± 48, 369 ± 50 and 322 ± 55 nl·min^{-1}, respectively. The S-Monovette flow system is able to keep up the flow for 4 days looking at the mean value and calculated standard deviation for day 1. However we are particularly interested in the first 24 hours.

3.3.4 Performace Plasmaflo hollow fiber sampling serum

In the hollow fiber adsorption experiments, the hollow fibers were tested in vitro in aqueous peptide solutions, however in a biomedical setting biofluids are more complex and more viscous. We asked ourselves whether a profile of serum analysed with SELDI-TOF-MS is similar or better, identical to that of the ultrafiltrate of the same serum sample.

The plasmaflo hollow fibers used in all experiments are taken from a hemodialyser device. In the clinic this device is used for separating plasma from blood cells and the performance of the permeability to plasma components is given by the manufacturer (Asahi Medical Co., Ltd., Tokyo, Japan) for various components separating one liter plasma. The percentages for total protein, albumin (68 kDa), IgG (150 kDa), IgA (160 kDa), IgM (971 kDa) and total-cholesterol were respectively 97.6, 96.5, 93.9, 95.7, 94.9, and 98.4%. So roughly 98% of the total proteins are ultrafiltrated by the plasma separator. This was also confirmed by a total protein test on ultrafiltrated serum with the plasmaflo hollow fiber probe (data not shown). To study the behaviour of the individual proteins we analysed serum and ultrafiltrate with the SELDI-TOF-MS.

The samples were spotted directly onto the CM10 protein chip without using denaturating solvent conditions.

With cluster analysis 64 peaks were detected in the range from 3-160 kDa **(figure 5)**. A significant difference for 6 peaks was found when comparing serum with ultrafiltrate ($p = 0.05$) with the Mann-Whitney U-test, these were for the M/Z ratios 9248, 9516, 22365, 39807, 51514, 79405 and 158099. The M/Z ratio's of 39807 and 79405 is probably transferrin. These 6 significant (10% of the total number of detected peaks) differences may be attributed to interactions of these proteins with the UF membrane and/or the topic reproducibility. Semmes et al. [282] assessed reproducibility of serum samples analysed across-laboratory and within laboratory. This group found that when pooled serum sample was analysed within different laboratories for 3 peaks, the normalized peak intensity was between 15-36%. This variation was comparable to the intra laboratory measurements of the same peaks, which is regarded as good and acceptable reproducibility [282].

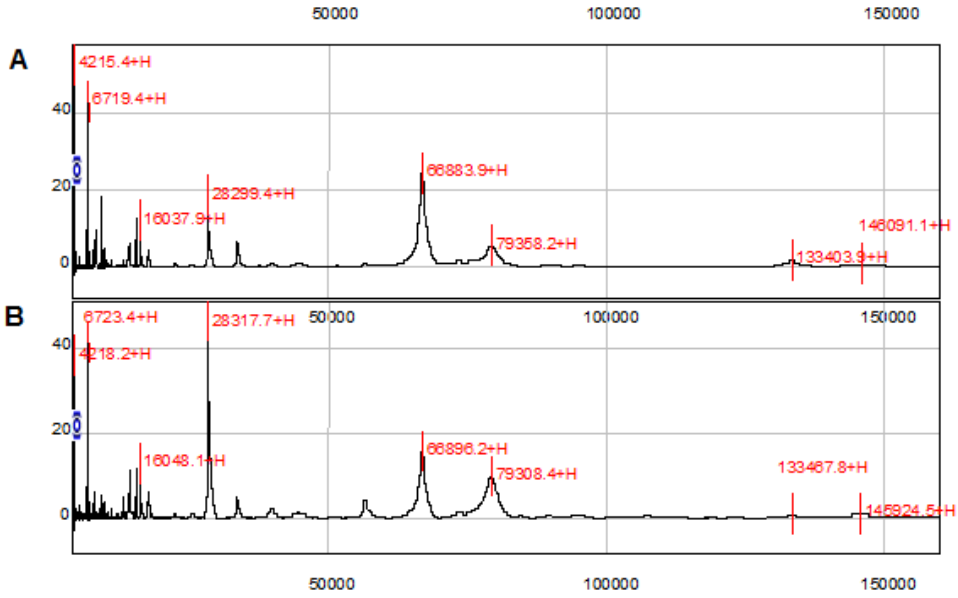

Figure 5 Representative spectra of proteins retained on the CM-10 proteins chip by SELDI-TOF-MS analysis for serum (**A**) and ultrafiltrate (**B**).

A major goal of proteomics is the determination of biomarkers whose measurements would effectively distinguish the onset of a defined disease state. Blood constantly perfuses tissues and therefore it might be assumed that the onset/presence of disease is reflected in the constituents of serum. The protein content of serum is dominated for 90% by 10 proteins with MW between 54-971 kDa; the high molecular weight fraction of the proteome. Of the remaining 10%, 12 proteins dominate for 90% the remaining 10%. In general, only 1% of the entire protein content of serum is of interest in proteomic studies [310], being the LMW fraction of the proteome. A separation step for removing the abundant HMW 'contaminating' proteins is not uncommon in the search for LMW biomarkers. But it is also known that the HMW protein albumin acts as a carrier and transport protein and binds physiologically important species such as hormones, cytokines, and lipoproteins. Thus if albumin is removed it is most likely that those proteins and peptides bound to the target protein are removed as well.

Hence an ideal fractionating/depletion method would remove abundant proteins but leave the peptides and proteins bound to them in solution. Turumalai et al. [310] succeeded in this set-up combining solvent conditions, disrupting protein-protein interactions, with UF; the LMW components that may bound to larger species were released and free to pass through the molecular weight cut-off (MWCO = 30 kDa) membrane [310]. This group showed that the mass spectrum of the sample ultrafiltrated (MWCO = 30 kDa) in the presence of acetonitrile shows many more peaks than obtained when serum is filtered without acetonitrile; also the spectrum of the ultrafiltrated serum show few peak above 6 kDa. They attribute this discrepancy to ion suppression effects because of HMW abundant serum proteins do not produce intense signals in the SELDI-TOF-MS analysis. They applied CHCA matrix that is used for the LMW fraction of the proteome. However the solvent conditions should not disturb the native, high order structure of a protein or protein-complex especially not when using bio-affinity chips.

The UCD set-up is developed for monitoring. Once filled, the coil may be segmented, and each segment after analysing will give together a time profile for one or multiple analytes. In addition the UCD could be placed for in vivo sampling near the tissue of interest, but also as purification method in the SELDI-TOF-MS analysis method. The SELDI-TOF-MS analysis is to illustrate that our UCD could be used in combination with this analysis method.

3.4 Conclusion

We have demonstrated in vitro, that hydrophilic and hydrophobic peptides can be sampled by means of plasmaflo UF without the loss of the recovery. We also showed that peptides could be stored in a coil for 6 days at 37˚C, profiles can be generated, without significant diffusion of the peptides in the coil and the S-Monovette pump can be used for continuous sampling for at least 4 days. The SELDI-TOF-MS analysis method is ideal because it can handle small volumes, in low concentrations and multiple analytes can be monitored in one sample. Combining UCD with SELDI-TOF-MS, high throughput analysis can be achieved and profiles can be generated for multiple analytes.

Overall can be concluded, our UCD has the potential being used for in vivo real time monitoring HWM analytes. Without inducing stress because of withdrawing multiple samples by needle injections and sampling can be performed in the subjects own naturalistic environment. When the coil is filled it can be sent by mail to an analysis facility. This article can be seen as pre-work for future in vivo experiments.

3.5 Acknowledgement

This research is supported by the Dutch Technology Foundation STW, applied science division of NWO and the Technology Program of the Ministry of Economic Affairs, grant number GPG 06038.

Chapter 4

Quantitative analyte collection by microfiltration or MetaQuant-microdialysis: optimizing perfusion rate, probe membranes and sample storage

Abstract

In situ microdialysis and microfiltration probes are convenient technologies for continuous sampling of a wide variety of analytes. Quantification, which is often a prerequisite for biomedical monitoring, can be achieved with ultraslow-microfiltration and ultraslow perfusion rates in conventional microdialysis. However ultraslow sampling results in unacceptable detection delay. MetaQuant-microdialysis (MQ-microdialysis) is a novel microdialysis technology introducing an additional carrier-flow that merge with the ultraslow dialysate immediately downstream the microdialysis membrane. Accordingly, the lag time is shortened and the final volume is increased for easier handling. We report hollow fiber membranes of the MQ-microdialysis and microfiltration probes to sample a variety of central nervous system (CNS) compounds (gaboxadol, citalopram, morphine) and the hormone corticosterone. At flow rates of 0.15 µl min-1 the regenerated-cellulose (RE) MQ-microdialysis probe showed recoveries >90% for all tested compounds. The polyethylene microfiltration probe coated with ethylenevinylalcohol (PE-EVAL) showed minimal citalopram and corticosterone absorption. Continuously collected microliter microfiltration -samples can be stored directly in a capillary coil for longer periods of time to allow later chemical analysis. Capillary fused silica tubing (FST) was the most suitable for storage as there was minimal evaporation of the aqueous solvent and minimal diffusion of corticosterone (sampled from rat plasma) assessed after storing at 4 and 37°C for 3 days and immediate analysis.

Keywords

microfiltration, microdialysis, nanoliter/microliter samples storage device, MetaQuant microdialysis

4.1 Introduction

Continuous in situ monitoring of molecular processes is a technological challenge. Monitoring in intensive care medicine and in freely moving humans and animals, is most often based on frequent (blood) sampling and sensor technologies. However the in situ application of biosensors is thus far modest, presumably because of the low sensitivity and the limited number of analytes that can be detected with this technology. More versatile are sampling technologies like microfiltration and microdialysis, allowing the application of a wide variety of detection methods. Of these, microdialysis is the most applied sampling technique having around 5,000 publications since its invention, 26 years ago [66]. Microfiltration has only 50 publications since its first description in 1987 [154]. Both microdialysis and microfiltration allow sampling of analytes with various molecular weights (MW), such as drugs, peptides and proteins depended on the molecular weight cut-off value (MWCO) of the hollow fiber membrane. The ideal in vivo sampling device is light-weighted, robust and can be attached to a freely moving subject. To accomplish the device must collect small samples (nl-µl range) continuously. Moreover the collected biosamples should be relatively clean, so minimum sample pretreatment is required for chemical analysis. microdialysis is a sampling technique based on the diffusion of the analytes across the hollow fiber membrane down their concentration gradient. This is in contrast with microfiltration, where an under-pressure drives the flow of the filtrate over the hollow fiber membrane. The sampling rate of either technique varies from 0.05 to 10 µl·min^{-1}. In conventional microdialysis (flow > 0.3 µl·min^{-1}) complicated calculations are necessary to determine (or -perhaps better- to estimate) the real levels of the analytes in the bio-matrix to account for recoveries below 100%. Such calculations can be avoided using ultraslow perfusion rates thereby achieving quantitative diffusion equilibrium [227]. microdialysis at flow rates as slow as 80-210 nl·min^{-1} was firstly described by Wages et al. [331] and Menacherry et al.[227] and on ultraslow-microdialysis combined with sensor technology was firstly reported by Kaptein et al. [159]. A major problem associated with ultraslow-microdialysis is the often unacceptable lag-time between

sampling and analysis, because of the dead volume of the devices and the connecting tubing. The latter problem is avoided with MetaQuant-microdialysis, where an additional flow (carrier flow; typical 0.9 µl·min^{-1}) merge with the ultraslow dialysate immediately downstream the microdialysis membrane (**figure 1A**). The carrier flow shortens the lag time and increases the final sample volume for easier handling. The notorious dilution of the dialysate requires sensitive analysis techniques but is known as it is instrumentally determined [52, 53].

The only commercially available microfiltration sampling device (Bioanalytical Systems inc., West Lafayette, IN, USA) is attached to a vacuum container allowing discontinuous sampling only [204]. Continuous microfiltration and subsequent storage (by an ultrafiltration collection device = UCD) of the samples without the need of manual sample treatment are ideal in freely moving subjects. A versatile technique using a capillary coil for storing the continuously collected samples for hydrophilic analytes [232] has been applied in vivo [271]. Minimal diffusion was shown for lactate, glucose [174] and poly-l-lysine [135], allowing storage at various temperatures (4°C and 37°C) for at least 5 days [135]. During storage the aqueous solvent should not evaporate through the capillary tubing, as this would lead to an uncontrollable concentration of the sample and formation of air bubbles. Here two collections coils, made from different materials, will be tested for evaporation. Diffusion of the lipophylic corticosterone hormone in plasma is assessed by storing the capillary coil at various temperatures.

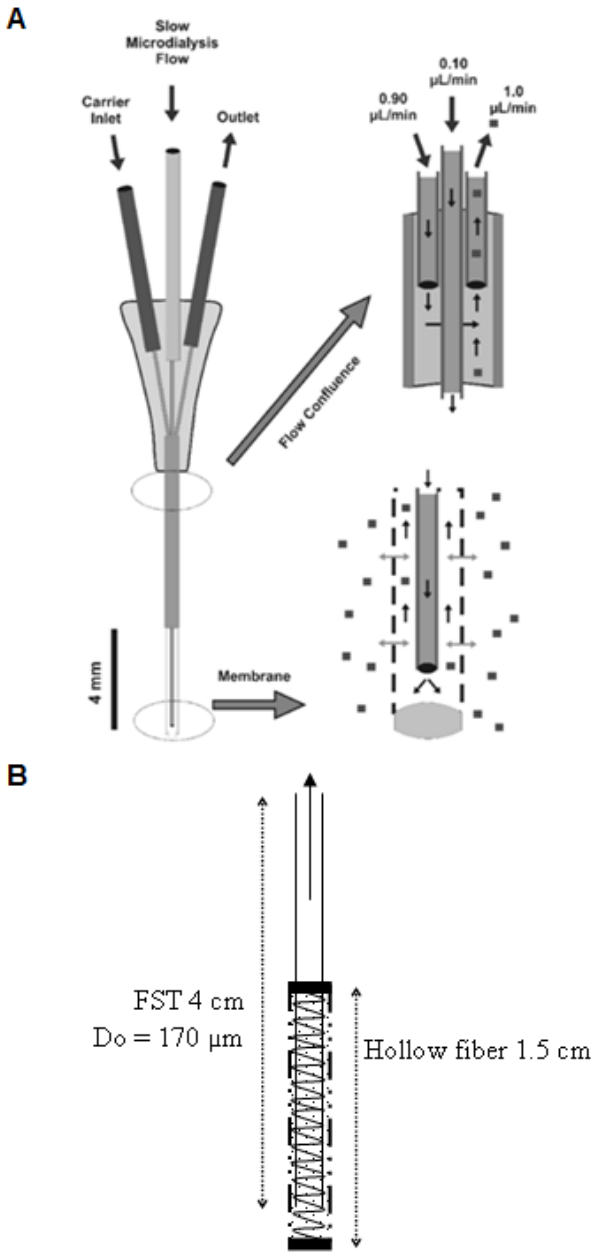

Figure 1 Schematic drawing of the MetaQuant-microdialysis (**A**) and microfiltration probe (**B**).

Table 1 Hollow fiber polymers used in the microfiltration sampling technique.

hollow fiber	plasmaphan P1LX	microPES TF10	plasmaflo OP-05W(L)	plasmacure PS-06	transvivo
company	Membrana	Membrana	Asahi medical	Kuraray	Transvivo
material	PP	PES	PE-EVAL	PS	PES
pore size (µm)	0.47	0.5	0.3*1	0.2	0.58
symmetric or a-sym	symmetric	asym inside > outside	symmetric	?	Asym Inside > outside
inner diameter (µm)	330	300	330	320	230
outer diameter (µm)	630	500	430	450	730
wall thickness (µm)	150	100	50	65	250
flow (µl·min^{-1})	0.3	0.3	0.3	0.3	0.3

*1 in literature sometimes the value of 3MDa is given [336]

Table 2 Hollow fiber polymers used in the MetaQuant-microdialysis sampling technique.

hollow fiber	MetaQuant-PES			MetaQuant-RE		
company	Brainlink			Membrana		
material	PES			RE		
pore size (kDa)	30			18		
symmetric or a-sym	symmetric			Symmetric		
inner diameter (µm)	200			200		
outer diameter (µm)	280			216		
wall thickness (µm)	40			8		
flow	total	carrier	slow	total	carrier	slow
(µl·min^{-1})	1.0	1.0	0.0	1.0	1.0	0.0
	1.0	0.5	0.5	1.0	0.5	0.5
	1.0	0.2	0.8	1.0	0.2	0.8
	1.05	0.15	0.9	1.05	0.15	0.9
	1.0	0.1	0.9	1.0	0.1	0.9

The present study was aimed to assess whether the MQ-microdialysis and microfiltration technique allow quantitative sampling of central nervous systems (CNS) compounds (gaboxadol, citalopram and morphine) with different physicochemical properties and the hormone corticosterone. The octanol partitioning values range from low to high: gaboxadol (-0.91 ± 0.31), morphine (1.21 ± 0.51), corticosterone (1.94 ± 0.38), and citalopram (3.57 ± 0.54) [303].

The MQ-microdialysis probes were made of polyethersulfone (PES) or regenerated cellulose (RE) with an active membrane length of 4 mm (**table 2**). Commercial hollow fibers of different sizes and materials were tested. The transport efficacy of the analytes is not only determined by the molecular weight (the MWCO value), but also by other characteristics of the hollow fiber membrane, such as the electric charge of the fiber surface, hydrophobic-hydrophilic properties, symmetric/asymmetric structure and thickness of the membrane. The sampling efficiency of corticosterone and citalopram was assessed with

five microfiltration membranes having various MWCO values and/or hollow fiber dimensions (**table 1**). We intend to apply the here optimized techniques to sample hormones bound to proteins and CNS compounds in living and freely moving organisms. Therefore the here explored microfiltration hollow fibers were selected because of their commercial availability and pore-size dimensions.

4.2 Materials & Methods

4.2.1 MQ-microdialysis probe

Stock solution of 1 mM citalopram, gaboxadol, morphine and corticosterone were made in ethanol and diluted with Ringer solution (147 mM NaCl, 3.0 mM KCl, 1.2 mM $CaCl_2$, and 1.2 mM $MgCl_2$) into standard solutions of 10 µM. The relative recoveries of the RE-MQ-microdialysis and PES-MQ-microdialysis probes (Brainlink B.V., Groningen, The Netherlands) were tested at 4-5 different ultraslow flow rates with standard solutions of gaboxadol, citalopram, morphine, and corticosterone. The MQ-microdialysis probes were connected via flexible PEEK tubing to a micro-perfusion pump (CMA 102); the line in and the line out were connected to a HPLC (Shimadzu LC10-AD) for analysis. The dynamics of MQ-microdialysis sampling system were studied by sampling firstly the Ringer solution for one hour, then placing of the MQ-microdialysis probe in the solution of interest for one hour and finally by replacing the probe in the Ringer solution.

4.2.2 Microfiltration probe

The present microfiltration probes are modified versions of those of Moscone et al. [232]. They are taken from plasmapheresis devices, except the Transvivo hollow fiber probes being developed as an intravenous catheter [40] (**table 1**). The membranes were cut into 15 mm long pieces and were provided with a hand-made stainless steel spring inside (Vogelsang, Hagen, Germany; 60 µm diameter, axis length 12 windings per centimeter) to prevent collapsing of the hollow fiber and to reduce the internal dead volume. Fused silica tubing (FST; TSP100170; 170 µm outer diameter, 100 µm inner diameter, Polymicro Technologies Inc., Phoenix, AZ) of approximately 4 cm was inserted into the probe up to 2

mm from the tip. Hollow fiber, spiral, and FST were fixated with cyanoacrylic glue (Ruplo B.V., Ten Boer, the Netherlands). The microfiltration probes were immersed in five standard concentrations: 1, 2, 5, 10, and 20 µM solution made from the MQ stock solution and diluted with Ringer. Each solution was sampled at 0.3 µl·min^{-1} for 40 minutes, starting with the lowest concentration.

4.2.3 HPLC conditions

The samples containing citalopram, gaboxadol, morphine or corticosterone were injected every 10 minutes, collected online into a 20 µl HPLC valve, separated over a HPLC column and analyzed by HPLC-UV at 254 nm. A C18 guard column was used for the analysis of citalopram. The mobile phase for citalopram consisted of 5% acetonitrile and 0.1% formic acid in ultrapure (UP; Barnstead easypure II) water using a flow rate of 0.5 ml·min^{-1}. Gaboxadol and morphine were separated on a BDS Hypersil C18 Thermo column with a C18 guard column attached. The mobile phases were 1.5% methanol and 300 mg·l^{-1} heptasulfonic acid for gaboxadol, and 20% acetonitrile, 300 mg·l^{-1} heptasulfonic acid with 0.05% acetic acid in UP water for morphine, respectively, using a flow 0.2 ml·min^{-1}. Corticosterone was separated over a C18 guard column (Aurora Borealis, Schoonebeek, the Netherlands) with a mobile phase consisting of 10% acetonitrile with a flow rate of 0.7 ml·min^{-1}.

4.2.4 Capillary collection coil – evaporation

FST (TSP320450; v = 1.439 ml, l = 18 m, inner diameter = 320 ± 6 µm and outer diameter = 435 ± 10 µm, Polymicro Technologies Inc., Phoenix) and CoEX (polyethylene tubing with an outer wall of PVC (BCOEX-T25; v = 1.445 ml, l = 11.5 m, inner diameter = 0.4 mm and outer diameter = 1.3 mm, Instech Solomon Scientific, USA) were tested for the evaporation of water. Firstly the tubing was weighted empty, then filled with UP and both sites of the tubing were glued with cyanoacrylic glue (Ruplo B.V., Ten Boer, The Netherlands). The coil was weighted automatically every minute for 5-6 days.

4.2.5 Capillary collection coil - diffusion study

FST (TSP300665, v = 229 µl, l = 330 cm, inner diameter 300 ± 6 µm and outer diameter 665 ± 15 µm, Polymicro Technologies Inc., Phoenix) was filled with fractions of 40 µl rat plasma samples at a flow of 1.0 µl·min^{-1} with known corticosterone concentrations. The fractions contained alternating lower and higher concentrations of corticosterone. After filling the collection coil, the pump was removed and both ends were sealed with cyanoacrylic glue (Ruplo B.V., Ten Boer, The Netherlands). Two collection coils were stored for 3 days at 4°C and 37°C and one coil was filled and analyzed immediately. For the analysis the capillary coil was fragmented afterwards into volume fractions of about 10 µl by cutting the capillary coil into pieces. Corticosterone was assessed with a radioimmunoassay.

4.2.6 Presentation of data and statistical analysis

In the MQ-microdialysis study for each analyte a new probe was used and for each probe the average recovery (± SEM, n = 3) was calculated. The recovery is the ratio of the measured peak height of the concentrations of the analyte in the solution of interest and that obtained of the MQ-microdialysis hollow fiber probe, when the dialyzable surface was closed with glue (**figure 2**). In the microfiltration study for each analyte a new hollow fiber was made and the average peak height was measured over 40 minutes. The recovery of microfiltration was the ratio of the average output putting the probe into the solution of interest and the average signal determined by sampling with FST with the same length encorporated in the microfiltration probe. In the evaporation study the length of the tubing was selected by keeping the total volume in the same range (1.47-1.51 ml). The average weight was calculated over 24 hours ± SD. Diffusion was determined by filling FST coil with rat plasma with known corticosterone concentrations. The coils were fragmented into volumes of 10 µl by cutting. Known corticosterone concentrations in vials were taking along to assess the effect of temperature.

4.3 Results & discussion

4.3.1 MQ-microdialysis

The recovery data based on ultraslow-flow rates for the assessment of the 3 CNS compounds sampled with the MQ-microdialysis made from RE are shown in **figure 2A, C-D**. **Figure 2B** shows the MQ-microdialysis data for corticosterone sampled with hollow fibers made from PES. Flow rates of 0.5 µl·min^{-1} and higher yield low recoveries for all tested compounds, which became near 100% at 0.1 and 0.2 µl·min^{-1}. These results are in line with in vivo studies for glucose and lactate showing near 100% recoveries at flow rates of 50-100 nl·min^{-1} applied in the rat striatum and of 100-300 nl·min^{-1} in the rat [160] and human subcutaneous compartment [52, 160, 308].

Next we tested how fast the maximum response is reached during MQ-microdialysis sampling. Ideally the sampling system should react instantaneous upon immersion in a solution. As shown in **figure 2F-J**, it takes one injection of compound (every 10 minutes) to reach maximal signal. Correcting this value for lag time, it takes about 2 minutes to reach maximum signal.

With the MQ-microdialysis devices made from PES and RE, using ultraslow-flow rates of 0.1 and 0.2 µl·min^{-1} recoveries near 100% were found when the sample contained 10-15 µM. Microdialysis sampling is most often used for the collection of hydrophilic compounds (such as monoaminergic neurotransmitters) and it appears difficult to sample hydrophobic analytes. Hydrophobic drugs and hormones exhibit high protein binding and their free (unbound) concentrations in the blood and in extracellular fluid are low. Hydrophobic analytes may also bind non-specifically to microdialysis probe-materials and tubing. The present experiments with the MQ-microdialysis system show that the tested CNS compounds and cortisosterone can be sampled quantitatively in µM concentrations, with both with PES and RE MQ-microdialysis probes.

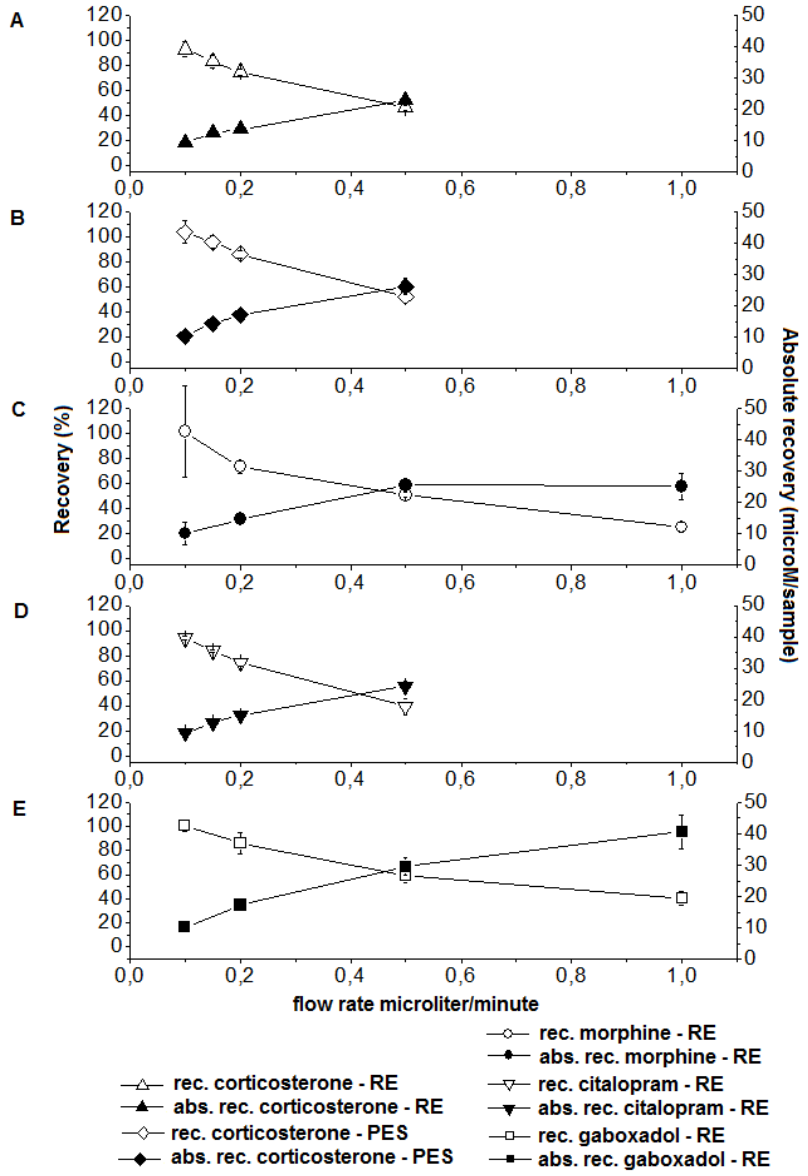

Figure 2 Analyte recoveries following MetaQuant-microdialysis at various flow rates (**A-E**). Using flow rates of 0.1 and 0.2 µl·min^{-1} yield near 100% recoveries for all CNS compounds and corticosterone using a RE-MQ-microdialysis (**figure A, C-E**) and corticosterone sampled with the PES-MQ-microdialysis (**B**). At 10-15 µM the recovery is 100% (**A-E**).

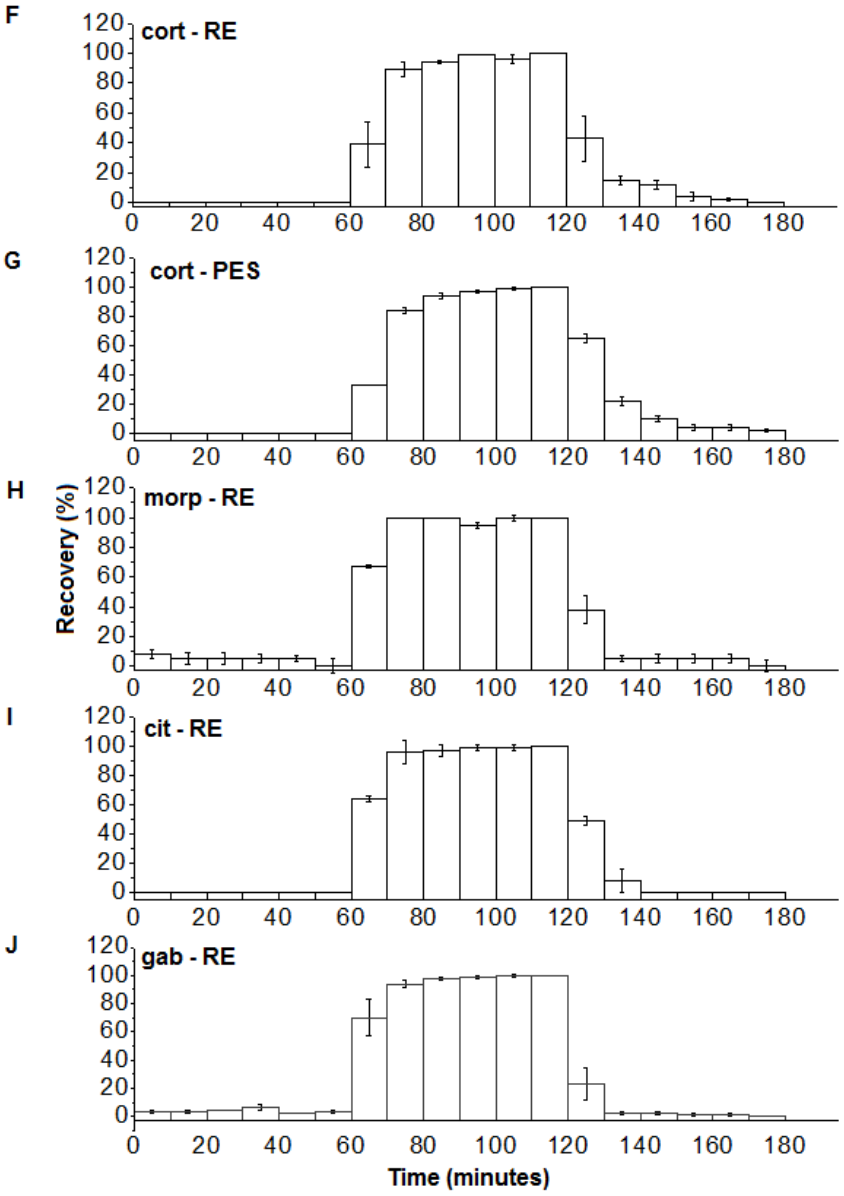

Figure 2 The dynamics (**F-J**) shows that one injection of each compound every 10 minutes the signal reaches it's maximum. Corticosterone – RE (**2F**), corticosterone – PES (**2G**), morphine – RE (**2H**), citalopram – RE (**2I**), and gaboxadol – RE (**2J**). Correcting this value for lag time, it takes about 2 minutes to reach maximum signal.

4.3.2 Microfiltration

The experiments were started by placing the probe in the lowest standard concentration. A continuous flow of 0.3 µl min^{-1} was used. Between the increasing concentrations, the probes were rinsed with UP water. Figure 3 illustrates the relative recovery of citalopram (**figure 3A**) and corticosterone (**figure 3B**). The plasmaflo (PE-EVAL) hollow fiber gave the best recovery for citalopram. Sampled at 5 µM the recovery reached 100%, and there is little absorption of citalopram at concentrations of 1 and 2 µM. The recoveries of the other tested hollow fiber membranes (PES, PS and PP) were far below 100%, presumably due to absorption of citalopram. The recoveries of corticosterone sampled with the plasmaflo (PE-EVAL), microPES (PES) and transvivo (PES) probes were 80-100%. With the transvivo the recovery for corticosterone rose instantaneously but remained at 70-80%. Recoveries of corticosterone below 10% were obtained with the hollow fibers made of PS and PP used in microfiltration. These low recoveries are probably due to hydrophobic interactions, as the other tested hollow fibers are more hydrophilic. A very hydrophobic peptide, poly-l-tryptophan [135], was absorbed by the microfiltration device made of PS as well [135]. Citalopram is more hydrophobic than corticosterone indicating that the polymer of the membrane does have an additional effect.

Figure 3 shows that plasmaflo (PE-EVAL), being the most hydrophilic hollow fiber, enables sampling of corticosterone and citalopram without significant absorption. The recovery of citalopram being the most hydrophobic CNS compound was lower than that of corticosterone. In contrast, the most hydrophobic hollow fiber membrane (PP) is unsuitable for sampling of both corticosterone and citalopram.

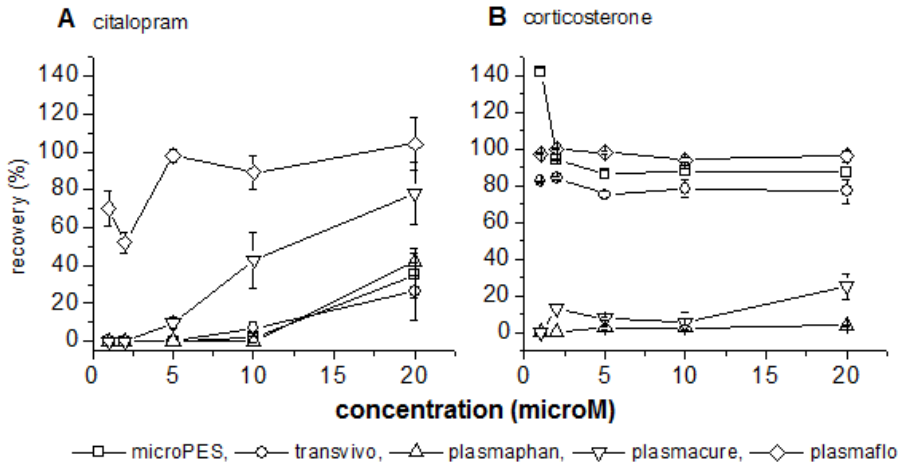

Figure 3 Recoveries of citalopram (**A**) and corticosterone (**B**) during microfiltration. Because of their low recoveries, plasmaphan and plasmacure were unsuitable for both compounds. Transvivo gave only low recoveries with citalopram. Micro-PES and transvivo were suitable for corticosterone sampling. Only the plasmaflo was suitable for both compounds.

We compared corticosterone absorption with one another for both sampling techniques. The hollow fibers manufactured of PES and PE-EVAL revealed recoveries of 80-100% (MQ-microdialysis slow flow = <0.15 µl·min^{-1}, l = 4 mm, and microfiltration flow = 0.3 µl·min^{-1}, l = 15 mm). Increasing the wall size of the PES hollow fiber led to lower recoveries. Probably corticosterone got trapped in the relatively thick wall of the transvivo hollow fiber membrane (250 µm). Literature suggests that microdialysis of lipophilic compounds is impaired [11] because of binding to the membranes. Araujo et al. [11] tested CMA20 microdialysis with a cut-off value of 20 kDa hollow fiber membranes (poly-arylethersulphone = PAES). At the lowest perfusion rate (1.0 µl·min^{-1}) the recovery was almost 60%. Mary et al. [220] assessed the CMA20 microdialysis probes with a MCWO of 20 kDa, and tested also 100 kDa hollow fiber (PES) to sample 3 lipophilic compounds. They concluded that with decreasing perfusion rate (lowest value = 1.0 µl·min^{-1}) and lipophylicity the recovery tends to increase; the highest measured recovery was 42% at 1.0 µl·min^{-1}. Sun and Stenken [297] tested the recovery of prostaglandin B2, leukotriene B4, and leukotrien C4 using a flow rate of 0.7 µl·min^{-1}. Hollow fiber materials, made from

polycarbonate (20 kDa), polyacrylonitrile (29 kDa), cuprophan (6 kDa) and PES (100 kDa) were tested and all their recoveries were below 50%. Measures to enhance the recovery of lipophilic analytes include the addition of albumin [156] or other binding agents [297] to the perfusate. In the above published microdialysis studies, the flow rates are all higher than 0.7 µl·min^{-1}, resulting in recoveries lower than 50% and that is in accordance with the results shown in **figure 2**.

4.3.3 Storage coils

Two storage coils, one made from FST (v = 1439 µl, l = 18m FST) and another from polyethylene with an outer-wall of PVC (v = 1445 µl, l = 11.5m CoEX tubing), were tested for evaporation (**figure 4A** and **4B**) and diffusion at various temperatures (**figure 5**). We used rat plasma samples with known corticosterone concentrations.

4.3.4 Evaporation of UP in CoEX and FST coil

Figure 4A shows nearly no weight loss over 6 days in the FST coil. In contrast the CoEX tubing filled with UP the SDs did not overlap pointing to significant evaporation. The volume loss of the FST tubing was ≈0.4 µl on day 6 days storing at room temperature. For the CoEX tubing the total volume loss was ≈4.9 µl, 10 times more than that of FST. The relative volume losses are still extremely low, because both coils were filled with 1439 µl and 1445 µl, respectively. Visual inspection of the CoEX tubing showed many empty spaces (presumably air bubbles). An aspect not investigated here in detail was that the UP water content is probably absorbed by the CoEX tubing; in this manner the weight of the coil may become minimally disturbed. Moscone et al. [232] wrapped the coil in a plastic bag to prevent evaporation. We conclude that FST is the best material for storing, because of its inertness and lack of absorption of UP water [232, 271].

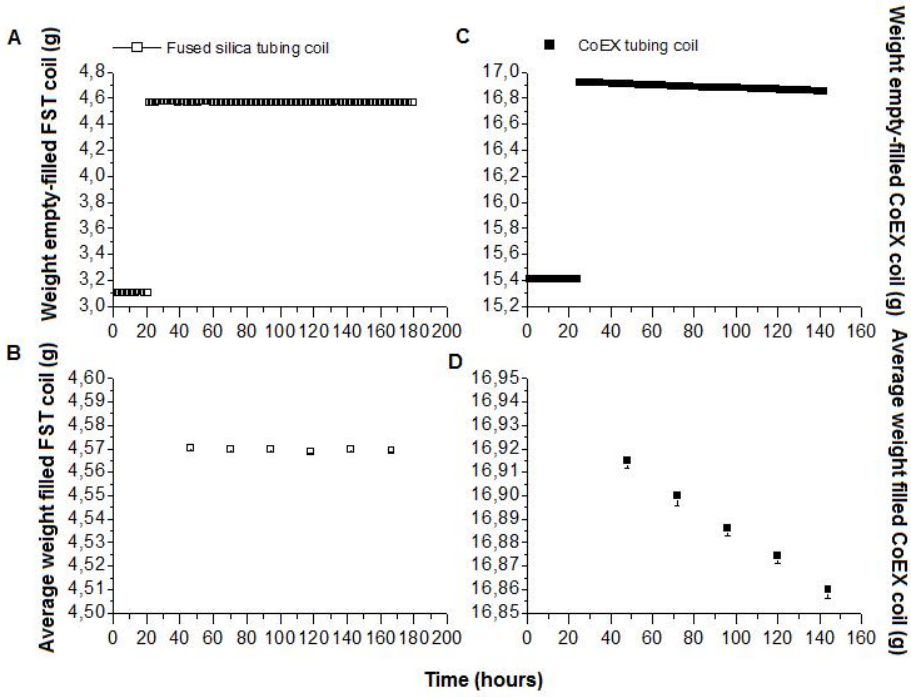

Figure 4 Evaporation of water from fused silica tubing (FST, **A-B**) and CoEX tubing (**C-D**). The weight of the empty and filled coil is given for FST (**A**) and the CoEX tubing (**B**). In figures **B** and **D** the filled coils are shown for FST and CoEX, respectively. The axis of **B** and **D** are kept in the same range. There was no significant decrease of the weight in FST, indicating negligible evaporation.

Storage effects of plasma in FST. After filling with rat plasma containing known concentrations of corticosterone, one coil was analyzed immediately (**figure 5A**) and the others after storage for 3 days at 4°C (**figure 5B**) or at 37°C (**figure 5C**). The in vitro filling of the coils was performed at room temperature, but in vivo the temperature will gradually increase to a 37°C. We investigated if temperature had an effect on the corticosterone concentration in plasma. Each plasma sample was collected during 40 minutes at a flow rate of 1.0 µl·min^{-1}. Fragments of (nearly) 10.0 µl were analyzed. It is nearly impossible to collect exactly 10.0 µl in a cut fragment, so the 1th and 4th fractions are mixtures of two adjacent plasma corticosterone samples, as illustrated in figure (**5A-5C**). The results shown in **figure 5D** indicate that the diffusion was insignificant in coils stored at 4°C and 37°C for at least 3

days. Thus FST coils are suitable for storage of plasma samples to assess time-profiles of corticosterone.

4.4 Conclusions

Every sampling technique has it own advantage(s) and/or limitation(s). Leegsma-Vogt et al. [193] and Garrison et al. [96] have recently reviewed and compared the microfiltration and microdialysis technologies critically.

A major advantage of microdialysis over microfiltration is that the microdialysis sampling technique can be applied in the brain. Another advantage is that with microdialysis drugs can be introduced locally via the perfusate. Drawbacks of conventional microdialysis are the maximal sampling frequency (sampling times less than 1 min are difficult to handle); the unknown recovery of the analyte, so only calculated (or estimated) levels are obtained. For instance, the dynamic net no flux (DNNF) has long been recognized as the "golden standard" in quantitative microdialysis methodologies. One disadvantage of DNNF is that more experimental data are necessary: meaning in practice that more animals are needed. Other disadvantages are that the compound of interest has to be added to the perfusate, which could introduce in vivo changes near the probe and the low consistency of the DNNF method [53]. Constant, -non-pulsating- flow at the nl·min^{-1} range is not well possible with current mechanical microdialysis pumps and the utility of microdialysis in large/freely moving animals is rather complex. Cooper et al. [50] have emphasized the potential of osmotic pumps in the conscious rat. The small and light weight under-pressure pump developed for ultraslow- microfiltration may serve as an alternative to the osmotic pumps [50]. The MQ variant of microdialysis circumvents important limitations of conventional microdialysis: i.e. the recovery becomes now near 100% so no calculations are required to determine the real level of analyte in the biomatrix and the time resolution is high due to the carrier flow.

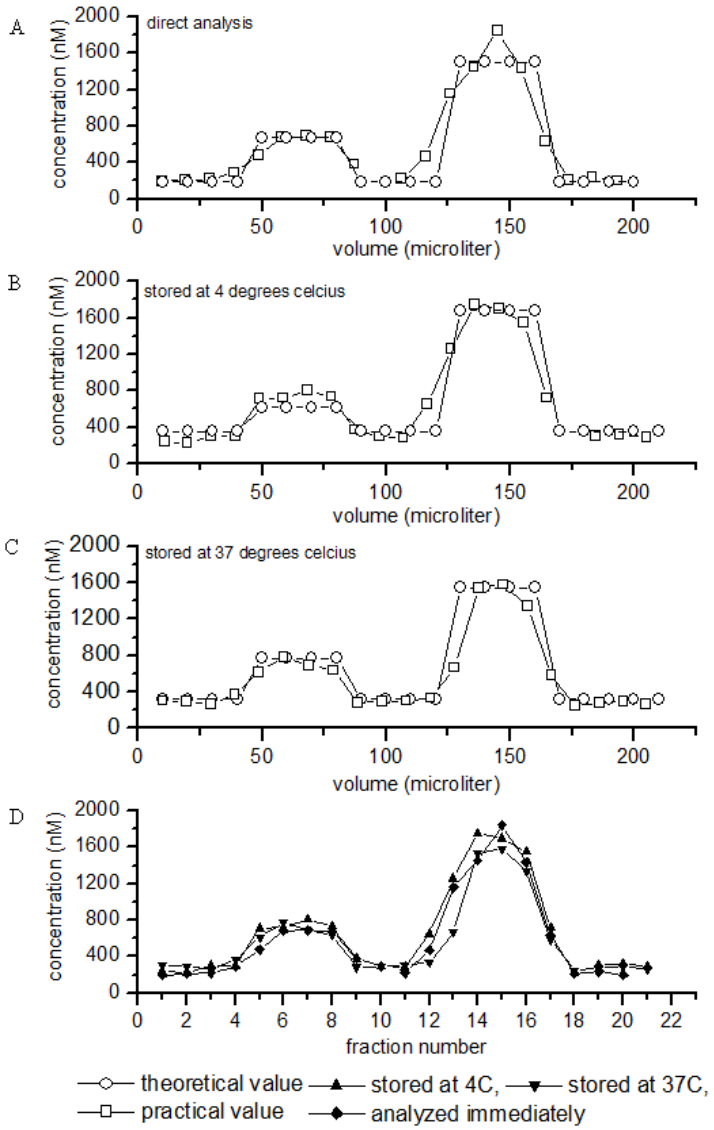

Figure 5 Storage effects of plasma in FST analyzed immediately (**A**), or stored at 4°C (**B**) and 37°C (**C**). Diffusion is insignificant in the coils stored at 4°C and 37°C for 3 days and temperature had no effect on the corticosterone concentrations.

The major advantages of the microfiltration sampling technique are the possibility of continuous –non pulsating- sampling, guaranteed recovery of 100% (when no absorption is taking place), the presence of the analyte in a most often stabilizing natural matrix and, when attached to an UCD and the possibility of sampling of freely moving subjects. Drawbacks of microfiltration sampling are that no drugs can be introduced locally through the probe and that microfiltration can only be applied when there is sufficient endogenous production of extracellular fluid [160].

Both microdialysis and microfiltration allow the collection of protein-containing and protein-free body fluids, depending on the MWCO value of the chosen hollow fiber membrane. Rosenbloom et al. [265] concluded, however, that the published MWCO value may not always predict the size of the proteins being recovered. Most reports on microdialysis and microfiltration use hollow fibers with a MWCO value < 20 kDa, but hollow fibers with MWCO with higher MWCO values have also been applied for microfiltration [135, 274] and microdialysis [266, 279]. A possible disadvantage of using a high MWCO value in microdialysis is the outflow of perfusion fluid via the hollow fiber membrane into the surrounding tissue, due to the hydraulic pressure in the outlet tubing. Such an effect may be avoided by adding high molecular weight osmotic agent to the perfusion fluid such as Dextran-60 [117, 137, 266]. microfiltration sampling might then be preferred.

There is a growing tendency to reduce sample volumes, provided by MQ- microdialysis and ultraslow- microfiltration sampling, because of the increasing sensitivity and specificity of current analytical technologies, such as microbore or capillary HPLC, capillary electrophoresis, mass spectrometry and (bio)-sensoring. On-line analysis with such versatile methods as HPLC or MS requires easy and preferentially automatic on-line sample handling that is most conveniently achieved with MQ- microdialysis. When on-line real time analysis is not required, ultraslow- microfiltration combined with storage in FST is to be preferred. Because of minimal diffusion, the thus stored samples can be -if required inter-continentally- shipped to be analyzed.

The present study emphasizes that quantitative bio-sampling with ultraslow- microdialysis or ultraslow- microfiltration is applicable to a wide variety of analytes and each technique has its own potential(s) and/or drawback(s). Our experiments may help to make to choose the proper probe and materials.

4.5 Acknowledgment

This research is supported by the Dutch Technology Foundation STW, applied science division of NWO and the Technology Program of the Ministry of Economic Affairs, grant number GPG 06038. We would also like to Bodo von Harten (Membrana GmbH, Wuppertal, Germany) and Jacob Kearns (Transvivo Inc., Napa, USA) for the hollow fiber gifts.

Chapter 5
Quantitative microdialysis using modified ultraslow-microdialysis; direct rapid and reliable determination of free brain concentrations with the MQ technique

This chapter is published in J. Neurosci.Methods, volume 178, numer 2, april 2009, page 249-254.

Abstract

The only method to quantify free extracellular levels of drugs in the brain of living animals is microdialysis. However, quantitative microdialysis has been hampered by methodological issues for decades. The problems arise from the need to establish the in vivo recovery for appropriate quantitation. In dealing with these issues the "Dynamic No Net Flux" (DNNF) method seemed to be the experimental method of choice. Major disadvantages were, however, the need for a very high degree of bioanalytical precision and accuracy and the need for a large number of animals. Moreover, today we know that the experimental data are not always straightforward.

To improve robustness and practicality of quantitative microdialysis sampling we modified the ultraslow-microdialysis approach. Ultraslow-microdialysis uses very low microdialysis flow rates (<200 nl·min^{-1}) which increase recovery (both in vivo and in vitro) to over 90%. However, new practical issues arise when attempting to work with these flow rates. The resulting very low volumes and long lag times make this method very impractical for general application. In the modified version, addition of a carrier flow after the dialysis process has been completed negates the problems of long lag times and low volumes. The resulting dilution of the dialysis sample concentration can simply be mathematically corrected.

In the current study we measured the free brain levels of two CNS compounds using the classic DNNF and the new modified ultraslow-microdialysis dialysis method.

Modified ultraslow-microdialysis was shown to generate robust data with the use of only small numbers of rats. The method is a promising tool for common straightforward screening of blood-brain barrier penetration of compounds into the brain.

Keywords

quantitative, microdialysis, ultraslow, recovery, brain, brain penetration, in vivo

5.1 Introduction

Measuring free brain levels of experimental CNS-active compounds in an early stage of their development is crucial to acquire vital information about their blood-brain barrier (BBB) penetrating properties. To date it is common practice to use total brain and/or CSF samples to ascertain this information. However, determination of CSF or total tissue levels yields data that may not necessarily reflect the actual free brain levels [61].

The only way to monitor free brain concentrations directly is by in vivo intracerebral microdialysis. This method provides access to the extracellular fluid (ECF) of the brain and enables repeated sampling in a living subject.

The basic mechanism of the microdialysis method, briefly, is to insert a small microdialysis probe into the brain and perfuse its lumen with a physiological solution. This probe contains a semi-permeable membrane that allows small molecules to penetrate the membrane down their concentration gradient. The effluent, the dialysate, is continuously collected into vials and analysis of the compound(s) of interest can, thus, be performed off-line. The concentration of a compound in the dialysate is therefore indicative of the free concentration in the extracellular fluid surrounding the semi-permeable membrane of the microdialysis probe. The rate and extent of concentration equilibration between the lumen of the probe and the fluid surrounding the semi-permeable membrane of the probe (concentration recovery) can be estimated in vitro, but never really reflects the probe's performance in vivo. Instead, it merely reveals the semi-permeable membrane transport characteristics as such. This is because in vivo concentration recovery is to a large extent affected by physiological processes in the tissue [64]. These processes, and hence in vivo recovery, may be time- and concentration dependent [35, 231]. Therefore the in vivo concentration recovery cannot be predicted simply from in vitro tests alone.

For many years the determination of the in vivo recovery, vital to obtain quantitative data, has proven to be a most eluding challenge. Quite a few

different methods have been developed, including both theoretical [35, 203, 231] and experimental approaches [30, 191, 210, 238, 272, 349]. These methods also range from simplified approaches with many incorporated assumptions to complex procedures with almost no assumptions [62]. Important experimental approaches are the retrodialysis by drug [272], the no-net-flux method (NNF), retrodialysis by calibrator [349], or by drug and calibrator [30] and the dynamic no net flux (DNNF [30]) method.

While the retrodialysis approach is relatively easy, it has to be assumed that in vivo concentration recovery is independent of concentration in the probes' surrounding, while also no time dependency is assumed, or at least a time-dependency being similar for the drug and calibrator. The DNNF almost has no assumptions. To date the only experimental method that takes into account time- and concentration-dependency of in vivo recovery. Instead of serial perfusion of individual animals with different concentrations via the probe like in the NNF, a group of animals are continuously perfused with one of the perfusion concentrations selected around the expected ECF concentrations. Different groups are perfused with different concentrations and the results are combined at each time point. Regression of the mean data points of the different groups at a particular point in time will give the actual concentration in the ECF with the associated in vivo concentration recovery value at that time [63, 343]. However, this is a very complex and time consuming protocol, with another important disadvantage from an ethical point of view: the requirement of a significantly larger number of experimental animals. Moreover, long standing experience with the use of the DNNF has made clear that often highly variable outcomes are generated (data not published).

In the current study we developed a new microdialysis sampling system. We adopted the principle that microdialysis extraction becomes quantitative (i.e. concentration recovery is close to 100%) when the dialysis flow rate is decreased from the more common rates of 1.5-2 $\mu l \cdot min^{-1}$ down to 0.1 $\mu l \cdot min^{-1}$ [54, 227].

In practice it is notoriously difficult in in vivo microdialysis experiments to work with flow rates as low as 0.1 $\mu l \cdot min^{-1}$, because of the long lag times (between probe and collection vial) and small sample volumes.

Therefore we modified the conventional microdialysis probe setup by introducing an additional flow (carrier flow) to merge with the ultraslow dialysate immediately downstream from the microdialysis membrane. The carrier fluid is delivered at a higher flow rate (typical 0.9 µl·min^{-1}) that shortens the lag time and increases final sample volume for easier handling. The obvious dilution of dialysate by the carrier fluid requires employment of sensitive techniques such as mass spectrometry. As the microdialysis exchange has already accomplished a close to 100% concentration recovery after leaving the dialysis membrane), the dilution of the sample by the carrier flow and collection systems can be mathematically corrected after analysis. This design greatly facilitates the ease of use of the sampling system in vivo [52]. In the current study, the new microdialysis sampling system, named MetaQuant, was validated for two known CNS compounds by evaluating rate and extent (dynamics) of their in vitro concentration recovery, and by comparing in vivo MQ data to those obtained by the DNNF.

5.2 Materials and methods

5.2.1 Specifications of a MQ probe

Figure 1 shows a diagram of the MQ-microdialysis probe (Brainlink B.V., Groningen, the Netherlands). An artificial cerebrospinal fluid (147 mM NaCl, 3.0 mM KCl, 1.2 mM $CaCl_2$, and 1.2 mM $MgCl_2$ containing 0.2 % albumin) is typically delivered at 0.1 µl·min^{-1} through the middle (yellow) inlet. The carrier flow (typically ultra-purified water) is delivered through the left (blue) inlet at 0.9 µl·min^{-1}. The right outlet thus yields a combined outflow of 1 µl·min^{-1}, consisting of 0.10 µl·min^{-1} dialysate with 100% recovery and 0.9 µl·min^{-1} water. The membranes used for the current application were made of polyethersulfone (PES) with a molecular weight cut-off of 18 kDa.

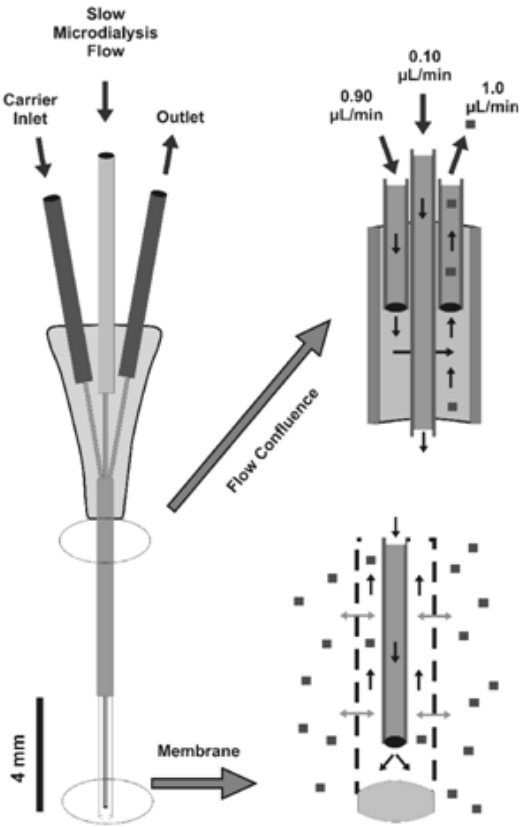

Figure 1 Diagram of a MetaQuant-Microdialysis probe.

5.2.2 In vivo experiments

For in vivo experiments, MQ probes were stereotaxically positioned in the medial prefrontal cortex of anesthetized male Wistar rats and fixed on the skull with dental polymer. Dialysis experiments on freely-moving animal were performed 24 h after introduction of the probes.

5.2.3 Recovery experiments

5.2.3.1 In vitro recovery

For the study of flow dependency and membrane dynamics, probes were positioned in 30 ml polypropylene beakers with membranes submerged in a stirred solution of 10 µM gaboxadol and citalopram at

ambient temperature. Samples were collected online into the HPLC loop and analysed using HPLC with UV detection. For confirmation of quantitative dialysis at low concentration, sampled were analysed using HPLC-MS/MS.

5.2.3.2 In vivo recovery by MQ

Gaboxadol or citalopram at concentration of 1 µM and 100 nM respectively was included in the dialysates perfusion fluid. Outlet concentrations were determined to evaluate the outward recovery of the compounds. The loss in the absence of systemic compound administered equals retrodialysis conditions (in vivo loss).

5.2.3.3 In vivo recovery by DNNF

DNNF experiments were performed using similar conditions as for MQ with regard to membrane, surgery and ringer composition. These probes were of a similar construction but without the dilution channel. The dialysis flow rate was set at 1.5 µl·min^{-1}. Experiments were performed 3 times to establish the flux compounds. To establish recovery for gaboxadol, the experiment was performed with 0, 1 and 10 µM of gaboxadol present in the perfusion fluid. For citalopram 0, 200 and 1000 nM was used as perfusion concentration. Dialysis conditions were allowed to stabilize for 2 hours prior to systemic drug adiministration.

For all experiments at any specific time point, the flux was determined, and the point of no net flux was determined to obtain the true extracellular concentration.

5.2.4 Bioanalysis

Compounds were analysed using either HPLC MS/MS (sciex API 4000, with shimadzu 10 ADvp HPLC equipment, analyst 1.4.2) or HPLC UV (Jasco In conjunction with shimadzu 10 ADvp).

5.3 Results

5.3.1 Gaboxadol

5.3.1.1 In vitro recovery

Decreasing the flow rate through the dialysis probe typically increased the relative recovery of gaboxadol from 40% at 1 µl·min^{-1} to over 90% at flow rates below 0.2 µl·min^{-1} **(figure 2A)**. At the same time, absolute amounts recovered decreased as the increase in recovery was outweighed by the decrease in volume.

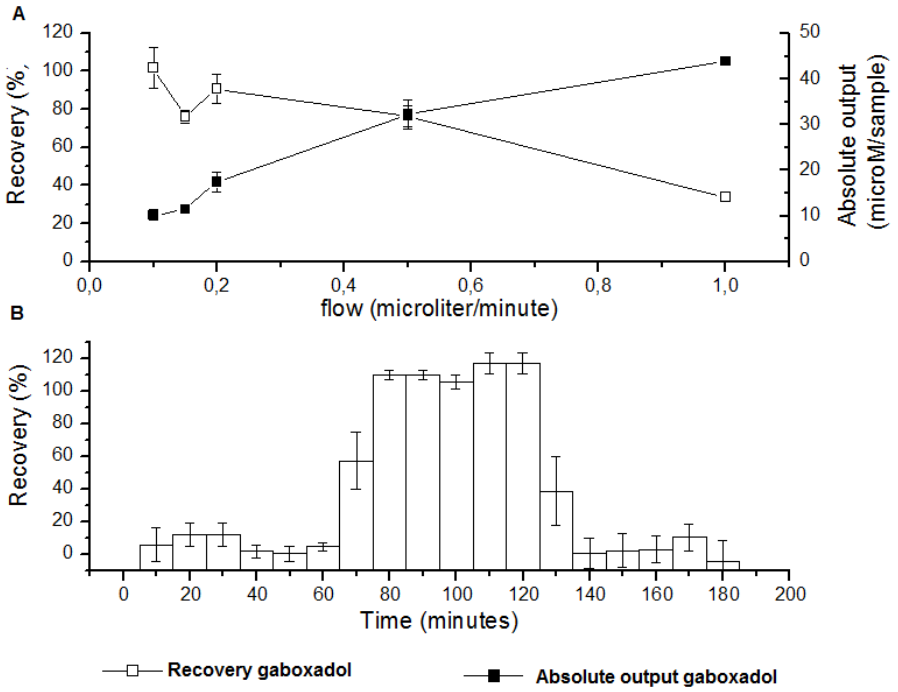

Figure 2 Comparison of absolute and relative recoveries in vitro **(A)**, and dynamics of the MQ system to instantaneous changes in external concentrations **(B)**.

The dynamic properties of changes in recovery to an instantaneously applied increase in external bulk fluid concentration, was characterized by a delay of 30% in the first 10 min. This means the MQ system takes 3

min to establish full recovery. When the probe was returned to blank solution some tailing was apparent in the first blank samples, again demonstrating a response delay of 3 min **(figure 2B)**.

5.3.1.2 In vivo loss

In vivo loss during slow flow no gaboxadol could be determined when 10 µM was infused through the probe at 100 nl·min^{-1}, which gave dilution and lower limit of quantification of the analysis would indicate that the probe had > 99% outward recovery in vivo[52].

In vivo loss during DNNF outward in vivo recovery for gaboxadol using DNNF at 1.5 µl·min^{-1} as determined at t = 0 was observed to be 17.4% ± 2.5 (SEM) (inclusion of 1 µM, n = 11) and 11.9 ± 3.3 (inclusion of 10 µM, n = 10).

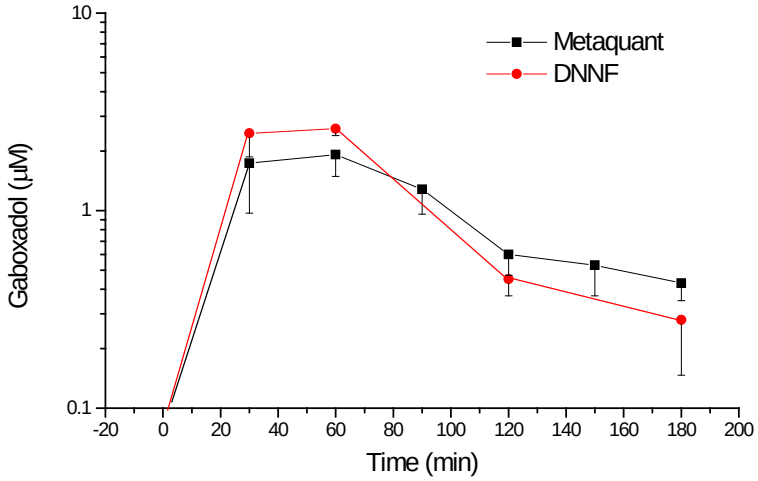

Figure 3 Free concentrations of gaboxadol in rat brain after administration of 10 mg·kg^{-1} s.c., quantitated with DNNF or MQ techniques.

5.3.1.3 In vivo DNNF

In vivo, the extracellular brain concentrations after administration of 10 mg·kg^{-1} subcutane reached approximately 2 µM, irrespective of whether quantitated by the DNNF or MQ method. Both methods also demonstrated a similar rapid elimination of gaboxadol **(figure 3)**.

5.3.2 Citalopram

5.3.2.1 In vitro recovery

Similar to gaboxadol, the in vitro recovery of citalopram was increased when flow rates were decreased from 1 µl·min^{-1} to 0.1 µl·min^{-1} **(figure 4B)**. At flow rates below 0.2 µl·min^{-1}, recoveries exceeded 90%, thus achieving quantitative microdialysis. Similar to gaboxadol, absolute levels concurrently decreased a factor two.

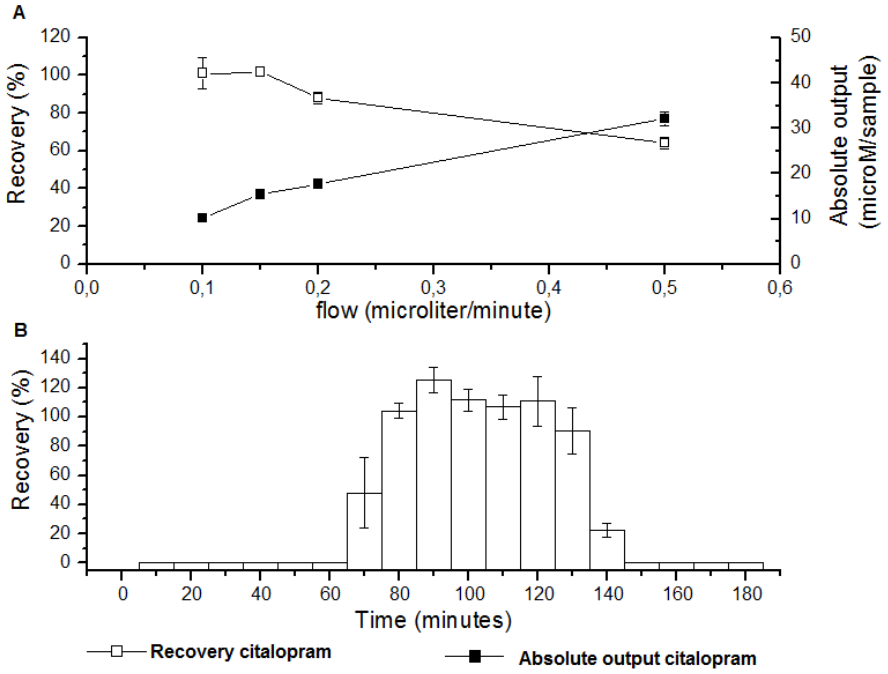

Figure 4 Comparison of absolute and relative recovery in vitro **(A)**, and dynamics of the sampling system to sudden changes in external concentrations **(B)**.

With citalopram the MQ system had a response delay of approximately 20% in the first 10 min sample, both to an instantaneous increase in external concentration as well as after returning the probe back into blank solution **(figure 4B)**.

5.3.2.2 In vivo loss

In vivo loss at slow flow with the MQ no citalopram could be determined in the dialysate when 100 nM was infused into the brain at 100 nl·min^{-1}. Given the lower limit of quantification of 1 nM and dilution factor of 10, the in vivo recovery should be at least above 90%.

In vivo loss during DNNF as quantified at t = 0 min using DNNF for a perfusion concentration of 200 nM was 28% ± 4.5% (n = 4). Interestingly, the outward recovery was only 9.1% ± 13% (n = 4) when the probe was perfused with 1000 nM.

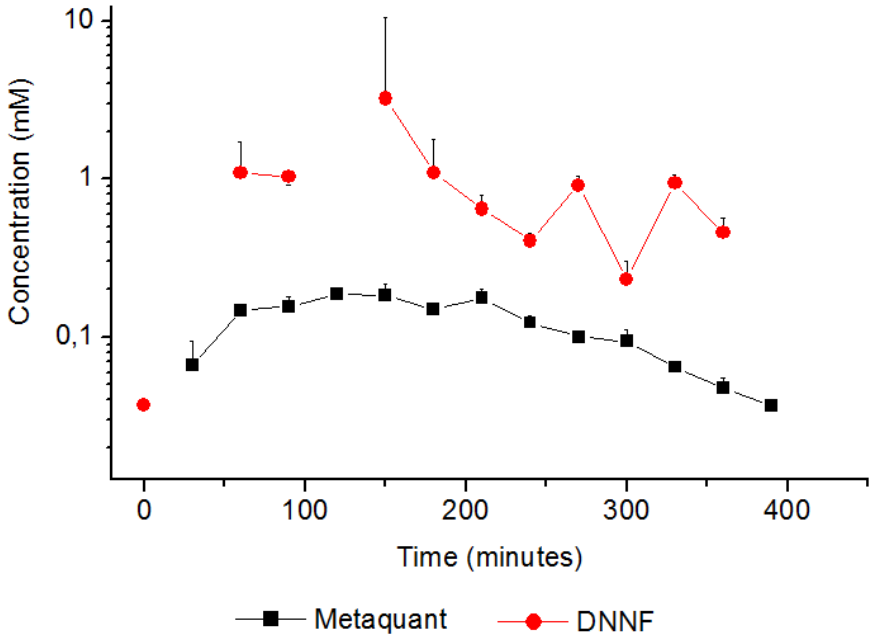

Figure 5 Free concentrations of citalopram in rat brain after administration of 7.5 mg·kg^{-1} subcutaneously, quantitated with DNNF or MQ techniques.

5.3.2.3 In vivo DNNF

In vivo, the extracellular brain concentrations after administration of 7.5 mg·kg^{-1} subcutaneously are different whether quantitated with DNNF or MQ methods. Whereas MQ data were consistent and reproducible, the data from DNNF formed a scattered pattern as the individual experimental groups yielded high levels of variation **(figure 5)**.

To plot extracellular concentrations from the DNNF experiment as a function of time it is necessary, for each time point, to fit a linear regression curve through the flux data from the different perfusate concentrations. This regression analysis sometimes generates negative brain concentrations, which obviously do not have a physiological meaning and therefore leave us with undesired gaps in the time plot. In addition, the estimated extracellular concentrations from the DNNF are more variable compared to MQ, also owing to their nature as aggregate statistic.

5.4 Discussion and conclusions

The research presented in this paper was designed to develop an advanced quantitative, highly practical and direct method for assessment of free brain concentrations of (new) CNS-targeted drugs. Quantitative microdialysis data are extremely useful in many research applications including, but not limited to, investigation of pharmacokinetic-pharmacodynamic relationships in drug development. The purpose of the current study was to improve the practical use of quantitative microdialysis, and thereby facilitate its worldwide deployment in experimental biomedical research.

To achieve these goals we modified the existing ultraslow-microdialysis method to take advantage of the very high in vivo recovery generated by ultraslow intra-luminal perfusion of the probe's membrane. The new element consisted of an after-probe-passage confluence of a carrier flow delivered at higher flow rate. While leaving the ultraslow-microdialysis exchange at the membrane unaltered, the higher flow rate of the combined outflows increased sample volume and decreased transit time between the probe's outlet and the collection vial. In the study reported here the performance of the modified ultraslow perfusion method was compared with DNNF in monitoring pharmacokinetic and pharmacodynamic parameters tested on two different pharmaco-therapeutic agents.

Although the DNNF method has since long been widely recognized as the "golden standard" among quantitative microdialysis methods, it comes with several major disadvantages.

The first disadvantage of the DNNF method is the required experimental design. For determination of every compound or compound dose a number of experimental groups (and hence often a large number of animals) is needed. This negates, at least in part, the inherent advantage of microdialysis for minimizing the use of living subjects. The method uses the principle of quantitating the extracellular levels by addition of the compound being studied to the perfusate in different concentrations. This implies that, besides the dialysis experiment without the compound of interest, a minimum of two more experimental groups are required in each of which the compound is added to the perfusate in a different concentration. This means that at least three experimental groups are required for every dose of a compound that is to be examined.

The second disadvantage of the DNNF method is caused by addition of the compound of interest into the perfusate, especially when the compound is added in the highest concentration. In that group a considerable net flow of compound into the brain occurs (typically between 10 and 20%). As most compounds being used are pharmacologically active, this is likely to induce changes in vasomotricity or blood-brain barrier permeability, and hence to distort the quantitation. Usually, the need to re-examine these issues in additional experiments further increases the total number of experimental animals required.

The third disadvantage is the low consistency of the method. In order to yield meaningful data on which inferences can be based, bioanalytical variation needs to be very small. In vivo recoveries, and hence actual extracellular levels, are quantified by flux differences into or out of the microdialysis probe. As recovery percentages typically range between 5 and 20% during regular flow microdialysis, the technique needs to be sufficiently sensitive to register small differences of only 5-20%. As normal bioanalytical variation up to 20% can be expected, this renders practical deployment of DNNF notoriously difficult.

Although generating quantitative results, handling of ultraslow-microdialysis at flow rates below 0.2 $\mu l \cdot min^{-1}$ is difficult on multiple levels and therefore too complex for standard application. When working with low volumes evaporation and longitudinal diffusion in

tubing become relevant issues that may skew dynamics of final readouts. Finally, an ultraslow flow cannot be combined with the use of swivels, because of their large intrinsic dead volumes. This would most likely preclude unattended in vivo experiments overnight.

However, all of the aforementioned disadvantages of ultraslow-microdialysis can be eliminated by the described after-probe-passage confluence of ultraslow and carrier flows. The dilution factor hence introduced can easily be corrected during bioanalysis.

5.4.1 In vitro recovery: flow dependence

Microdialysis is typically performed at flow rates of 1-2 µl·min^{-1}. At these flow rates extraction is far from quantitative, even in vitro. In addition, in vivo, the extraction is modified by perimembrane processes in the tissue like absorption, metabolism and uptake. These factors all contribute to the overall in vivo recovery, which hampers the predictive power of in vitro tests.

The relative recovery of a microdialysis membrane is known to be increased when the flow rate is decreased. Here we demonstrated that in vitro recoveries approached 100% at flow rates below 0.2 µl·min^{-1}.

5.4.2 In vitro dynamics

A meaningful detection method should rapidly respond to instantaneous changes in concentration of the external medium. Although only scarcely reported in literature [301] and limited to polar compounds like the monoaminergic neurotransmitters, these delaying effects are actually even more relevant when apolar compounds (like CNS-targeted ones) are being monitored.

Gaboxadol and citalopram are both specifically designed to penetrate the brain. Likewise their polarity is low with octanol partitioning factors above 3. Given their physicochemical properties, these compounds are prone to non-specific interactions with sampling materials, effects that should always be validated. We demonstrated here a functional delay of three min when probes were switched from blank ringer to standard solution or vice versa. Overall, it should be realized that there is a functional lag time of the system, apart from the regular lag time for

transport of sample through the outlet tubing to the collection vial, which might be caused by non-specific interactions with the membrane, tubing or probe material. As the time delay due to interaction with the dialysis "hard-ware" can be determined in vitro, it may accordingly be corrected for.

5.4.3 In vivo experiments

In vivo loss (retrodialysis conditions) confirmed the quantitative extraction of the compounds by the ultraslow dialysis method at flow rates of 100 nl·min^{-1}. Conversely, in vivo recoveries during DNNF as estimated by outflux prior to systemic drug administration of gaboxadol were around 11 and 17% for 1 and 10 μM, respectively.

In vivo, both sampling methods registered similar free brain concentrations of gaboxadol after subcutaneous administration of 10 mg·kg^{-1}. This supports their mutual validity. Arguably the DNNF method required approximately four times more animals and generated data with more variation. For citalopram however, the DNNF and MQ methods generated different results.

Citalopram data from the MQ method, but not from DNNF, were regular and even followed the same pattern as the free plasma concentrations in the later phases of the time plot. These findings are indicative of a compound that fully penetrates the brain by passive transport. Whereas quantitative extraction of citalopram using low flow dialysis was confirmed in vitro as well as in vivo, the DNNF method was hindered by undesired gaps in the time plot caused by negative brain concentrations generated by the regression analysis. In vivo recovery of citalopram was lower when citalopram was added to the perfusate at a higher concentration, namely 28% versus 9%. This indicates that influx of citalopram into the brain modulates the recovery.

5.5 Conclusion

The present study shows that modified ultraslow-microdialysis ("MQ") technique can be employed to quantify free brain levels without the need to perform complex approaches with an aggregate statistic. In

addition, it is important to note that this approach requires considerably fewer animals and even created a more robust measurement.

We therefore conclude that the modified ultraslow-microdialysis technique is an advanced quantitative method for assessment of free brain concentrations of (new) CNS-targeted drugs that has great potential for worldwide deployment in experimental biomedical research. Further validation by testing its performance on more compounds is both necessary and warranted the effort.

Chapter 6
Microfiltration sampling in rats and in cows: toward a portable device for continuous glucocorticoid hormone sampling

Abstract

To monitor temporal patterns of glucorticoids hormones in living animals most often blood samples are collected. Blood sampling is invasive and subjects may find it -in particular- unpleasant when multiple samples are collected. We have developed a microfiltration collection device (MCD) sampling continuously, pulse-free, over a selected period of time, with minimum invasiveness as the device is inserted with only one vena puncture. The MCD consist of a hollow fiber membrane (probe), capillary collection coil and flow creator. Three biocompatible hollow fibers were assessed on flow rate in rats, by placing the probe intraperitoneal, subcutane, and intravascular coated without and with heparin. The probe made from polyethylene coated with ethylenevinylalcohol-heparin conveyed the best results and had the most benefit of the heparin coating. Consequently this probe was built into a collection device and tested in cows. Cortisol (protein bound and free) could be monitored in cows over a period of 7 hours. This device has several major advantages compared to manual blood collection: minor stress is induced by the application of the device; it has a low weight and can therefore be used in freely moving subjects being in their own surrounding; it can be sterilized and can be manufactured as a disposable, and the filled MCD can be shipped by regular mail to specialized laboratory facility for analysis.

Keywords

continuous sampling system, in vivo, monitoring, cortisol, proteins

6.1 Introduction

To monitor metabolic processes in living organisms most often blood samples are being collected but other body fluids may be sampled as well, e.g. saliva, cerebrospinal fluid (CSF) and urine. Occasionally subcutaneous and intraperitoneal sampling has been applied. Sampling of blood is invasive and subjects may find it in particular unpleasant when multiple samples are collected. Another disadvantage is that blood sampling must be performed in a hospital or laboratory setting. We have developed a device to collect biomedical samples continuously in freely moving subjects [135, 271]. Our microfiltration collection device (MCD, **figure 1A-B**) allows sampling over a selected period of time with minimum invasiveness, as the device has to be inserted once.

During microfiltration a body fluid, containing the analytes of interest, is transferred over a semi-permeable hollow fiber membrane due to a pressure gradient over the probe membrane. The maximum size of particles that pass the membrane is not only determined by the molecular weight cut-off value (MWCO) of the hollow fiber membrane, but also by the configuration and the charge of the analyte. The trans-membrane pressure gradient together with the fluid restriction (inserted in the under-pressure pump, see **figure 1A-B**) different flow rates can be realised. In vivo the membrane surface area, the hydraulic resistance, protein layer on the membrane surface, and environment may influence the flow rate as well. Previously our group optimised ultraslow-microfiltration for hydrophilic analytes such as lactate, glucose, proteins and various drugs [135, 271].

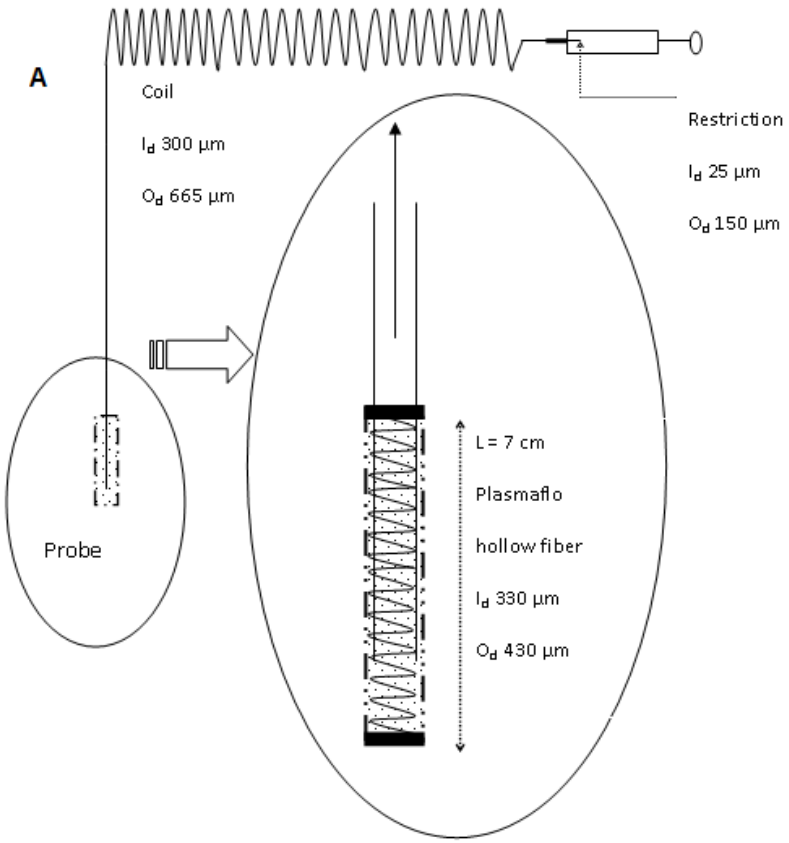

Figure 1A: Schematic design of the microfiltration collection device (MCD). The hollow fiber membrane (probe) is connected to the fused silica collection coil and the coil is connected to the 9 mL S- Monovette negative-pressure pump. Only the probe is placed in vivo.

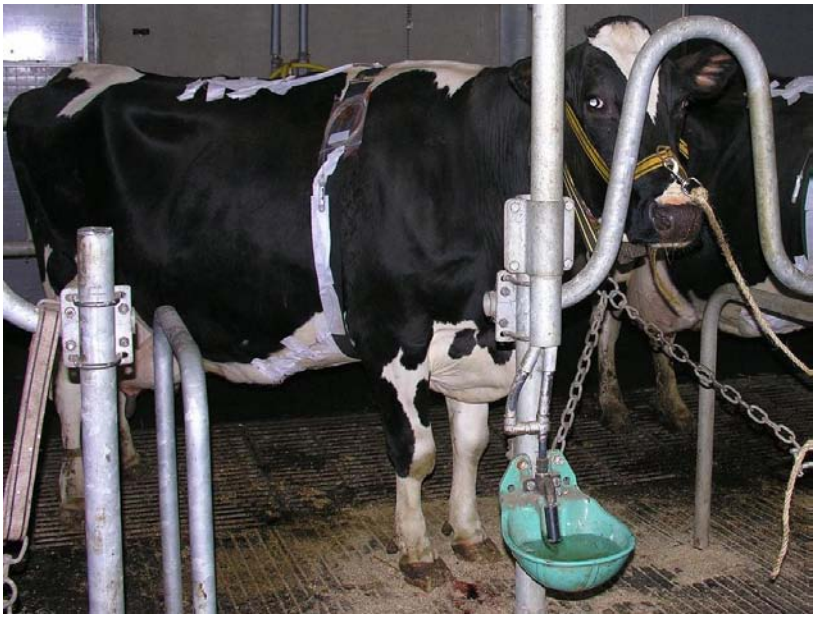

Figure 1B Magnification of the microfiltration probe consisting of the hollow fiber membrane, spring and restriction tube. The restriction specification gives in combination with the negative-pressure according to Poiseuilles law.

Figure 1C MCD attached to the cow sampling the udder vein.

The current study focuses on microfiltration-sampling of the lipophylic stress hormones cortisol (in cows and human) and corticosterone (in rodents). Both glucocorticoid hormones are released into the blood stream following activation of the hypothalamic-pituitary-adrenal axis and exhibit circadian rhythms. To explore the temporal patterns of glucocorticoid hormones, multiple blood samples have to be collected. In blood cortisol is bound to the proteins transcortin and albumin for 90%, with molecular weights of 48 kDa and 68 kDa, respectively. Only 10% is free cortisol and biologically active. For the measurement of total (protein-bound plus free) cortisol it is necessary to sample albumin and transcortin also. We tested (unpublished results) the polyethylene microfiltration probe coated with ethylenevinylalcohol probes (not coated with heparin) and noticed that sampling was not possible longer than 1.5-2 hours. Therefore we investigated other hollow fiber membranes **(table 1)** and assessed also if a heparin-coating improved microfiltration.

All tested hollow fiber membranes posses MWCO values ≥0.3 µm, so 98% of the high molecular weight (HMW) blood proteins are microfiltered [135]. To our knowledge there are only five other studies describing the sampling of proteins [130-133, 274] using microfiltration. These sampling techniques are comparable with ours, but without the storage device. A similar device was tested in broiler chickens sampling continuously the low molecular weight (LMW) analytes glucose and lactate [271]. The hollow fiber used in the latter sampling device had a MWCO value of 20 kDa and allowed sampling over a time period of eight hours intravenously (IV) and subcutaneously (SUB).

Table 1 Specifications of the microfiltration hollow fibers, provided by the manufacturing companies. The three hollow fibers were all tested without and with heparin coating.

specifications	plasmaflo OP-05W(L)	microPES TF10	transvivo
company	Asahi	Membrana	Transvivo
hollow fiber material	polyethylene coated with ethylenevinyl-alcohol	polyethersulfone	polyethersulfone
pore size (μm)	0.3	0.5	0.58
outer diameter (μm)	430	500	730
inner diameter (μm)	330	300	230
wall thickness (μm)	50	100	250

The present study shows that the microfiltration hollow fibers can be placed in various locations in vivo in rats: SUB, IV, and intraperitoneal (IP) and in the cow (IV). To our knowledge IP-microfiltration has not been described before. We optimised the MCD for cortisol (in the cow) and corticosterone (in the rat) by assessing microfiltration flow rates during long-term sampling. The probe with the best performance on sampling rats IV was implemented in the MCD and tested in cows.

6.2 Experimental

6.2.1 Animals

Approval for carrying out the animal experiments was obtained from the ethical committees of the University of Groningen (experiments with rats) and the Animal Sciences Group of Wageningen UR (at Lelystad, the Netherlands). A minimal number of animals were used and efforts were done to avoid suffering.

Male Wistar rats (280-350 g, Harlan, Zeist, the Netherlands) were used for the experiments. Rats were individually housed in plastic cages (30x30x40) and had ad libitum access to food and water. The adult dairy cows were older than 4 years and of the Holstein Friesian breed. They were taken out of the flock for a working day and restraint by a neck band in a stable. They were fed by silage and hay and were able to drink water. These cows are not conditioned on handling by people as milking is performed by a robot.

6.2.1.1 Surgery rats

Rats were anesthetized using isoflurane (2%, 400 ml·min$_{-1}$ N_2O, 400 ml·min$_{-1}$ O_2), the probes were inserted via a sterile catheter (BD Insyte, 14 Ga, Becton Dickinson) SUB and IP. The IV-probe was inserted into the isolated right jugular vein. During the whole experiment rats were kept anesthetized. Via the tail vein 25 IU of heparin was given and the rats were kept at 37°C during the whole experiment. For each experiment a fresh hollow fiber was used and flushed with ultra-pure water with 50 IU·ml^{-1} of heparin. After inserting the probe 30 minutes fractions are collected into vials and weighed. The increased weight divided by the sampling time lead to a calculated flow rate (µl·min^{-1}).

6.2.1.2 MCD sampling cows IV assessing cortisol

In the cow experiments, the microfiltration probe was inserted in the udder vein via a sterile intravascular catheter (Braunule MT, 4/G12, l = 8 cm, B. Braun, Germany). The MCD was inserted in a plastic bag and the bag was attached to an elastic belt wrapped around the cow **(figure 1B)**. Before sampling cows in vivo, restrictions were tested in vitro. The coils were emptied into different fractions by attaching the coil to a syringe pump (Harvard) using a flow rate of 4 µl·min^{-1} in opposite flow direction compared to in vivo sampling. Comparing sampling time, flow rate determined in vitro and the criterion of clear microfiltration-samples (indicating absence of blood and wounds) versus ultrapure (UP) water volume in the collection coil gave insight if the device worked properly. Cortisol and corticosterone were quantified with a radioimmunoassay. Biofilm sediment onto the membrane was investigated with the electron microscope [311].

6.2.2 Microfiltration probes and MCD materials

6.2.2.1 Microfiltration sampling rats – IP, SUB and IV

The microfiltration probe was composed of a hollow fiber connected, via fused silica tubing (FST), to a syringe pump and samples were manually collected by emptying the syringe every 30 minutes. The hollow fiber was cut into a 4 cm piece, for SUB and IP sampling. A fiber of 1.5 cm was placed in the jugular vein. A spiral spring (stainless steel wire, diameter = 60 µm, 170-200 µm spiral diameter, Vogelsang, Hagen, Germany) was inserted into the hollow fiber to prevent hollow fiber collapsing and also to reduce the dead volume of the probe. FST (TSP100170; outer diameter = 170 µm, inner diameter = 100 µm, l = 30 cm, Polymicro Technologies Inc, Phoenix, AZ) was inserted into the probe up to 2 mm from the tip. Hollow fiber, spiral, and fused silica tubing were fixated with cyanoacrylic glue (Ruplo B.V., Ten Boer, the Netherlands). The microfiltration probe was connected to a syringe pump with a flow rate of 0.3 µl·min^{-1}, collecting volume fractions of 30 minutes for 5-6 hours.

6.2.2.2 MCD sampling cows

The MCD **(figure 1A)** consisted of: the plasmaflo OP-05W(L) microfiltration heparin coated-hollow fiber (Asahi Medical Co., Ltd., Tokyo, Japan), FST coil (Polymicro Technologies Inc., Phoenix, AZ) and the monovette under-pressure pump Sarstedt B.V.). The hollow fiber was cutted into 7 cm long piece and a spiral (stainless steel wire, diameter = 60 µm, 170-200 µm spiral diameter, Vogelsang, Hagen, Germany) was inserted into the probe to prevent collapsing. FST of 30 cm (TSP100170; inner diameter = 100 µm, outer diameter = 170 µm) is inserted until a distance from 2 mm into the tip of the hollow fiber. The collection coil (FST300665; inner diameter = 300 µm, outer diameter = 665 µm), connecting the sampling part to the under pressure pump, had a length of 600 cm, storing a volume of 424 µl. With a flow rate in the range of 0.5-0.7 µl·min^{-1}, 606-848 minutes (= 10-14 hours) can be sampled continuously. The pump consists of a disposable syringe (Monovette 9 ml, Sarstedt B.V.). In the pump a 5.7 cm restriction FST tubing (TSP025150; inner diameter = 25 µm, outer diameter = 150 µm)

is glued to ensure a constant flow rate of 0.637 µl·min^{-1} as calculated by Poiseuilles law [232].

The different sampling parts are linked together with cyanoacrylic glue (Ruplo BV, Ten Boer, the Netherlands). The entire system was filled with UP water and tested in vitro on continuous sampling and assessing flow rate, before sampling in vivo.

6.2.3 Heparin coating procedure hollow fibers

Multilayer thin films were deposited on the cleaned hollow fibers using a dip technique, at room temperature, from a polyelectrolyte solution. First, the fibers were dipped in an aqueous solution (2 mg·ml^{-1}) of polyethyleneimine in borate buffer pH 9 for 20 min, rinsed with water, dried with a stream of nitrogen and then dipped into an aqueous solution (10 IU·ml^{-1}) of heparin for 60 min, followed by rinsing with water and drying with a stream of nitrogen. This procedure was repeated once, resulting in self-organized multilayer films [300].

6.2.4 Temperature effects on cortisol levels

During sampling the MCD will warm up till body temperature as de device is attached near to the skin. Also implementation of immediate analysis is not always an option, therefore collection coils need to be stored in the fridge. The effect of storage at different temperatures assessing cortisol deterioration was determined by storing cow plasma samples for 0-100 hours at 4°C and at 37°C to mimic body temperature. The plasma sample was collected in a separate experiment and fractionated storing the vials under different experimental conditions.

6.2.5 Presentation of data

In vivo flow rates in rats were tested by sampling fraction of 30 minutes with a flow rate of 0.3 µl·min^{-1}. Fractions were weighted and divided by sampling time (n = 3) calculating the theoretical flow rate value. Each probe -heparin coated and uncoated- tested IP, SUB and IV, one EM photograph was made. Four different cows were sampled and the in vivo flow rate was calculated by total volume of biofluid in the collection coil divided by sampling time.

6.3 Results and discussion

6.3.1 Semi-continuous microfiltration sampling in rats

Figure 2 shows the flow rates of microfiltration probes placed IV **(upper)**, SUB **(middle)** and IP **(lowest)**. Overall taken, the heparin coated probes sampled better than the uncoated probes. A heparin coating is usually aimed to improve haemocompatibility [334], so assessing heparin coated hollow fibers SUB and IP seems peculiar. The improved performance in these compartments is likely due to residual bleeding that is unavoidable following the insertion of the probe, but may also to be attributed to the property of heparin to inhibit sticking of other proteins to the probes. As expected the flow rates of the IV placed probes exhibit the largest difference between uncoated/coated probes. The PE-EVAL-heparin coated microfiltration probe performance is superior over the PES (transvivo and microPES) probes, possibly an effect of the PE-EVAL probes being more hydrophilic. In the SUB compartment the rate of sampling declines over 6 hours in contrast to the IP compartment **(figure 2)**. Less extracellular fluid is available SUB and because of this the SUB placed probe is more susceptible to a declining flow rate due to the anesthesia reinforced dehydration. Kissinger et al. concluded from their microfiltration experiments that an anesthesized rat produces almost no SUB fluid in contrast to a freely moving rat that has free access to water and food [172]. Unlike possible side effects due to anaesthesia, microfiltration is still not applicable in some tissues, including the rat brain or human subcutaneous compartments, even at ultraslow flow rates of 50-100 $nl \cdot min^{-1}$, presumably because of insufficient production of extracellular fluid [160].

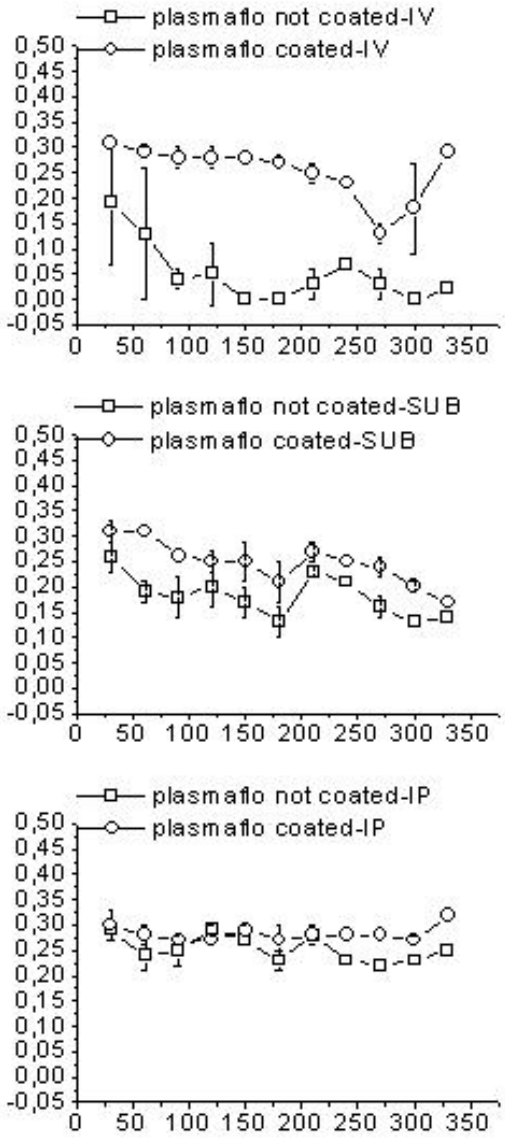

Figure 2A Flow rates during in vivo sampling in the anaesthetized rats: effects of location and plasmaflo probe sampling intravascular **(upper)**, subcutaneous **(middle)**, and intraperitoneal **(lowest).**

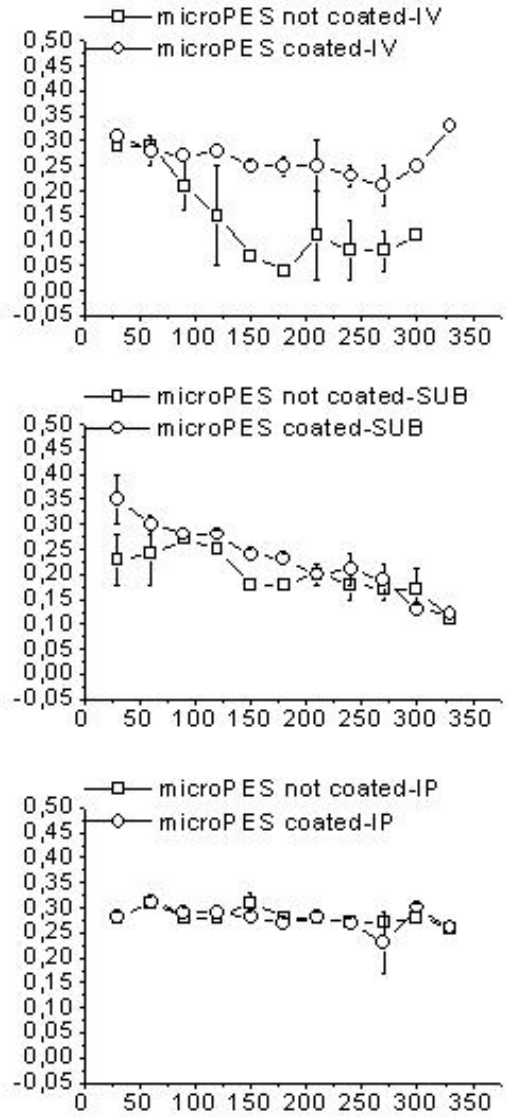

Figure 2B Flow rates during in vivo sampling in the anaesthetized rats: effects of location and microPES probe sampling intravascular **(upper)**, subcutaneous **(middle)**, and intraperitoneal **(lowest).**

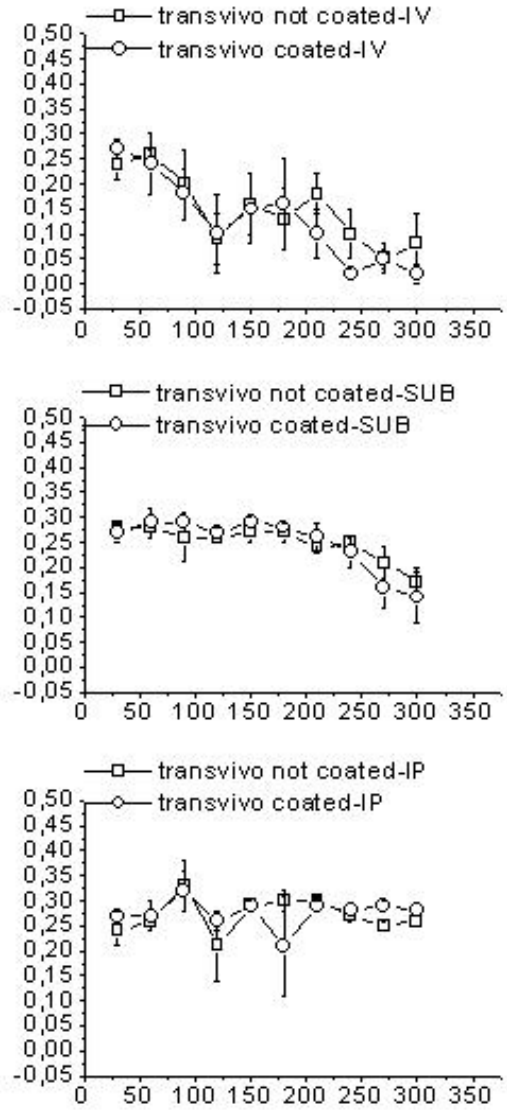

Figure 2C Flow rates during in vivo sampling in the anaesthetized rats: effects of location and transvivo probe sampling intravascular **(upper)**, subcutaneous **(middle)**, and intraperitoneal **(lowest).**

6.3.2 Biofouling

Biofilm sedimentation was investigated with the electron microscope. The photographs **(figure 3)** affirmed that the coated plasmaflo hollow fiber is the least prone to biofouling. The electron microscope photographs did not show clear differences in biofouling between the heparin coated/uncoated hollow fiber membranes. So the positive effects of heparin coating could not be supported by biofouling, as revealed with electron-microscopy. Not only heparin coating, but also the small MWCO of the plasmaflo may reduce biofouling as compared to the other membranes. Several haemodialysis studies described hollow fiber biofouling [80, 117, 330]. Our study led us to the conclusion that membranes with the smaller pore sizes provide superior sampling stability in blood, as compared to membranes with larger pore sizes. Presumably proteins clog easier to the hollow fiber membranes with bigger pores [80, 117].

Biocompatibility studies (fiber-tissue reaction) are often performed SUB in rats. Clark et al. [47] harvested hollow fiber membranes made from polysulfone (PS) and cellulose diacetate (pore sizes = 0.2 µm) after 3, 6 and 12 weeks of implantation. All probes showed extensive deterioration, but most decayed was the PS membrane with a pore size of 0.65 µm. The mixed ester cellulose with pore size 0.2 µm and regenerated cellulose (MWCO = 3 kDa) retained their integrity for 12 weeks without deterioration over time [47]. Although the PS fiber (MWCO = 10 kDa) remained intact, it was infiltrated by inflammatory cells and, together with the regenerated cellulose fiber (MWCO = 13 kDa), had the thinnest fibrotic capsule formation [47]. Expanded polytetrafluoroethylene (e-PTFE) membranes were harvested after 4, 10 and 21 days of implantation [353]. This material did not deteriorate (pore size or MWCO value was not mentioned). Brauker et al. [32] tested the same material in a similar setup with a pore size large enough to be completely penetrated by host cells (0.8-8 µm) comparing this with PTFE membranes with pore size of 0.02 µm. The membranes with larger pores were invaded 80-100 fold more with vascular residues. Apparently, probes with the larger pores have a larger surface that can be reached by proteins. These studies taken together indicate that both pore size (especially big pores)

and polymer characteristics should be considered for future biomedical applications of microfiltration probes.

The heparin coated plasmaflo probes convey the best performance on flow rate **(figure 2)** and biofilm deposit **(figure 3)**. Consequently we continued, testing the heparin coated plasmaflo probe building it into a MCD examining MCD sampling time and flow rate in vivo in cows.

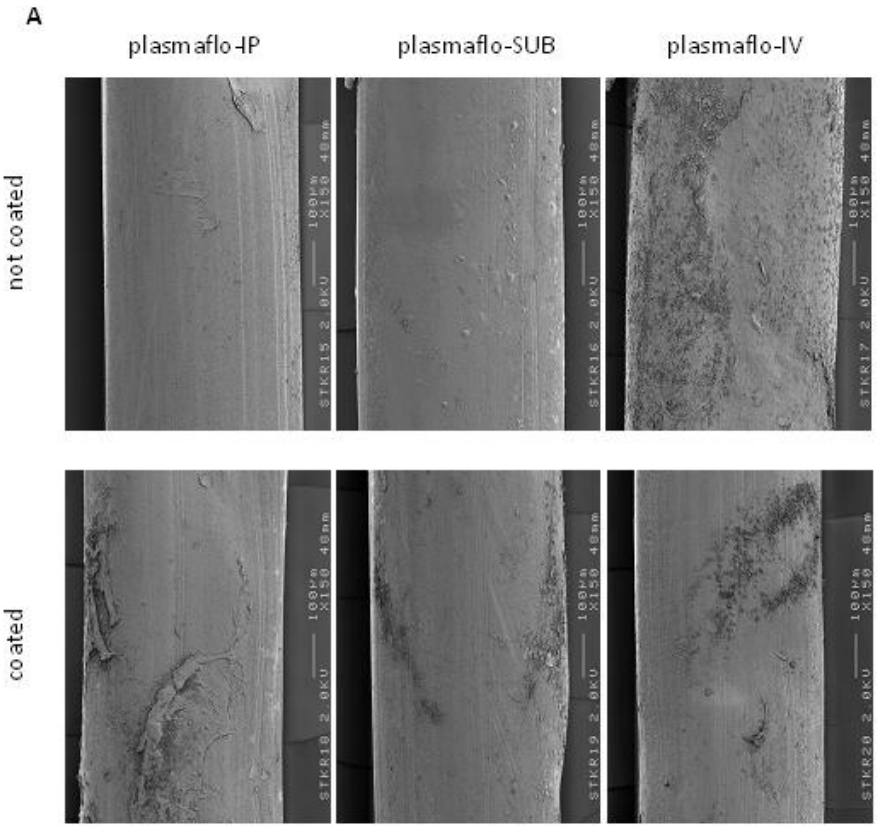

Figure 3A Plasmaflo probe quality following in vivo sampling in the anaesthetized rats: effects of location and probe membranes. Locations tested were intraperitoneal **(left)**, subcutaneously **(middle)**, and intravascular (right), assessing none coated **(upper panels)** versus heparin coated **(lower panels)**. Photograps plasmaflo **(A)**, microPES **(B)** and transvivo **(C)** probes.

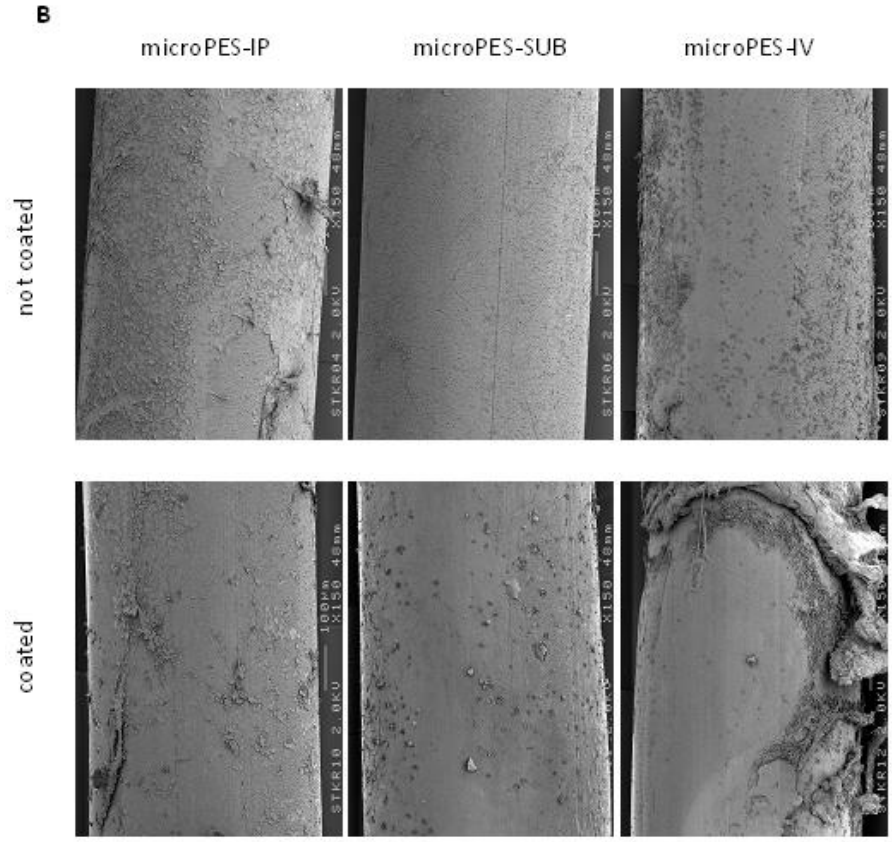

Figure 3B MicroPES probe quality following in vivo sampling in the anaesthetized rats: effects of location and probe membranes. Locations tested were intraperitoneal **(left)**, subcutaneously **(middle)**, and intravascular (right), assessing none coated **(upper panels)** versus heparin coated **(lower panels)**. Photograps plasmaflo **(A)**, microPES **(B)** and transvivo **(C)** probes.

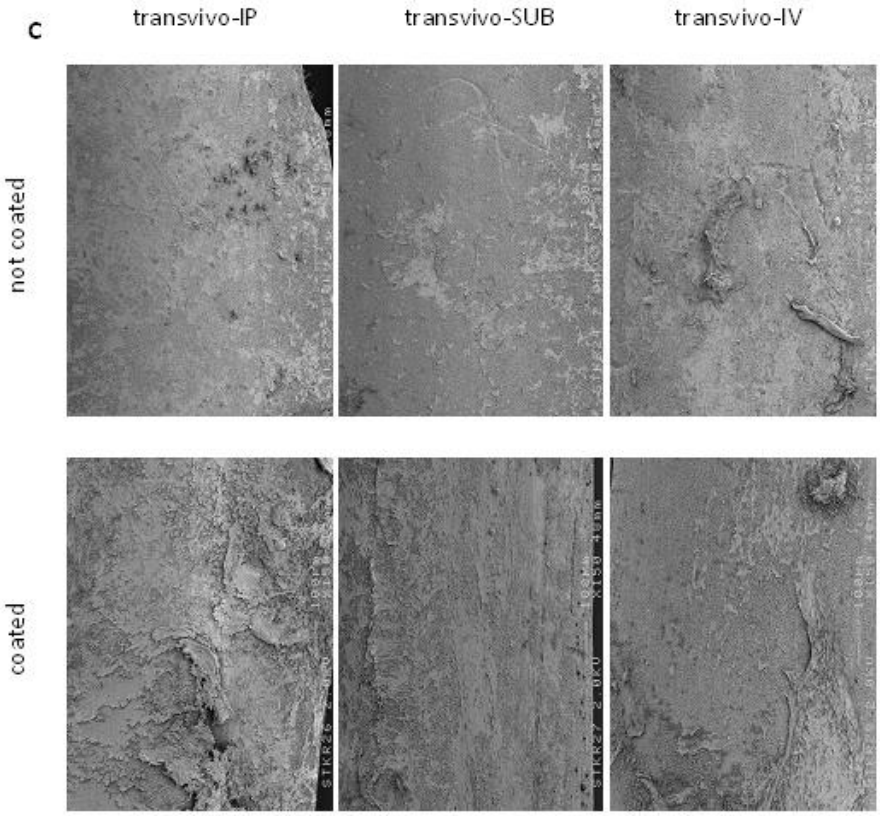

Figure 3C Transvivo probe quality following in vivo sampling in the anaesthetized rats: effects of location and probe membranes. Locations tested were intraperitoneal **(left)**, subcutaneously **(middle)**, and intravascular (right), assessing none coated **(upper panels)** versus heparin coated **(lower panels)**.

6.3.3 Temperature and cortisol levels in the MCD

No significant temperature effect was seen on the levels of cortisol in plasma **(figure 4)**. Previously our group assessed diffusion and decay of corticosterone in fractions of rat plasma factions stored in a collection coil. Corticosterone diffusion in a complex plasma samples was insignificant and corticosterone did not deteriorate by storing the coil for 3 days at 4°C and 37°C (submitted manuscript). The MCD is thus applicable for both glucocorticoids.

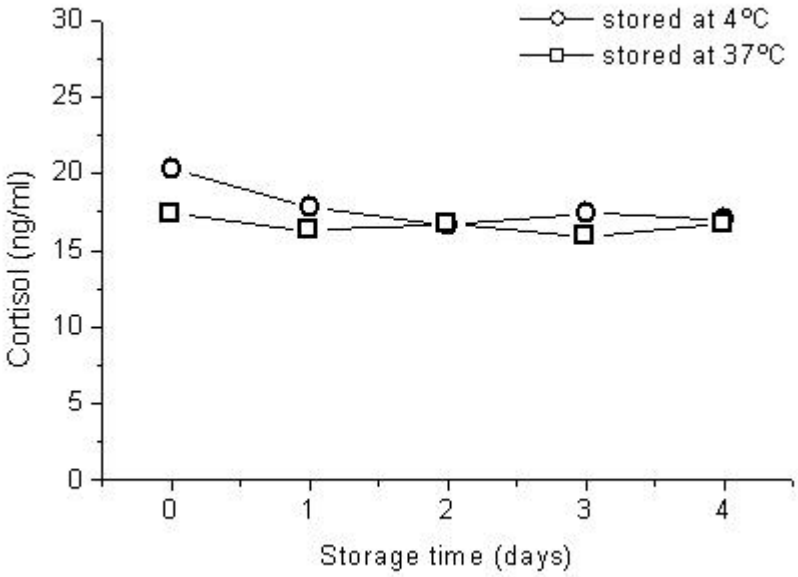

Figure 4 Time and temperature effects on the levels of cortisol stored in vials.

6.3.4 In vivo versus in vitro flow

The flow rate is specified by the restriction dimension along with pressure gradient over the probe membranes and the viscosity of the sampled biofluid. The used restrictions were tested in vitro and induced a flow rate 0.7 µl·min^{-1}. The restriction is 5.7 ± 0.1 cm long and the internal diameter is 25 ± 2 µm. Calculating the expected flow rates by taking into account an average diameter (± SD) of the restriction, resulted in values with an interval of 448-882 nl·min^{-1} (n = 15; average 649 nl·min^{-1}). The in vivo flow rates were significantly lower compared to the in vitro flow rates. The collected bio-fluid viscosity is bigger than UP water and therefore the flow resistance is higher over the hollow fiber membrane and in the capillary collection tubing. **Figure 5** shows the different flow rates as determined by the restriction used in cow 4. The restriction was tested alone in vitro continuously as shown in **figure 5A**. Then the restriction from **figure 5A** was build into an MCD and tested in vitro over 6 time frames with a maximum of 24 hours. The in vivo value is shown in **figure 5C**. The flow rates of the experiments

shown in **figure A, B and C** (± SEM) were 702 ± 9.2, 622 ± 20.7, and 476 nl·min^{-1}, respectively. As shown in **figure D**, the flow is still 559 ± 36.9 nl·min^{-1} after 8.2 days in vitro. MCD using an ultraslow flow rate in Broiler chickens sampling SUB and IV using a hollow fiber made of acrylonitrilsodium methallyl sulfonate with a MWCO of 20 kDa the in vitro tested flow of 50 nl·min^{-1} was not significantly different from the in vivo flow of 46 nl·min^{-1} [271]. These studies emphasize that the in vitro installed flow rates based on ultra-pure water do predict the flow rates in probes with smaller pore sizes well, but do not predict precisely the in vivo flow rates when using probes with big pores.

6.3.5 MCD in the cow

Four cows were sampled with the MCD. At first we tried to place the MCD in the jugular vein, but this vein was unsuited as this vein could not be fixed properly: it “roles”. Fixating the neck gives immobilization stress and, moreover, the neck is a relatively sensitive area so, the placement of the probe through an intravascular catheter could produce additional stress, thus affecting circulating cortisol levels. The udder vein is an attractive alternative for sampling: the vein is at the surface of the skin in a less sensitive location and the cow is used to handling this area.

At first a plain plasmaflo (without heparin coating) was tested in vivo. Sampling lasted only 1.5-2 hours (data not shown). The MCD tested in the cows were ended manually at the end of a working day. All the heparin coated MCD’s allowed sampling up to 7 hours **(figure 5)**, thus significantly longer then the heparin uncoated plasmaflo hollow fiber.

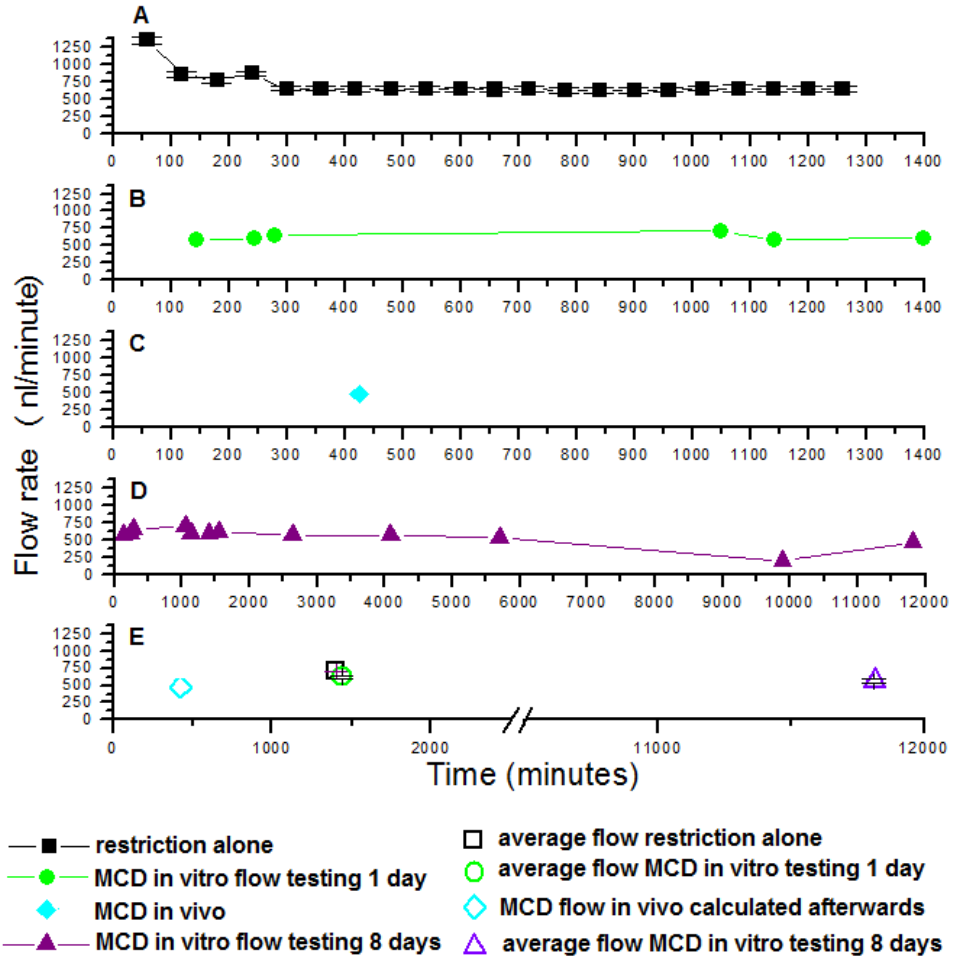

Figure 5 Flow rate assessed for the restriction alone **(A)**, build into a MCD device sampling ultra-pure water in vitro **(B)** and in **figure C** the in vivo calculated flow rate for maximum time frame of 1 day and 8.2 days in **figure D** for MCD tested in vitro. The average calculated flow rates from figure A, B, and the in vivo value of C are plotted in **figure E**.

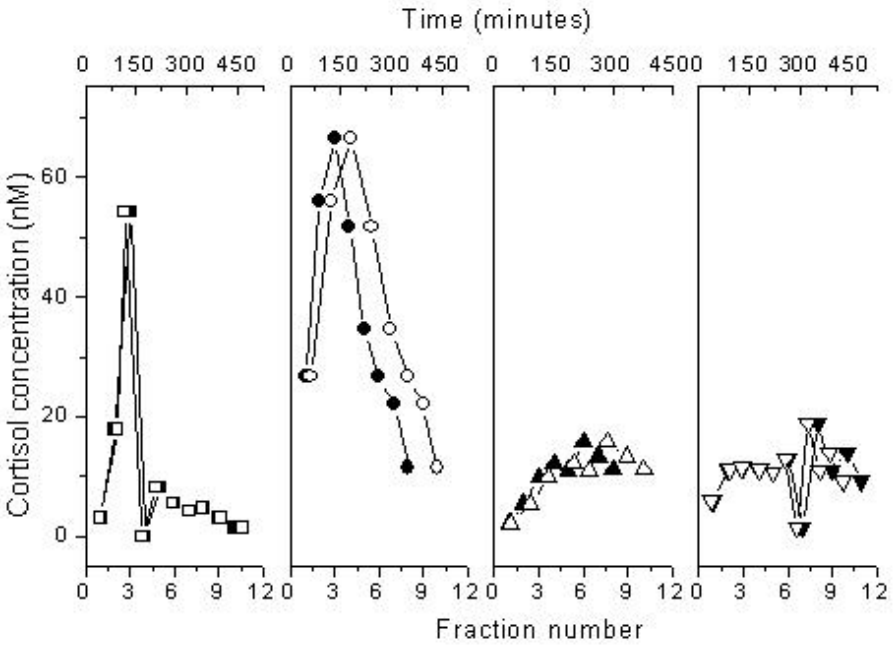

Figure 6 The levels of cortisol in storage coil applied to cows 1, 2, 3, and 4 (for the details of each cow see text), shown in **A**, **B**, **C**, and **D**, respectively. The volume fractions are given on the lower X-axis; the upper x-axis shows the corresponding sampling time.

The time profiles of cortisol are plotted in **figure 6**. At the lower x-axis the numbers of subsequent fractions of 15-25 µl of biofluid obtained from in the storage coil are shown. The upper x-axis shows the time as calculated by dividing the fraction volumes by the flow rate. Alternately the flow rate is calculated by the total biofluid volume (by weight) divided by duration of microfiltration, thus assuming a constant flow. The flow rates were for cow 1, 2, 3 and 4, respectively 603, 472, 523, and 476 nl·min^{-1}.

Cow 1 convey a short lasting cortisol peak **(figure 6A)**, presumably induced by the stress of fixation as we first tried to implant the probe in the jugular vein. Cows 2 and 3 were sampled simultaneously (data in **figure 6B-C**). In addition to the IV-MCD, a SUB-MCD was placed. Cow 2 was more restless and therefore the fixation of SUB-MCD was more difficult than with cow 3. In both cows SUB-MCD sampling was unsuccessful at a flow rate of 0.7 µl·min^{-1}. The cortisol profile from cow 2 depicts a "hill" of cortisol levels over a long time interval. Cows 3 and 4 were rather calm in line with the cortisol profiles shown in **figures 6C** and **6D**. Hopster et al. [123] collected single blood samples in cows used to handling between 1600 and 1700 hours for 6 successive days and multiple samples with 15 minute intervals between 1100 and 1200 hours in cows not accustomed to intensive handling. In the first experiment average cortisol value was 2.6 ± 0.24 ng·ml^{-1} for the latter experiment cortisol values ranged from 4.5 to 22.6 ng·ml^{-1}. Handling experience and personal traits of the cows differed in the latter experiment, which may influence cortisol levels.

Cow 1, 3, and 4 were calm and basal cortisol levels lie in range of 0-6 ng·ml^{-1}. Cow 2 was stressed and this is affirmed by the cortisol profile. Hopster et al. [123] concluded that one puncture -taken within 1 minute- does not affect cortisol concentration but successive jugular puncture may induce an increase in cortisol concentration. The Holstein-Friesian cows used in this experiment were not accustomed to handling and are sampled for 7 hours with one venapuncture. With this device more insight is gathered about cortisol over a longer time-frame as compared with the study performed by Hopster et al. [123].

6.4 Conclusions

Microfiltration have only occasionally been applied since it first description in 1987 [154]. It was applied in various species. In rat [197, 204-206], cat [146], dog [28], horse [58, 291], pig [309], chicken [271], mouse [152], and sheep [145, 153, 289] microfiltration has been applied to various tissues, including subcutaneous tissue [28, 58, 145, 146, 152, 204-206, 206, 271, 291], adipose tissue, blood [196, 197, 271, 309], bone [145, 153, 289] and muscle [145, 153, 289, 291] to monitor LMW compounds such as lactate [196, 197, 271, 307, 309], glucose [146, 152, 196, 197, 271, 307, 309] and electrolytes like magnesium, calcium, phosphorus, chloride, sodium,

potassium [145, 153, 205, 291] and other analytes/drugs [28, 58, 204, 206]. Only one study described microfiltration in human subcutaneous tissue monitoring glucose and lactate [307]. Most hollow fiber membranes have MWCO values <20 kDa, excluding macromolecules like proteins, peptides and cellular elements. However macro-analytes such as cytokines and peptide hormones may be interesting to sample systemically and/or in local tissues [120, 129, 130, 132, 133, 138, 208, 346]. All in vivo studies sampling was discontinuous except for the MCD study applied in Broiler chickens [271].

The present study illustrates the potential of the MCD sampling rats and cows with minimum invasiveness as the device is applied with one manual action. In Broiler chickens [271] the small hydrophilic analytes - glucose and lactate- were collected continuously with a LMW cut-off value hollow fiber membrane (20 kDa). Application of the MCD induces minor if any stress and allows continuous sampling for periods of at least 7 hours. The MCD has the potential to be used for in vivo monitoring of many compounds with different chemical properties, including the stress hormone cortisol with short biological halve lives bound to big proteins. Here we measured total glucocorticoid levels because of the very small free fraction of the hormones. Improving analytical power, sampling of free LMW analytes using appropriate membranes might also become possible. Further advantages of the MCD are its low weight, it can be sterilized, it can be manufactured as a disposable and the filled MCD can be shipped by regular mail to specialized laboratory facilities for analysis.

6.5 Acknowledgements

This research is supported by the Dutch Technology Foundation STW, applied science division of NWO and the Technology Program of the Ministry of Economic Affairs, grantnumber 06038.

Chapter 7
Microdialysis and microfiltration: technology and cerebral applications for energy substrates

Abstract

This chapter is divided into four parts:

1. Sampling methods, detection technologies and quantitative aspects of monitoring cerebral energy substrates in living brain, mainly glucose and lactate, are reviewed, including microdialysis, microfiltration and biosensors. Advances in the field, including MQ sampling, in situ microsensors, as well as choice of hollow fiber materials for microdialysis and microfiltration are discussed.

2. Experimental and clinical applications of cerebral microdialysis of glucose and lactate are reviewed. Effects of drugs, physiological stimuli and pathological conditions (e.g. hypoxia, ischemia, head or spinal cord trauma) are discussed. For clinical monitoring in neurointensive care low time resolution microdialysis at 0.5-1 minute intervals and on-line detection by a biosensor assay may provide immediate information on the metabolic consequences of therapeutic interventions during surgery.

3. The significance of intracerebral trafficking of glucose and lactate in the interstitial space is reviewed and addressed in some detail in an experimental study. In this study ultraslow-microdialysis and reverse dialysis of exogenous glucose or lactate is used to assess the relative glucose and lactate turnover at steady state. It is concluded that only about 12% of the brain's energy substrates traffick through this compartment.

4. Finally, an attempt is made to link our current knowledge of brain energy metabolism to physiological events in the brain. In this context we discuss the concepts of metabolic and potential brain energy. Potential brain energy, defined as the large ionic gradients over neuronal membranes and storage of glutamate and GABA in synaptic vesicles, is considered a necessary condition for fast neuronal processing, whereas metabolic brain energy (i.e. consumption of glucose and oxygen) serves to restore high levels of potential energy.

Keywords

energy substrates, glucose, lactate, in vivo, sampling techniques , microdialysis, microfiltration, biosensors, clinical microdialysis, brain, metabolic energy, potential energy

7.1 Introduction and scope

The brain consumes -relative to its weight- a major part of an organism's energy budget. The human brain, for instance, 2-3% of the whole body weight, consumes more than 20% of the energy of the entire organism at physical rest [15]. So it is obvious that the unique properties of the brain, e.g. to register, store and integrate sensory information, and to initiate and control behaviour, e.g. motor activity and thinking, requires much energy. Already in 1890, the tight coupling between neuronal activities and cerebral blood flow, known as functional hyperemia, was recognized [267], and this coupling has been suggested to enable the extra supply of energy substrates. More than 50% of the brain's energy consumption is used for excitatory synaptic activity and action potentials [15]. This does not necessarily imply that only glucose directly derived from the circulation is used for excitatory transmission, since – theoretically – also indirect glucose use via lactate uptake from the intercellular space and subsequent consumption by neurons and/or glia may play a role, as will be discussed. Brain energy stores are insufficient to support brain activity for longer than a few minutes [5, 15, 180], as was estimated following experimental interruption of cerebral blood flow. If glucose and oxygen are derived from the blood circulation or formed from rapidly turning over cerebral intermediates, always (approximately) 6 molecules oxygen per molecule glucose are consumed. If there are intermediate substrates leaving the brain (as may occur with lactate), or if the contribution of brain energy reserves (e.g. glycogen, predominantly localized in astroglia) are significant, this ratio becomes less or more than 6, respectively. Under normoxic conditions the ratio is about 5.5, indicating that a small, albeit significant, fraction of the glucose is not aerobically metabolized, either stored as glycogen [99, 113, 241] or leaving the brain (by the bloodstream) [195, 229]. The possible importance of glycogen as an in vivo energy source must be questioned. For instance 20 minutes of visual stimulation does not result in detectable glycogen utilization in the visual cortex [241]. The various cerebral cells, including several types of neurons and glia, are differently positioned towards the blood circulation.

In most current models energy substrates enter the cells following passage of the blood-brain-barrier, consisting of endothelial cells, to glia and some neurons. Several brain cells have no direct connection to blood vasculature and the energy substrates will reach these cells only indirectly, i.e. their energy supply depends entirely on the transport via cells that have direct access to the blood. Both aerobically or an-aerobically intra-cellular production of energy metabolites leave the cell via trans-membrane transport directly or indirectly, i.e. via other cells, presumably most often glia. Trafficking of substrates between cells is thus considered essential to meet the energy demand of the brain. Accordingly, it has been hypothesized that neuronal energy consumption depends, at least in part, on the release of glucose and lactate from astroglia cells and on subsequent diffusion to neurons via the brain intercellular space (ICS; [72, 87, 88, 100, 127, 217, 222, 247, 248, 278 69]).

Monitoring of brain energy substrates requires invasive surgery, as the brain is situated inside the skull. Non-invasive approaches like imaging can only be performed in dedicated locations and is thus unsuitable for frequent or continuous monitoring. Technologies allowing continuous or near-continuous monitoring include biosensor technology, microdialysis, UF. In the present text we prefer the term microfiltration instead of UF as this is more conform the microdialysis terminology. Microdialysis has a long history in studying effects of drugs, behaviour and cerebral ischemia on the release of neurotransmitters, monoamines, and metabolites such as amino acids and other small endogenous compounds. Microfiltration is a sampling technique not used so often but in certain experiments microfiltration is to be preferred over microdialysis.

The importance of estimating glucose and lactate concentration in the ICS has been recognized by several authors [67, 82, 83, 90, 186, 187, 212, 215, 224, 283, 326]. Partly recovered concentrations of energy substrates as measured with conventional microdialysis (typically using flow rates of 2 $\mu l \cdot min^{-1}$), are sufficient for monitoring relative changes in the extracellular levels of lactate, pyruvate and glucose during experimental interventions. This is indeed the common practice in most studies investigating the effects of drugs **(table 1)**, behaviour or physiological conditions **(table 2)** or changes as a result of hypoxia-ischemia or trauma **(table 3)**. In these

studies, microdialysis parameters are allowed to stabilize after insertion of the probe, and then concentrations of the analytes measured in the dialysate are expressed as percent change from baseline (100% = baseline levels). However when the aim is to relate extracellular glucose or lactate levels to absolute turnovers of tissue energy metabolism, the real, absolute concentrations must be known to obtain a meaningful interpretation of the data. The present survey gives examples of studies based on both relative and absolute levels of the energy substrates and metabolites.

There are as yet no reports on either estimates of the turnover rate of glucose and lactate of the ICS or of the proportion of energy substrates that is transferred via this compartment. Quantification of intra-cerebral transport and transport of energy substrates and products to and from the blood circulation may shed light on the metabolic organization of brain energy consumption. Current approaches to assess brain energy dynamics include isotope dilution of labelled substrates as detected either with conventional radioactive tracers or with modern imaging technologies, such as magnetic resonance spectroscopy and positron emission tomography (PET). The current review is emphasizing another quantitative approach, which is based on intra-cerebral and intravascular sampling with microdialysis and microfiltration. Both intra-cerebral microdialysis and microfiltration inform only about the fate of substrates in the direct vicinity of the probe and do not inform about the condition of the whole brain. Whole brain metabolism can be studied by measuring arterio-venous differences.

Here we describe the essentials of both the intra-cerebral and intravascular sampling and (biosensor) detection technologies, some approaches for obtaining quantitative in vivo results and some new experimental data that allow estimates of the turnover rates of glucose and lactate in the rat brain ICS. We provide an overview of experimental and clinical applications of cerebral glucose/lactate monitoring (mainly conventional microdialysis with off-line or on-line detection).

Finally a general discussion on the implications of recent findings for current theories on intra-cerebral trafficking of energy substrates and products is given.

7.2 On monitoring methodologies

7.2.1 Microdialysis

The basic principle of microdialysis is to mimic the function of capillary blood vessels by perfusing a dialysis tube implanted into the tissue with a physiological fluid. The dialysate reflects the composition of the extracellular fluid containing substances that can diffuse through the pores of the hollow fiber membrane. The driving force of microdialysis is thus the concentration gradients of the various constituents between the extracellular fluid and the perfusion fluid over the membrane of the probe and thus no fluid is withdrawn from the sampling site. The first rather primitive version of the present microdialysis was developed by Delgado et al. [66] in 1972 and Ungerstedt and Pycock described the first microdialysis for in vivo measurements in 1974 [321]. There are several variables to be considered when applying microdialysis. These include the flow rate and type of the perfusion fluid, length of the hollow fiber membrane, the molecular weight cut-off (MWCO) value of the membrane, and the hollow fiber material. The hollow fiber is the analogue between microdialysis and microfiltration sampling techniques, which will be discussed in the next section.

Conventional microdialysis systems consist of a pump, probe/catheter, and a small vial to collect the dialysate (off-line system) or an analytical device directly coupled to the outlet of the probe (on-line system). The microdialysis probe may be placed inside the brain or in peripheral tissue and fluids (cerebrospinal, bile and blood) or other matrices. Most microdialysis probes are concentric with a diameter in the range of 0.15-0.4 mm but there are looped shaped microdialysis probes as well. The perfusate is an aqueous solution that mimics the surrounding biomatrix, so no osmotic differences exist over the probe membrane. The diffusion of the analytes over the probe membrane is dependent on the concentration gradient. While the perfusion fluid passes the membrane, molecules up to a certain molar mass diffuse inwards (recovery) or outwards (delivery) the probe. Thus microdialysis can be used for both collecting substances in the dialysate as well as delivering them into the surrounding tissue (= retrodialysis).

In far most microdialysis studies, perfusion rates of 0.5-2 µl·min^{-1} have been used for in vivo sampling with relative recoveries far less than 100%. The term relative recovery describes the ratio between the concentration of the substance in the dialysate and (peripheral) tissue. Therefore complicated procedures are required to calculate (or estimate) the real concentration of the analyte in the tissue compartment. There are many different methods for calibration and thus for determination of the relative recovery, method for e.g. of flow rate variation, no-net-flux, dynamic-no-net-flux, retrodialysis and adding an endogenous reference substance. All these calibration procedures have nicely been reviewed by Plock and Kloft [250]. Quantitative microdialysis, without calibrating the microdialysis device, is also possible by applying an ultraslow-perfusion rate, which results in similar concentrations of the analyte in the dialysate and the tissue compartment. Kaptein et al. [160], Ekberg et al. [76] and Rosdahl et al. [262] proved that using ultraslow-microdialysis the recovery is exactly 100% in contrast to the lower recoveries obtained with conventional microdialysis. Rosdahl described in 1998 one of the first experiments using ultraslow-microdialysis in human skeletal muscle and adipose tissue. Complete equilibrium was reached in both tissues using a flow rate 0.16 µl·min^{-1}. Also the measured concentrations of glucose, glycerol, and urea were in good agreement with expected extracellular tissue-specific levels [262]. Kaptein et al. compared in anesthetized rats subcutaneous ultraslow-microdialysis with microfiltration sampling (see also next section) at sampling rates of 100 and 300 nl·min^{-1} [160]. Glucose and lactate concentrations were exactly the same for both sampling techniques. Ekberg et al. [76] measured glucose, lactate, pyruvate and glycerol using four different flow rates: 0.3 (= ultraslow-microdialysis), 1, 2, and 5 µl·min^{-1} in interstitial fluid of subcutaneous adipose tissue. The conclusion was that the ultraslow-microdialysis flow rate of 0.3 µl·min^{-1} resulted in recoveries near 100% with decreasing recoveries at higher perfusion rates.

To make sampling possible at low flow rates, without a substantial loss of perfusion fluid into the tissue (= ultrafiltration fluid loss), some groups add a colloid to the perfusion fluid [108, 263]. Rosdahl et al. [263] placed microdialysis probes in the abdominal subcutaneous adipose tissue and

femoral muscle testing 4 different perfusion rates: 0.16, 0.33, 0.66, and 1.6 µl·min^{-1} in humans. Their perfusion fluid was a modified Krebs-Henseleit bicarbonate buffer (KHB) [264] or a KHB supplemented with dextran-70. Without dextran perfusion fluid was lost and adding dextran (40 g·L^{-1}) to the perfusion fluid, a good balance was observed between the expected and collected volumes [263]. Hamrin et al. investigated also this phenomenon, adding dextran to the perfusate, but varying the position of the outlet tubing was also investigated. By varying the position of the outlet tubing mechanical/hydrostatic pressure is changed, which is is a function of the outlet tubing length and diameter, fluid viscosity, and flow rate as stated by Poiseuille's law. The sample volumes were significantly smaller when the dialysis membranes was influenced by maximal hydrostatic pressure compared with minimal hydrostatic pressure [108]. Both studies used microdialysis probes with a MWCO value of 20 kDa [108, 263]. Membrane technology is improving, thus hollow fiber membranes with a larger MWCO value can be purchased. Microdialysis probes up to 3000 kDa allows monitoring of a wider range of compounds, including e.g. cytokines, proteins, peptides [10, 117, 120, 136, 138, 266, 279]. However due to the increased porosity of the hollow fiber, ultra-filtration fluis loss can become a problem and over time, the physiology of the tissue being sampled can be altered. Trickler and Miller [312] proved that adding bovine serum albumin (0-10% m/v BSA) to the perfusion fluid ultrafiltration loss was reduced (MWCO = 20 and 100 kDa using flow rates of 0.5-5 µl·min^{-1}). Hillman et al. [117] investigated ultra-filtration fluid loss using an ultraslow-flow of 0.3 µl·min^{-1} with perfusion fluid without and with BSA (3.5%) or with Ringer-dextran 60 (RD60-Braun Medical AB, Stockholm, Sweden). Addition of BSA or dextran reduced fluid loss.

Another problem of using ultraslow-flow rates is the very long lag times between sampling and analysis. In addition, evaporation may occur during the long collection times, which concentrates the samples. These disadvantages can be circumvented by using the MQ (MQ) sampling technique (patented by Brainlink B.V.), which allows fast transport of dialysate together with high recoveries of the analytes. In this type of microdialysis probe an extra fluid line is added, which rapidly transports the dialysate sample to the detection device [52] **(see figure**

1A). A drawback of the additional fluid line is that extra care should be taken to keep the sampling system sterile.

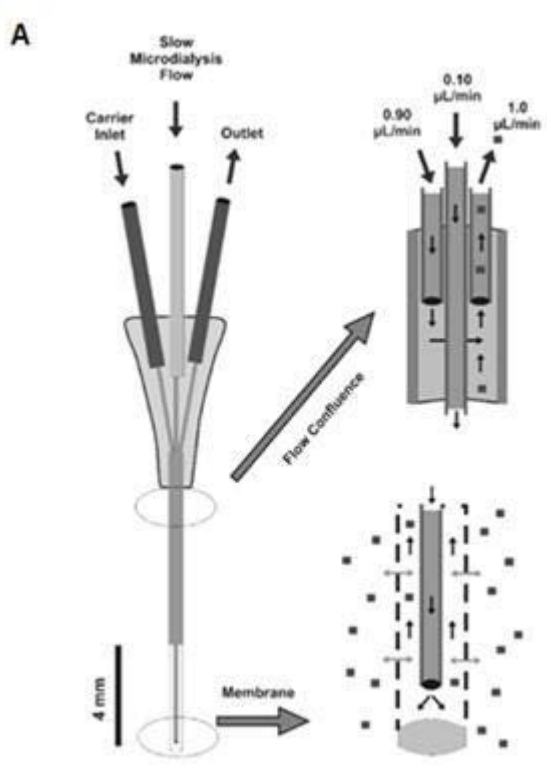

Figure 1A Schematic drawing of MQ-microdialysis system. The slow flow into the hollow fiber membrane generates a dialysate, which is rapidly diluted and removed by a second, fast "dilution" flow resulting in a "total flow" out of the probe.

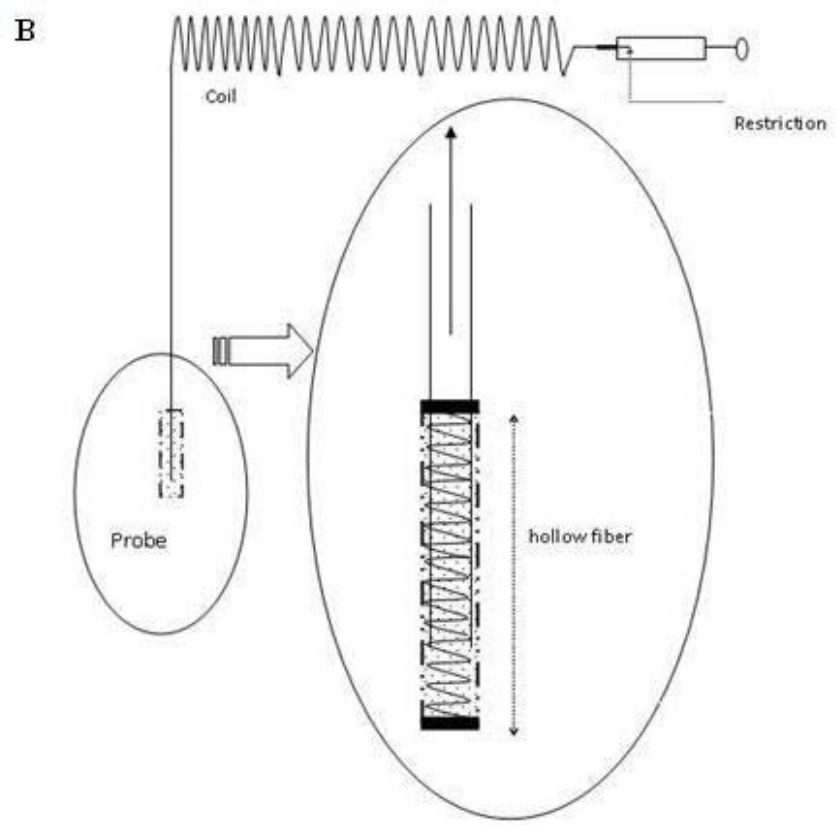

Figure 1B A schematic drawing of the UCD, with enlargement of the hollow fiber membrane, containing: a spiral, which prevents the hollow fiber from collapsing. The spiral extends into the restriction tube, which defines the flow rate in combination with the under-pressure container used (**B**).

7.2.2 Microfiltration

The driving force of the flow rate in microfiltration is a pressure gradient over the probe membrane, pulling sample from the in vivo environment. Microfiltration samples are undiluted (100% recovery), small (µl or nl size) and sterile. **Figure 1B**. is showing a typical microfiltration device schematically. Two different types of microfiltration probes have been developed of which only one is commercially available (Bioanalytical Systems, Lafayette, Ind., USA). The commercial probe consists of one or more hollow fiber loops with

various hollow fiber lengths (ranging from 2-12 cm). The sampling rate is relatively high, in the range of 0.5-2 µl·hour^{-1}·cm^{-1} hollow fiber membrane.

A previously described non-commercial probe is much smaller: a concentric probe of 4 cm using a ultraslow-sampling rate of 100 nl·min^{-1}, applied subcutaneously in conscious rats [232]. In this study the microfiltration probe was connected to a long capillary, enabling continuous sampling for 30 hours and simultaneous storage of the samples in that collection coil. This integrated device is called the ultra-filtration collection device (UCD; **figure 1B**). With this device time-profiles can be created without manual intervention and the profile can be stored in de collection device until further analysis up to several days without significant diffusion artifacts. The collection coil is made of fused silica tubing, because a significant evaporation of the collected fluid through several other tested materials was observed. Due to the small size of the probe, the probe can be introduced in vivo via adapted needles with minimal tissue damage and without using anesthetics.

The amount of fluid withdrawn from the tissue compartment is usually relatively small, so the chemical balances of the extracellular fluid (ECF) is not or minimally disturbed and in most tissues the body fluid is easily replenished. Because microfiltration concentrations are (nearly) identical to those in the ECF concentrations, the measured levels of the analyte reflect rather accurately the metabolic changes in the intercellular spaces [232]. It appeared, however, that the volume of the ECF may differ between animal species, condition of the organism and the investigated organ. For instance, Kissinger et al. mentioned that an anesthetized rat produces almost no microfiltration fluid, whereas a rat, that has free access to water and food and is moving around, produced plenty of microfiltration fluid [172]. Our own studies in anesthetized rats revealed that over a period of 6-7 hours and the syringe pump set at a flow rate of 0.3 µl·min^{-1}, the ultra-filtration flow rate is declining with a subcutaneous placed probe, but remained stable with a probe positioned intraperitoneal. In contrast to most peripheral tissues, microfiltration was not possible in rat brain striatum, not even when applying syringe pump flow rates of 50-100 nl·min^{-1} [160]. Apparently, the ECF production and/or supply in the rat brain was too low. Whether

this limitation applies to the human brain is unlikely, considering the high percentage –as compared to the rat brain– of extracellular volume (see below) of more than 30% of the total brain volume. So in rat [197, 204-206], cat [146], dog [28], horse [58, 291], pig [309], chicken [271], mouse [152], and sheep [145, 153, 289] microfiltration has been applied to other tissues, including subcutaneous tissue [28, 58, 145, 146, 152, 204-206, 206, 271, 291], adipose tissue, blood [196, 197, 271, 309], bone [145, 153, 289] and muscle [145, 153, 289, 291] to monitor LMW compounds such as lactate [196, 197, 271, 307, 309], glucose [146, 152, 196, 197, 271, 307, 309] and electrolytes like magnesium, calcium, phosphorus, chloride, sodium, potassium [145, 153, 205, 291] and other analytes/drugs [28, 58, 204, 206]. Only one study described microfiltration in human subcutaneous tissue monitoring glucose and lactate [307]. Most hollow fiber membranes have MWCO values <20 kDa, excluding macromolecules like proteins, peptides and cellular elements. However macro-analytes such as cytokines and peptide hormones may be interesting to sample systemically and/or in local tissues [120, 129, 130, 132, 133, 138, 208, 346].

7.2.3 On-line detection: Biosensors

An electrochemical biosensor is defined as a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element, which is retained in direct spatial contact with an electrochemical transduction element [304]. The biological recognition system translates information from the biochemical domain, usually an analyte concentration, into a chemical or physical output signal with a defined sensitivity. The main purpose of the recognition system is to provide the sensor with a high degree of selectivity for the analyte of interest. The transducer part, also called the detector, sensor, or electrode, of the sensor serves to transfer the signal from the output domain of the recognition system to, mostly, the electrical domain. An electrochemical biosensor is a biosensor with an electrochemical transducer. It is considered to be a chemically modified electrode as a conducting material is coated with a film encapsulating selective proteins. Although a biosensor with different transducer types, e.g. electrochemical, optical, piezoelectric or thermal type, show common features. The amperometric enzymatic glucose biosensors are most

extensively studied. Here the enzyme glucose oxidase is immobilized at a charged electrode and glucose concentrations can be monitored by the change in electric current flow caused by the enzyme-catalyzed production of hydrogen peroxide [1, 29, 52] or -less often- by the consumption of oxygen [12]. Biosensors are usually covered with a thin membrane that has several functions, reduction of interference and mechanical protection of the sensing element [309, 338, 341], but some are made without membrane (needle-type) and called microsensors [128, 237]. Biosensors are most often in the format of a needle coated with the selective enzymes that can be directly inserted in the ICS of any organ or a bodyfluid. The problem is then the reactivity of the tissue: such biosensors become rapidly encapsulated in vivo. To avoid such problems the sensor is combined with microdialysis or microfiltration. The direct environment of the sensor remains constant in a relatively clean matrix. The best studied biosensors are sensors for glucose [68, 71, 91, 92, 102, 103, 157, 213, 214, 249, 251, 257, 308, 309, 332, 348, 352] and lactate [68, 71, 89, 91, 92, 103, 249, 251, 309, 332];[157, 348, 352], but sensors for glutamate [268];[352], and pyruvate [95] are also described with applications in mammalian brain and blood [309], via an extracorporeal shunt [68, 92, 251] or in vitro preparations, including single cells, cell cultures or tissue slices [195] and reviewed by Pancrazio et al. [242]. Finally, ion-sensitive electrodes for the influx or efflux of Ca^{2+} , Na^{+}, and K^{+} ions have been widely used in both in vivo and in vitro preparations [34, 158, 164, 195, 288]. Preferably, the biosensor should be applied non-invasively, however such biosensors lack accuracy and researchers are thus focusing on invasive biosensors. Due to the high risk of thrombosis, embolism and septicemia, only a few intravascular sensors have been described [12] and tested [18, 345]. However most glucose sensors developed for continuous monitoring do not measure blood glucose directly, but rely instead on the measurement of the glucose in the interstitial fluid of subcutaneous tissue.

7.2.4 Hollow fiber materials

The maximum size of particles that are transported over the hollow fiber membrane is defined by the MWCO value of the hollow fiber membrane. Nevertheless the hollow fiber material and build-up, the configuration and the charge of the analyte of interest may have additional effect if the analyte of interest is transported over the hollow

fiber membrane. Hollow fibers are made from polymers with different compositions.

The hollow fiber is the interface between the device and the in vivo environment and thus can elicit a material-tissue/blood interaction. Introduction of the hollow fiber probe in vivo causes little disruption in the tissue, but this might not be the case for implanting the hollow fiber for longer periods of time. The invasiveness may differ depending on the location and the material of the hollow fiber. In general, an inflammatory response can be observed in all tissues. Several articles have addressed this topic in the context of microfiltration, microdialysis sampling and/or implanted biosensors [20, 21, 139, 338, 339, 341]. Considering the enormous amount of papers published in the field of microdialysis, microfiltration and biosensoring, relatively few studies compared systemically several hollow fiber membranes for biofouling and/or biocompatibility (tissue reactions to the implanted hollow fiber membrane).

Implantation of the microfiltration probe made from polyacrylonitrile in sheep muscle, caused inflammation and degeneration of the surrounding tissue; the longer time elapsed the worse the magnitude of inflammation [139]. No local edema was seen within 2 days implanting microfiltration of the same material intramuscular in horses [291]. Subcutaneous placement of polyacrylonitrile microfiltration in rats no inflammatory response was observed 6 weeks after implantation [148]. Most studies investigating hollow fiber-tissue reactions test subcutaneously in rats, for e.g.: Clark et al. [47] tested different hollow fiber membranes and harvested the fibers after 3, 6 and 12 weeks. The membranes made from polysulfone and cellulose diacetate, both with pore size 0.2 μm, were extensively degraded. Even more degraded was the polysulfone fiber with a pore size of 0.65 μm. The mixed ester cellulose with pore size 0.2 μm and regenerated cellulose (MWCO = 3 kDa) retained their integrity even after 12 weeks and degradation did not increase over time [47]. Although the polysulfone with MWCO = 10 kDa remained intact, it was infiltrated by inflammatory cells and had together with the regenerated cellulose fiber (MWCO = 13 kDa) the thinnest fibrotic capsule formation [47]. Expanded polytetrafluoroethylene (e-PTFE) was harvested after 4, 10 and 21 days by Zhao et al. [353]. This

material was very stable and not degraded (pore size or MWCO value was not mentioned). Brauker et al. [32] tested the same material in a similar setup with a pore size large enough to be completely penetrated by host cells (0.8-8 μm) comparing this with PTFE membranes with pore size of 0.02 μm. The larger pore size membranes had 80-100 fold more vascular structures.

Only a few studies investigated the brain tissue after implantation of a probe [24, 43, 169, 284]. Benveniste and Diemer [24] investigated cellular reactions to implantation of a dialysis fiber (Diaflo Hollow fiber, MWCO = 50 kDa, Amicon). Within 2 days the tissue adjacent to the hollow fiber (a 50 μm border zone) exhibited edema, minor hemorrhages and accumulations of polymorphononuclear leucocytes followed by astrocyte hypertrophy [78]. Eosinophilic neurons, indicating cell death, were occasionally present within 100-150 μm from the implanted probe, but otherwise normal neuropil surrounded the hollow fiber.

An advantage of subcutaneous and brain sampling is the avoidance of the complement cascade. In microdialysis anticoagulant can be added to the perfusate to reduce or lower the complement cascade, this can't be done with microfiltration.

Several hemodialysis studies have described hollow fiber biofouling interactions [80, 117, 330]. Hollow fibers with smaller pore sizes have greater stability in blood, than membranes with larger pore sizes. Probably proteins can easier clog the hollow fiber membranes with bigger pore size [80, 117]. This conclusion may also apply to subcutaneously implanted fibers, not because of clogging, but because cells adhere more easily to larger pores.

For microfiltration studies in pig heart -lactate/glucose changes by myocardial ischemia-, and rat carotid artery and jugular vein -glucose/lactate changes by cerebral ischemia [197]-, heparin was administered intravenously [309]. For intravenous microfiltration sampling in chickens, administration of heparin was not necessary [271]. The microfiltration probes in these studies were all made of acrylonitrilsodium methallyl sulfonate with MWCO = 20 kDa.

7.3 Applications

7.3.1 Levels in brain ICS and blood circulation

A number of studies has estimated absolute concentrations of glucose and/or lactate in rat brain with concentrations ranging from 0.35-3.3 mM for glucose [224] and 0.35-1.1 mM for lactate [67, 187, 283,256]. Some of these studies have directly compared extracellular glucose levels in different brain areas by the zero-net-flux method (based on perfusing with different concentrations of glucose) and showed that striatal levels are lower (0.71 mM) than in hippocampus (about 1.0 mM) [224] and that fasting overnight decreased interstitial glucose in hypothalamus from 1.42 to 0.84 mM [221].

For clinical studies, normal levels of glucose, lactate, pyruvate and lactate/pyruvate ratio's have been determined in the human brain: 1.7-2.1 mM for glucose, 2.9-3.1 mM for lactate, 155-166 μM for pyruvate and lactate/pyruvate ratio's of 19-23 and lactate/glucose ratio's of 1.2-1.8 [2, 190, 253, 277]. The ratio of blood/interstitial glucose has been extensively debated in the literature. Tiessen et al. [308] studied the relationship of glucose levels in adipose tissue, loose connective tissue of the abdominal subcutis with blood glucose levels in healthy volunteers and found significantly different ratio's. Glucose levels measured in subcutaneous loose connective tissue, but not those in adipose tissue, matched plasma glucose levels [308]. The controversy on the ratio of blood/subcutaneous interstitial glucose may thus be explained by the position of the probe/device in the subcutis. Rhemrev-Boom et al. [257] refined the technique and developed a portable lightweight biosensor for continuous, subcutaneous glucose monitoring in humans [257]. The device was validated for its accuracy, linearity, sensitivity, selectivity and stability during ex vivo and in vivo experiments. These biosensors could be used up to 3 days in continuous mode [257].

In the present context the most important conclusion is that brain ICS glucose levels are at least a factor 3 (in human brain) or 5-10 (in rat brain) lower than blood levels, whereas brain ICS lactate is usually somewhat higher than blood lactate levels (in both rat and human brain).

Table 1 Effects of drugs on cerebral glucose and/or lactate changes monitored by microdialysis or implanted sensors in experimental animals (rats).

drug	drug adm	position micro-dialysis probe	glucose	lactate	assay	ref
pento-barbital	i.p.	hippocampus Striatum	increase (+15-76%)	decrease (-17%)	off-line on-line glucose sensor	39, 209, 240
ketamine	i.p.	hippocampus	increase (+85%)		off-line	39
chloral hydrate	i.p.	striatum	increase (+150%) no change in response to hypoxia	hypoxia-induced-increase reduced	on-line, amperometric implanted glucose sensor	83, 212
probenecid (blocks lactate carrier)	local	striatum		increase (+50%)	on-line, fluorometric	175, 187, 302
alpha-cyano-4-hydroxycinnamate (blocks lactate transport)	local	cortex		increase ^{14}C-lactate uptake inhibited	off-line	228, 354
high K^+	local	striatum N. Accumb	decrease (-23-70%)	increase (+70-85%)	on-line, fluorometric Off-line	55, 302, 316
veratridine	local	striatum n.accumb.	decrease (-86-94%) decrease (-75%)	Increase (+100%)	on-line glucose sensor	83, 240, 316
tetrodotoxin (TTX)	local	striatum n. accumb	increase (+34%) increase (+93%)	attenuation of ECT-induced increase no change	on-line, amperometric, fluorometric, NMR spectroscopy	83, 170, 185, 316

drug	drug adm	position micro-dialysis probe	glucose	lactate	assay	ref
ouabain	local	cortex		inhibits trauma-induced increase	off-line	162
glutamate	local	cortex	decrease (-48%)	increase (+600-700%)	off-line on-line, amperometric	4, 228, 260
N-methyl-D-aspartate (NMDA)	local	striatum		increase	on-line, fluorometric	186
kainic acid (KA)	local, i.p.	striatum		increase	on-line, fluorometric	185, 344
D,L-threo-hydroxyaspartate (THA)	local	striatum		increase (+200%)	on-line, amperometric	91
dihydrokainate (DHK)		medial prefrontal cortex amygdala		increased basal release, inhibited foot-shock induced increase	on-line, fluorometric	317
AP-5, AP-7 (NMDA receptor antagonists)	local	hippocampus striatum		inhibits ECS/stress-induced increase	on-line, fluorometric	177, 186
MK-801 (non-competitive NMDA receptor antagonist)	local	hippocampus Striatum		no effect on tail-pinch induced increase	on-line, amperometric	84

drug	drug adm	position micro-dialysis probe	glucose	lactate	assay	ref
CNQX (AMPA receptor antagonist)	local	cerebellum	no change	inhibits mossy fiber stimulation-induced increase	on-line, amperometric	37
kynurenic acid	local	Cortex		inhibits trauma- or ischemia-induced increase	off-line	161, 162
pilocarpine	i.p.	cortex	perturbation (± 44-48%)	increase (+379%)	off-line	287
isoprenaline	local	striatum	increase	no change	on-line, amperometric	91
propanolol	local	striatum	decrease (-17%)		on-line, amperometric	91
cAMP	local	striatum	increase		on-line, amperometric	91
diazepam	i.p.	amygdala		attenuation of stress-induced increase	on-line, fluorometric	315, 318
apomorphine	i.p.	n. accumb.	increase (+41%)	increase (+30%)	on-line, fluorometric	316
bromocriptine	i.p.	n. accumb.	increase (+110%)	no change	on-line, fluorometric	316
haloperidol	i.p.	striatum hippocampus		increase	on-line, fluorometric	70
8-OH-DPAT (5-HT1A agonist)	i.p.	hypothalamus	decrease		off-line	329
methampheta	i.p.	striatum		increase	off-line	293

drug	drug adm	position micro-dialysis probe	glucose	lactate	assay	ref
mine.		Prefrontal cortex				
3,4-Methylene-dioxymethamphetamine (MDMA, "Ecstasy")	s.c. i.v.	striatum	increase (+39-123%)	increase (up to +87%)	off-line on-line, amperometric	57, 102, 106
estradiol	i.v.	cortex	no change	attenuation of ischemia or glutamate-induced increase	off-line	228, 259
cyanide, malonate, MPP^+ etc. (mitochondrial toxins)	local	cortex Striatum	decrease (-46%)	increase (+375-434%)	off-line NMR	49, 155, 246

7.3.2 Effects of drugs and physiological stimuli

A variety of approaches have been used to study changes of local cerebral glucose/lactate -reflecting increased metabolism- as a result of increased neuronal activity. Local neuronal activity has been manipulated by various drugs, including pentobarbital or ketamine-xylazine induced anesthesia, local application of depolarizing agents (high K^+, veratridine), agents blocking action potentials (e.g.. TTX), glutamate or glutamate receptor agonists (i.e. NMDA, AMPA) or antagonists (e.g. MK801, AP5 or NBQX) or systemic application of convulsive drugs (e.g. kainic acid, pilocarpine) or drugs of abuse (e.g. amphetamines). Effects of these agents and the references are listed in **table 1**. Other stimuli used include tail pinch, restraint stress, foot

shock, electroconvulsive shock, swimming or exercise, learning & memory tasks or local electrophysiological stimulation **(table 2)**. Effects of drugs have been studied in control animals and in animals exposed to stimuli listed in **table 2**. Finally, microdialysis of glucose and/or lactate has been extensively applied in acute brain injury models to study patterns of metabolic perturbations and effects of neuroprotective treatments **(table 3)**.

Of the conditions listed in **table 2** one of the most recent and detailed studies on the coupling between neuronal activity and energy metabolism is the study by [37], which specifically addressed the question whether lactate production is triggered by glutamate uptake or glutamate receptor activation using climbing fiber stimulation of cerebellar Purkinje cells. In the latter study glucose and lactate was monitored simultaneously every 30 seconds using an on-line flow injection-biosensor based detection system. Electrical stimulation of climbing fibers caused rapid lactate increases of about 30% in the molecular layer of the cerebellar cortex, but no changes in dialysate glucose levels within 30 seconds of stimulation. The stimulus-induced lactate increases could be blocked by the AMPA antagonist CNQX and were independent of glycogen breakdown, indicating that the lactate increases are directly derived from glucose uptake and coupled to neuronal activity through AMPA receptors, which are localized both postsynaptically on neurons (Purkinje cells) and on astrocytes.

Further, effects of drugs of abuse, including the amphetamine derivatives methamphetamine [294] and 3,4-methylenedioxymethamphetamine (MDMA, "ecstasy") [56, 57, 102, 106] on brain energy metabolism have been investigated using microdialysis of glucose and lactate. Systemic MDMA administration causes rapid glucose and lactate rises in striatum. The brain glucose changes are related to transient blood glucose elevations, whereas brain lactate remains elevated over a more prolonged period. These metabolic responses seem to be mediated by serotonin and action on 5-HT2 receptors as discussed in Gramsbergen and Cumming [102]. Others, however, reported decreased blood glucose by high doses of MDMA using female Dark Agouti rats [290].

Table 2 Glucose-lactate monitoring following physiological stimuli in rats.

stimulus	position microdialysis probe	glucose	lactate	time Resolution Assay	Ref
tail Pinch	striatum hippocampus medial prefrontal cortex	perturbations (first decrease, then increase) ± 5-15%	increase +23-93%	On-line 2.5 min., continuous	84, 86, 91, 299
restraint stress	striatum hippocampus medial prefrontal cortex amygdala	perturbations (first decrease, then increase) ± 10-125%	increase +57-135%	On-line 2.5-5 min, continuous	60, 78, 84, 175, 178, 299, 315
acoustic Stimulation	medial prefrontal cortex		increase +23%	On-line continuous	299
swimming / exercise	hippocampus striatum hypothalamus	decrease -26-42%	increase +12-45%	On-line 2-5 min	175, 332
learning & memory	hippocampus	decrease -11-48%		Off-line 5 min.	223, 224, 226
food shock	Medial prefrontal cortex amygdala		increase +50-59%	On-line continuous	317, 318
electrocon-vulsive shock	striatum, hippocampus	increase 2-fold	increase +47%	On-line 2 min, continuous Off-line 15 min.	55, 60, 175, 178, 184

7.3.3 Clinical microdialysis-monitoring energy substrates

Over the last two decades cerebral microdialysis of glucose and lactate (and pyruvate and glutamate) has been extensively used for monitoring neurointensive care patients. These studies are still primarily aimed at studying the possible use of microdialysis parameters for the early

detection of adverse metabolic changes during or after neurosurgical interventions. Two major categories of patients have been studied: a) traumatic brain injury (TBI) and b) subarachnoid hemorrhage (SAH). Several reviews on clinical microdialysis in neurointensive care have appeared [115, 116, 322]. For clinical decision making during neurological/neurosurgical intensive care, the microdialysis method must fulfill several demands: the microdialysis should be standardized to allow comparison of data between hospitals, the biochemical variables should be analysed and displayed at the bedside, the data should be quantitative and the biochemical variables should cover important aspects of cerebral metabolism, and each microdialysis probe reflects interstitial biochemical values in small surrounding volume, the locations of the probe in relation of the lesion must be documented and defined [80].

Table 3 Glucose-lactate monitoring in experimental models of acute brain injury.

acute brain injury Model	position microdialysis probe	glucose	lactate	time resolution Assay	ref.
hypoxia (rat) repetitive 3 min 4 min 15 min	hippocamp. striatum cortex cortex	perturb. no change decrease increase +210%	increase +76% increase +1160%	on-line cont. on-line 1 min. glucose sensor on-line 3 min	157, 212, 285, 326
hypoxia (rat) 90 min 30 min	hippocamp. cortex	decrease -48% -75-85%	increase +158% +153%	off-line 30 min. off-line 15 min	109, 122, 355
global ischemia 5 min (gerbil) 10 min (rat) 14 min (pig)	cortex	decrease - 80%	increase +200-260%	off-line 10 min on-line cont. MRS	51, 161, 175, 325
focal ischemia (rat,	cortex striatum	decrease <-70%	increase + 100-	off-line 15 min	3, 79, 93,

acute brain injury Model	position microdialysis probe	glucose	lactate	time resolution Assay	ref.
gerbil) (monkey)	cortex	core: <-82% penumbra: -31%	500% core: +245% penumbra: +148%	off-line 15 min	94, 202
focal ischemia (rat) (cat)	striatum cortex	decrease <-80% - 8-38% PID: -5%	increase +320% + 51% PID:+ 26%	on-line 1 min on-line 0.5 min	103, 124
traumatic b rain Injury (rat)	cortex	no change, decrease (-13-50%) or increase	increase +97-600% (+volume insult)	off-line 10-30 min	6, 23, 44, 163, 183, 199, 218, 252, 270, 305
traumatic brain injury (rat)	thalamus	No change	Increase (+ hypoxia)	off-line 15 min	97, 98
epilepsy (rat)	striatum hippocamp.	see kainic acid, pilocarpine **(table 1)** and electroconvulsive chok **(table 2)**			

Hutchinson et al. [136] investigated different microdialysis hollow fiber lengths, relative recoveries at different perfusion flow rates, the effect of freezing and thawing on glutamate analysis, and compared glucose, lactate, pyruvate and glutamate assays by a bedside enzyme analyzer with results obtained by HPLC analyses. Two microdialysis probes made of polyamide were placed in the frontal cortex of patients with head injury or poor grade subarachnoid hemorrhage. The reference catheter (l = 10 mm, flow rate = 0.3 μl·min^{-1}) and a probe with different lengths (l = 10 mm or 30 mm, tested flow rates 0.1, 0.3, 0.5, 0.7, 0.9, 1.1, 1.3 and

1.5 µl·min^{-1}) perfused with Ringer or normal saline. It was concluded that adjacent microdialysis probes of the same length and using the same perfusion fluid, and flow rates revealed equivalent results. Using different compositions of the perfusion fluid or freezing/thawing of the samples caused significant variation in the results. Probe lengths (10 mm) had major effect on the relative recovery which was near 70% at 0.3 µl·min^{-1} and 30% at 1.0 µl·min^{-1}. Hillman and co-workers [117-119, 138] performed a series of elegant clinical studies. Patients who became comatose after subarachnoid hemorrhage or traumatic brain injury were inserted with paired microdialysis polyethersulfone membranes (l = 20 mm, flow rate = 0.3 µl·min^{-1}) with MWCO values of 10 kDa and 100 kDa. The analytes of interest were glucose, glycerol, lactate, and pyruvate [138]. In addition, the recovery of the HMW interleukin-6 and total protein values were determined [117]. The overall conclusion was that microdialysis probes with a 100 kDa MWCO value can be used in the clinic, allowing sampling of cytokines and other macromolecules [117]. Properties of the 20 kDa and 100 kDa hollow fiber were investigated for glucose, glutamate, glycerol, lactate, pyruvate, urea and total proteins [119]. The fluid recovery was similar for both catheters; however significantly more protein was recovered with the 100 kDa catheter. The recoveries for glycerol and pyruvate were similar, while minor differences were observed for glutamate and glucose. A significant difference between the catheter types was observed for lactate (and pyruvate) recovery, which affects the lactate/pyruvate ratio [119].

The importance of probe location was investigated by Engström et al. [80]. This group compared cerebral energy metabolism, as an indicator of secondary excitotoxic injury and cell membrane degradation close to the focal traumatic lesions (= penumbra zones) and in remote (apparently intact) brain regions of the ipsi-lateral and contra-lateral hemispheres. Except for pyruvate the values of all biochemical variables (glucose, lactate, pyruvate, glutamate, and glycerol) and the lactate/pyruvate (L/P) ratio were significantly different in the penumbra zone when compared with mean values found in "normal" tissue ipsilateral to the parenchymal damage and in contra-lateral normal tissue ($p < 0.001$). In the penumbra zone a slow normalization of the L/P ratio and levels of glutamate and glycerol was observed. In normal tissue these parameters

remained within normal limits. Concluding that data obtained from intracerebral microdialysis can be correctly interpreted only if the locations of the catheters as they relate to focal brain lesions are visualized. A "biochemical penumbra zone" surrounds focal traumatic brain lesions. It remains to be proven whether therapeutic interventions can protect the penumbra zone from permanent damage.

In most of the clinical microdialysis studies, samples are analysed off-line at time intervals of 30 minutes or more, which makes the technique less effective for clinical monitoring and decision making during surgery or in a neurointensive care setting. A major improvement in this respect has been the introduction of rapid sampling microdialysis (e.g. every 0.5-1 min) coupled to flow injection analysis with flow-through biosensors for glucose and lactate [27, 244], (see also below). In a clinical setting, microdialysis is often combined with other multi-module parameters (e.g. O_2, pH, electrophysiology, PET), thereby providing a broader and more reliable basis for interpretation of neurochemical changes and clinical decision making [6, 48, 121, 167, 168, 189, 269, 327, 350, 351]. Since hyperglycemia after acute human brain insults (stroke, SAH or head trauma) has been associated with a worse clinical outcome or increased risk of death, a strict insulin-therapy to maintain normal blood glucose levels has been proposed and evaluated in clinical trials. However, maintaining strict euglycemia by glucose-potassium-insulin infusions in stroke patients was not associated with significant clinical benefit [104] and intensive insulin therapy to control blood glucose in SAH [166, 273] or head trauma patients [328] caused potentially dangerous decreases in cerebral glucose, despite normoglycemia. In addition, it has been suggested that lower blood glucose levels may trigger peri-infarct depolarizations (PIDs), which may contribute to infarct expansion [81, 296]. Dynamic metabolic changes related to PIDs have recently been reported in the cat and human brain using rapid sampling microdialysis (0.5-1 min) coupled to flow-injection analysis with enzymatic-amperometric detection [244]. Fast metabolic perturbations (within a few seconds) related to spreading-depression-like events cannot be detected by conventional off-line detection techniques, but may be relevant early signs of adverse metabolic events or compromised local cerebral blood flow. A recent review [116] on the potential of cerebral microdialysis

monitoring for the early detection of adverse events in neurointensive care was rather positive. It emphasized that no other bedside technology allows the simultaneous monitoring of brain levels of lactate, glucose, glycerol, various amino acids and, last but not least, O_2 and CO_2 (the latter parameters not by microdialysis). Because these levels exert transient changes, a single measurement does not provide clinically useful information, so only continuous monitoring informs adequately on the dynamics of metabolic processes. The current clinical brain monitoring is still in a validation phase rather than a routine diagnostic tool. One of the obstacles for routine use of clinical microdialysis is the too laborious practice of the current monitoring technique, including handling, collecting and analyzing the samples in a 24-hour setting, as required in a neurointensive care unit. Introduction of automated high throughput technologies is necessary for a wider application and appreciation of microdialysis and microfiltration (see below) in intensive care medicine.

7.3.4 Experimental microfiltration - monitoring brain energy substrates

Thus far no articles have been published on microfiltration in the human blood or brain. The rat brain experiments were as yet not successful [160]. As emphasized human brain microfiltration may be still an option as the human brain ICF is substantially larger than that of the rat. Microfiltration has been used to investigate whole brain metabolism by placing a microfiltration probe in the aorta and jugular vein, thus measuring arterial-venous differences for glucose and lactate, after brain injury and lactate infusion [196]. The injury experiments [197] show an increased release of lactate following increased intracranial pressure induced by an inflating balloon. Concomitant uptake of glucose from the blood circulation was decreased. The infusion experiments with lactate showed that the brain may both release and accumulate lactate. The cerebral influx or efflux of lactate depends on the relative concentrations of lactate in the blood and the adjacent brain. The lactate transport is essentially concentration driven, as could be expected considering the molecular properties of the various lactate transporters. Immediately after the infusion there was a net influx of lactate: so the arterial levels were higher than the venous concentration of lactate. The

reverse was true when after prompt ending of the infusion of lactate: there was an apparent cerebral release of lactate into the circulation [196]. Computer modeling suggests that only a minor fraction of the accumulated lactate is metabolized [195]. The results suggest that the majority of lactate moving into the brain is not used as an energy substrate, and that lactate does not replace glucose as an energy source. Instead, the authors propose the concept of a lactate pool in the brain that can be filled and emptied in accordance with the blood lactate concentration, which does not serve as a major substrate for cerebral energy metabolism under normoglycemic conditions at least in anesthetized animals [195, 196].

7.4 Intracerebral trafficking of glucose and lactate

7.4.1 Strategy

We give an example of how to estimate ICS steady state levels and the amounts of lactate and glucose diffusing through the ICS of the striatum of freely moving rats. This approach is based on the application of ultraslow-microdialysis (previous sections), assuming a 100% recovery in vivo. Here we propose a simple approach to estimate turnover rates based on the loss of glucose or lactate added to the perfusate at steady state levels. In practice, the decrease in the concentrations of lactate or glucose in the influx and efflux were measured at a constant perfusion rate of 100 nl·min^{-1}. This approach assumes that higher levels of glucose do not determine the rate of glucose consumption. Such assumption is justified, among others, because acute hyperglycemia does not affect cerebral glucose consumption in conscious rats [73] [239]. During slow perfusion rate new steady state levels are achieved rapidly. The difference between the amounts infused (by retrodialysis) and collected per time unit reflects the sum of diffusion and consumption of the substrates in the ICS and brain tissue surrounding the microdialysis-probe. Because passive diffusion may also contribute to the loss of added glucose or lactate, the present approach reveals maximal turnover rates of glucose or lactate in the ICS. Assuming a normal ICS compartment of 18% of total rat brain tissue volume [173, 215], we calculated the maximal ICS turnover of glucose and lactate per g wet

weight tissue. On-line ultraslow-microdialysis of glucose and lactate was performed by application of adapted micro-sensor technology as described previously [254].

7.4.2 The experiment in brief

Male Wistar rats were anaesthetized, the body temperature was controlled, and the rats were fixated in a stereo-tactic frame. The microdialysis probe was implanted into the left striatum and secured with screws and dental acrylate. The animals were allowed to recover for 24 h before measuring. Lactate and glucose were monitored continuously using flow-through nanoliter biosensors as described [254]. On completion of testing, rats were killed by overdose of sodiumpentobarbital for histological examination of probe placement. Animals showing signs of tissue damage other than probe track, or where the probe was placed outside the striatum were discarded from statistical analysis. Values represent means +SEM. Statistical significance of the differences was evaluated by using the Mann-Whitney U–test or Student t test ($p < 0.05$).

7.4.3 Calculations

The calculations are based on the model as defined in **figure 2**. The basic idea is that under steady state conditions the outflow of glucose and lactate via the dialysate is the sum of endogenous glucose and lactate released from brain tissue into the ICS and the fraction of exogenous glucose or lactate added to the inflowing perfusate, which did not diffuse into ICS. We assume that the fraction of exogenous glucose or lactate which is released directly into the blood circulation is small, since this is only possible via its cellular transporters [197]. Further we assume 100% recovery at ultraslow-microdialysis (= 100 nl·min^{-1}).

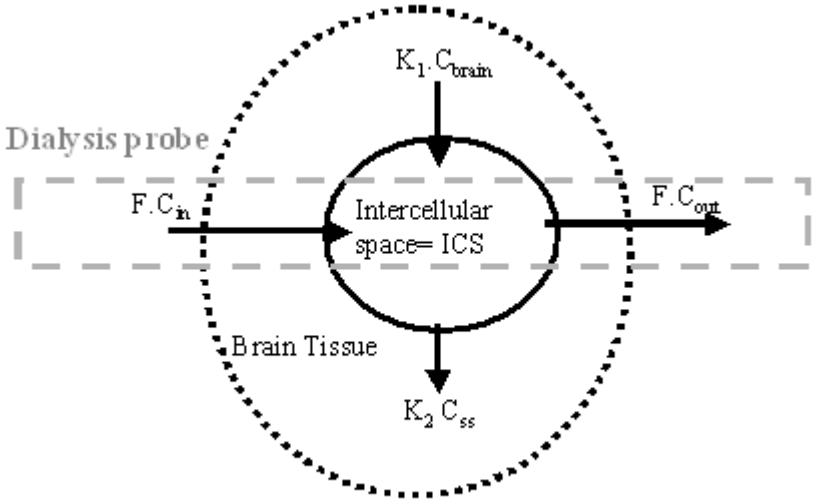

Figure 2 Compartmental model used for the calculations. For definitions of the used parameters see section 7.3.3.

Under steady-state conditions the following equation defines the amounts of lactate or glucose is transporte per time unit into and out of the dialysis probe. At such low perfusion rates we assume that the concentration of glucose and lactate in the (out-flowing) dialysate is the same as that in the ICS, because the diffusion over the probe membrane is not limiting.

$$k_1 * C_{brain} = k_2 * C_{ss} + F * C_{out} = (k_2 + F)\, C_{ss} \qquad \textbf{[equation 1]}$$

in which,

k_1 = a transport constant (or composition of various constants; arbitrarily defined) of brain glucose or lactate into the probe;

C_{brain} = apparent (both cellular and intercellular) concentration of the substrates in the brain;

So the term $k_1 * C_{brain}$ equals the amount of lactate or glucose that is released (per L) from brain tissue per time unit reaching the probe.

k_2 = transport constant from the ICS into the brain tissue; at ultraslow-microdialysis we consider the probe as part of the ICS, without limitations of diffusion of glucose or lactate over the probe membrane;

C_{ss} = measured concentration under steady state conditions (without added glucose or lactate to the inflowing perfusate;

C_{out} = C_{ss} concentration glucose or lactate measured in the dialysate when no glucose or lactate was added to the perfusate.

The product $k_2 * C_{ss}$ is the amount of lactate or glucose that diffuses per time unit (minute) from the probe into brain tissue.

F = flow rate (in the present experiments, set at 100 nl·min^{-1});

When glucose or lactate are added to the perfusate (Cin) and assuming that the rates of inflow and the outflow are identical (there is no loss of fluid to or gain of fluid from brain tissue, which is reasonable because both inflow and outflow were set identical with the perfusion pumps), and (new) steady states are reached, the amounts of the substrates transported or diffused per time unit can be described as:

$$k_1 * C_{brain} + F * C_{in} = (k_2 + F) * C_{out}, \quad \textbf{[equation 2]}$$

C_{in} = concentration glucose or lactate added to the dialysis solution;

Substituting the term $k_1 * C_{brain}$ by combining equation [1] and [2] results in:

$$(k_2 + F) * C_{ss} + F * C_{in} = F * C_{out} + k_2 * C_{out}$$

that can be rewritten as:

$$k_2 = F * (C_{in}-C_{out} + C_{ss}) / (C_{out}-C_{ss}) \quad \textbf{[equation 3]}$$

The constant k_2 reflects the combined rates of transport out of the extracellular compartment due to diffusion, carrier mediated transport and possibly also metabolism.

This rate constant depends on the volume of the probe, which is considered part of the extracellular compartment. The volume of the probe (V_{prob}) is 0.48 µl. The volume of the ICS of the rat striatum is 180 µl·g^{-1} of brain tissue (18% v/v; e.g. Korf and Postema, 1988 [181]. We expressed the relative energy metabolism in glucose equivalents (in mM·g^{-1}·min^{-1}); so the turnover of lactate equals ½ that of glucose. Based upon these assumptions, the flux (or turnover) of glucose or lactate per gram brain tissue (in mM·g^{-1}·min^{-1}) can thus be calculated as:

$$\text{Flux (or Turnover ICS)} = (180/0.48)\, C_{ss}\, (k_2 + F).$$

The percentage of metabolic trafficking via the ICS (R %) is calculated as the ratio between the calculated Flux (= turnover ICS) and the known Cerebral Metabolic Rate (CMR) for glucose in the rat striatum (0.82 µM·g^{-1}·min^{-1}; the mean value of the values given by Barbelivien et al., 1999 [17], Duelli et al., 2000 [74], Horinaka et al., 1997 [125] * 100% as follows:

$$R(\%) = [(\text{Turnover ICS in glucose equivalents})/(\text{CMR})] * 100\%$$

[equation 4]

7.4.4 Results

Examples of recordings of typical individual experiments are shown in **figure 3**, upper panel. Rat striatal steady state concentrations of ICS (C_{ss}) glucose (n = 10) and lactate (n = 6) were measured up to 6 days following the implantation of a microdialysis probe (figure 3 lower panel. The lowest levels (+ SEM) of glucose were measured at days 2, 3 and 4 and were 0.35 + 0.07, 0.18 + 0.04 and 0.31 + 0.05 mM, respectively. In contrast, the lactate levels (+ SEM) did not significantly vary over 4 days; these levels were at day 2, 3 and 4, 0.64 + 0.03, 0.68 + 0.03 and 0.58 + 0.02 mM, respectively.

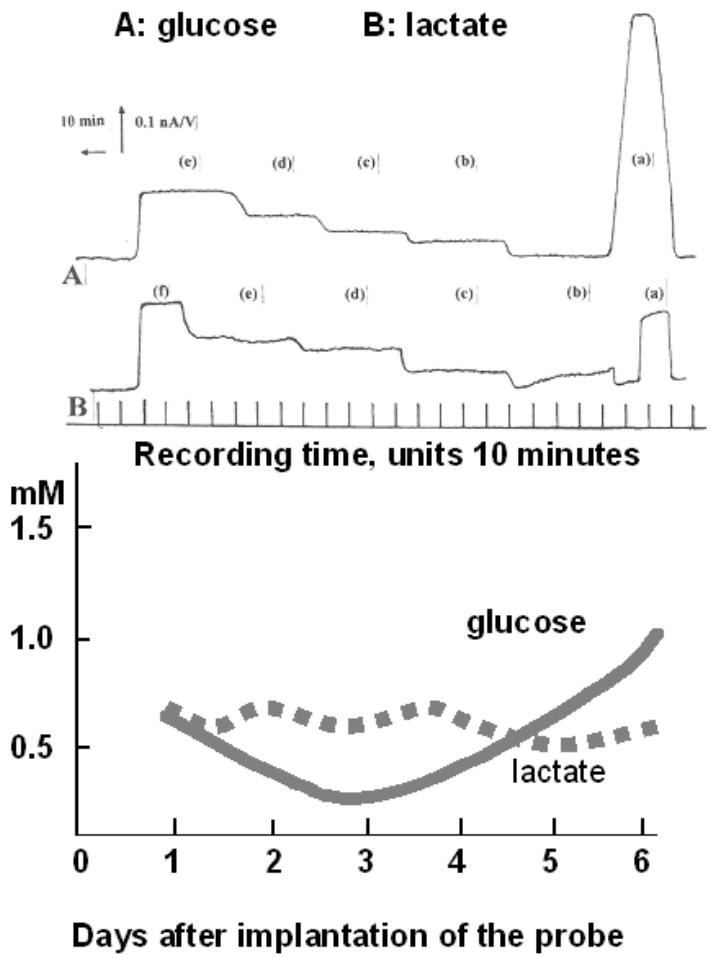

Figure 3 Striatal recordings with ultraslow-microdialysis coupled to a microsensor in freely moving rats. Upper panel: Representative recordings of glucose (**A**) and lactate (**B**) steady state levels following infusion of exogenous glucose or lactate at day 3 or 4 after implantation of the probe. (a): standard solutions of 5 mM glucose or 2.5 mM lactate and striatal levels following infusion (via the probe) of glucose 1.0, 2.0, 2.5, 5.0 mM (b, c, d, e) or lactate 0.5, 1.5, 3.0, 4.0, 5.0 mM (b, c, d, e, f). Lower panel: Glucose and lactate baseline levels measured up to 6 days following implantation of the probe. Data are mean ± SEM of 6 rats with SEM between the thick-ness of the lines.

The turnover rates of glucose (n = 6) and lactate (n = 6) were estimated by infusing glucose (1.0, 2.0, 2.5 or 5.0 mM) or lactate (0,5, 1.5, 3.0, 4.0 or 5.0 mM) at day 3 and the other substrate at day 4. The lactate or glucose data collected at day 3 (n = 3 for each substrate) and day 4 (n = 3 for each substrate) were pooled and the transport constant k_2 and R (%) were calculated for both glucose and lactate in each individual rat using equations [3] and [4], respectively. The k_2 values were 0.13 $g^{-1} \cdot min^{-1}$ for glucose and 0.2 $g^{-1} \cdot min^{-1}$ for lactate. There was no significant correlation between added substrate and calculated turnover rate (R%) of glucose or lactate. The total turnover rate of glucose and lactate expressed in glucose equivalents was estimated to be about 11.5% of total energy consumption, of which 3.4% trafficked as glucose and 8.1% as lactate. These relative turnover rates of glucose and lactate were significantly different **(table 4)**. Thus, more than 88% of the rat brain's energy substrates must be taken up directly from the brain's capillaries or from adjacent glial cells via gap junctions.

Table 4 Steady state levels, absolute turnover rates and relative turnover percentages of interstitial glucose and lactate in rat brain. Ultraslow-microdialysis was applied in the striatum of freely moving rats 3-4 days after implantation of the dialysis probe. Data are mean ± SEM, n = 6.

analyte	glucose	lactate	total in glucose equivalents
steady state levels C_{ss} (mM)	0.23 ± 0.12	0.66 ± 0.36	
turnover ICS ($\mu Mol \cdot g^{-1} \cdot min^{-1}$)	0.03 ± 0.008	0.13 ± 0.03	0.095 ± 0.02
relative turnover (%)	3.40 ± 0.99	8.14 ± 2.39*	11.54 ± 2.25

* in glucose equivalents, different from glucose $p<0.01$; Mann Whitney-U test

7.4.5 Discussion of ICF glucose and lactate levels

The concentrations ranging from 0.20-0.90 mM for glucose and 0.5-0.73 mM for lactate in the striatum of freely moving rats are among the lowest reported (see introduction). The lowest ICS levels of glucose were measured at days 2, 3 and 4 after probe implantation, whereas increasingly higher levels were measured at days 5 and 6. These observations are comparable with those found by Fellows et al. [83], who reported a 75% reduction of ICS glucose levels over 72 h following implantation of the probe, which were attributed to a disturbed glucose metabolism following implantation of the probe. According to these authors, the blood-brain-barrier is resealed within 2 h, whereas the local cerebral blood flow and glucose utilization in tissue surrounding the probe normalizes within 24 h. Groothuis et al. [105] reported, however, that the blood-brain-barrier permeability is affected bi-phasically: a prompt increase shortly after insertion, followed by a second increase several days following placement of the probe. Measurements at longer time intervals are not recommended because of gliosis, that starts in the vicinity of the probe already within 2 days after implantation [25, 78]. Although the blood-ICS-barrier may fluctuate over time, we never observed glucose values approaching blood levels, demonstrating that the barrier remained largely intact throughout the experiments. In contrast to glucose, no significant deviations in lactate content were observed over the 4 days after probe implantation. Following implantation the cellular composition of the brain tissue surrounding the probe will change already from 2 day with relatively more glial cells or glial processes as compared to day 0 or 1 as discussed above. Therefore it is unlikely that ICS lactate levels directly reflect local cellular metabolism in this experiment. Rather these steady state levels are determined by the characteristics of the bidirectional transporters and the average intracellular lactate levels. Brain tissue levels of lactate may remain relatively constant, because even under normoxic conditions lactate leaves the brain, particularly when local levels e.g. in glia adjacent to the vascular bed, tend to become higher than blood plasma levels [197].

7.4.6 About the method of turnover rate estimation

A key assumption in our model is that the rate constants of disappearance of glucose or lactate from the dialysate at ultraslow-microdialysis and from the ICS are similar. Moreover, we assumed that these constants are not affected by the concentrations of the substrates. Indeed, the calculated k_2 values were concentration-independent, thereby suggesting that these values could be applied at steady state perfusion without addition of substrates. The present experimental design does not distinguish between passive diffusion or active metabolism of the added substrates. If diffusion and (already saturated) carrier-mediated transport are the most prominent routes of disappearance of the substrates, the calculated k_2 values should be concentration-independent, in particular at high concentrations of added glucose or lactate (e.g. 5 mM glucose or 5 mM lactate). Direct diffusion from the probe to blood is highly unlikely, considering the large concentration gradient of glucose that has to be overcome at any of the presently applied concentrations of glucose. In case of the lactate perfusion experiments, lactate may diffuse to the blood circulation, but assuming that the blood-brain barrier is intact, only via transport through other cells. The present experiments do not distinguish between transport unrelated to local metabolism or transport of lactate out of the brain. A direct transfer of glucose and lactate to the brain ICS is unlikely, although ICS glucose and lactate levels -after a delay- may follow blood levels as assessed by conventional microdialysis or other techniques (e.g. measuring arterio-venous differences over brain). Fray et al. [89] measured reductions in extracellular glucose in rat striatum following exposure to veratridine, but did not give a turnover estimate of ICS glucose. Considering an apparent half-life of extracellular glucose of about 7.5 minutes, a steady state concentrations of about 0.35 mM and an extracellular space of 20%, we estimate the relative turnover rate in that study about 10% higher than our estimates in the here presented study. Possibly veratridine affects both the size of the extracellular compartment (probably decreased) and cellular processing of glucose (probably increased) as compared to our experiments.

7.4.7 Implications of the extracellular turnover rates

The consumption of energy of the rat striatum is estimated to be 0.82 μmol·g^{-1}·min^{-1} [17, 74, 125]. The present results suggest that about 12% of the energy metabolites traffic through the ICS, implying that the majority of energy substrates do not traffic through this compartment. Both behavioural and pharmacological investigations show that ICS levels of glucose and lactate can easily be influenced and that these effects might well be related to neuronal and glia activity (see our previous sections). The present study emphasizes that such induced variations does not necessarily reflect cerebral metabolism quantitatively and may only represent a specific brain compartment. Knowledge of contributing cells could be useful for a more precise interpretation of the relationship between metabolic en physiological brain activities. For a further discussion on these subjects see the following sections.

7.5 Interpretations and concepts

7.5.1 On the significance of lactate trafficking

In the central nervous system lactate is formed under both aerobic and anaerobic conditions. The question is whether the generated lactate following neural excitation by glutamate is immediately metabolized. According to the so called astrocyte-neuron lactate shuttle hypothesis lactate is formed following glutamate uptake in glia, then released into the intercellular compartment and subsequently used by neurons [247, 248]. In several recent reports [16, 326] it has been assumed that lactate serves as a major substrate for oxidative metabolism during nerve cell activation in vivo. In this context, the interpretation of the biosensor study of Hu and Wilson [127, 128] is crucial. In that study the perforant pathway of the rat was simulated for 5-seconds once or repeatedly and the time course of extracellular lactate, oxygen and glucose in the dentate gyrus of the rat brain was measured with rapidly responding biosensors. A single stimulation showed an initial drop of all the analytes, followed by transient increases. After repeated stimulations extra-cellular lactate was substantially increased, with a concomitant decrease of glucose and little, if any change of oxygen. This study and several others e.g [60], [184, 187, 326], (see also **tables 1-2**) have shown that lactate is formed under

conditions of enhanced neural activity which is -at least in part- associated with lower extra-cellular glucose. The time-course of hippocampus lactate following a single stimulation (lasting 5-seconds) [127, 128], and detected with the biosensors is very similar to that seen with continuous flow analysis of micro-dialysates in rats, subjected to a single electroconvulsive stimulus with an intact entorhinal-hippocampal glutamatergic pathway [184]. Modelling [187] shows that such a time-course can be best described by a very rapid (nearly immediate) increase of intracellular lactate that is subsequently released into the extracellular compartment via a carrier mediated process. To appreciate the results of in vivo studies, a few cautionary remarks should be made. It should be realized that ICS lactate levels are the net result of appearance and disappearance, which is dependent on the rates of lactate influx and efflux across the blood brain barrier, lactate influx and efflux across the membranes of neurons and glial cells, lactate formation from glucose and glycogen, lactate oxidation in the different brain cells, diffusion to and from the site of the probe or sensor, and also the volume of the ICS in brain and finally, the blood lactate concentration. Similar factors determine ICS glucose and oxygen levels, with the obvious differences in transport capacity and diffusion capability. Changes in extracellular space are emphasized below as an example, but the other factors need also careful consideration. Both electroconvulsive shock [187] and electrical stimulation [127, 128] of cerebral neuronal pathways are uncommon (and perhaps even artificial) conditions, as in an intact brain it is unlikely that all neurons of a pathway fire synchronously and at the same rate. In our opinion [176] the study of Hu and Wilson [127] does not support the idea that a substantial proportion of the lactate formed following neural activity, is immediately used as an aerobic substrate. Essential for a quantitative interpretation of the biosensor recordings is that the diffusion of analytes remains constant in the extra-cellular compartment. Shrinkage of the extra-cellular compartment, as a consequence of neuronal activity and associated swelling, has been reported in models of cerebral ischemia (see e.g. [173, 355] and status epilepticus [286]. Since shrinkage of the extra-cellular space affects lactate diffusion towards and into the dialysis probe, we conclude that the initial dip in lactate as reported in the Hu and Wilson study is probably

not due to oxidative metabolism of lactate, but rather to changes in lactate diffusion. Hu and Wilson [127, 128] reported that the slow transient decrease in the levels of extracellular glucose following stimulation is concomitant with a longer lasting increase in lactate levels and constant oxygen levels. This may indeed point to the possible utilization of lactate instead of glucose as an oxidative substrate 10 minutes after the onset of stimulation. In any case, however, the possible oxidative metabolism of lactate is a relatively slow pathway.

A consistent interpretation of all the mentioned studies is that most of the glucose used through the glycolytic pathway, proceeds directly through oxidative metabolism, and that some glucose is converted into lactate, in particular during high neuronal activity. In vivo estimates of the turnover of lactate and glucose in the freely moving rat suggest that a minor proportion of these substrates traffic through the extra-cellular space (previous section). It is likely that a substantial proportion of cerebrally formed lactate disappears (un-metabolized) via the blood circulation [196]. This may also explain the well-known discrepancy between glucose and oxygen uptake and metabolism in brain during activation [195].

Our results (previous section) taken together suggest that under physical rest less than 12% of the glucose and lactate consumed by the brain is transported via the ICS. It can be argued that our experiments are done at low activity of e.g. the glutaminergic neurotransmission, thus underestimating the contribution of ICS glucose and lactate to energy metabolism in the activated brain. Rat striatal ICS-lactate is increased during physical exercise to a (maximum) increase of only 30% [60] (see for other references **table 2**). At least part of the increase was mediated by glutamatergic innervations [177, 180]. These observations suggest that the lactate response is due to both released glutamate and glutamate receptor mediated signaling. A recent paper by Caesar et al. [37] measuring fast changes of glucose and lactate in the cerebellar cortex upon mossy fiber stimulations is essentially in line with the conclusions by Attwell and Laughlin [15], since application of the AMPA antagonist NBQX completely blocked the stimulation induced lactate rises. This study [37] emphasizes that glutamate release does not necessarily cause lactate formation in glia, but rather that lactate is primarily formed by

activation of the postsynaptic neuron. In neurons postsynaptic to glutamate nerve endings often a high affinity lactate transporter (MCT2) is localized [26]. This high affinity transporter has often been considered to support the hypothesis that neurons can efficiently accumulate lactate, released from glia for aerobic metabolism. Alternatively, we propose that such a high affinity transporter may also serve to export neuronal lactate to the ICS instead, in order to keep intracellular lactate at a low level. A new concept of brain metabolism that acknowledges these findings may include a less strict cellular localization of sequential metabolic processes. Rather, the cellular heterogeneity of the brain should be taken into account to explain simultaneously operating metabolic pathways.

7.5.2 Brain energy and brain events in space and time

In the introduction we referred to the suggested tight coupling between neuronal activity and cerebral blood flow or increased energy metabolism. In this section we will discuss in some detail the question for which brain processes (increased) energy metabolism is required, and will draw the conclusion that the coupling between energy utilization and brain function may not be as strict as usually assumed. A more complete discussion on this issue has recently been published [179].

7.5.2.1 Neurotransmission and action potentials

Action potentials and glutamatergic and γ-amino butyric acid (GABA)-ergic neurotransmission are generally considered the fastest processes enabling intercellular communication. A single action potential lasts 2-5 ms and may be followed by a refractory period of about 4 ms. Firing frequencies of cerebral neurons range from less than 1 up to 200 Hz; the latter frequency points to action potentials of 5 ms duration. Fast excitatory and inhibitory neurotransmission by glutamate and GABA are also in the millisecond range. Attwell and Gibb [14] summarized the kinetic properties and affinities of the AMPA and NMDA receptors and of glutamate transporters, and concluded that glutamate transmission requires only a few milliseconds to proceed. Similarly, the inhibitory action of GABA by opening Cl^- channels via the $GABA_a$ (and $GABA_c$)-receptor requires less than 5 ms. Neurotransmitters are stored in

synaptic vesicles. Vesicular glutamate uptake is ATP-dependent [295]. ATP and Ca^{2+} are required for the fusion of vesicles with the pre-synaptic membrane [134] and release of vesicular transmitter is in the range of m*s* [85, 219]. Assuming that released GABA and glutamate are predominantly metabolized and that 1 ATP is required for vesicular uptake and storage, the cerebral (metabolic) turnover of these amino acids indicates how much ATP is required for fast neurotransmission. This has been estimated to be about 2% of the total energy budget of the brain [14]. If we assume that the amino acid terminals occupy less than 1% of the brain volume, the excitatory nerve terminals use a major proportion of local metabolic energy to keep the vesicles filled up. Similarly, in GABA-ergic terminals a major part of energy metabolism may be required for vesicular transmitter storage. In addition to ionotropic glutamate and GABA receptors, fast neurotransmission is also possible through nicotinic acetylcholine, strychnine-sensitive glycine and $5HT_3$ serotonin receptors [42].

The large trans-membrane gradients of ions and fast transmitters allow synaptic release and postsynaptic effects within 2 to 10 m*s* with a delay in transmitter synthesis and vesicular accumulation.

7.5.2.2 Nerve activity and in vivo metabolic response

Physiological stimuli or electrical stimulation of nerve fibers in vivo or in vitro have been used to explore the consequences of enhanced impulse flow on energy metabolism. There is little question, whether during (or following) neuronal activation the consumption of glucose and oxygen is increased.

A decrease in brain (cerebellum and hippocampus) oxygen tensions (presumably reflecting increased consumption) has been observed within a few seconds after excitatory stimulation. Such studies show that the coupling of oxygen consumption and electrophysiological activity is not linear. The energy consumption (glucose and/or oxygen) is only marginally increased at relatively low stimulation frequencies and non-linearly at higher stimulation frequencies. The site of increased energy consumption differs often from that of direct activation [323]. One explanation could be the relatively slow transport of glucose as compared to oxygen. Another possibility is the consumption of lactate or

acetate as an alternative energy substrate [7, 112]. Several experiments have shown fast anaerobic glucose metabolism following enhanced neuronal activity (previous section) [100, 127, 184, 187]. Cerebral lactate transporters are essentially bidirectional, thus questioning the idea of a strong coupling between neuronal lactate metabolism and glutamatergic transmission. So the intercellular movement of lactate is mainly concentration-driven. And a directed flow of lactate into particular neurons is highly unlikely [176]. Accordingly, a large proportion of lactate leaves the brain un-metabolized when lactate levels in capillary blood are lower than the lactate levels in adjacent cells.

The Na^+-K^+-membrane-pumps do not always have fast access to mitochondrial energy substrates (e.g. ATP). Thompson et al. [306] assessed the relationship between the temporal and spatial changes in blood flow and oxygen changes with a locally placed oxygen electrode. After increased focal activity fast negative dips of oxygen tension following visual stimulation in the cat dentate gyrus or cerebral cortex were observed near the stimulating electrode. During widespread activity, the oxygen dip was less pronounced, occurred later and predominantly increases in oxygen tension were seen. The latter changes are rather slow as compared to cellular activity in the visual cortex. As in the case of cerebral blood flow, the late overshoot in metabolic activity occurs always several seconds after the enhanced activity of neurons that triggered these changes. The initial dip in O_2 tension occurring very soon following neuronal activity may be attributed to the utilization of oxygen that is not compensated by the blood supply and diffusion through the tissue. Changes in the cellular and intercellular volume may also explain at least some of the changes in the oxygen signal [8, 176]. The dip shows early deoxygenating of hemoglobin within 100 m*s*, but no changes in CBF or blood volume [8]. Together, these studies emphasize that after neuronal activation the consumption of freely dissolved oxygen and other energy substrates starts within 100 ms.

7.5.2.3 Potential versus metabolic energy

The shortest physiological activities involved in inter-neuronal communication in the peripheral and central nervous system are action potentials and excitatory and inhibitory neurotransmission

predominantly by glutamate and GABA, lasting 2-10 m*s*. Activities, such as reflexes, speaking, recognition and complicated information processing of the brain does most often require less than 100 m*s*. Psycho-physiological studies illustrate that fast physiological processes do not primarily depend on extra-cerebral energy substrate recruitment. Moreover, consumption of energy reserves (such as glycogen) is too slow to be available for immediate use. Changes in cerebral glucose uptake and cerebral blood flow, which helps delivering and removing energy substrates to and from the brain, are also relatively slow processes. However, an increased O_2 consumption is already observed within 100 m*s* following neuronal activity and is therefore most sensitive indicator of increased cerebral energy utilization. The brain is best prepared for fast activities in the ms range, when the trans-membrane gradients of the ions (Na^+, K^+ and Cl^-) are kept maximal and when the vesicle stores (of GABA and glutamate) are maximally filled. These concentration gradients are the exclusive prerequisite to perform psycho-physiological functions and may be denoted potential energy of the brain. Brain energy metabolism must be primarily considered a restorative activity: i.e. to restore and maintain the potential energy of the brain at the highest level.

7.6 Concluding remarks

Quantitative microdialysis of glucose and lactate in rat and human brain has revealed much lower glucose levels in the brain ICS than in blood, whereas ICS lactate levels are elevated or similar as compared to blood levels. The various issues discussed in the present paper are shown in **figure 4**. In many of the neuronal activation paradigms tested in experimental animals, lactate elevations are faster and more pronounced than glucose perturbations, but the fastest detected changes (e.g. at 30 seconds intervals) are still slow as compared to the electrophysiological events, which occur on a millisecond time scale. Activation-induced ICS lactate and glucose changes are predominantly mediated through ionotropic glutamate receptors (NMDA and AMPA type). By using ultraslow-microdialysis coupled to biosensors for glucose and lactate and perfusion with exogenous glucose or lactate, we determined the turnover rates of glucose and lactate in the brain ICS of

freely moving rats and found that less than 12% of total glucose metabolism can be attributed to diffusion of glucose (3 - 4%) and lactate (8%) via the intercellular compartment.

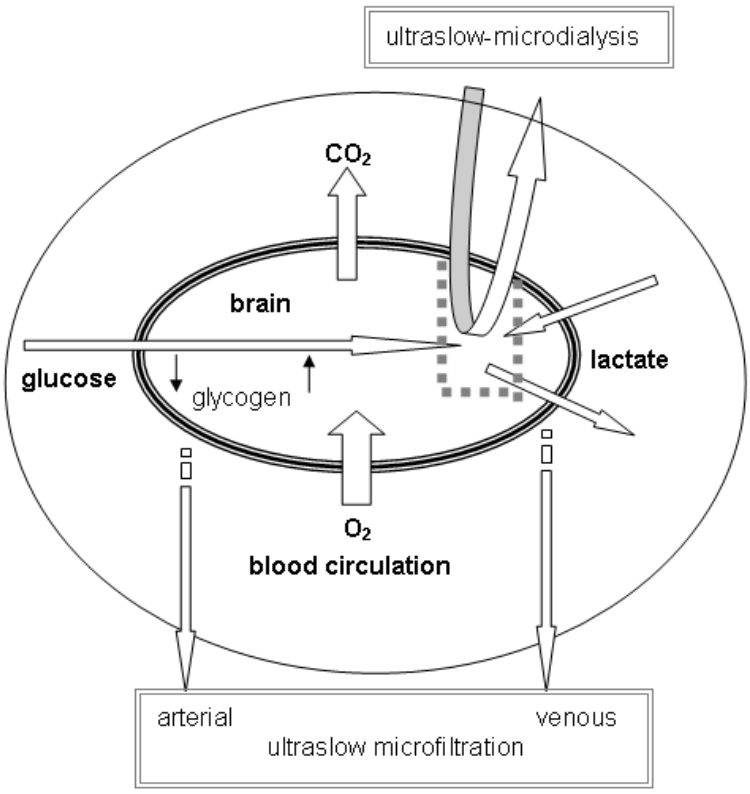

Figure 4 Intercellular trafficking of glucose and lactate as studied with ultraslow intravascular microfiltration and ultraslow intracerebral microdialysis. Most of the glucose taken up by the brain is directly transported to the oxidative pathways in both neurons and glial cells. A minor part of glucose is stored temporarily as glycogen in astroglia, which can be released as lactate. Lactate produced in the brain, predominantly by astroglia will be oxidized (minor part), predominantly by neurons and oligodendroglia or transported out of the brain (major part). Depending on the concentration gradient of lactate over the blood-brain barrier, lactate enters or leaves the brain.

If activated neurons would preferably consume lactate produced by astrocytes, a directed lactate flow from glia to neurons and a high

turnover rate would be expected, which is clearly not the case under normal conditions. Steady state levels of lactate but not of glucose, is essentially independent on the cell types surrounding the probe, suggesting a dominant role of transporters, rather than cellular metabolism. In this review we emphasize that lactate transporters on both neurons and glia are essentially bidirectional and that ICS lactate is derived from both (postsynaptic) neurons and glia. Under normal conditions elevated ICS lactate is predominantly removed by the blood circulation. It remains to be shown whether under conditions of increased neuronal activity (e.g. convulsions or exercise) or limited glucose supply (e.g. hypoglycaemia), ICS lactate turnover rates are increased. Advances in nanosensor technology may in the future allow simultaneous, real-time recording of intra- and extracellular glucose and lactate at the synaptic level and studies of substrate flow through neuronal or glial transporters. However, such studies can only be performed in vitro with no blood supply and abnormal concentrations of energy metabolites in the ICS.

For clinical studies the recently low time resolution microdialysis sampling at 0.5 or 1 minute intervals and on-line detection by a FIA-biosensor set-up is most promising, since this may allow early interventions to prevent expansion of ischemic injury. In such studies the position of the probe(s) in relation to the trauma, ischemic core or operated blood vessels (in SAH patients) is of course of paramount importance.

Finally, we discussed brain energy metabolism at a more theoretical level and provided arguments to distinguish potential and metabolic brain energy. Potential brain energy is considered a necessary condition for fast neuronal processing, whereas metabolic brain energy serves to restore high levels of potential energy.

7.7 Acknowledgement

This research is supported by the Dutch Technology Foundation STW, applied science division of NWO and the Technology Program of the Ministry of Economic Affairs, grant numbers GPG 06038 and GGN 4680. Support of the Commission of the European Communities, Director

General for DG XII Science, Research and Development, Biomed 2 Program PL-972726.

Chapter 8
Summary and general discussion

Important aspects investigated in this thesis are how to obtain biological samples from intact organisms, and how to store and analyse hormones and some pharmaceutical compounds. Part of the investigations is aimed to develop a continuous monitoring device sampling the glucocorticoids corticosterone and/or cortisol in a freely moving subject. Glucocorticoids are hormones released from the adrenal cortex often as the result of stress experiences of the subject. In addition they are subject to a generally consistent circadian rhythm, although considerable intra- and inter-individual differences exist. The pharmaceutical compounds serve as examples to assess whether our methodologies could be used as a convenient approach to monitor pharmacokinetics. To explore the temporal patterns of peptides, drugs or hormones, conventionally multiple blood samples have to be collected which is stressful and invasive. As alternative sampling methods we explored microfiltration and microdialysis applied at very low perfusion rates.

In **chapter 2** an overview is given about ultraslow-microfiltration since its invention in 1987. Microfiltration is a sampling technique based on the principle of slow withdrawal of body fluid from an organism that is filtrated when passing a semi-permeable membrane. The driving force in microfiltration is under-pressure. In microdialysis the analyte is collected in a dialysate that is obtained after the infusion of a physiological solution (perfusate) through a hollow fiber with a semi-permeable membrane. The concentration gradient of the analytes over a hollow fiber membrane is the driving force in microdialysis. At very low perfusion rate the concentrations of the analytes in the perfusate and in the body compartment outside the probe become identical (100%).

More than 5,000 experimental studies on microdialysis have been published since its introduction. In contrast only about 50 scientific papers on microfiltration have appeared. The clinical potential of both techniques was recognised from the early onset. Microdialysis is applied in clinical and preclinical research and was initially developed for sampling the brain. Microfiltration is predominantly applied in experimental procedures in preclinical research. Application of microfiltration in the brain is not possible, at least not in the rat. The

extracellular compartment is too small to allow withdrawal of even minor amounts of body fluid (50 nl·min^{-1}).

Microfiltration hollow fiber membranes with different specifications were assessed for non-specific binding in **chapter 3** (in vitro) and **chapter 4** (in vitro and in vivo). The glucocorticoids corticosterone and cortisol are rather lipophilic and bind easily to microfiltration and/or microdialysis fiber membranes. In addition the undesired and non-specific binding a variety of drugs (gaboxadol, citalopram, morphin), small peptides (poly-l-lysine and poly-l-tryptophan) to the hollow fibers was investigated. The microfiltration polyethylene probe coated with ethylenevinylalcohol (PE-EVAL; plasmaflo) showed minimal adsorption of poly-l-lysine, poly-l-tryptophane, citalopram and corticosterone (recoveries near 100%). The performance for the tested analytes is better than the other tested hollow fiber membranes (polyethersulfone (microPES, and transvivo), polysulfone (plasmacure), polypropylene (plasmaphan) on non-specific binding. The plasmaflo is the most hydrophilic and the plasmaphan the most hydrophobic membrane. Concluding that hydrophilic, as well as, hydrophobic analytes can be sampled with plasmaflo hollow fiber membrane. We continued testing this probe in vivo together with the PES membranes –microPES and transvivo. These two hollow fiber membranes performed not as poor as polypropylene (plasmaphan) and polysulfone (plasmacure) on non-specific binding in **chapter 6**.

In **chapter 6** the microfiltration hollow fibers were placed in various locations: subcutaneously, intraperitoneally, and intravenously in rats. The hollow fibers were coated with and without heparin (plasmaflo (polyethylene coated with ethylenevinylalcohol), microPES (polyethersulfone), and transvivo (polyethersulfone). The plasmaflo probe coated with heparin showed the best performance, particularly in blood. Therefore this probe was connected with a collection device and further tested in cows sampling total cortisol. The microfiltration collections device allowed continuous sampling for a period of at least 7 hours. In contrast with heparin uncoated plasmaflo hollow fiber, sampling lasted only 1.5-2 hours.

In conventional microdialysis perfusion rates over 1 µl·min^{-1} and higher have been used, resulting in recoveries far from 100%. Complicated

procedures and computations are required to calculate (or –perhaps better- to estimate) the real concentration of the analyte. Quantitative microdialysis, without calibrating the microdialysis device, can be realized by applying an ultraslow perfusion rate (<0.3 µl·min^{-1}), resulting in similar concentrations for the analyte in the dialysate and the tissue compartment. Using ultraslow perfusion rate the recovery is near 100%. A disadvantage of ultraslowflow perfusion rate is consequently the long lag times between sampling and subsequent chemical analysis, and the small sample volumes. These disadvantages are avoided by using the MetaQuant microdialysis sampling technique, which is a modified conventional microdialysis probe. In the metaquant microdialysis probe an additional flow (carrier flow) is induced to merge with the ultraslow dialysate immediately downstream from the microdialysis probe membrane. The carrier fluid is delivered at a higher flow rate (typical 0.9 µl·min^{-1}) that shortens lag time and increases final sample volume for easier handling. The obvious dilution of the dialysate by the carrier fluid requires sensitive analysis techniques such as mass spectrometry. Because at ultraslow-microdialysis the exchange of the analyte over the probe membrane is close to 100% and the dilution of the sample by the carrier flow and collection is known, the quantity of the analyte of interest can easily be corrected mathematically after analysis.

Chapter 4 and **chapter 5** describe the testing of two different MetaQuant hollow fiber membranes, sampling with 4-5 different flow rates (0.1 - 1.0 µl·min^{-1}). At flow rates of 0.15 µl·min^{-1} and lower the regenerated-cellulose (RE) and polyethersulfone probes showed recoveries >90% for all tested compounds (citalopram, morphine, gaboxadol and corticosterone). The MQ-microdialysis probe is extremely practical as the lag times are short and in vivo obtained concentrations are quantitative.

A major advantage of microfiltration over microdialysis was the high recoveries, guaranteed to be near 100%, but with the introduction of the quantitative MQ-microdialysis technique this advantage is lost. Another advantage of the MetaQuant technique is that pharmaceuticals can be introduced locally via the perfusate. But the MQ-microdialysis posses also some disadvantages. The utility of mechanical pumps and sampling

in large and freely moving animals is rather complex. Mechanical pumps are rather big and heavy to carry and need substantial power supply to work properly over longer periods of time.

Both microdialysis and microfiltration allow the collection of protein-containing and protein-free body fluids, depending on the MWCO value of the chosen hollow fiber membrane. Most reports on microdialysis and microfiltration use hollow fibres with a molecular weight cut-off (MWCO) value <20 kDa. However there is an increasing interest in sampling proteins. A possible disadvantage of using a high MWCO value in microdialysis is the outflow of perfusion fluid via the hollow fiber membrane into the surrounding tissue, due to the hydraulic pressure in the outlet tubing (= perfusion ultra-filtration loss). Such effect may be avoided by adding high molecular weight osmotic agent to the perfusion fluid such as Dextran-60.

In microfiltration it is possible to sample continuously without mechanical pump pulsations, the presence of the analyte in a most often stabilizing natural matrix, and when attached to a collection device, sampling of freely moving subjects. Necessary conditions for good functioning of the collection device (a long capillary) applied in freely moving subjects, is that the longitudinal diffusion of the analyte of interest is not significant and the analyte must be stable at body temperature. Glucose and lactate -hydrophilic and small analytes- have continuously been collected with a similar device with a small cut-off value of 20-30 kDa and sampling with a flow rate of 50 $nl \cdot min^{-1}$. Glucose/lactate and protein-bound cortisol, diffusion was not significant through the capillary coil and/or the analytes did not deteriorate at body temperature.

Disadvantages of microfiltration sampling are that no drugs can be introduced locally through the probe and microfiltration can only be applied when there is sufficient endogenous production of extracellular fluid. There is another disadvantage to consider, that concerns the in vivo flow rates. In previous studies in broiler chickens it was observed that in vitro flow rates (fixed at 50 $nl \cdot min^{-1}$), were lower in vivo (46 $nl \cdot min^{-1}$); a non-significant difference. In the cow experiments we noticed that an in vitro flow rate of 700 $nl \cdot min^{-1}$, was about 250 $nl \cdot min^{-1}$ lower in vivo. This difference was significant and probably due to the

higher viscosity of blood filtrate. Viscosity of the blood filtrate obtained by the plasmafo hollow fiber membrane with a cut-off value of 3 MDa is substantially higher than that of the blood filtrate collected at a cut-off of 20-30 kDa.

In both techniques the hollow fiber membrane is the interface between the sampling devices and the organism. Relatively few studies have systemically investigated hollow fiber membranes materials and dimensions for biofouling and/or biocompatibility. Introduction of the hollow fiber probe in vivo causes minor acute mechanical tissue damage, but after longer time periods of implantation the damage may increase. The emerging tissue damage may depend on the location and the material of the hollow fiber. In general, in all tissues an inflammatory response will sooner or later be observed. Our experiments performed in the rats, led us to the conclusion that membranes with the smaller pore sizes provide superior sampling stability in blood, as compared to membranes with larger pore sizes. Presumably proteins clog the hollow fiber membranes with wider pores more easily. This may also apply to subcutaneously implanted microfiltration probes, not on account of clogging, but because proteins and cells adhere more easily to larger pores.

Chapter 7 is describing an extensive overview on the biomedical application of microdialysis and microfiltration in the monitoring of cerebral energy substrates. The focus is, mainly on glucose and lactate. However the experimental study described in this chapter to assess the relative glucose and lactate turnover rate at steady state could also be applied to other analytes, for instance to study cerebral pharmacokinetics quantitatively. The approach is based on the loss of the analyte added to the perfusate of the microdialysis probe at steady state levels. In the present context it was assumed that higher levels of glucose do not affect the rate of glucose consumption. Such an assumption is justified, among others, because acute hyperglycemia does not affect cerebral glucose consumption in conscious rats. We described a model to calculate turnover rates of lactate and glucose and concluded that only a minor fraction of total energy metabolism of the brain is carried by transport of energy substrates through the extracellular compartment.

This thesis describes microdialysis and microfiltration sampling and storage of a wide variety of analytes, ranging from low molecular weight hydrophilic (lactate and glucose) and hydrophobic (cortisol, cortisosterone, various drugs) compounds up to peptides (polytryptophan, polylysine) and voluminous proteins (e.g. binding corticosterone or cortisol). The suitability of various hollow fiber membranes with cut-off values ranging from 20 kDa up to 0.3 µm (3 MDa) was tested. As a proof-of-principle it was demonstrated that the microfiltration collection and storage device is applicable to a wide range of analytes –small and big- versus -hydrophilic and hydrophobic- in freely moving subjects. The in vivo experience is thus far limited, so further validation of the microfiltration-collection device is necessary to achieve optimal performance in freely moving subjects. In particular more compounds, hollow fibers, longitudinal diffusion and deterioration of compounds deserve further attention. Moreover, comparison of results with conventional sampling techniques (e.g. frequent blood sampling) is also necessary before widespread applications. Probes with a high cut-off value may be beneficial in the distribution, regulation, concentrations and functioning of proteins. Peptidess are important in cell-to-cell communication in immune defence, inflammatory processes and wound healing. But also in the search of biomarkers and therapeutic targets in cancer therapy this device may be valuable. Microfiltration as well as the MQ microdialyis approach requires considerably fewer animals and more data is gathered with one microfiltration and/or MQ-microdialysis experiment.

There is a growing tendency to reduce sample volumes, because of the increasing sensitivity and specificity of current analytical technologies, such as microbore or capillary HPLC, capillary electrophoresis, mass spectrometry and (bio)-sensoring. The MQ-microdialysis and microfiltration sampling technique provide small samples. On-line analysis with such versatile methods as HPLC or MS requires easy and preferentially automatic on-line sample handling that is most conveniently achieved with the MQ-microdialysis. When on-line real time analysis is not required, microfiltration combined with storage in capillary coil is to be preferred. Because of minimal diffusion, the thus stored samples can be -if required inter-continentally- shipped to be

analysed. In theory the capillary coil could also be attached to an analytical device. Fractionating the biofluid content into fragments and analysing each fragment on the analyte(s) of interest.

The present thesis shows that every technique has its own potential(s) and/or drawback(s), in a certain experimental set-up. The investigations presented in the present thesis may help to make to choose the proper probe and materials.

Chapter 9
Samenvatting voor de geïnteresseerde leek

Artsen en onderzoekers zijn soms geïnteresseerd in het meten van stofjes in het lichaam over een bepaalde tijdsperiode in vrij bewegende individuen, dit kan met behulp van twee monstername-technieken.

In dit proefschrift worden twee monstername-technieken beschreven: microfiltratie en microdialyse. Enerzijds zijn de technieken vergelijkbaar, anderzijds totaal verschillend. De methodiek waar de voorkeur naar uitgaat is onder andere afhankelijk van de opzet van het experiment. Wat wil je meten en waar wil je dit stofje (analiet) meten (bloed, hersenen, onder de huid, of in weefsels). In dit proefschrift zet ik beide technieken uiteen.

De belangrijkste vraag in dit proefschrift is: Is het mogelijk om monsters uit het lichaam (*in vivo*) te nemen, deze monsters tegelijkertijd met behoud van tijdverloop op te slaan en pas nadien te analyseren.

Een deel van dit onderzoek is gericht op het monsteren van de glucorticoiden; corticosteron (knaagdieren) en cortisol (mensen). Glucocorticoiden worden ook wel stresshormonen genoemd. In een normale fysiologische situatie volgt het stresshormoon een vierentwintig-uurs ritme met een piek in de vroege ochtend die daarna weer terugkeert naar een basale waarde. Tevens schiet het stresshormoon omhoog bij het ervaren van stress. Het ritme van het stresshormoon is doorgaans zeer consistent, maar verschillen kunnen voorkomen zowel binnen een individu als tussen individuen. Wil je het glucorticoide-ritme in kaart brengen, dan moet je normaal gesproken over vierentwintig uur om de zoveel tijd een bloedmonster nemen. Zowel mensen als dieren vinden dit doorgaans geen prettige handeling en kunnen dit als een stressor ervaren waardoor de glucorticoidwaarde negatief beïnvloed kan worden.

Niet alleen wil ik in dit proefschrift laten zien dat je endogene stoffen (stoffen die voorkomen in het lichaam) als eiwitten, peptiden, en andere hormonen kunt monsteren, maar ook exogene stoffen zoals geneesmiddelen en andere farmaceutische middelen (gaboxadol, citalopram en morfine).

In **hoofdstuk 2** wordt een overzicht gegeven van zeer langzame (ultraslow) microfiltratie sinds de eerste beschrijving in 1987.

Microfiltratie is een monsternametechniek waarbij lichaamsvloeistof uit het lichaam wordt getrokken over een membraan (=filter met poriën erin) in de vorm van een "holle draad" (= hollow fiber). De hollow fiber wordt in het lichaam gebracht, dit kan zijn in de bloedbaan, onder de huid of in een ander weefsel. Vacuüm is de drijvende kracht voor het ontrekken van de lichaamsvloeistof over de membraan. Achter het membraan bevindt zich een capillaire opvangslang welke op zijn beurt langzaam gevuld wordt, met aan het eind de vacuümpomp.

In microdialyse wordt het analiet waarin men geïnteresseerd is verzameld door het inbrengen (infusie) van een fysiologische vloeistof door een hollow fiber en het opvangen van het dialysaat. Het dialysaat is de vloeistof dat je opvangt met het analiet waarin je geïnteresseerd bent. Er wordt dus geen vloeistof onttrokken uit het lichaam. De drijvende kracht van microdialyse is het bewerkstelligen van een concentratie-gradiënt over het membraan. Wanneer er een concentratieverschil ontstaat, dus bij microdialyse binnen en buiten de hollow fiber, zal de omgeving waarin de hollow fiber zich bevindt, het concentratie-verschil willen opheffen. Dit betekent dat analieten van een hoge concentratie zich verplaatsen naar waar de concentratie laag is. Dit proces heet ook wel diffusie. Zodoende worden analieten over het membraan getransporteerd. Diffusie is een langzaam proces. De hoeveelheid beweging van een analiet per tijdseenheid is afhankelijk van de grootte van het analiet (een groter analiet diffundeert langzamer), de temperatuur (een hogere temperatuur zorgt voor een snellere diffusie), en een stroperige omgeving (viscositeit) zal de diffusie snelheid van een analiet afremmen. Dus bij een zeer langzame perfusie snelheid zal het analiet binnen en buiten de hollow fiber sneller in evenwicht komen. In niet-leken-taal: de recovery is dan 100%.

In de vakliteratuur zijn meer dan 5.000 experimenten beschreven die gaan over microdialyse. Tegen slechts 50 die gaan over microfiltratie, waarin het percentage overzichtartikelen verhoudingsgewijs relatief groot is. Vanaf de eerste beschrijving van microdialyse en microfiltratie werd ingezien dat de klinische toepassingsmogelijkheden zeer groot zijn. Echter is de toepassing twee a drie decennia later nog steeds zeer gering in de kliniek.

Microdialyse is in eerste instantie ontwikkeld om het brein te kunnen monsteren, wat niet mogelijk is met microfiltratie (tenminste in het brein van de rat). Dit is dan ook een van de belangrijkste verschillen tussen microdialyse en microfiltratie. Er is te weinig vloeistof buiten de cellen (extracellulair) aanwezig om hele kleine hoeveelheden lichaamsvloeistof te kunnen monsteren. Zelfs wanneer er gemonsterd wordt met een zeer lage snelheid van 50 nanoliter per minuut. Een druppel water heeft een volume van 50 microliter (= 50.000 nanoliter).

Verschillende hollow fiber membranen worden getest in **hoofdstuk 3** (*in vitro* = in glas) en **hoofdstuk 4** (*in vitro* en *in vivo* (= in het lichaam) op a-specifieke binding. Glucorticoiden zijn lipofiele stoffen. Lipofiele stoffen zijn stoffen die goed oplossen in vetten en oliën en niet houden van water (hydrofoob). Veel hollow fiber membranen zijn synthetisch en hebben een hydrofoob karakter. Doordoor zouden analieten waarin je geïnteresseerd bent kunnen binden aan het membraan (a-specifieke binding) en dus wordt de analieten-waarde negatief beïnvloed. De meest hydrofiele membraan (Plasmaflo = polyethyleen gecoat met ethyleen vinyl alcohol) presteerde het best in tegenstelling tot de meest hydrofobe membraan (Plasmaphan = polypropyleen). Hieruit wordt de conclusie getrokken dat de plasmaflo membraan zowel hydrofiele als hydrofobe analieten kan monsteren. Dit membraan is op zijn beurt verder getest in het lichaam (**hoofdstuk 6**)samen met twee polyethersulfone membranen (microPES en transvivo). Deze membranen vertoonden a-specifieke binding, maar de recovery waarde was groot genoeg.

In conventionele microdialyse wordt er gemonsterd met perfusie snelheden van 1 microliter per minuut (20 druppels water per minuut) of hoger, resulterend in zeer lage recoveries. Om terug te kunnen rekenen naar de werkelijke waarde moeten er zeer gecompliceerde berekeningen en methodieken worden uitgevoerd. Kwantitatieve microdialyse kan gerealiseerd worden, dus zonder je microdialysemethode te kalibreren, door een zeer lage perfusiesnelheid toe te passen van 300 nanoliter per minuut. De concentratie van het analiet is dan zowel buiten als binnen de hollow fiber gelijk (recovery = 100%). Het nadeel van het werken met een zeer kleine flow is toename in de dode tijd. Hiermee wordt bedoeld dat het punt van waar je

monstert tot waar je uiteindelijk je waarde kunt aflezen een vloeistof volume aanwezig is van bijvoorbeeld 200 microliter. Het duurt dan 11 uur voordat je monster op het punt is aangekomen waar je de waarde kunt aflezen. Een ander nadeel is dat het op te vangen volume zeer klein is, dus verdamping van de vloeistof kan een probleem zijn. Een microdialysemonster is meestal een monster van 30 minuten, dat komt dus overeen met een volume van 9 microliter. De MetaQuant microdialyse techniek is in staat quantitatief te meten met een lage dode tijd, doordat de zeer langzame flow wordt verdund tot 1 microliter per minuut. Het werkvolume is dus groter en de recovery 100%.

In **hoofdstuk 4** en **hoofdstuk 5** worden MetaQuant microdialyseprobes gemaakt van regenerated cellulose en polyethersulfone en getest op recovery voor de verschillende flowsnelheden in de range van 0,1 – 1,0 microliter per minuut. Bij het gebruiken van een flowsnelheid van 0,15 microliter per minuut en lager waren de opbrengsten 90% en hoger voor de volgende geteste stoffen: citalopram, morfine, gaboxadol en corticosteron.

Het grote voordeel van microfiltratie is het verkrijgen van hoge recoveries. Met de komst van de MetaQuant wordt dit voordeel tenietgedaan. Een ander voordeel van de MetaQuant methode is, dat je aan het perfusaat farmaceutische middelen kan toevoegen. Maar de MetaQuant bezit ook nadelige eigenschappen. Je verdunt je oplossing en daarom moet de analysemethode gevoelig genoeg zijn. Tevens ben je voor de flow inductie een mechanische pomp nodig en dus een stopcontact. Een individu zal dus nooit vrij kunnen bewegen in zijn eigen omgeving.

Zowel met microdialyse als met microfiltratie kun je eiwitrijke als wel eiwitarme lichaamsvloeistoffen monsteren. Dit is afhankelijk van de hollow fiber en de grootte van de poriën in het membraan (de zogenaamde molecular weight cut-off value = MWCO). Meestal worden er membranen gebruikt met een MWCO waarde kleiner dan 20 kDa. Je vangt dan een eiwitarme lichaamsvloeistof op. Er is een toename in interesse naar membranen met een grotere MWCO waarde. Echter wanneer je zo'n membraan gebruikt in microdialyse dan is de kans groot dat het perfusaat, via het membraan het lichaam invloeit (ultrafiltratievloeistof -verlies). Dit verschijnsel kan tegen gegaan

worden door de osmotische waarde van het perfusaat te verhogen door bijvoorbeeld Dextran-60 toe te voegen.

Bij microfiltratie is het mogelijk om continu te monsteren zonder het gebruik van een mechanische pomp. Tevens is het analiet waarin je geïnteresseerd bent opgelost in zijn eigen stabiliserende matrix en kan er continue gemonsterd worden in een vrij bewegend individu. Daartoe wordt (lichaams-)vloeistof langzaam, maar wel continu, aangezogen en gaat daarna door een zeer lang capillair met een doorsnede van minder dan 0.5 mm en een lengte van enkele meters. Een voorwaarde is wel dat het analiet waarin je geïnteresseerd bent niet longitudinaal diffundeert door de opvangcapillair en het analiet niet kapot mag gaan tijdens het monsteren en/of bewaren in de opvangslang.

Cortisol is in het lichaam gebonden aan twee grote eiwitten: transcortine en albumine, slechts 10% is ongebonden en biologisch actief. Cortisol diffusie was niet significant bij het opslaan van plasma in de opvangslang en er vond geen afbraak plaats tijdens het bewaren bij 37 of 4 graden Celsius gedurende 3 dagen (**hoofdstuk 4**)

Een nadeel van microfiltratie is dat er lokaal geen stoffen via de hollow fiber het apparaat kunnen worden geïntroduceerd zoals bij microdialyse waar je een perfusie-vloeistof toedient. Additioneel moet er voldoende vloeistof aanwezig zijn om te kunnen monsteren, omdat je netto vloeistof van het lichaam onttrekt. In het verleden is microfiltratie ook toegepast voor het monsteren van glucose en lactaat in onder andere kippen. Beiden analieten zijn relatief klein en houden van water (hydrofiel). Destijds werd er gemonsterd met een ingestelde flowsnelheid van 50 nanoliter per minuut. De werkelijke flow was 46 nanoliter per minuut, een niet significant verschil. In de koeien-experimenten beschreven in **hoofdstuk 6** was de ingestelde flow 700 nanoliter per minuut. Echter bij het monsteren van de koe bleek de flow 250 nanoliter per minuut lager te zijn. Een significant verschil, waarschijnlijk veroorzaakt door de hogere viscositeit van het opgevangen microfiltraat. Het membraan toegepast in de koeienexperimenten heeft een MWCO waarde heeft van 3MDa een factor 100 groter dan de membraan gebruikt in de kippen-experimenten.

Bij beide monsterafname-technieken is het membraan van de hollow fiber de overgang tussen het monstername systeem en het lichaam. Relatief weinig studies hebben systematisch onderzoek verricht naar holle vezel membranen en de vervuiling ervan door het in contact staan met het lichaam. Tevens heb je te maken met de uitwerking van het lichaam op het lichaamsvreemde membraan (biocompatibiliteit). Bij het inbrengen van de membraan in het lichaam wordt er altijd schade veroorzaakt. In het algemeen kun je stellen dat alle weefsels een ontstekingsreactie vertonen. In onze experimenten uitgevoerd in ratten, waarbij de hollow fiber onder de huid, in de buikholte en in de bloedbaan werd gebracht, bracht ons tot de conclusie dat membranen met grote poriën minder stabiel zijn vergeleken met membranen met kleine poriën. Zeer waarschijnlijk kunnen eiwitten makkelijker hechten/vast blijven zitten aan grote poriën.

Hoofdstuk 7 is gewijd aan de biomedische toepassing van microdialyse en microfiltratie in het monitoren van energie-substraten in de hersenen. De focus ligt met name op glucose en melkzuur (lactaat). Tevens wordt een experimentele studie beschreven om de relatieve omzetsnelheid van glucose en lactaat te bepalen. Deze gedachtegang zou ook op kunnen opgaan voor andere analieten in het brein. De methodiek is gebaseerd op het verlies van analieten toegevoegd aan het perfusaat bij steady state levels. Hierbij wordt aangenomen dat hoge glucoseconcentraties de omzetting niet beïnvloedt. Deze aanname wordt onder andere gerechtvaardigd doordat bij acute hyperglycemie de consumptie van glucose niet wordt beïnvloed. Ik beschrijf in dit hoofdstuk een model om de omzettingssnelheid voor glucose en lactaat te bepalen en concludeer dat een kleine fractie van het totale energie metabolisme gedragen wordt door het transport van energie-substraten door het extracellulaire compartiment.

Dit proefschrift beschrijft twee monsterafname technieken, namelijk microdialyse en microfiltratie, en het bewaren van verschillende analieten, variërend van kleine (glucose en lactaat) tot grote analieten (peptiden, cortisol gebonden aan albumine en transcortine), van hydrofiele (glucose en lactaat) tot hydrofobe analieten (cortisol, corticosteron, en andere geneesmiddelen). Het toepassen van

membranen met kleine en grote poriën, variërend van 18 kDa – 3.000 kDa.

Het microfiltratie collectie systeem kan toegepast worden in een groot scala van analieten -klein en groot-, versus –hydrofiel en hydrofoob- in vrij bewegende individuen. Het aantal uitgevoerde *in vivo* experimenten is limiterend en vereist verdere validatie en optimalisatie. Met name moeten meer analieten en hollow fiber membranen worden getest op longitudinale diffusie in de opvangcapillair en op mogelijke degradatie/stabiliteit van analieten tijdens het opslaan. Tevens moeten dergelijke analieten vergeleken worden met de conventionele monstername-systemen om goed inzicht te krijgen over de toepasbaarheid. Probes met een grote poriën kunnen inzicht geven in distributie, regulatie, concentratie en het functioneren van proteïnen. Peptiden zijn met name interessant in cel-cel communicatie tijdens een immunologische afweer reactie, ontstekingprocessen en het helen van wonden. Verder zijn peptiden ook interessant in de zoektocht naar biomarkers en therapeutische aanknooppunten in kankertherapie.

De algemene tendens is dat de gevoeligheid en specificiteit van detectiesystemen toeneemt. Microdialyse en microfiltratie monstername-technieken kunnen hierin voorzien.

Een verder voordeel van de combinatie van continue monsterafname middels microfiltratie en het beschreven opslagsysteem is dat de opgeslagen monsters per post verstuurd kunnen worden. In theorie zou het zelfs kunnen dat de opvangslang in zijn geheel wordt gekoppeld aan een analytisch apparaat en in fracties wordt leeg gedrukt. In iedere fractie kan dan het analiet bepaald worden waarin men geïnteresseerd is.

Iedere techniek heeft zijn eigen voordeel en/of nadeel in een bepaalde experimentele vraagstelling. De bevindingen beschreven in dit proefschrift kunnen van waarde zijn voor het maken van de beste keuze.

Chapter 10
References

(1) Abel P., Muller A., Fischer U. Experience with an implantable glucose sensor as a prerequisite of an artificial beta cell. 43 ed. **1984**:577-84.

(2) Abi-Saab WM, Maggs DG, Jones T, et al. Striking differences in glucose and lactate levels between brain extracellular fluid and plasma in conscious human subjects: effects of hyperglycemia and hypoglycemia. *J. Cereb. Blood Flow Metab* **2002**; 22(3):271-9.

(3) Alessandri B, Basciani R, Langemann H, et al. Chronic effects of an aminosteroid on microdialytically measured parameters after experimental middle cerebral artery occlusion in the rat. *J. Clin. Neurosci.* **2000**; 7(1):47-51.

(4) Alessandri B, Landolt H, Langemann H, Gregorin J, Hall J, Gratzl O. Application of glutamate in the cortex of rats: a microdialysis study. *Acta Neurochir. Suppl* **1996**; 67:6-12.

(5) Allen NJ, Karadottir R, Attwell D. A preferential role for glycolysis in preventing the anoxic depolarization of rat hippocampal area CA1 pyramidal cells. *J. Neurosci.* **2005**; 25(4):848-59.

(6) Alves OL, Bullock R, Clausen T, Reinert M, Reeves TM. Concurrent monitoring of cerebral electrophysiology and metabolism after traumatic brain injury: an experimental and clinical study. *J. Neurotrauma* **2005**; 22(7):733-49.

(7) Ames A, III. CNS energy metabolism as related to function. *Brain Res. Brain Res. Rev.* **2000**; 34(1-2):42-68.

(8) Ances BM. Coupling of changes in cerebral blood flow with neural activity: what must initially dip must come back up. *J. Cereb. Blood Flow Metab* **2004**; 24(1):1-6.

(9) Andren P., Farmer T., Klintenberg R. Endogenous release and metabolism of neuropeptides utilizing in vivo microdialysis microelectrospray mass spectrometry. **2002**:193-213.

(10) Ao X, Stenken JA. Microdialysis sampling of cytokines. *Methods* **2006**; 38(4):331-41.

(11) Araujo BV, Silva CF, Haas SE, Dalla CT. Microdialysis as a tool to determine free kidney levels of voriconazole in rodents: a model to study the technique feasibility for a moderately lipophilic drug. *J. Pharm. Biomed Anal* **2008**; 47(4-5):876-81.

(12) Armour JC, Lucisano JY, McKean BD, Gough DA. Application of chronic intravascular blood glucose sensor in dogs. *Diabetes* **1990**; 39(12):1519-26.

(13) Ash SR, Rainier JB, Zopp WE, et al. A subcutaneous capillary filtrate collector for measurement of blood chemistries. *ASAIO J.* **1993**; 39(3):M699-M705.

(14) Attwell D, Gibb A. Neuroenergetics and the kinetic design of excitatory synapses. *Nat. Rev. Neurosci.* **2005**; 6(11):841-9.

(15) Attwell D., Laughlin S.B. An energy budget for signaling in the grey matter of the brain. 21 ed. **2001**:1133-45.

(16) Aubert A, Costalat R, Magistretti PJ, Pellerin L. Brain lactate kinetics: Modeling evidence for neuronal lactate uptake upon activation. *Proc. Natl. Acad. Sci. U. S. A* **2005**; 102(45):16448-53.

(17) Barbelivien A, Bertrand N, Besret L, Beley A, MacKenzie ET, Dauphin F. Neurochemical stimulation of the rat substantia innominata increases cerebral blood flow (but not glucose use) through the parallel activation of cholinergic and non-cholinergic pathways. *Brain Res.* **1999**; 840(1-2):115-24.

(18) bdel-Hamid I., Atanasov P., Wilkins E. Development of a needle-type biosensor for intravascular glucose monitoring. 313 ed. **1995**:45-54.

(19) Belmaker RH, Agam G. Major depressive disorder. *N. Engl. J. Med.* **2008**; 358(1):55-68.

(20) Ben-Yoseph O, Boxer PA, Ross BD. Oxidative stress in the central nervous system: monitoring the metabolic response using the pentose phosphate pathway. *Dev. Neurosci.* **1994**; 16(5-6):328-36.

(21) Ben-Yoseph O, Boxer PA, Ross BD. Noninvasive assessment of the relative roles of cerebral antioxidant enzymes by quantitation of pentose phosphate pathway activity. *Neurochem. Res.* **1996**; 21(9):1005-12.

(22) Bengtsson J., Jansson B., Hammarlund-Udenaes M. On-line desalting and determination of morphine, morphine-3-glucuronide and morphine-6-glucuronide in microdialysis and plasma samples using column switching and liquid chromatography/tandem mass spectrometry. 19 ed. **2005**:2116-22.

(23) Bentzer P, Davidsson H, Grande PO. Microdialysis-based long-term measurements of energy-related metabolites in the rat brain following a fluid percussion trauma. *J. Neurotrauma* **2000**; 17(5):441-7.

(24) Benveniste H, Diemer NH. Cellular reactions to implantation of a microdialysis tube in the rat hippocampus. *Acta Neuropathol. (Berl)* **1987**; 74(3):234-8.

(25) Benveniste H, Huttemeier PC. Microdialysis--theory and application. *Prog. Neurobiol.* **1990**; 35(3):195-215.

(26) Bergersen LH. Is lactate food for neurons? Comparison of monocarboxylate transporter subtypes in brain and muscle. *Neuroscience* **2007**; 145(1):11-9.

(27) Bhatia R, Hashemi P, Razzaq A, et al. Application of rapid-sampling, online microdialysis to the monitoring of brain metabolism during aneurysm surgery. *Neurosurgery* **2006**; 58(4 Suppl 2):ONS-20.

(28) Bidgood T.L., Papich M.G. Plasma and interstitial fluid pharmacokinetics of enrofloxacin, its metabolite ciprofloxacin, and marbofloxacin after oral administration and a constant rate intravenous infusion in dogs. 28 ed. **2005**:329-41.

(29) Bindra D.S., Zhang Y., Wilson G.S., et al. Design and in vitro studies of a needle-type glucose sensor for subcutaneous monitoring. 63 ed. **1991**:1692-6.

(30) Bouw MR, Hammarlund-Udenaes M. Methodological aspects of the use of a calibrator in in vivo microdialysis-further development of the retrodialysis method. *Pharm. Res.* **1998**; 15(11):1673-9.

(31) Brask A, Goranovic G, Bruus H. Theoretical analysis of the low-voltage cascade electro-osmotic pump. *Sens. Actuators B Chem.* **2003**; B92(1-2):127-32.

(32) Brauker JH, Carr-Brendel VE, Martinson LA, Crudele J, Johnston WD, Johnson RC. Neovascularization of synthetic membranes directed by membrane microarchitecture. *J. Biomed. Mater. Res.* **1995**; 29(12):1517-24.

(33) Brodin E, Lindefors N, Ungerstedt U. Potassium evoked in vivo release of substance P in rat caudate nucleus measured using a new technique of brain dialysis and an improved substance P-radioimmunoassay. *Acta Physiol. Scand. Suppl.* **1983**; 515:17-20.

(34) Buck RP, Lindner E. Tracing the history of selective ion sensors. *Anal. Chem.* **2001**; 73(3):88A-97A.

(35) Bungay PM, Morrison PF, Dedrick RL. Steady-state theory for quantitative microdialysis of solutes and water in vivo and in vitro. *Life Sci.* **1990**; 46(2):105-19.

(36) Burhop K.E., Johnson R.J., Simpson J., Chenoweth D.E., Borgia J. Biocompatibility of hemodialysis membranes: evaluation in an ovine model. 121 ed. **1993**:276-93.

(37) Caesar K, Hashemi P, Douhou A, et al. Glutamate receptor-dependent increments in lactate, glucose and oxygen metabolism evoked in rat cerebellum in vivo. *J. Physiol* **2008**; 586(5):1337-49.

(38) Camara MS, Mastandrea C, Golier JA. Chemometrics-assisted simple UV-spectroscopic determination of carbamazepine in human serum and comparison with reference methods. *J. Biochem. Biophys. Methods* **2005**; 64:153-66.

(39) Canal CE, McNay EC, Gold PE. Increases in extracellular fluid glucose levels in the rat hippocampus following an anesthetic dose of pentobarbital or ketamine-xylazine: an in vivo microdialysis study. *Physiol Behav.* **2005**; 84(2):245-50.

(40) Caricato A, Pennisi M, Mancino A, et al. Levels of vancomycin in the cerebral interstitial fluid after severe head injury. *Intensive Care Med.* **2006**; 32(2):325-8.

(41) Cellar N., Burns S., Meiners J., Chen H., Kennedy R. Microfluidic Chip for Low-Flow Push-Pull Perfusion Sampling in Vivo with On-Line Analysis of Amino Acids. 77 ed. **2005**:7067-73.

(42) Chebib M, Johnston GA. The 'ABC' of GABA receptors: a brief review. *Clin. Exp. Pharmacol. Physiol* **1999**; 26(11):937-40.

(43) Chen KC. Effects of tissue trauma on the characteristics of microdialysis zero-net-flux method sampling neurotransmitters. *J. Theor. Biol.* **2006**; 238(4):863-81.

(44) Chen T, Qian YZ, Di X, Rice A, Zhu JP, Bullock R. Lactate/glucose dynamics after rat fluid percussion brain injury. *J. Neurotrauma* **2000**; 17(2):135-42.

(45) Cheng C, Kaptein WA, Gruendig B, Yuen Y, Korf J, Renneberg R. Continuous lactate measurement that combines a portable ultrafiltration storage device with an enzyme sensor. *Anal. Lett.* **2000**; 33(11):2153-68.

(46) Cheregi M., Matachescu C., Moscone D., Ciucu A. Glucose and lactate biosensors coupled with microdialysis probe for continuous monitoring. 4 ed. **1996**:9-17.

(47) Clark H, Barbari TA, Stump K, Rao G. Histologic evaluation of the inflammatory response around implanted hollow fiber membranes. *J. Biomed. Mater. Res.* **2000**; 52(1):183-92.

(48) Clausen T, Khaldi A, Zauner A, et al. Cerebral acid-base homeostasis after severe traumatic brain injury. *J. Neurosurg.* **2005**; 103(4):597-607.

(49) Clausen T, Zauner A, Levasseur JE, Rice AC, Bullock R. Induced mitochondrial failure in the feline brain: implications for understanding acute post-traumatic metabolic events. *Brain Res.* **2001**; 908(1):35-48.

(50) Cooper JD, Heppert KE, Davies MI, Lunte SM. Evaluation of an osmotic pump for microdialysis sampling in an awake and untethered rat. *J. Neurosci. Methods* **2007**; 160(2):269-75.

(51) Corbett R, Laptook A, Kim B, Tollefsbol G, Silmon S, Garcia D. Maturational changes in cerebral lactate and acid clearance following ischemia measured in vivo using magnetic resonance spectroscopy and microdialysis. *Brain Res. Dev. Brain Res.* **1999**; 113(1-2):37-46.

(52) Cremers T, Ebert B. Plasma and CNS concentrations of Gaboxadol in rats following subcutaneous administration. *Eur. J. Pharmacol.* **2007**; 562(1-2):47-52.

(53) Cremers TI, de Vries MG, Huinink KD, et al. Quantitative microdialysis using modified ultraslow microdialysis: Direct rapid and reliable determination of free brain concentrations with the MetaQuant technique. *J. Neurosci. Methods* **2008**.

(54) Cremers TI, Wiersma LJ, Bosker FJ, den Boer JA, Westerink BH, Wikstrom HV. Is the beneficial antidepressant effect of coadministration of pindolol really due to somatodendritic autoreceptor antagonism? *Biol. Psychiatry* **2001**; 50(1):13-21.

(55) Darbin O, Carre E, Naritoku D, Risso JJ, Lonjon M, Patrylo PR. Glucose metabolites in the striatum of freely behaving rats following infusion of elevated potassium. *Brain Res.* **2006**; 1116(1):127-31.

(56) Darvesh AS, Gudelsky GA. Activation of 5-HT2 receptors induces glycogenolysis in the rat brain. *Eur. J. Pharmacol.* **2003**; 464(2-3):135-40.

(57) Darvesh AS, Shankaran M, Gudelsky GA. 3,4-Methylenedioxymethamphetamine produces glycogenolysis and increases the extracellular concentration of glucose in the rat brain. *J. Pharmacol. Exp. Ther.* **2002**; 301(1):138-44.

(58) Davis J.L., Salmon J.H., Papich M.G. Pharmacokinetics and tissue fluid distribution of cephalexin in the horse after oral and i.v. administration. 28 ed. **2005**:425-31.

(59) de Boer J., Plijter-Groendijk H., Visser K.R., Mook G.A., Korf J. Continuous monitoring of lactate during exercise in humans using subcutaneous and transcutaneous microdialysis. 69 ed. **1994**:281-6.

(60) De Bruin LA, Schasfoort EM, Steffens AB, Korf J. Effects of stress and exercise on rat hippocampus and striatum extracellular lactate. *Am. J. Physiol* **1990**; 259(4 Pt 2):R773-R779.

(61) de Lange EC, Danhof M. Considerations in the use of cerebrospinal fluid pharmacokinetics to predict brain target concentrations in the clinical setting: implications of the barriers between blood and brain. *Clin. Pharmacokinet.* **2002**; 41(10):691-703.

(62) de Lange EC, de Boer AG, Breimer DD. Methodological issues in microdialysis sampling for pharmacokinetic studies. *Adv. Drug Deliv. Rev.* **2000**; 45(2-3):125-48.

(63) de Lange EC, Marchand S, van den BD, et al. In vitro and in vivo investigations on fluoroquinolones; effects of the P-glycoprotein efflux transporter on brain distribution of sparfloxacin. *Eur. J. Pharm. Sci.* **2000**; 12(2):85-93.

(64) de Lange E.C.M., Danhof M., de Boer A.G., Breimer D.D. Methodological considerations of intracerebral microdialysis in pharmacokinetic studies on drug transport across the blood-brain barrier. 25 ed. **1997**:27-49.

(65) de Weerth C., Zijl RH, Buitelaar JK. Development of cortisol circadian rhythm in infancy. *Early Hum. Dev.* **2003**; 73(1-2):39-52.

(66) Delgado JM, DeFeudis FV, Roth RH, Ryugo DK, Mitruka BM. Dialytrode for long term intracerebral perfusion in awake monkeys. *Arch. Int. Pharmacodyn. Ther.* **1972**; 198(1):9-21.

(67) Demestre M, Boutelle M, Fillenz M. Stimulated release of lactate in freely moving rats is dependent on the uptake of glutamate. *J. Physiol.* **1997**; 499 (Pt 3):825-32.

(68) Dempsey E, Diamond D, Smyth MR, et al. In vitro optimisation of a microdialysis system with potential for on-line monitoring of lactate and glucose in biological samples. *Analyst* **1997**; 122(2):185-9.

(69) Dienel GA, Hertz L. Glucose and lactate metabolism during brain activation. *J. Neurosci. Res.* **2001**; 66(5):824-38.

(70) Dijk S, Krugers HJ, Korf J. The effect of theophylline and immobilization stress on haloperidol-induced catalepsy and on metabolism in the striatum and hippocampus, studied with lactography. *Neuropharmacology* **1991**; 30(5):469-73.

(71) Dong Y, Wang L, Shangguan D, et al. Analysis of glucose and lactate in hippocampal dialysates of rats during the operant conditioned reflex using microdialysis. *Neurochem. Int.* **2003**; 43(1):67-72.

(72) Dringen R, Wiesinger H, Hamprecht B. Uptake of L-lactate by cultured rat brain neurons. *Neurosci. Lett.* **1993**; 163(1):5-7.

(73) Duckrow RB, Bryan RM, Jr. Regional cerebral glucose utilization during hyperglycemia. *J. Neurochem.* **1987**; 48(3):989-93.

(74) Duelli R, Maurer MH, Staudt R, Heiland S, Duembgen L, Kuschinsky W. Increased cerebral glucose utilization and decreased glucose transporter Glut1 during chronic hyperglycemia in rat brain. *Brain Res.* **2000**; 858(2):338-47.

(75) Ehwald R. Affinity assay in the microdialysis cell - a general principle for on-line measuring biosensors. 71 ed. **2004**:24-8.

(76) Ekberg NR, Wisniewski N, Brismar K, Ungerstedt U. Measurement of glucose and metabolites in subcutaneous adipose tissue during hyperglycemia with microdialysis at various perfusion flow rates. *Clin. Chim. Acta* **2005**; 359(1-2):53-64.

(77) Ekberg NR, Wisniewski N, Brismar K, Ungerstedt U. Measurement of glucose and metabolites in subcutaneous adipose tissue during hyperglycemia with microdialysis at various perfusion flow rates. *Clin. Chim. Acta* **2005**; 359(1-2):53-64.

(78) Elekes O, Venema K, Postema F, Dringen R, Hamprecht B, Korf J. Evidence that stress activates glial lactate formation in vivo assessed with rat hippocampus lactography. *Neurosci. Lett.* **1996**; 208(1):69-72.

(79) Enblad P, Frykholm P, Valtysson J, et al. Middle cerebral artery occlusion and reperfusion in primates monitored by microdialysis and sequential positron emission tomography. *Stroke* **2001**; 32(7):1574-80.

(80) Engstrom M, Polito A, Reinstrup P, et al. Intracerebral microdialysis in severe brain trauma: the importance of catheter location. *J. Neurosurg.* **2005**; 102(3):460-9.

(81) Fabricius M, Fuhr S, Bhatia R, et al. Cortical spreading depression and peri-infarct depolarization in acutely injured human cerebral cortex. *Brain* **2006**; 129(Pt 3):778-90.

(82) Fellows LK, Boutelle MG. Rapid changes in extracellular glucose levels and blood flow in the striatum of the freely moving rat. *Brain Res.* **1993**; 604(1-2):225-31.

(83) Fellows LK, Boutelle MG, Fillenz M. Extracellular brain glucose levels reflect local neuronal activity: a microdialysis study in awake, freely moving rats. *J. Neurochem.* **1992**; 59(6):2141-7.

(84) Fellows LK, Boutelle MG, Fillenz M. Physiological stimulation increases nonoxidative glucose metabolism in the brain of the freely moving rat. *J. Neurochem.* **1993**; 60(4):1258-63.

(85) Felmy F, Neher E, Schneggenburger R. The timing of phasic transmitter release is Ca2+-dependent and lacks a direct influence of presynaptic membrane potential. *Proc. Natl. Acad. Sci. U. S. A* **2003**; 100(25):15200-5.

(86) Fillenz M, Lowry JP. Studies of the source of glucose in the extracellular compartment of the rat brain. *Dev. Neurosci.* **1998**; 20(4-5):365-8.

(87) Forsyth R., Fray A., Boutelle M., Fillenz M., Middleditch C., Burchell A. A role for astrocytes in glucose delivery to neurons? 18 ed. **1996**:360-70.

(88) Forsyth RJ. Astrocytes and the delivery of glucose from plasma to neurons. *Neurochem. Int.* **1996**; 28(3):231-41.

(89) Fray AE, Boutelle M, Fillenz M. Extracellular glucose turnover in the striatum of unanaesthetized rats measured by quantitative microdialysis. *J. Physiol* **1997**; 504 (Pt 3):721-6.

(90) Fray AE, Boutelle M, Fillenz M. Extracellular glucose turnover in the striatum of unanaesthetized rats measured by quantitative microdialysis. *J. Physiol.* **1997**; 504 (Pt 3):721-6.

(91) Fray AE, Forsyth RJ, Boutelle MG, Fillenz M. The mechanisms controlling physiologically stimulated changes in rat brain glucose and lactate: a microdialysis study. *J. Physiol* **1996**; 496 (Pt 1):49-57.

(92) Freaney R, McShane A, Keaveny TV, et al. Novel instrumentation for real-time monitoring using miniaturized flow systems with integrated biosensors. *Ann. Clin. Biochem.* **1997**; 34 (Pt 3):291-302.

(93) Frykholm P, Hillered L, Langstrom B, Persson L, Valtysson J, Enblad P. Relationship between cerebral blood flow and oxygen metabolism, and extracellular glucose and lactate concentrations during middle cerebral artery occlusion and reperfusion: a microdialysis and positron emission tomography study in nonhuman primates. *J. Neurosurg.* **2005**; 102(6):1076-84.

(94) Fuxe K, Kurosawa N, Cintra A, et al. Involvement of local ischemia in endothelin-1 induced lesions of the neostriatum of the anaesthetized rat. *Exp. Brain Res.* **1992**; 88(1):131-9.

(95) Gajovic N, Beinyamin G, Warsinke A, Scheller FW, Heller A. Operation of a miniature redox hydrogel-based pyruvate sensor in undiluted deoxygenated calf serum. *Anal. Chem.* **2000**; 72(13):2963-8.

(96) Garrison KE, Pasas SA, Cooper JD, Davies MI. A review of membrane sampling from biological tissues with applications in pharmacokinetics, metabolism and pharmacodynamics. *Eur. J. Pharm. Sci.* **2002**; 17(1-2):1-12.

(97) Geeraerts T, Friggeri A, Mazoit JX, Benhamou D, Duranteau J, Vigue B. Posttraumatic brain vulnerability to hypoxia-hypotension: the importance of the delay between brain trauma and secondary insult. *Intensive Care Med.* **2008**; 34(3):551-60.

(98) Geeraerts T, Ract C, Tardieu M, et al. Changes in cerebral energy metabolites induced by impact-acceleration brain trauma and hypoxic-hypotensive injury in rats. *J. Neurotrauma* **2006**; 23(7):1059-71.

(99) Gibbs ME, Anderson DG, Hertz L. Inhibition of glycogenolysis in astrocytes interrupts memory consolidation in young chickens. *Glia* **2006**; 54(3):214-22.

(100) Gjedde A, Marrett S, Vafaee M. Oxidative and nonoxidative metabolism of excited neurons and astrocytes. *J. Cereb. Blood Flow Metab* **2002**; 22(1):1-14.

(101) Goldstein DS, Kopin IJ. Evolution of concepts of stress. *Stress.* **2007**; 10(2):109-20.

(102) Gramsbergen JB, Cumming P. Serotonin mediates rapid changes of striatal glucose and lactate metabolism after systemic 3,4-methylenedioxymethamphetamine (MDMA, "Ecstasy") administration in awake rats. *Neurochem. Int.* **2007**; 51(1):8-15.

(103) Gramsbergen JB, Skjoth-Rasmussen J, Rasmussen C, Lambertsen KL. On-line monitoring of striatum glucose and lactate in the endothelin-1 rat model of transient focal cerebral ischemia using microdialysis and flow-injection analysis with biosensors. *J. Neurosci. Methods* **2004**; 140(1-2):93-101.

(104) Gray CS, Hildreth AJ, Sandercock PA, et al. Glucose-potassium-insulin infusions in the management of post-stroke hyperglycaemia: the UK Glucose Insulin in Stroke Trial (GIST-UK). *Lancet Neurol.* **2007**; 6(5):397-406.

(105) Groothuis DR, Ward S, Schlageter KE, et al. Changes in blood-brain barrier permeability associated with insertion of brain cannulas and microdialysis probes. *Brain Res.* **1998**; 803(1-2):218-30.

(106) Gudelsky GA, Yamamoto BK. Actions of 3,4-methylenedioxymethamphetamine (MDMA) on cerebral dopamineric, serotonergic and cholinergic neurons. *Pharmacol. Biochem. Behav.* **2007**.

(107) Hakim R.M., Lowrie E.G. Hemodialysis-associated neutropenia and hypoxemia: the effect of dialyzer membrane materials. 32 ed. **1982**:32-9.

(108) Hamrin K, Rosdahl H, Ungerstedt U, Henriksson J. Microdialysis in human skeletal muscle: effects of adding a colloid to the perfusate. *J. Appl. Physiol* **2002**; 92(1):385-93.

(109) Harada M, Okuda C, Sawa T, Murakami T. Cerebral extracellular glucose and lactate concentrations during and after moderate hypoxia in glucose- and saline-infused rats. *Anesthesiology* **1992**; 77(4):728-34.

(110) Hasegawa T, Iwasaki Y, Ishihara K. Preparation and performance of protein-adsorption-resistant asymmetric porous membrane composed of polysulfone/phospholipid polymer blend. *Biomaterials* **2001**; 22(3):243-51.

(111) Heim C, Ehlert U, Hellhammer DH. The potential role of hypocortisolism in the pathophysiology of stress-related bodily disorders. *Psychoneuroendocrinology* **2000**; 25(1):1-35.

(112) Hertz L, Dienel GA. Lactate transport and transporters: general principles and functional roles in brain cells. *J. Neurosci. Res.* **2005**; 79(1-2):11-8.

(113) Hertz L, Peng L, Dienel GA. Energy metabolism in astrocytes: high rate of oxidative metabolism and spatiotemporal dependence on glycolysis/glycogenolysis. *J. Cereb. Blood Flow Metab* **2007**; 27(2):219-49.

(114) Higuchi A, Shirano K, Harashima M, et al. Chemically modified polysulfone hollow fibers with vinylpyrrolidone having improved blood compatibility. *Biomaterials* **2002**; 23(13):2659-66.

(115) Hillered L, Persson L, Nilsson P, Ronne-Engstrom E, Enblad P. Continuous monitoring of cerebral metabolism in traumatic brain injury: a focus on cerebral microdialysis. *Curr. Opin. Crit Care* **2006**; 12(2):112-8.

(116) Hillered L, Vespa PM, Hovda DA. Translational neurochemical research in acute human brain injury: the current status and potential future for cerebral microdialysis. *J. Neurotrauma* **2005**; 22(1):3-41.

(117) Hillman J, Aneman O, Anderson C, Sjogren F, Saberg C, Mellergard P. A microdialysis technique for routine measurement of macromolecules in the injured human brain. *Neurosurgery* **2005**; 56(6):1264-8.

(118) Hillman J, Aneman O, Persson M, Andersson C, Dabrosin C, Mellergard P. Variations in the response of interleukins in neurosurgical intensive care patients monitored using intracerebral microdialysis. *J. Neurosurg.* **2007**; 106(5):820-5.

(119) Hillman J, Milos P, Yu ZQ, Sjogren F, Anderson C, Mellergard P. Intracerebral microdialysis in neurosurgical intensive care patients utilising catheters with different molecular cut-off (20 and 100 kD). *Acta Neurochir. (Wien.)* **2006**; 148(3):319-24.

(120) Hillman J, Milos P, Yu ZQ, Sjogren F, Anderson C, Mellergard P. Intracerebral microdialysis in neurosurgical intensive care patients utilising catheters with different molecular cut-off (20 and 100 kD). *Acta Neurochir. (Wien.)* **2006**; 148(3):319-24.

(121) Hlatky R, Valadka AB, Goodman JC, Robertson CS. Evolution of brain tissue injury after evacuation of acute traumatic subdural hematomas. *Neurosurgery* **2004**; 55(6):1318-23.

(122) Homola A, Zoremba N, Slais K, Kuhlen R, Sykova E. Changes in diffusion parameters, energy-related metabolites and glutamate in the rat cortex after transient hypoxia/ischemia. *Neurosci. Lett.* **2006**; 404(1-2):137-42.

(123) Hopster H, van der Werf JT, Erkens JH, Blokhuis HJ. Effects of repeated jugular puncture on plasma cortisol concentrations in loose-housed dairy cows. *J. Anim Sci.* **1999**; 77(3):708-14.

(124) Hopwood SE, Parkin MC, Bezzina EL, Boutelle MG, Strong AJ. Transient changes in cortical glucose and lactate levels associated with peri-infarct depolarisations, studied with rapid-sampling microdialysis. *J. Cereb. Blood Flow Metab* **2005**; 25(3):391-401.

(125) Horinaka N, Artz N, Cook M, et al. Effects of elevated plasma epinephrine on glucose utilization and blood flow in conscious rat brain. *Am. J. Physiol* **1997**; 272(4 Pt 2):H1666-H1671.

(126) Hsieh Y., Zahn J. Glucose recovery in a microfluidic microdialysis biochip. B107 ed. **2005**:649-56.

(127) Hu Y, Wilson GS. A temporary local energy pool coupled to neuronal activity: fluctuations of extracellular lactate levels in rat brain monitored with rapid-response enzyme-based sensor. *J. Neurochem.* **1997**; 69(4):1484-90.

(128) Hu Y, Wilson GS. Rapid changes in local extracellular rat brain glucose observed with an in vivo glucose sensor. *J. Neurochem.* **1997**; 68(4):1745-52.

(129) Huang C., Wang C., Kawai M., Barnes S., Elmets C. Surfactant sodium lauryl sulfate enhances skin vaccination. Molecular characterization via a novel technique using ultrafiltration capillaries and mass spectrometric proteomics. 5 ed. **2006**:523-32.

(130) Huang CM, Ananthaswamy HN, Barnes S, Ma Y, Kawai M, Elmets CA. Mass spectrometric proteomics profiles of in vivo tumor secretomes: capillary ultrafiltration sampling of regressive tumor masses. *Proteomics.* **2006**; 6(22):6107-16.

(131) Huang CM, Nakatsuji T, Liu YT, Shi Y. In vivo tumor secretion probing via ultrafiltration and tissue chamber: implication for anti-cancer drugs targeting secretome. *Recent Patents. Anticancer Drug Discov.* **2008**; 3(1):48-54.

(132) Huang CM, Wang CC, Barnes S, Elmets CA. In vivo detection of secreted proteins from wounded skin using capillary ultrafiltration probes and mass spectrometric proteomics. *Proteomics.* **2006**; 6(21):5805-14.

(133) Huang CM, Wang CC, Kawai M, Barnes S, Elmets CA. In vivo protein sampling using capillary ultrafiltration semi-permeable hollow fiber and protein identification via mass spectrometry-based proteomics. *J. Chromatogr. A* **2006**; 1109(2):144-51.

(134) Huang YH, Bergles DE. Glutamate transporters bring competition to the synapse. *Curr. Opin. Neurobiol.* **2004**; 14(3):346-52.

(135) Huinink KD, Venema K, Roelofsen H, Korf J. In Vitro Sampling and Storage of Proteins with an Ultrafiltration Collection Device (UCD) and Analysis with Absorbance Spectrometry and SELDI-TOF-MS. *Analyst* **2005**; 130:1168-74.

(136) Hutchinson PJ, O'Connell MT, Al Rawi PG, et al. Clinical cerebral microdialysis: a methodological study. *J. Neurosurg.* **2000**; 93(1):37-43.

(137) Hutchinson PJ, O'Connell MT, Al-Rawi PG, et al. Clinical cerebral microdialysis: a methodological study. *J. Neurosurg.* **2000**; 93(1):37-43.

(138) Hutchinson PJ, O'Connell MT, Nortje J, et al. Cerebral microdialysis methodology--evaluation of 20 kDa and 100 kDa catheters. *Physiol Meas.* **2005**; 26(4):423-8.

(139) Imsilp K, Whittem T, Koritz GD, Zachary JF, Schaeffer DJ. Inflammatory response to intramuscular implantation of polyacrylonitrile ultrafiltration probes in sheep. *Vet. Res.* **2000**; 31(6):623-34.

(140) Ishihara K, Fukumoto K, Iwasaki Y, Nakabayashi N. Modification of polysulfone with phospholipid polymer for improvement of the blood compatibility. Part 2. Protein adsorption and platelet adhesion. *Biomaterials* **1999**; 20(17):1553-9.

(141) Ishihara K, Hasegawa T, Watanabe J, Iwasaki Y. Protein adsorption-resistant hollow fibers for blood purification. *Artif. Organs* **2002**; 26(12):1014-9.

(142) Ishihara K, Ziats NP, Tierney BP, Nakabayashi N, Anderson JM. Protein adsorption from human plasma is reduced on phospholipid polymers. *J. Biomed. Mater. Res.* **1991**; 25(11):1397-407.

(143) Jackson G, Atkinson L, Clark M, Crook B, Armstrong P, Oram S. Diagnosis of coronary artery disease by estimation of coronary sinus lactate. *Br. Heart J.* **1978**; 40(9):979-83.

(144) Jakubowski J., Hatcher N., Sweedler J. Online microdialysis-dynamic nanoelectrospray ionization-mass spectrometry for monitoring neuropeptide secretion. 40 ed. **2005**:924-31.

(145) Janle E., Cregor M., Sojka J.E. Interstitial fluid calcium, magnesium and phosphorus concentrations in bone, muscle and subcutaneous tissue sampled with ultrafiltration probes. 19 ed. **2001**:81-5.

(146) Janle EM, Clark T, Ash SR. Use of an Ultrafiltrate Sampling Probe to Control Glucose Levels in a Diabetic Cat: Case Study. *Curr. Sep.* **1992**; 11:3-6.

(147) Janle EM, Cregor M. Ultrafiltrate and microdialysis DL probe in vitro recoveries: electrolytes and metabolites. *Curr. Sep.* **1996**; 15(1):31-4.

(148) Janle E.M., Kissinger P.T. Microdialysis and ultrafiltration sampling of small molecules and ions from in vivo dialysis fibers. 14 ed. **1993**:159-65.

(149) Janle EM, Kissinger PT. Short Interval Monitoring of Glucose in Zucker Diabetic Fatty (ZDF) Rats. *Curr. Sep.* **1995**; 14(2):58-63.

(150) Janle EM, Kissinger PT. Monitoring physiological variables with membrane probes. *Acta Astronaut.* **1998**; 43(3-6):87-99.

(151) Janle E.M., Kissinger P.T. Monitoring physiological variables with membrane probes. 43 ed. **1998**:87-99.

(152) Janle EM, Ostroy S, Kissinger PT. Monitoring the Progress of Streptozotocin Diabetes in the Mouse with the Ultrafiltrate Probe. *Curr. Sep.* **1992**; 11:17-9.

(153) Janle EM, Sojka JE. Use of ultrafiltration probes in sheep to collect interstitial fluid for measurement of calcium and magnesium. *Contemp. Top. Lab. Anim Sci.* **2000**; 39(6):47-50.

(154) Janle-Swain E, Van Vleet JF, Ash SR. Use of a capillary filtrate collector for monitoring glucose in diabetics. *ASAIO Trans.* **1987**; 33(3):336-40.

(155) Jenkins BG, Brouillet E, Chen YC, et al. Non-invasive neurochemical analysis of focal excitotoxic lesions in models of neurodegenerative illness using spectroscopic imaging. *J. Cereb. Blood Flow Metab* **1996**; 16(3):450-61.

(156) Jensen SM, Hansen HS, Johansen T, Malmlof K. In vivo and in vitro microdialysis sampling of free fatty acids. *J. Pharm. Biomed Anal* **2007**; 43(5):1751-6.

(157) Jones DA, Ros J, Landolt H, Fillenz M, Boutelle MG. Dynamic changes in glucose and lactate in the cortex of the freely moving rat monitored using microdialysis. *J. Neurochem.* **2000**; 75(4):1703-8.

(158) Kahlert S, Reiser G. Glial perspectives of metabolic states during cerebral hypoxia--calcium regulation and metabolic energy. *Cell Calcium* **2004**; 36(3-4):295-302.

(159) Kaptein WA, Kemper RH, Ruiters MH, Venema K, Tiessen RG, Korf J. Methodological aspects of glucose monitoring with a slow continuous subcutaneous and intravenous ultrafiltration system in rats. *Biosens. Bioelectron.* **1997**; 12(9-10):967-76.

(160) Kaptein WA, Zwaagstra JJ, Venema K, Korf J. Continuous ultraslow microdialysis and ultrafiltration for subcutaneous sampling as demonstrated by glucose and lactate measurements in rats. *Anal. Chem.* **1998**; 70(22):4696-700.

(161) Katayama Y, Kawamata T, Kano T, Tsubokawa T. Excitatory amino acid antagonist administered via microdialysis attenuates lactate accumulation during cerebral ischemia and subsequent hippocampal damage. *Brain Res.* **1992**; 584(1-2):329-33.

(162) Kawamata T, Katayama Y, Hovda DA, Yoshino A, Becker DP. Lactate accumulation following concussive brain injury: the role of ionic fluxes induced by excitatory amino acids. *Brain Res.* **1995**; 674(2):196-204.

(163) Kawamata T, Katayama Y, Hovda DA, Yoshino A, Becker DP. Lactate accumulation following concussive brain injury: the role of ionic fluxes induced by excitatory amino acids. *Brain Res.* **1995**; 674(2):196-204.

(164) Kennedy RT, Kauri LM, Dahlgren GM, Jung SK. Metabolic oscillations in beta-cells. *Diabetes* **2002**; 51 Suppl 1:S152-S161.

(165) Kennedy RT, Watson CJ, Haskins WE, Powell DH, Strecker RE. In vivo neurochemical monitoring by microdialysis and capillary separations. *Curr. Opin. Chem. Biol.* **2002**; 6(5):659-65.

(166) Kerner A, Schlenk F, Sakowitz O, Haux D, Sarrafzadeh A. Impact of hyperglycemia on neurological deficits and extracellular glucose levels in aneurysmal subarachnoid hemorrhage patients. *Neurol. Res.* **2007**; 29(7):647-53.

(167) Kett-White R, Hutchinson PJ, Al-Rawi PG, Gupta AK, Pickard JD, Kirkpatrick PJ. Adverse cerebral events detected after subarachnoid hemorrhage using brain oxygen and microdialysis probes. *Neurosurgery* **2002**; 50(6):1213-21.

(168) Khaldi A, Zauner A, Reinert M, Woodward JJ, Bullock MR. Measurement of nitric oxide and brain tissue oxygen tension in patients after severe subarachnoid hemorrhage. *Neurosurgery* **2001**; 49(1):33-8.

(169) Khan A.S., Michael A.C. Invasive consequences of using micro-electrodes and microdialysis probes in the brain. 22 ed. **2003**:503-8.

(170) Khandelwal P, Beyer CE, Lin Q, Schechter LE, Bach AC. Studying rat brain neurochemistry using nanoprobe NMR spectroscopy: a metabonomics approach. *Anal Chem* **2004**; 76(14):4123-7.

(171) King SL, Hegadoren KM. Stress hormones: how do they measure up? *Biol. Res. Nurs.* **2002**; 4(2):92-103.

(172) Kissinger C, Peters S, Zhu Y. New method for automating sample collection from in vivo ultrafiltration probes. *Curr. Sep.* **2003**; 20:3 (2003):97-102.

(173) Klein HC, Krop-Van GW, Go KG, Korf J. Prediction of specific damage or infarction from the measurement of tissue impedance following repetitive brain ischaemia in the rat. *Neuropathol. Appl. Neurobiol.* **1993**; 19(1):57-65.

(174) Kobayashi N, Kazui M, Ikeda T. Rapid, real-time sampling of R-84760 in blood by in vivo microdialysis with tandem mass spectrometry. *J. Pharm. Biomed. Anal.* **2000**; 21(6):1233-42.

(175) Korf J. Intracerebral trafficking of lactate in vivo during stress, exercise, electroconvulsive shock and ischemia as studied with microdialysis. *Dev. Neurosci.* **1996**; 18(5-6):405-14.

(176) Korf J. Is brain lactate metabolized immediately after neuronal activity through the oxidative pathway? *J. Cereb. Blood Flow Metab* **2006**; 26(12):1584-6.

(177) Korf J, de Boer J. Lactography as an approach to monitor glucose metabolism on-line in brain and muscle. *Int. J. Biochem.* **1990**; 22(12):1371-8.

(178) Korf J, de Boer J, Baarsma R, Venema K, Okken A. Monitoring of glucose and lactate using microdialysis: applications in neonates and rat brain. *Dev. Neurosci.* **1993**; 15(3-5):240-6.

(179) Korf J, Gramsbergen JB. Timing of potential and metabolic brain energy. *J. Neurochem.* **2007**; 103(5):1697-708.

(180) Korf J, Klein HC, Venema K, Postema F. Increases in striatal and hippocampal impedance and extracellular levels of amino acids by cardiac arrest in freely moving rats. *J. Neurochem.* **1988**; 50(4):1087-96.

(181) Korf J, Postema F. Rapid shrinkage of rat striatal extracellular space after local kainate application and ischemia as recorded by impedance. *J. Neurosci. Res.* **1988**; 19(4):504-10.

(182) Korf J, Tiessen RG, Venema K, Rhemrev MM. Biosensors for continuous glucose and lactate monitoring. *Ned. Tijdschr. Geneeskd.* **2003**; 147(25):1204-8.

(183) Krishnappa IK, Contant CF, Robertson CS. Regional changes in cerebral extracellular glucose and lactate concentrations following severe cortical impact injury and secondary ischemia in rats. *J. Neurotrauma* **1999**; 16(3):213-24.

(184) Krugers HJ, Jaarsma D, Korf J. Rat hippocampal lactate efflux during electroconvulsive shock or stress is differently dependent on entorhinal cortex and adrenal integrity. *J. Neurochem.* **1992**; 58(3):826-30.

(185) Kuhr WG, Korf J. Extracellular lactic acid as an indicator of brain metabolism: continuous on-line measurement in conscious, freely moving rats with intrastriatal dialysis. *J. Cereb. Blood Flow Metab* **1988**; 8(1):130-7.

(186) Kuhr WG, Korf J. N-methyl-D-aspartate receptor involvement in lactate production following ischemia or convulsion in rats. *Eur. J. Pharmacol.* **1988**; 155(1-2):145-9.

(187) Kuhr WG, van den Berg CJ, Korf J. In vivo identification and quantitative evaluation of carrier-mediated transport of lactate at the cellular level in the striatum of conscious, freely moving rats. *J. Cereb. Blood Flow Metab* **1988**; 8(6):848-56.

(188) Lam H, Davies M, Lunte CE. Vacuum ultrafiltration sampling for determination of plasma protein binding of drugs. *J. Pharm. Biomed. Anal.* **1996**; 14(12):1753-7.

(189) Landolt H, Langemann H, Mendelowitsch A, Gratzl O. Neurochemical monitoring and on-line pH measurements using brain microdialysis in patients in intensive care. *Acta Neurochir. Suppl (Wien.)* **1994**; 60:475-8.

(190) Langemann H, Alessandri B, Mendelowitsch A, Feuerstein T, Landolt H, Gratzl O. Extracellular levels of glucose and lactate measured by quantitative microdialysis in the human brain. *Neurol. Res.* **2001**; 23(5):531-6.

(191) Larsson CI. The use of an "internal standard" for control of the recovery in microdialysis. *Life Sci.* **1991**; 49(13):L73-L78.

(192) Lazar I.M., Ramsey R.S., Jacobson S.C., Foote R.S., Ramsey J.M. Novel microfabricated device for electrokinetically induced pressure flow and electrospray ionization mass spectrometry. 892 ed. **2000**:195-201.

(193) Leegsma-Vogt G, Janle E, Ash SR, Venema K, Korf J. Utilization of in vivo ultrafiltration in biomedical research and clinical applications. *Life Sci.* **2003**; 73(16):2005-18.

(194) Leegsma-Vogt G, Rhemrev-Boom MM, Tiessen RG, Venema K, Korf J. The potential of biosensor technology in clinical monitoring and experimental research. *Biomed. Mater. Eng* **2004**; 14(4):455-64.

(195) Leegsma-Vogt G, van der Werf S, Venema K, Korf J. Modeling cerebral arteriovenous lactate kinetics after intravenous lactate infusion in the rat. *J. Cereb. Blood Flow Metab.* **2004**; 24(10):1071-80.

(196) Leegsma-Vogt G, Venema K, Korf J. Evidence for a lactate pool in the rat brain that is not used as an energy supply under normoglycemic conditions. *J. Cereb. Blood Flow Metab* **2003**; 23(8):933-41.

(197) Leegsma-Vogt G, Venema K, Postema F, Korf J. Monitoring arterio-venous differences of glucose and lactate in the anesthetized rat with or without brain damage with ultrafiltration and biosensor technology. *J. Neurosci. Res.* **2001**; 66(5):795-802.

(198) Leggas M., Zhuang Y., Welden J., Self Z., Waters C., Stewart C. Microbore HPLC method with online microdialysis for measurement of topotecan lactone and carboxylate in murine CSF. 93 ed. **2004**:2284-95.

(199) Lewen A, Hillered L. Involvement of reactive oxygen species in membrane phospholipid breakdown and energy perturbation after traumatic brain injury in the rat. *J. Neurotrauma* **1998**; 15(7):521-30.

(200) Liddle GW. An analysis of circadian rhythms in human adrenocortical secretory activity. *Trans. Am. Clin. Climatol. Assoc.* **1966**; 77:151-60.

(201) Lin H, Decuypere E, Buyse J. Oxidative stress induced by corticosterone administration in broiler chickens (Gallus gallus domesticus) 1. Chronic exposure. *Comp Biochem. Physiol B Biochem. Mol. Biol.* **2004**; 139(4):737-44.

(202) Lin JY, Chung SY, Lin MC, Cheng FC. Effects of magnesium sulfate on energy metabolites and glutamate in the cortex during focal cerebral ischemia and reperfusion in the gerbil monitored by a dual-probe microdialysis technique. *Life Sci.* **2002**; 71(7):803-11.

(203) Lindefors N, Amberg G, Ungerstedt U. Intracerebral microdialysis: I. Experimental studies of diffusion kinetics. *J. Pharmacol. Methods* **1989**; 22(3):141-56.

(204) Linhares MC, Kissinger PT. Capillary ultrafiltration: in vivo sampling probes for small molecules. *Anal. Chem.* **1992**; 64(22):2831-5.

(205) Linhares MC, Kissinger PT. Determination of endogenous ions in intercellular fluid using capillary ultrafiltration and microdialysis probes. *J. Pharm. Biomed. Anal.* **1993**; 11(11-12):1121-7.

(206) Linhares MC, Kissinger PT. Pharmacokinetic monitoring in subcutaneous tissue using in vivo capillary ultrafiltration probes. *Pharm. Res.* **1993**; 10(4):598-602.

(207) Linthorst AC, Reul JM. Stress and the brain: solving the puzzle using microdialysis. *Pharmacol. Biochem. Behav.* **2008**; 90(2):163-73.

(208) Liu YT, Huang CM. In Vivo Sampling of Extracellular {beta}-Thymosin by Ultrafiltration Probes. *Ann. N. Y. Acad. Sci.* **2007**.

(209) Lonjon M, Risso JJ, Palmier B, Negrin J, Darbin O. Effects of hypothermic deep-anaesthesia on energy metabolism at brain and peripheral levels: a multi-probe microdialysis study in free-moving rat. *Neurosci. Lett.* **2001**; 304(1-2):21-4.

(210) Lonnroth P, Jansson PA, Smith U. A microdialysis method allowing characterization of intercellular water space in humans. *Am. J. Physiol* **1987**; 253(2 Pt 1):E228-E231.

(211) Lourido J, Ederoth P, Sundvall N, Ungerstedt U, Nordstrom CH. Correlation between blood glucose concentration and glucose concentration in subcutaneous adipose tissue evaluated with microdialysis during intensive care. *Scand. J. Clin. Lab Invest* **2002**; 62(4):285-92.

(212) Lowry JP, Demestre M, Fillenz M. Relation between cerebral blood flow and extracellular glucose in rat striatum during mild hypoxia and hyperoxia. *Dev. Neurosci.* **1998**; 20(1):52-8.

(213) Lowry JP, Miele M, O'Neill RD, Boutelle MG, Fillenz M. An amperometric glucose-oxidase/poly(o-phenylenediamine) biosensor for monitoring brain extracellular glucose: in vivo characterisation in the striatum of freely-moving rats. *J. Neurosci. Methods* **1998**; 79(1):65-74.

(214) Lowry JP, O'Neill RD, Boutelle MG, Fillenz M. Continuous monitoring of extracellular glucose concentrations in the striatum of freely moving rats with an implanted glucose biosensor. *J. Neurochem.* **1998**; 70(1):391-6.

(215) Lund-Andersen H. Transport of glucose from blood to brain. *Physiol Rev.* **1979**; 59(2):305-52.

(216) Mader R., Brunner M., Rizovski B., et al. Analysis of microdialyzates from cancer patients by capillary electrophoresis. 19 ed. **1998**:2981-5.

(217) Magistretti PJ, Pellerin L, Rothman DL, Shulman RG. Energy on demand. *Science* **1999**; 283(5401):496-7.

(218) Marklund N, Clausen F, Lewander T, Hillered L. Monitoring of reactive oxygen species production after traumatic brain injury in rats with microdialysis and the 4-hydroxybenzoic acid trapping method. *J. Neurotrauma* **2001**; 18(11):1217-27.

(219) Markram H, Lubke J, Frotscher M, Sakmann B. Regulation of synaptic efficacy by coincidence of postsynaptic APs and EPSPs. *Science* **1997**; 275(5297):213-5.

(220) Mary S, Muret P, Makki S, et al. Assessment of the recovery of three lipophilic psoralens by microdialysis: an in vitro study. *International Journal of Pharmaceutics* **1998**; 161(1):7-13.

(221) Mayer CH, Fink H, Rex A, Voigt JP. Changes in extracellular hypothalamic glucose in relation to feeding. *Eur. J. Neurosci.* **2006**; 24(6):1695-701.

(222) McKenna MC, Tildon JT, Stevenson JH, Hopkins IB, Huang X, Couto R. Lactate transport by cortical synaptosomes from adult rat brain: characterization of kinetics and inhibitor specificity. *Dev. Neurosci.* **1998**; 20(4-5):300-9.

(223) McNay EC, Canal CE, Sherwin RS, Gold PE. Modulation of memory with septal injections of morphine and glucose: effects on extracellular glucose levels in the hippocampus. *Physiol Behav.* **2006**; 87(2):298-303.

(224) McNay EC, Fries TM, Gold PE. Decreases in rat extracellular hippocampal glucose concentration associated with cognitive demand during a spatial task. *Proc. Natl. Acad. Sci. U. S. A* **2000**; 97(6):2881-5.

(225) McNay EC, Gold PE. Extracellular glucose concentrations in the rat hippocampus measured by zero-net-flux: effects of microdialysis flow rate, strain, and age. *J. Neurochem.* **1999**; 72(2):785-90.

(226) McNay EC, McCarty RC, Gold PE. Fluctuations in brain glucose concentration during behavioral testing: dissociations between brain areas and between brain and blood. *Neurobiol. Learn. Mem.* **2001**; 75(3):325-37.

(227) Menacherry S, Hubert W, Justice JB, Jr. In vivo calibration of microdialysis probes for exogenous compounds. *Anal. Chem.* **1992**; 64(6):577-83.

(228) Mendelowitsch A, Ritz MF, Ros J, Langemann H, Gratzl O. 17beta-Estradiol reduces cortical lesion size in the glutamate excitotoxicity model by enhancing extracellular lactate: a new neuroprotective pathway. *Brain Res.* **2001**; 901(1-2):230-6.

(229) Mintun MA, Vlassenko AG, Rundle MM, Raichle ME. Increased lactate/pyruvate ratio augments blood flow in physiologically activated human brain. *Proc. Natl. Acad. Sci. U. S. A* **2004**; 101(2):659-64.

(230) Morf WE, Guenat OT, de Rooij NF. Partial electroosmotic pumping in complex capillary systems Part 1: Principles and general theoretical approach. *Sens. Actuators B Chem.* **2001**; B72(3):266-72.

(231) Morrison PF, Bungay PM, Hsiao JK, Ball BA, Mefford IN, Dedrick RL. Quantitative microdialysis: analysis of transients and application to pharmacokinetics in brain. *J. Neurochem.* **1991**; 57(1):103-19.

(232) Moscone D, Venema K, Korf J. Ultrafiltrate sampling device for continuous monitoring. *Med. Biol. Eng. Comput.* **1996**; 34(4):290-4.

(233) Mpholo M., Smith C.G., Brown A.B.D. Low voltage plug flow pumping using anisotropic electrode arrays. B92 ed. **2003**:262-8.

(234) O'Brien K., Esguerra M., Miller R., Bowser M. Monitoring Neurotransmitter Release from Isolated Retinas Using Online Microdialysis-Capillary Electrophoresis. 76 ed. **2004**:5069-74.

(235) Odland RM, Kizziar R, Rheuark D, Simental A. The effect of capillary ultrafiltration probes on skin flap edema. *Otolaryngol. Head Neck Surg.* **2003**; 128(2):210-4.

(236) Odland RM, Rheuark D, Ispirescu S, Kizziar R. Effect of tissue ultrafiltration on skin flap survival. *Otolaryngol. Head Neck Surg.* **2004**; 131(3):296-9.

(237) Oldenziel WH, Dijkstra G, Cremers TI, Westerink BH. In vivo monitoring of extracellular glutamate in the brain with a microsensor. *Brain Res.* **2006**; 1118(1):34-42.

(238) Olson RJ, Justice JB, Jr. Quantitative microdialysis under transient conditions. *Anal. Chem.* **1993**; 65(8):1017-22.

(239) Orzi F, Lucignani G, Dow-Edwards D, et al. Local cerebral glucose utilization in controlled graded levels of hyperglycemia in the conscious rat. *J. Cereb. Blood Flow Metab.* **1988**; 8(3):346-56.

(240) Osborne PG, Niwa O, Kato T, Yamamoto K. On-line, continuous measurement of extracellular striatal glucose using microdialysis sampling and electrochemical detection. *J. Neurosci. Methods* **1997**; 77(2):143-50.

(241) Oz G, Seaquist ER, Kumar A, et al. Human brain glycogen content and metabolism: implications on its role in brain energy metabolism. *Am. J. Physiol Endocrinol. Metab* **2007**; 292(3):E946-E951.

(242) Pancrazio JJ, Whelan JP, Borkholder DA, Ma W, Stenger DA. Development and application of cell-based biosensors. *Ann. Biomed. Eng* **1999**; 27(6):697-711.

(243) Pariante CM, Lightman SL. The HPA axis in major depression: classical theories and new developments. *Trends Neurosci.* **2008**; 31(9):464-8.

(244) Parkin M, Hopwood S, Jones DA, et al. Dynamic changes in brain glucose and lactate in pericontusional areas of the human cerebral cortex, monitored with rapid sampling on-line microdialysis: relationship with depolarisation-like events. *J. Cereb. Blood Flow Metab* **2005**; 25(3):402-13.

(245) Parrot S., Sauvinet V., Xavier J., et al. Capillary electrophoresis combined with microdialysis in the human spinal cord: A new tool for monitoring rapid peroperative changes in amino acid neurotransmitters within the dorsal horn. 25 ed. **2004**:1511-7.

(246) Pazdernik T, Cross R, Nelson S, Kamijo Y, Samson F. Is there an energy conservation "system" in brain that protects against the consequences of energy depletion? *Neurochem. Res.* **1994**; 19(11):1393-400.

(247) Pellerin L, Bouzier-Sore AK, Aubert A, et al. Activity-dependent regulation of energy metabolism by astrocytes: an update. *Glia* **2007**; 55(12):1251-62.

(248) Pellerin L, Magistretti PJ. Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization. *Proc. Natl. Acad. Sci. U. S. A* **1994**; 91(22):10625-9.

(249) Perdomo J, Hinkers H, Sundermeier C, Seifert W, Martinez MO, Knoll M. Miniaturized real-time monitoring system for L-lactate and glucose using microfabricated multi-enzyme sensors. *Biosens. Bioelectron.* **2000**; 15(9-10):515-22.

(250) Plock N, Kloft C. Microdialysis--theoretical background and recent implementation in applied life-sciences. *Eur. J. Pharm. Sci.* **2005**; 25(1):1-24.

(251) Rabenstein K, McShane AJ, McKenna MJ, Dempsey E, Keaveny TV, Freaney R. An intravascular microdialysis sampling system suitable for application in continuous biochemical monitoring of glucose and lactate. *Technol. Health Care* **1996**; 4(1):67-76.

(252) Reinert M, Schaller B, Widmer HR, Seiler R, Bullock R. Influence of oxygen therapy on glucose-lactate metabolism after diffuse brain injury. *J. Neurosurg.* **2004**; 101(2):323-9.

(253) Reinstrup P, Stahl N, Mellergard P, Uski T, Ungerstedt U, Nordstrom CH. Intracerebral microdialysis in clinical practice: baseline values for chemical markers during wakefulness, anesthesia, and neurosurgery. *Neurosurgery* **2000**; 47(3):701-9.

(254) Rhemrev-Boom MM, Jonker MA, Venema K, Jobst G, Tiessena R, Korf J. On-line continuous monitoring of glucose or lactate by ultraslow microdialysis combined with a flow-through nanoliter biosensor based on poly(m-phenylenediamine) ultra-thin polymer membrane as enzyme electrode. *Analyst* **2001**; 126(7):1073-9.

(255) Rhemrev-Boom MM, Korf J, Venema K, Urban G, Vadgama P. A versatile biosensor device for continuous biomedical monitoring. *Biosens. Bioelectron.* **2001**; 16(9-12):839-47.

(256) Rhemrev-Boom MM, Leegsma-Vogt G, Venema K, Postema F, Korf J. Turn-over of extracellular glucose and lactate in the rat striatum estimated by equilibrium microdialysis. *J. Cereb. Blood Flow Metab.* **2005**; 25 Supplement Brain 05, poster 89.

(257) Rhemrev-Boom MM, Tiessen RG, Jonker AA, Venema K, Vadgama P, Korf J. A lightweight measuring device for the continuous in vivo monitoring of glucose by means of ultraslow microdialysis in combination with a miniaturised flow-through biosensor. *Clin. Chim. Acta* **2002**; 316(1-2):1-10.

(258) Rhemrev-Boom MM, Tiessen RG, Venema K, Korf J. Biosensor device and ultrafiltration sampling for continuous in vivo monitoring of glucose. *biocybernetics and biomedical engineering* **1999**; 19(1):97-104.

(259) Ritz MF, Schmidt P, Mendelowitsch A. Acute effects of 17beta-estradiol on the extracellular concentration of excitatory amino acids and energy metabolites during transient cerebral ischemia in male rats. *Brain Res.* **2004**; 1022(1-2):157-63.

(260) Ros J, Jones D, Pecinska N, et al. Glutamate infusion coupled with hypoxia has a neuroprotective effect in the rat. *J. Neurosci. Methods* **2002**; 119(2):129-33.

(261) Rosano GM, Kaski JC, Arie S, et al. Failure to demonstrate myocardial ischaemia in patients with angina and normal coronary arteries. Evaluation by continuous coronary sinus pH monitoring and lactate metabolism. *Eur. Heart J.* **1996**; 17(8):1175-80.

(262) Rosdahl H, Hamrin K, Ungerstedt U, Henriksson J. Metabolite levels in human skeletal muscle and adipose tissue studied with microdialysis at low perfusion flow. *Am. J. Physiol.* **1998**; 274(5 Pt 1):E936-E945.

(263) Rosdahl H., Ungerstedt U., Henriksson J. Microdialysis in human skeletal muscle and adipose tissue at low flow rates is possible if dextran-70 is added to prevent loss of perfusion fluid. 159 ed. **1997**:261-2.

(264) Rosdahl H., Ungerstedt U., Jorfeldt L., Henriksson J. Interstitial glucose and lactate balance in human skeletal muscle and adipose tissue studied by microdialysis. 471 ed. **1993**:637-57.

(265) Rosenbloom AJ, Sipe DM, Weedn VW. Microdialysis of proteins: Performance of the CMA/20 probe. *J. Neurosci. Methods* **2005**; 148(2):147-53.

(266) Rosenbloom AJ, Sipe DM, Weedn VW. Microdialysis of proteins: performance of the CMA/20 probe. *J. Neurosci. Methods* **2005**; 148(2):147-53.

(267) Roy CS, Sherrington CS. On the Regulation of the Blood-supply of the Brain. *J. Physiol* **1890**; 11(1-2):85-158.

(268) Rutherford EC, Pomerleau F, Huettl P, Stromberg I, Gerhardt GA. Chronic second-by-second measures of L-glutamate in the central nervous system of freely moving rats. *J. Neurochem.* **2007**; 102(3):712-22.

(269) Sakowitz OW, Stover JF, Sarrafzadeh AS, Unterberg AW, Kiening KL. Effects of mannitol bolus administration on intracranial pressure, cerebral extracellular metabolites, and tissue oxygenation in severely head-injured patients. *J. Trauma* **2007**; 62(2):292-8.

(270) Salci K, Nilsson P, Goiny M, Contant C, Piper I, Enblad P. Low intracranial compliance increases the impact of intracranial volume insults to the traumatized brain: a microdialysis study in a traumatic brain injury rodent model. *Neurosurgery* **2006**; 59(2):367-73.

(271) Savenije B, Venema K, Gerritzen MA, Lambooij E, Korf J. Minimally invasive technique based on ultraslow ultrafiltration to collect and store time profiles of analytes. *Anal. Chem.* **2003**; 75(17):4397-401.

(272) Scheller D, Kolb J. The internal reference technique in microdialysis: a practical approach to monitoring dialysis efficiency and to calculating tissue concentration from dialysate samples. *J. Neurosci. Methods* **1991**; 40(1):31-8.

(273) Schlenk F, Graetz D, Nagel A, Schmidt M, Sarrafzadeh AS. Insulin-related decrease in cerebral glucose despite normoglycemia in aneurysmal subarachnoid hemorrhage. *Crit Care* **2008**; 12(1):R9.

(274) Schneiderheinze JM, Hogan B.L. Selective in vivo and in vitro sampling of proteins using miniature ultrafiltration sampling probes. *Anal. Chem.* **1996**; 68(21):3758-62.

(275) Schramm W, Smith RH. An ultrafiltrate of saliva collected in situ as a biological sample for diagnostic evaluation. *Clin. Chem.* **1991**; 37(1):114-5.

(276) Schramm W, Smith RH, Craig PA. Methods of simplified saliva collection for the measurement of drugs of abuse, therapeutic drugs, and other molecules. *Ann. N. Y. Acad. Sci.* **1993**; 694:311-3.

(277) Schulz MK, Wang LP, Tange M, Bjerre P. Cerebral microdialysis monitoring: determination of normal and ischemic cerebral metabolisms in patients with aneurysmal subarachnoid hemorrhage. *J. Neurosurg.* **2000**; 93(5):808-14.

(278) Schurr A, Rigor BM. Brain anaerobic lactate production: a suicide note or a survival kit? *Dev. Neurosci.* **1998**; 20(4-5):348-57.

(279) Schutte RJ, Oshodi SA, Reichert WM. In vitro characterization of microdialysis sampling of macromolecules. *Anal. Chem.* **2004**; 76(20):6058-63.

(280) Sefton MV, Gemmell CH, Gorbet MB. What really is blood compatibility? *J. Biomater. Sci. Polym. Ed* **2000**; 11(11):1165-82.

(281) Selvaganapathy P, Ki Y, Renaud P, Mastrangelo C. Bubble-free electrokinetic pumping. *J. Microelectromech. Syst.* **2002**; 11(5):448-53.

(282) Semmes OJ, Feng Z, Adam BL, et al. Evaluation of serum protein profiling by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry for the detection of prostate cancer: I. Assessment of platform reproducibility. *Clin. Chem.* **2005**; 51(1):102-12.

(283) Shram NF, Netchiporouk LI, Martelet C, Jaffrezic-Renault N, Bonnet C, Cespuglio R. In vivo voltammetric detection of rat brain lactate with carbon fiber microelectrodes coated with lactate oxidase. *Anal. Chem.* **1998**; 70(13):2618-22.

(284) Shuaib A, Xu K, Crain B, et al. Assessment of damage from implantation of microdialysis probes in the rat hippocampus with silver degeneration staining. *Neurosci. Lett.* **1990**; 112(2-3):149-54.

(285) Silver IA, Erecinska M. Extracellular glucose concentration in mammalian brain: continuous monitoring of changes during increased neuronal activity and upon limitation in oxygen supply in normo-, hypo-, and hyperglycemic animals. *J. Neurosci.* **1994**; 14(8):5068-76.

(286) Slais K, Vorisek I, Zoremba N, Homola A, Dmytrenko L, Sykova E. Brain metabolism and diffusion in the rat cerebral cortex during pilocarpine-induced status epilepticus. *Exp. Neurol.* **2008**; 209(1):145-54.

(287) Slais K, Vorisek I, Zoremba N, Homola A, Dmytrenko L, Sykova E. Brain metabolism and diffusion in the rat cerebral cortex during pilocarpine-induced status epilepticus. *Exp. Neurol.* **2008**; 209(1):145-54.

(288) Smith PJ, Hammar K, Porterfield DM, Sanger RH, Trimarchi JR. Self-referencing, non-invasive, ion selective electrode for single cell detection of trans-plasma membrane calcium flux. *Microsc. Res. Tech.* **1999**; 46(6):398-417.

(289) Sojka JE, Adams SB, Rohde C, Janie EM. Surgical implantation of ultrafiltration probes in ovine bone and muscle. *J. Invest. Surg.* **2000**; 13(5):289-94.

(290) Soto-Montenegro ML, Vaquero JJ, Arango C, Ricaurte G, Garcia-Barreno P, Desco M. Effects of MDMA on blood glucose levels and brain glucose metabolism. *Eur. J. Nucl. Med. Mol. Imaging* **2007**; 34(6):916-25.

(291) Spehar A., Tiedje L., Sojka J.E., Janle E.M., Kissinger P.T. Recovery of endogenous ions from subcutaneous and intramuscular spaces in horses using ultrafiltrate probes. 17 ed. **1998**:47-51.

(292) Stahl N, Mellergard P, Hallstrom A, Ungerstedt U, Nordstrom CH. Intracerebral microdialysis and bedside biochemical analysis in patients with fatal traumatic brain lesions. *Acta Anaesthesiol. Scand.* **2001**; 45(8):977-85.

(293) Stephans SE, Whittingham TS, Douglas AJ, Lust WD, Yamamoto BK. Substrates of energy metabolism attenuate methamphetamine-induced neurotoxicity in striatum. *J. Neurochem.* **1998**; 71(2):613-21.

(294) Stephans SE, Whittingham TS, Douglas AJ, Lust WD, Yamamoto BK. Substrates of energy metabolism attenuate methamphetamine-induced neurotoxicity in striatum. *J. Neurochem.* **1998**; 71(2):613-21.

(295) Storm-Mathisen J. Localization of transmitter candidates in the brain: the hippocampal formation as a model. *Prog. Neurobiol.* **1977**; 8(2):119-81.

(296) Strong AJ, Boutelle MG, Vespa PM, Bullock MR, Bhatia R, Hashemi P. Treatment of critical care patients with substantial acute ischemic or traumatic brain injury. *Crit Care Med.* **2005**; 33(9):2147-9.

(297) Sun L, Stenken JA. Improving microdialysis extraction efficiency of lipophilic eicosanoids. *J. Pharm. Biomed. Anal.* **2003**; 33(5):1059-71.

(298) Takamura Y, Onoda H, Inokuchi H, Adachi S, Oki A, Horiike Y. Ramsey,J.M.van den Berg,A, (Eds) Proceedings of μTAS. Dordrecht: Kluwer Academic Publishers, **2001**.

(299) Takita M, Mikuni M, Takahashi K. Habituation of lactate release responding to stressful stimuli in rat prefrontal cortex in vivo. *Am. J. Physiol* **1992**; 263(3 Pt 2):R722-R727.

(300) Tan Q, Ji J, Barbosa MA, Fonseca C, Shen J. Constructing thromboresistant surface on biomedical stainless steel via layer-by-layer deposition anticoagulant. *Biomaterials* **2003**; 24(25):4699-705.

(301) Tao R, Hjorth S. Differences in the in vitro and in vivo 5-hydroxytryptamine extraction performance among three common microdialysis membranes. *J. Neurochem.* **1992**; 59(5):1778-85.

(302) Taylor DL, Urenjak J, Zilkha E, Obrenovitch TP. Effects of probenecid on the elicitation of spreading depression in the rat striatum. *Brain Res.* **1997**; 764(1-2):117-25.

(303) Tetko IV, Gasteiger J, Todeschini R, et al. Virtual computational chemistry laboratory--design and description. *J. Comput. Aided Mol. Des* **2005**; 19(6):453-63.

(304) Thevenot DR, Toth K, Durst RA, Wilson GS. Electrochemical biosensors: recommended definitions and classification. *Biosens. Bioelectron.* **2001**; 16(1-2):121-31.

(305) Thomale UW, Griebenow M, Mautes A, et al. Heterogeneous regional and temporal energetic impairment following controlled cortical impact injury in rats. *Neurol. Res.* **2007**; 29(6):594-603.

(306) Thompson JK, Peterson MR, Freeman RD. Separate spatial scales determine neural activity-dependent changes in tissue oxygen within central visual pathways. *J. Neurosci.* **2005**; 25(39):9046-58.

(307) Tiessen R.G., Kaptein W.A., Venema K., Korf J. Slow ultrafiltration for continuous in vivo sampling: application for glucose and lactate in man. 379 ed. **1999**:327-35.

(308) Tiessen RG, Rhemrev-Boom MM, Korf J. Glucose gradient differences in subcutaneous tissue of healthy volunteers assessed with ultraslow microdialysis and a nanolitre glucose sensor. *Life Sci.* **2002**; 70(21):2457-66.

(309) Tiessen RG, Tio RA, Hoekstra A, Venema K, Korf J. An ultrafiltration catheter for monitoring of venous lactate and glucose around myocardial ischemia. *Biosens. Bioelectron.* **2001**; 16(3):159-67.

(310) Tirumalai RS, Chan KC, Prieto DA, Issaq HJ, Conrads TP, Veenstra TD. Characterization of the low molecular weight human serum proteome. *Mol. Cell. Proteomics* **2003**; 2(10):1096-103.

(311) Toes GJ, van Muiswinkel KW, van OW, et al. Superhydrophobic modification fails to improve the performance of small diameter expanded polytetrafluoroethylene vascular grafts. *Biomaterials* **2002**; 23(1):255-62.

(312) Trickler WJ, Miller DW. Use of osmotic agents in microdialysis studies to improve the recovery of macromolecules. *J. Pharm. Sci.* **2003**; 92(7):1419-27.

(313) Tsai T. Concurrent measurement of unbound genistein in the blood, brain and bile of anesthetized rats using microdialysis and its pharmacokinetic application. 1073 ed. **2005**:317-22.

(314) Tsai TH. Assaying protein unbound drugs using microdialysis techniques. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2003**; 797(1-2):161-73.

(315) Uehara T, Kurata K, Sumiyoshi T, Kurachi M. Immobilization stress-induced increment of lactate metabolism in the basolateral amygdaloid nucleus is attenuated by diazepam in the rat. *Eur. J. Pharmacol.* **2003**; 459(2-3):211-5.

(316) Uehara T, Sumiyoshi T, Itoh H, Kurachi M. Dopamine D1 and D2 receptors regulate extracellular lactate and glucose concentrations in the nucleus accumbens. *Brain Res.* **2007**; 1133(1):193-9.

(317) Uehara T, Sumiyoshi T, Itoh H, Kurachi M. Role of glutamate transporters in the modulation of stress-induced lactate metabolism in the rat brain. *Psychopharmacology (Berl)* **2007**; 195(2):297-302.

(318) Uehara T, Sumiyoshi T, Matsuoka T, et al. Enhancement of lactate metabolism in the basolateral amygdala by physical and psychological stress: role of benzodiazepine receptors. *Brain Res.* **2005**; 1065(1-2):86-91.

(319) Unger JK, Haltern C, Dohmen B, Rossaint R. Maximal flow rates and sieving coefficients in different plasmafilters: effects of increased membrane surfaces and effective length under standardized in vitro conditions. *J. Clin. Apheresis.* **2002**; 17(4):190-8.

(320) Ungerstedt U. Microdialysis, a new bioanalytical sampling technique. *Curr. Sep.* **1986**; 7:43-6.

(321) Ungerstedt U, Pycock C. Functional correlates of dopamine neurotransmission. *Bull. Schweiz. Akad. Med. Wiss.* **1974**; 30(1-3):44-55.

(322) Ungerstedt U., Rostami E. Microdialysis in neurointensive care. 10 ed. **2004**:2145-52.

(323) Vafaee MS, Gjedde A. Spatially dissociated flow-metabolism coupling in brain activation. *Neuroimage.* **2004**; 21(2):507-15.

(324) Valette P, Thomas M, Dejardin P. Adsorption of low molecular weight proteins to hemodialysis membranes: experimental results and simulations. *Biomaterials* **1999**; 20(17):1621-34.

(325) Valtysson J, Persson L, Hillered L. Extracellular ischaemia markers in repeated global ischaemia and secondary hypoxaemia monitored by microdialysis in rat brain. *Acta Neurochir. (Wien.)* **1998**; 140(4):387-95.

(326) van der Kuil JH, Korf J. On-line monitoring of extracellular brain glucose using microdialysis and a NADPH-linked enzymatic assay. *J. Neurochem.* **1991**; 57(2):648-54.

(327) Vespa P, Bergsneider M, Hattori N, et al. Metabolic crisis without brain ischemia is common after traumatic brain injury: a combined microdialysis and positron emission tomography study. *J. Cereb. Blood Flow Metab* **2005**; 25(6):763-74.

(328) Vespa P, Boonyaputthikul R, McArthur DL, et al. Intensive insulin therapy reduces microdialysis glucose values without altering glucose utilization or improving the lactate/pyruvate ratio after traumatic brain injury. *Crit Care Med.* **2006**; 34(3):850-6.

(329) Voigt JP, Nwaiser B, Rex A, Mayer C, Fink H. Effect of 5-HT1A receptor activation on hypothalamic glucose. *Pharmacol. Res.* **2004**; 50(3):359-65.

(330) von Baeyer H, Lajous-Petter A, Debrandt W, Hampl H, Kochinke F, Herbst R. Surface reactions on blood contact during haemodialysis and haemofiltration with various membrane types. *J. Neurosci. Methods* **1988**; 36:215-29.

(331) Wages SA, Church WH, Justice JB, Jr. Sampling considerations for on-line microbore liquid chromatography of brain dialysate. *Anal. Chem.* **1986**; 58(8):1649-56.

(332) Wang L, Dong Y, Yu X, et al. Analysis of glucose and lactate in dialysate from hypothalamus of rats after exhausting swimming using microdialysis. *Biomed. Chromatogr.* **2002**; 16(7):427-31.

(333) Wang Z, Zhang Z, Fu Z, Xiong Y, Zhang X. A flow-injection ultrafiltration sampling chemiluminescence system for on-line determination of drug-protein interaction. *Anal. Bioanal. Chem.* **2003**; 377(4):660-5.

(334) Wendel HP, Ziemer G. Coating-techniques to improve the hemocompatibility of artificial devices used for extracorporeal circulation. *Eur. J. Cardiothorac. Surg.* **1999**; 16(3):342-50.

(335) Wilson GS, Gifford R. Biosensors for real-time in vivo measurements. *Biosens. Bioelectron.* **2005**; 20(12):2388-403.

(336) Winter CD, Iannotti F, Pringle AK, Trikkas C, Clough GF, Church MK. A microdialysis method for the recovery of IL-1beta, IL-6 and nerve growth factor from human brain in vivo. *J. Neurosci. Methods* **2002**; 119(1):45-50.

(337) Wisniewski N. Characterization of mass transport through implantable biosensor membranes using microdialysis. **2001**:204.

(338) Wisniewski N, Klitzman B, Miller B, Reichert WM. Decreased analyte transport through implanted membranes: differentiation of biofouling from tissue effects. *J. Biomed. Mater. Res.* **2001**; 57(4):513-21.

(339) Wisniewski N, Moussy F, Reichert WM. Characterization of implantable biosensor membrane biofouling. *Fresenius. J. Anal. Chem.* **2000**; 366(6-7):611-21.

(340) Wisniewski N, Rajamand N, Adamsson U, et al. Analyte flux through chronically implanted subcutaneous polyamide membranes differs in humans and rats. *Am. J. Physiol. Endocrinol. Metab.* **2002**; 282(6):E1316-E1323.

(341) Wisniewski N, Reichert M. Methods for reducing biosensor membrane biofouling. *Colloids Surf. B Biointerfaces.* **2000**; 18(3-4):197-219.

(342) Xie F, Bruntlett CS, Zhu Y, Kissinger CB, Kissinger PT. Good preclinical bioanalytical chemistry requires proper sampling from laboratory animals: automation of blood and microdialysis sampling improves the productivity of LC/MSMS. *Anal. Sci.* **2003**; 19(4):479-85.

(343) Xie R, Hammarlund-Udenaes M, de Boer AG, de Lange EC. The role of P-glycoprotein in blood-brain barrier transport of morphine: transcortical microdialysis studies in mdr1a (-/-) and mdr1a (+/+) mice. *Br. J. Pharmacol.* **1999**; 128(3):563-8.

(344) Yager JY, Armstrong EA, Miyashita H, Wirrell EC. Prolonged neonatal seizures exacerbate hypoxic-ischemic brain damage: correlation with cerebral energy metabolism and excitatory amino acid release. *Dev. Neurosci.* **2002**; 24(5):367-81.

(345) Yang Q., Atanasov P., Wilkins E. An integrated needle-type biosensor for intravascular glucose and lactate monitoring. 10 ed. **1998**:752-7.

(346) Yang S, Huang CM. Recent advances in protein profiling of tissues and tissue fluids. *Expert. Rev. Proteomics.* **2007**; 4(4):515-29.

(347) Yao T. Flow-injection biosensor system for in vivo analysis of biomolecules in the extracellular space of brain. 21 ed. **2004**:59-62.

(348) Yao T., Yano T., Nishino H. Simultaneous in vivo monitoring of glucose, L-lactate, and pyruvate concentrations in rat brain by a flow-injection biosensor system with an on-line microdialysis sampling. 510 ed. **2004**:53-9.

(349) Yokel RA, Allen DD, Burgio DE, McNamara PJ. Antipyrine as a dialyzable reference to correct differences in efficiency among and within sampling devices during in vivo microdialysis. *J. Pharmacol. Toxicol. Methods* **1992**; 27(3):135-42.

(350) Zauner A, Doppenberg E, Woodward JJ, et al. Multiparametric continuous monitoring of brain metabolism and substrate delivery in neurosurgical patients. *Neurol. Res.* **1997**; 19(3):265-73.

(351) Zauner A, Doppenberg EM, Woodward JJ, Choi SC, Young HF, Bullock R. Continuous monitoring of cerebral substrate delivery and clearance: initial experience in 24 patients with severe acute brain injuries. *Neurosurgery* **1997**; 41(5):1082-91.

(352) Zhang FF, Wan Q, Li CX, et al. Simultaneous assay of glucose, lactate, L-glutamate and hypoxanthine levels in a rat striatum using enzyme electrodes based on neutral red-doped silica nanoparticles. *Anal. Bioanal. Chem.* **2004**; 380(4):637-42.

(353) Zhao S, Pinholt EM, Madsen JE, Donath K. Histological evaluation of different biodegradable and non-biodegradable membranes implanted subcutaneously in rats. *J. Craniomaxillofac. Surg.* **2000**; 28(2):116-22.

(354) Zielke HR, Zielke CL, Baab PJ. Oxidation of (14)C-labeled compounds perfused by microdialysis in the brains of free-moving rats. *J. Neurosci. Res.* **2007**; 85(14):3145-9.

(355) Zoremba N, Homola A, Rossaint R, Sykova E. Brain metabolism and extracellular space diffusion parameters during and after transient global hypoxia in the rat cortex. *Exp. Neurol.* **2007**; 203(1):34-41.

Dankwoord

Mijn promotietraject is bijna volbracht, dit is het laatste stukje tekst wat ik schrijf om mijn proefschrift af te maken. Vijfenhalf jaar (2003-2009) heb ik mij bezig gehouden met het AIO-schap, verdeelt over twee verschillende werkplekken: de Biologische Psychiatrie en de Biomonitoring and Sensoring groep. Deze periode is wel onderbroken geweest door twee keer een zwangerschaps- en bevallingsverlof.

Tijdens mijn promotietraject had ik slechts één begeleider, Jaap Korf, een persoon met ideeen voor tien. Jaap, je bent een duizendpoot die overal een positieve draai aan weet te geven. Ik wil je hartelijk bedanken voor alles en ik vond de samenwerking zeer prettig!

Er is heel wat af geknutseld of ook wel geprutst op de zevende verdieping. Kor Venema was de opperprutser. Ik heb je erg gemist sinds je met pensioen bent, je rust, je kennis en je grapjes.

Petra Bakker was onmisbaar voor de cortisol/corticosteron bepalingen. Petra bedankt en het ga je goed! Speciaal wil ik Marjolein, mijn voormalig kamergenoot, bedanken voor al de gezellige momenten. Verder wil ik Wim van Oeveren bedanken voor het uitvoeren van de heparine coatings, zonder deze coating was het niet mogelijk geweest om 8 uur te monsteren.

Tweeduizendenzes was voor mij een bewogen jaar: Kor ging met pensioen, ik kreeg een baby en ik bleef als enige prutser achter op de zevende verdieping. Mede door het naderende eind van de Biologische Psychiatrie, moest ik keuzes gaan maken: of ik verhuisde mee naar het nieuwe lab of ik moest iets anders gaan verzinnen. Marieke van der Hart overtuigde mij om te komen werken voor de Biomonitoring en Sensoring groep. Marieke bedankt voor de tip, ik heb er tot op de dag van vandaag geen dag spijt van gehad.

Bij de Biomonitoring en Sensoring groep is Thomas Cremers mijn tweede begeleider en copromotor geworden. Thomas, Gunnar en Ben, heel erg bedankt dat ik bij B&S en BOL mocht komen werken. Aan alle andere (soms voormalig) BOL, BL en B&S medewerkers: Aloys, André, Carlos, Christa, Corry, Daphne, Eliaha, Elvira, Gerrit, Harm, Jan, Jan-Paul, Jeanette, Jelle, Jeroen, Joost, Kim, Klaas-Jan, Korrie, Luthea, Marieke, Marius, Martin, Miranda, Rikje, Robert, Saskia, Si, Theo, Tietie, Ulrike, Wahono, en Wei, jullie zijn een gezellig club mensen en ik ga met veel plezier naar mijn werk.

Ondanks dat ik officieel geadopteerd werd door B&S, heb ik feitelijk gezien met weinig collega's van B&S samengewerkt. Suzan Postma en Karola Jansen-van Zelm jullie zijn hartstikke goed, bedankt voor jullie hulp. Karola wat vond ik het fijn dat je meeging naar Lelystad, mijn dank is groot.

De STW heeft het beschreven onderzoek mogelijk gemaakt. Twee keer per jaar kwamen we bij elkaar, de gebruikers en mijn mede AIO-partner Truus Posthuma-Trumpie. Iedereen bedankt voor het mogelijk maken van dit project!

Tevens wil ik de leescommisie-leden bedanken voor het goedkeuren van dit manuscript; Prof.dr. R. Vonk, Prof.dr. J. Strubbe en Prof.dr. B. H. C. Westerink.

Aan mijn paranimfen:

Jolanda we kennen elkaar van de HLO, al hadden we daar weinig contact met elkaar. Naarmate we ouder worden, wordt onze vriendschap hechter en dat over een periode van 10 jaar. Je bent een schat en ik ben blij dat jij mijn paranimf bent.

Jan Willem op 8 juni 2009 zijn we ruim 10 jaar bij elkaar. Je brengt me rust en samen hebben we twee hele lieve en mooie jongens: Zep & Tren, waar ik dolgelukkig mee ben en van word. Jullie zijn mijn schatjes!

Curriculum vitae

De auteur van dit proefschrift werd op 30 maart 1976 geboren te Meppel. In 1992 behaalde ze haar MAVO-diploma aan het Reestdalcollege te Meppel. In datzelfde jaar werd begonnen met de Middelbaar Laboratorium Opleiding (MLO), Medische richting, te Groningen. Na 3 jaar MLO stroomde ze in, in het tweede jaar van de Hoger Laboratorium Opleiding (HLO), richting Medisch, te Groningen. Naast de Medische richting is ook de afstudeerrichting Biotechnologie gevolgd. Het examen voor beide studies werd afgelegd in 2000. In datzelfde jaar werd begonnen met de verkorte opleiding Biomedische Wetenschappen aan de VU te Amsterdam, waarvan ze haar diploma behaalde in februari 2003. In september 2003 werd zij aangenomen als AIO bij de Biologische Psychiatrie te Groningen wat resulteerde in dit proefschrift. Vanaf februari 2009 is zij trial manager bij Brains On-Line B.V. en general manager bij Brainlink B.V. te Groningen.