Chapter 2
Recovery and Calibration Techniques: Toward Quantitative Microdialysis

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Abstract One of the most important questions in microdialysis is how to relate the concentrations in the microdialysate to the true concentrations outside the probe as in vitro recovery was not equal per se to the recovery in vivo. Many calibration techniques and approaches have been proposed, with different levels of practicality and usefulness, to obtain quantitative information on extracellular concentrations as a function of time. Today, we have build up enough experience to know how to obtain quantitative data using the microdialysis technique. With that, it is the only technique that provides data on the unbound concentration of compounds in extracellular fluid spaces in the body, being highly important information in drug development as the unbound concentrations are the drivers for the effect.

2.1 Introduction

Microdialysis technique was developed about 30 years ago, actually as improvement of the push–pull technique to obtain information on the extracellular fluid in the brain (brainECF), mostly focused on neurotransmitter functioning. By the use of a microdialysis, probe with a tip consisting of a semipermeable membrane and a continuous flush of the microdialysis probe with perfusion fluid, small enough molecular weight molecules diffuse from the outside to the inside of the probe

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according to their concentration gradient, providing the dialysate fluid to be collected in intervals and analyzed on its content.

Then, questions arose about the possible impact of the microdialysis probe on its surrounding tissue. Quite a few studies have dedicated to investigate the changes of the periprobe tissue and its functionality. It was concluded that provided that surgery is carefully performed and experimental conditions are well chosen, the microdialysis probe had only minimal influence on the periprobe tissue.

After the application of the microdialysis technique was broadened to the pharmacokinetic research field, being quantitative by nature, from this field the question rose about the relationship between the concentrations measured in the dialysate in relation to the concentrations around the microdialysis tip. This ratio was termed recovery and was first estimated in vitro, by measuring the recovery of the compound of interest in the dialysate from a bulk solution with known concentration. This value was used to correct in vivo microdialysis concentrations to obtain what was supposed to be the free concentrations around the microdialysis probe. With time, it became clear that in vitro recovery was not equal per se to the recovery in vivo.

Thus, one of the most important questions in microdialysis is how to relate the concentrations in the microdialysate to the true concentrations outside the probe. In other words: to which extent is the drug recovered by the dialysate (or lost if a certain drug is added to the perfusate) and how fast? This chapter deals with the concept of recovery, and factors that will influence recovery, followed by theoretical and experimental approaches to determine the extracellular concentrations around the microdialysis probe, and pharmacokinetic data analysis. Finally, some emerging techniques and applications will be discussed.

2.2 Recovery

2.2.1 The Concept of Recovery

The term recovery describes the relation between concentrations of the drug in the periprobe fluid and those in the dialysate. These concentrations will differ from each other because a constant flow of the perfusate prevents the establishment of true concentration equilibrium between the periprobe fluid and the dialysate. Two types of recoveries can be distinguished: (1) absolute recovery (or mass recovery), which is defined as the amount of drug that is extracted by the dialysate as a function of time, and (2) relative recovery (or concentration recovery) being defined as the concentration of the drug in the dialysate divided by its uniform concentration in the periprobe fluid. In general, the term “recovery” is used to refer to concentration recovery. The recovery is defined as

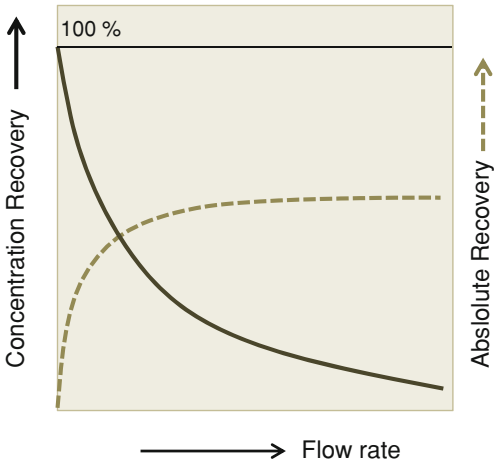

Fig. 2.1 At least in vitro the relative or concentration recovery is inversely dependent on the flow rate and often linearly dependent on the concentration in the periprobe fluid. The absolute (mass) recovery increases with flow rate up to a maximum when the mass flux of molecules become rate limiting

$$R = (C_{in} - C_{dial})/C_{in}$$

In this equation, C_{in} and C_{dial} are the concentrations of a compound in the perfusate and in the dialysate, respectively. This equation should be adapted if concentrations are added to the perfusate, as in some of the calibration methods discussed later in this chapter, i.e., the retrodialysis method, the no-net-flux, and the dynamic-no-net-flux method.

2.2.2 *In Vitro Recovery*

A number of parameters influence absolute and concentration recovery (Hsiao et al. 1990). These parameters include perfusate flow rate, temperature, characteristics of the semipermeable membrane, probe geometry, surface of the semipermeable membrane, perfusate composition, tubing characteristics, and length, as well as the characteristics of the drug. These factors can be investigated in vitro, knowing the concentration of the drug in the bulk solution.

Flow rate. The in vitro recovery is inversely dependent on the *flow rate* and often linearly dependent on the concentration in the periprobe fluid (Fig. 2.1). Most investigators now use a flow rate that ranges from 0.1 to 5 µl/min, with a typical value being 2 ul/min.

Membrane surface. Then, an increase of the *dialysis membrane surface* will increase the recovery (Fig. 2.2). This will be a linear increase with small surfaces, and dependent on drug characteristics. Note that, at larger surfaces the increase in recovery will start to lag behind the increase in surface because the concentration difference between periprobe fluid and dialysate traversing along the semipermeable membrane will gradually diminish with the longer length of the membrane

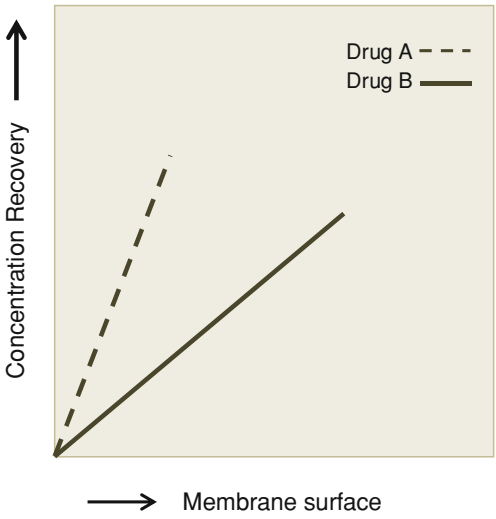

Fig. 2.2 Then, an increase of the dialysis membrane surface will increase the recovery. This will be a linear increase with small surfaces, where a huge concentration-gradient is maintained between periprobe fluid and dialysate concentrations. Further, the recovery depends on drug and membrane characteristics

Temperature. As diffusion increases with higher *temperature*, a higher temperature will increase recovery. As probes in vivo are exposed to body temperature, it is important that testing of the probe characteristic in vitro also occur at this temperature.

Membrane material. Different membrane materials can be used. For self-made probes, the choice among the different membranes is wide, e.g., those used for artificial kidneys. Commercially available probes have limited choice of membrane material (De Lange 1997). It is important to investigate the rate toward and extent of equilibrium of concentration recovery of the drug for a selected membrane (Tao and Hjorth 1992). If the drug has interaction with the membrane, it may lead to delayed response of the dialysate concentration to changes in the external medium (Fig. 2.3). The faster the changes occur in the external medium (read here in vivo periprobe concentrations), the more important this aspect is. The problem of interaction of a drug with the membrane material is more often encountered for lipophilic drugs (Carneheim and Stahle 1991).

Tubing material and dimensions. Tubing material from the microdialysis probe to the sample collector should ideally have no interaction with the drug as this may have a profound effect on the relation between the concentration of the drug found in the dialysate and the periprobe fluid concentrations, similar to effects of interaction with the probe membrane (Lindberger et al. 2002). The inner diameters of the tubing connections, however, may be of importance with respect to build up of fluid pressure and thereby fluid loss over the semipermeable membrane. This may be prevented by using inlet tubing (from perfusion pump to the probe) with an inner diameter smaller than that of the probe itself, and an outlet tubing (from probe to collection site) with an inner diameter being larger than that of the probe.

Perfusion medium composition. Many different perfusion medium composites in microdialysis studies have been used in the past (De Lange et al. 1997).

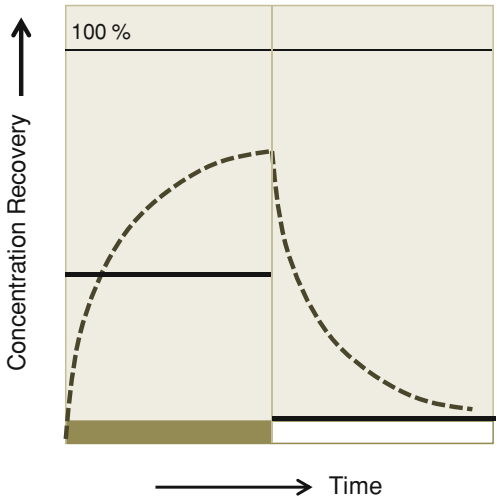

Fig. 2.3 It is important to investigate the rate toward and extent of equilibrium of concentration recovery of the drug for a selected membrane as the drug may interaction with the membrane (as well as tubing). This may lead to a delayed ("blunted") response of the dialysate concentration to changes in the external medium. The faster the changes occur in the external medium (read here in vivo periprobe concentrations), the more important this aspect is. Drugs that interact with probe membrane and/or tubing are called "sticky" drugs. Care should be taken to avoid a blunted response

The most commonly used today is the minimal perfusion medium, consisting of 140 mM sodium, 3.0 mM potassium, 1.2 mM calcium, 1.0 mM magnesium, and 147 mM chloride.

To prevent or reduce the interaction of a drug with the probe membrane and/or tubing (or improve the rate and/or extent of concentration recovery), several additions to the perfusion medium have been proposed (Carneheim and Stahle 1991). The addition of bovine serum albumin has often been proven useful to that end, and 0.2–0.5 % appears to be enough in many cases. Higher concentrations will increase the osmotic value of the perfusion medium, which may lead water movement from the external medium into the dialysate, a condition that should be avoided. To enhance recovery of lipophilic compounds, the addition of intralipid and encapsin has been suggested based on in vitro studies (Ward et al. 2003).

Another approach was the use of trapping agents such as native beta-cyclodextrin and a water-soluble beta-cyclodextrin polymer to study the temporal in vitro concentration response for the lipophilic drug carbamazepine to concentration changes outside of the probe (Ao et al. 2006). The in vitro concentration response thereby was no longer delayed.

Then, with time the microdialysis technique has been expanded from small molecules toward larger ones. In the light of having pharmacokinetic information on biological the work reviewed by Duo et al. (2006) is of interest. They reported on the inclusion of affinity-based trapping agents into the microdialysis perfusion fluid to improve the recovery via the binding reaction of low molecular weight

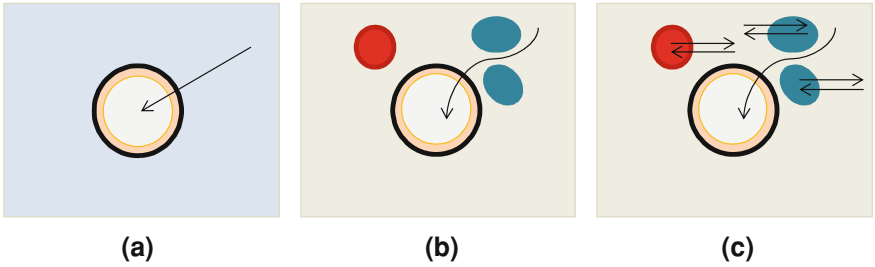

Fig. 2.4 The relation between dialysate concentrations and the true extracellular (in vivo) concentration of a drug is dependent on conditions in the tissue sampled and cannot be calculated from "calibrations" based on in vitro probe recoveries where only diffusion through the buffer and through the membrane takes place (**a**). In the in vivo situation, the recovery of the drug into the dialysate is influenced by diffusion through the tissue with tortuosity (**b**), and in virtually all of the cases also by extra-intracellular and capillary exchange (**c**)

hydrophobic analytes and larger analytes such as peptides and proteins. Antibody-immobilized bead microdialysis sampling enhancement was investigated for various endocrine hormones (amylin, GLP-1, glucagon, insulin, and leptin). The antibody-bead enhancement approach allowed for recovery enhancements that ranged between 3- and 20-fold for these peptides. Using the enhanced recovery approach, endocrine peptides at pM concentrations could be quantified. Then, non-antibody based enhancement agents using bovine serum albumin-heparin conjugates covalently bound to polystyrene microspheres were presented for the cytokine, tumor necrosis factor-alpha (TNF-alpha). Unlike antibodies, heparin provides the advantage of being reusable as an enhancement agent and served to improve the relative recovery of TNF-alpha by threefold.

2.2.3 In Vivo Recovery

It has been common practice in microdialysis studies for probes to be "calibrated" in bulk solution and to use these in vitro recoveries values to estimate in vivo extracellular concentrations. However, the relation between dialysate concentrations and the true extracellular concentration of a drug is dependent on conditions in the tissue sampled and cannot be calculated from in vitro probe recoveries (Fig. 2.4). In vivo, effective diffusion of the drug through the extracellular fluid of a tissue, uptake into cells, metabolic conversion rate, active transport across membranes, extent of tissue vascularization and blood flow all may play a role. Scheyer et al. (1994) investigated recovery of carbamazepine and carbamazepine epoxide, and found differences between the in vitro and human in vivo recovery, due to tissue factors. Another example is provided by the study of Ross et al. (2006) on factors that determine the in vivo recovery of salicylate and norepinephrine.

2.2.4 Mathematical Framework

The mathematical framework for in vivo concentration recovery from tissue, developed by Bungay et al. (1990), provided lots of insight into the contribution of different transport processes to the in vivo recovery. The mathematical model was developed given steady-state concentrations of the compound of interest. They approached the recovery process by division into three components, by means of diffusion in (1) probe lumen, (2) dialysis membrane, and (3) the periprobe environment Diffusion in probe lumen is limiting only with the use of very low flow rates. Diffusion through the dialysis membrane is limiting only when transport through the periprobe environment is rapid.

For the mathematical framework, it was assumed that the probe is inserted in tissue with intimate contact between tissue and outer surface of the probe, for which diffusion through tissue for hydrophilic drugs takes place through the extracellular fluid, and for which transport processes are linear in the studied concentration range. Other assumptions may lead to other expressions, which according to the authors are nevertheless likely to fit within the general mass transport laws. For in vivo concentration recovery the following equation can be used, viewing the transport of a molecule from the tissue to the dialysate being determined by a series of mass transfer resistances:

$$E_d = (C_{\text{out}} - C_{\text{in}})/(\text{brain}_{\text{ECF}} - C_{\text{in}}) = 1 - \exp\{-1/[F\,(R_d + R_m + R_e)]\}.$$

Here E_d = in vivo recovery (or dialysate extraction fraction); C_{out} = dialysate concentration; C_{in} = perfusate concentration; F = flow rate of the perfusate; R = mass transfer resistance with subscripts d = dialysate, m = membrane and e = external medium. For a probe in tissue it generally holds that $R_e \gg R_m \gg R_d$, leaving only the R_e to be further considered:

$$R_e = \{(K_0/K_1)/(2\,r_{\text{o}}\,L D_e\,\varphi_{\text{e}})\}\,\Gamma$$

Here K_0 and K_1 are modified Bessel functions, r_0 is the radius of the probe, L is the length of the semipermeable part of the membrane, D_{e} is the diffusion coefficient for the extracellular phase, φ_{e} is the accessible volume fraction of the extracellular phase, and Γ is the profile depth parameter. The profile depth parameter is defined as:

$$\Gamma = \sqrt{\{D_e/\left(k_{\text{ep}} + k_{\text{em}} + k_{e\rightarrow im}\right)\}}$$

in which k_{ep}, k_{em}, and $k_{\text{e}\rightarrow\text{im}}$ represent first-order rate constants for efflux to the microvasculature, irreversible extracellular metabolism, and the composite of irreversible intracellular metabolism and extracellular–intracellular exchange respectively. Thus, it can be understood that under steady-state conditions all processes that contribute to elimination of a drug will affect in vivo concentration recovery. In contrast, influx of the drug does not affect the in vivo concentration recovery, but plays a role in determining the actual concentrations of the drug in the extracellular fluid.

As a steady-state concentration of endogenous compounds and drugs in vivo is more exception than a rule, Morrison et al. (1991) developed time-dependent equations for the dialysate concentration and for concentration profiles about the probe. The model predictions were compared with in vivo observations. It was found that microvasculature transport and metabolism play major roles in determining microdialysate transient responses. The higher rates of metabolism and/or capillary transport the shorter was the time required to approach steady-state recovery. This indicates that for substances characterized by low permeabilities and negligible metabolism, experimental situations exist that are predicted to have very slow approaches to microdialysis steady state.

2.3 In Vivo Methods to Obtain Quantitative Microdialysis Data

As in vitro recovery will not be equal to in vivo recovery, special quantification methods are needed to determine the actual relation between dialysate concentrations and those in the external medium which in vivo is the extracellular fluid.

2.3.1 Zero and Ultraslow Flow Rate Method

The lower the flow rate, the more the dialysate concentrations will approach the external medium concentration (Jacobson et al. 1985). In vivo, if steady-state concentrations are present in the tissue, flow rates can be sequential changed from high to low. The resulting dialysate concentrations can be related to the flow rate, and by regression will provide the true extracellular concentrations at zero flow rate. The ultraslow microdialysis was proposed by Kaptein et al. (1998), for endogenous glucose and lactate. It is however, very difficult to properly handle very low volumes of dialysate, while also the analytical methodology may be too insensitive to measure the very low amounts of drugs in the microdialysis samples.

2.3.2 No-Net-Flux or Difference Method

The principle of the "no-net-flux" (NNF) or "difference"method was developed by Lonnroth et al. (1987) for endogenous glucose, but was not yet named as such at that time, but later by Menacherry and Hubert (1992) in their study on cocaine. The method involves consecutive perfusion of the microdialysis probe with different concentrations, if steady-state conditions of the drug are present in the tissue. The resulting dialysate concentrations are measured and the difference

between the perfusion medium concentration and the dialysate concentration is calculated and plotted as a function of the perfusion medium concentration. Regression analysis of the results yields the perfusate concentration, which is in equilibrium with the surrounding tissue.

With this method, no assumptions on periprobe behavior of the drug have to be made due to the fact that at NNF conditions no mass-transfer of the drug from further positions to the probe is taking place. Stahle (1991) used this approach to compare in vitro and in vivo recoveries of theophylline and caffeine, two highly identical compounds. It was found that the recovery in vitro was virtually identical for caffeine and theophylline. The in vivo recovery of theophylline, however, was significantly smaller than the recovery of caffeine in brain, liver, muscle, and adipose tissue. The difference in recovery was significantly larger in the brain than in other tissues.

2.3.3 Extended or Dynamic No-Net-Flux Method

Quantitative determination of extracellular drug concentration under transient conditions can be obtained using the extended version of the NNF approach proposed by Olson and justice (1993). The method provides the extracellular concentration and the in vivo probe recovery as a function of time. The technique is based on the NNF method for steady-state conditions, but differs in the use of a between-group rather than a within-group design. Instead of serial perfusion of individual animals with different concentrations via the probe, a group of animals are continuously perfused with one selected perfusion concentration. Different groups receive 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 extracellular concentration with the associated in vivo concentration recovery value at that time. An important observation of Olson and Justice (1993), using this approach, was that the administration of a neuroactive drug may alter the in vivo recovery of neurotransmitters. Although this is a powerful experimental setup, more experimental animals are needed, which in part reduces the advantage of minimizing the use of living experimental animals by the microdialysis technique.

2.3.4 Internal Standard Technique

Another approach to determine in vivo recovery for every dialysate sample during the experiment is the use of an internal standard, which is added to the perfusate during the course of the experiment (Larsson 1991; Yokel et al. 1992; Scheller and Kolb 1991).

The internal standard should match the characteristics of the drug as close as possible, so that the concentration loss of the internal standard will predict the concentration recovery of the drug. In vitro, the recoveries of both drugs can be measured, and the ratio between the values for internal standard and drug can be determined. With the assumption that the obtained ratio in vitro would remain the same in vivo, it can be used to calculate in vivo recovery of the drug as a function of time. Recovery of the drug of interest (R_{drug}) is defined as:

$$\text{In vitro } R_{drug} = \left[C_{in,drug} - C_{dial,drug}\right] / C_{in,drug}$$

The recovery of the internal standard is defined as:

$$\text{In vitro } R_{IS} = \left[C_{in,IS} - C_{dial,IS}\right] / C_{in,IS}$$

Then, loss of the internal standard (L_{IS}) is defined as:

$$\text{In vivo } L_{IS} = \left[C_{inIS} - C_{dial,IS}\right] / C_{in,IS}$$

Assuming that the ratio of recovery of the drug and internal standard in vitro will be the same in vivo, and that in vivo loss and recovery of the internal standard is equal to the in vivo gain of the internal standard, the concentration of the drug in the extracellular fluid ($C_{ECF,drug}$) can be calculated by:

$$C_{ECF,drug=}C_{dial,drug} \times 1/[\text{In vivo } L_{IS}] \times [\text{In vitro } R_{drug}/\text{In vitro } R_{IS}]$$

This method would be suited to determine changes in recovery if brought about by factors that decrease probe efficiency, such as the formation of air bubbles on the inside of the semipermeable membrane or occlusion of membrane pores by cells or sticky drugs. However, in vivo, effective diffusion of the internal standard and drug is assumed to be equal. This may not be realistic as illustrated by Stahle et al. (1991) for theophylline and caffeine, for which a difference in in vivo recovery was found using the NNF method. Moreover, the difference found was dependent on the tissue, with the highest deviation in brain. Also interaction of the internal standard with the drug should be ruled out. This stresses prudence to be exercised in the use of internal standards. Other investigators also used and validated this method against the no-net-flux method.

2.3.5 Retrodialysis or Reverse Dialysis of the Drug

In reverse dialysis or retrodialysis, the drug itself may be added to the perfusate and its in vivo loss may be used as a measure for in vivo recovery (Wang et al. 1991, 1993). It is based on the calculation of in vivo recovery of a drug on the basis of its loss from the perfusate during so-called retrodialysis, assuming that in vivo recovery equals in vivo loss, and was used to estimate the concentration of drug in the extracellular fluid (Fig. 2.5).

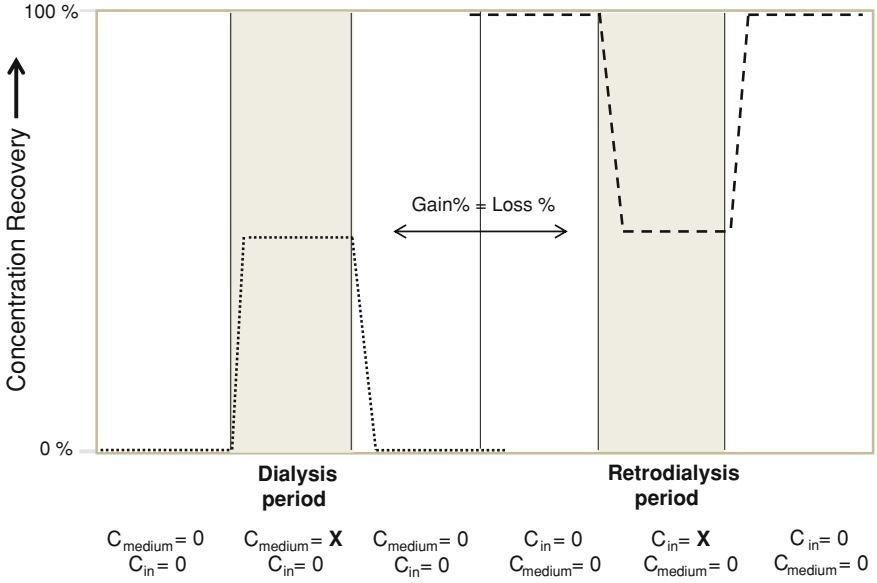

Fig. 2.5 Microdialysis probes should first be investigated in vitro by measuring the loss of drug using drug-containing perfusate or the gain of drug using drug-containing sample solutions. This will reveal drug recovery as being fully governed by simple diffusion and probe performance (membrane characteristics, potential interaction of the drug with the membrane of the probe, and/or tubing). When significant interaction of the drug and microdialysis probe and/or tubing is observed, one has to search for other probe and/or tubing materials without significant interaction(s). Furthermore, as the recovery determined in vitro may differ from the recovery in humans. Its actual value therefore needs to be determined in every in vivo experiment

This measurement has to be conducted before administration of the drug to the body. It is a relatively easy and useful approach, and is therefore most often used, but should be validated, as has been shown by a tissue specific asymptotic profile in recovery that results under increasing concentration gradient (LeQuellec 1995).

2.3.6 Combined Retrodialysis by Drug and Calibrator

A stronger approach, a combination of internal standard (calibrator) and retrodialysis, has been developed by Bouw and Hammarlund-Udenaes (1998). In vivo recovery of morphine was determined by morphine itself, retrodialysis by drug, and by the calibrator nalorphine, retrodialysis by calibrator. The calibrator is valuable as a quality control during the whole experiment. For morphine, this method has been compared with the DNNF method in a study on blood–brain barrier transport of morphine as discussed later in this chapter.

2.3.7 Endogenous Reference Technique

Glucose was the first endogenous compound used as an endogenous reference or internal recovery marker (Hashimoto et al. 1998). It was assumed that the concentration of glucose in the extracellular fluid of skin tissues is the same as that in plasma and that the in vivo recovery ratio of glucose to heir drug tranilast by microdialysis is the same as that estimated in vitro. Stringberg and Lonnroth (2000) validated the use of urea as an internal reference for the calibration of microdialysis catheters, for both subcutaneous and muscle measurements. The equilibration calibration of urea, glucose, and lactate, and the internal reference (3H-glycerol retrodialysis) calibration of glycerol were performed in both the muscle and fat, and compared with those taken from the arterial plasma. The interstitial fluid concentrations of glucose, lactate, and glycerol can were found to be calculated from the knowledge of plasma and microdialysate content of urea together with the recovery in vitro. To reduce burden of microdialysis procedures in critically ill patient, Schwalbe et al. (2006) evaluated and validated the use of urea as an endogenous reference compound to determine relative in vivo recovery of anti-infectives, with linezolid in this study being used as model drug. Concentration- and flow rate-dependence were evaluated in vitro to determine the recovery of urea. The in vivo recovery of urea was correlated with in vivo recovery of linezolid obtained by the traditional retrodialysis method, with reasonable correlation.

2.3.8 Some Method Comparisons

Le Quellec et al. (1995), investigated the NNF and the reverse dialysis methods. First, the accuracy of the No-Net-Flux method to estimate in vivo recovery was compared in two situations: diffusion from the probe into the dialysis medium and diffusion from the outer medium into the probe.

The point of no net transport was used to estimate the concentration surrounding the probe. Neither differences between extracellular concentrations (intercept values) nor differences between recoveries were observed. Then, the reverse dialysis method was tested to estimate the relative loss of drug from the perfusate when the probe was placed in a drug-free medium. Finally, comparisons of the behavior of the drug diffusion across the membrane under increasing gradient conditions have shown an asymptotic profile, specific of the tissue; blood, muscle, and adipose tissue. It was found that (in line with predictions from the mathematical model of Bungay) that the faster a drug was removed by microvascular transport (blood > muscle > adipocytes), the higher was the recovery, until the perfusate concentration reached a threshold value where the transport process became gradient limited and no more tissue limited.

The urea reference technique was compared with the NNF and the retrodialysis technique for glucose and paracetamol as model compounds healthy volunteers (Brunner et al. 2000). For glucose, recovery values, calculated by the urea reference technique differed significantly from those values, which were assessed by the no-net-flux technique whereas for paracetamol recovery values did not differ significantly, albeit high intramethod variability was observed. Thus, the authors could not confirm the hypothesis that recovery values calculated by the urea reference technique provide equivalent results compared with standard calibration techniques.

For microdialysis studies in the skin, laboratory-made linear probes are often used, which requires careful assessment of the linear probes' performance to ensure validity of the data obtained using this technique. Zheng et al. (2007). For sinomenine, in vitro and vivo recovery was determined using different lengths of the dialysis membrane and different perfusion flow rates. In vitro recovery of sinomenine from the microdialysis probe was independent of concentration, stable over an 8-h period. Comparable in vitro recoveries were obtained by different established approaches including recovery estimation by gain, loss, and the NNF method. Recovery by loss was used to study the in vivo recovery of sinomenine from rat subcutaneous tissue. The performance of the microdialysis system was stable over an 8-h study, resulting identical in vivo recovery values for the retrodialysis and the NNF method.

2.3.9 Modified Ultraslow Microdialysis

The microdialysis technique is to date still of enormous value in obtaining information on unbound (therewith highly relevant) drug concentrations in the tissue of choice. However, obtaining quantitative data by calibration of the microdialysis probe recovery by the earlier described calibration methods includes assumptions, is time-consuming and is not without limitations. To improve robustness and practicality of quantitative microdialysis sampling, Cremers et al. (2009) modified the ultraslow microdialysis approach. Ultraslow microdialysis uses very low microdialysis flow rates (<200 nl/min) 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 which negates the problems of long lag times and low volumes. The resulting dilution of the dialysis sample concentration can simply be mathematically corrected. To show the potential of this approach, Cremers et al. (2009) measured the free brain levels of two CNS compounds using the classic DNNF and the new modified ultraslow dialysis method. Modified ultraslow microdialysis was shown to generate robust data with the use of only small numbers of rats. Currently lots of experience is built on quantitatively measuring blood–brain barrier

penetration of compounds into the brain. Although researchers needs to become skilled in using technique, it is very promising as it circumvents the assumptions and limitations of calibration methods, and may be used for a much more straightforward screening of drugs.

2.4 Considerations in Experimental Conditions

Appropriate experimental conditions are a prerequisite for obtaining valuable quantitative data (De Lange et al. 1997). The relationship between dialysate concentrations and those in the extracellular fluid of the periprobe tissue, the recovery of the drug, depends on periprobe processes governing the actual concentration of the drug at that site. These include pressure gradients, extracellular–microvascular exchange, metabolism, and tissue diffusion of the drug. Experimental conditions, also between a control and treated group of subjects, or even disease conditions may influence these processes and may therefore have an impact on the actual in vivo recovery.

2.4.1 Static and Pulsatile Pressure

As already described for in vitro recovery, it has been considered that a higher flow rate will increase the fluid pressure inside the probe, which *may* result in net transport of fluid across the dialysis membrane and thereby counteract the diffusion of the drug into the dialysis fluid). In a study of Siaghy et al. (1999), this is exemplified by a microdialysis study in the heart, in which it was shown that mass transfer across a microdialysis membrane is dependent on the direction of the transmembrane pressure gradient and increases with heart rate, and must be taken into account in calculation of extracellular fluid concentrations.

2.4.2 Anesthesia

Pan et al. (1995) evaluated the effects of chloral hydrate and pentobarbital sodium on the basal extracellular concentrations of dopamine (DA) and norepinephrine (NA) as well as their in vivo extraction fraction (relative recovery) by the NNF method with conscious rats as controls. There were no significant effects of anesthesia on the basal extracellular concentrations of DA and NA. In vivo recovery of DA was not influenced by anesthesia, while for NA a higher recovery was observed for the conscious group.

2.4.3 Blood Flow

Kurosawa et al. (1991) found that changes in cerebral blood flow do not directly affect in vivo recovery of extracellular lactate from the rat brain through microdialysis probe. Relative radioactivity loss of (14C)-lactate from perfusate medium through a microdialysis probe was continuously measured in vivo as an indicator of relative recovery of extracellular lactate through the probe because both the relative recovery of lactate and the relative hot loss of (14C)-lactate through a microdialysis probe were similar to each other in vitro. The relative hot loss of (14C)-lactate decreased in parallel with decreases in the striatal blood flow, while it did not significantly change in response to increases in the blood flow up to 200 % of control. These results demonstrate that recovery of extracellular lactate in vivo through the microdialysis probe was not directly influenced by changes in the cerebral blood flow.

Clough et al. (2002) investigated the impact of changes in local blood flow on the recovery of a small, diffusible molecule (sodium fluorescein) from the extravascular tissue space of the human skin, by microdialysis in vivo, under conditions of basal, reduced (noradrenaline), and increased (glyceryl trinitrate) blood flow. Whereas loss of tracer from the delivery probe appeared unaffected by changes in local blood flow, retrieval of fluorescein by the second probe was directly related to blood flux, measured using scanning laser Doppler imaging. They concluded that clearance of solute by the blood will have a significant impact on microdialysis probe recovery and that, in the skin, the magnitude of this clearance is directly related to blood flow.

2.4.4 Transporter Functionality and Metabolism

Intracerebral microdialysis probe recovery (extraction fraction) may be influenced by several mass transport processes, including efflux and uptake exchange between brain and blood. The mdr1a-encoded P-glycoprotein is an active pump being expressed among other tissue linings, on the luminal face of the cells constituting the blood–brain barrier. Thereby, P-glycoprotein will counteract brain penetration of its widely diverse substrates as implicated by large differences in brain distribution of a number of P-glycoprotein substrates observed between mdr1a (−/−) and wild-type mice. Intracerebral microdialysis has been applied in mdr1a (−/−) and wild-type mice and the importance of quantification of microdialysate data will be exemplified beneath for two drugs for which in vivo recovery values were influenced by differences in active elimination of these drugs by absence versus presence of mdr1a-encoded P-glycoprotein. Using the NNF and DNNF techniques this was shown for Rhodamine-123 (De Lange et al. 1998), morphine (Xie et al. 1999) and sparfloxacin (De Lange et al. 2000). In all cases, the in vivo recovery values were lower for the mdr1a (−/−) mice. This is in line with the theoretical

mathematical framework on in vivo concentration recovery developed by Bungay et al. (1990), which indicates lower in vivo recovery values in case of absence of an eliminating process (absence of Pgp) compared with presence of this elimination process.

Sun et al. (2001) investigated further the effect of capillary efflux transport inhibition on the determination of probe recovery during in vivo microdialysis in the brain. The effect of inhibiting transport on probe recovery was investigated for two capillary efflux transporters, Pgp and an organic anion transporter, with potentially different membrane localization and transport mechanisms. Fluorescein/probenecid and quinidine/LY-335979 were chosen as the substrate/inhibitor combinations for organic anion transport and P-glycoprotein-medicated transport, respectively. Probenecid decreased the probe recovery of fluorescein in the frontal cortex, in line with a reduction of the total brain elimination rate constant of fluorescein. In contrast, the microdialysis recovery of quinidine, delivered locally to the brain via the probe perfusate, was not sensitive to P-glycoprotein inhibition by systemically administered LY-335979, a potent and specific inhibitor of P-glycoprotein. Recovery of difluorofluorescein, an analog of fluorescein, was also decreased by probenecid in the frontal cortex but not in the ventricle cerebrospinal fluid. These studies suggest that only in certain circumstances will efflux inhibition at the blood–brain barrier and blood–cerebrospinal fluid barrier influence the microdialysis probe recovery, and this may depend upon the substrate and inhibitor examined and their routes of administration, the localization and mechanism of the membrane transporter, as well as the microenvironment surrounding the probe.

Stenken et al. (1997) investigated the impact of metabolism on microdialysis recovery of phenacetin and antipyrine in the liver, by local infusion of these drugs with/without intravenous administration of an inhibitor of metabolism. In the rat, the results indicated that microdialysis recoveries measured after inhibition of a concentration-dependent kinetic process via pharmacological challenge will change only when the kinetic process that is being challenged is large compared to the contribution of all concentration-dependent kinetic processes, including other metabolism routes, capillary exchange, or uptake that remove the analyte from the tissue space. It is concluded that the microdialysis recovery of a substance from the liver is not generally affected by liver metabolism.

2.4.5 Composition and Temperature of the Perfusate

Perfusion media used in microdialysis experiments vary widely in composition and pH. Ideally the composition, ion strength, osmotic value, and pH of the perfusion solution should be as close as possible to those of the extracellular fluid of the dialyzed tissue. Extracellular fluids mostly contain only very small concentrations of proteins. But, in some cases, proteins have been added to the perfusion medium to prevent sticking of the drug to the microdialysis probe and

tubing connections Also, the composition of the perfusate can be changed with the intention to study the effects on the system under investigation. Deviations in ion composition have been shown to affect brain dialysate levels of neurotransmitters and drugs. De lange et al. (1994) have shown that a non-physiological (hypotonic) perfusion medium used in daily repeated experiments to measure blood–brain barrier permeability resulted in a substantial increase of the dialysate levels of the hydrophilic drug atenolol with days, presumably reflecting increased blood–brain barrier permeability.

Most investigators use a perfusion medium at room temperature before entering the probe. As a result, a temperature gradient exists between the probe and its environment. This may have an effect on tissue processes and consequently on the results. De Lange et al. (1995) used a subcutaneous cannula by which the perfusion fluid could equilibrate to body temperature before entering the cortical brain probe. The results on area under the concentration–time curve values for acetaminophen following intravenous administration obtained with "prewarmed" isotonic and hypotonic perfusate were compared to those obtained with perfusate at room temperature. A temperature effect was observed only for the use of the hypotonic perfusate, with a twofold higher dialysate area under the concentration–time curve value obtained with the room temperature perfusion medium). It was hypothesized that the periprobe tissue, already "stressed" by the hypotonic condition, loses its capability to compensate temperature effects. This indicates that perfusate temperature may be especially important in pathological circumstances. However, it is recommended to perform all microdialysis experiments with perfusion fluids at body temperature.

2.4.6 Interactions of Probe Material with Drug or Tissue

Jensen et al. (2007) investigated the application of microdialysis for sampling of free fatty acids (FFA). In vitro and in vivo studies were performed. The recovery of oleic acid was found to be dependent on the concentration of analyte in the medium surrounding the membrane, but this addition of BSA to the perfusion fluid resulted, however, in a concentration-independent recovery. This indicated an interaction of the FFA with the microdialysis membrane and or tubing. However, in vitro, it was observed that not for glycerol but for FFA's the recovery of FFA's declined indicating clogging of the membrane pores by these compounds.

Other examples of changes in transport of compounds over microdialysis membrane exist, and may also point to tissue reactions at the implantation site (Wisniewski et al. 2001), upon long-term use (Mou et al. 2010; Georgieva et al. 1993). Changes in periprobe tissues (Georgieva et al. 1993) while also tissue compatibility has been reported (De Lange et al. 1995, 1997).

2.5 Considerations in Data Analysis

Finally, as concentration–time profiles of drugs can be obtained by the microdialysis method, pharmacokinetic parameters can be derived. To that end, it should be realized that microdialysate data represent "average" concentrations obtained within the sampling interval, typically during a period between 5 and 20 min. So, this should be considered in the description of the concentration–time profiles (Ståhle 1992). For pharmacokinetic data, in most cases, the use of the interval midpoint to relate the dialysate concentrations to is valid. However, adjustments are required when the half-life of a process, such as absorption or elimination, is short in comparison with the sampling interval. Calculations of half-life and slopes are similar to standard methods for equal sample intervals. Calculation of area-under-the-curve and clearance values may be even more accurate for microdialysis data than for normal sampling at discrete time-points. This is because of the time-integral character of the microdialysis technique as well as the typical more frequent data points. Ståhle (1992) used a general method to deal with multicompartment models. Then, adjusted time points are not equidistant although sample intervals are equal. This is because the slope will be different in different portions of the natural logarithmic of the concentrations depicted versus time. Then, the time point T, measured from the initial time-point t_i of the sampling interval at which the extracellular concentration coincides with the dialysate concentration should be found. With the assumption that the data can be locally approximated by a mono-exponential function the following relation can be derived:

$$T/\underline{^{a}}t = [\ln(q) - \ln(1 - e^{-q})]/q$$

Here $q = K\underline{^{a}}t$, with k being the slope of the log(conc) versus time profile. When $\underline{^{a}}t \ll t2$, $T/\underline{^{a}}t$ will be about 0.5, the interval midpoint. For longer sampling intervals, $T/\underline{^{a}}t$ will shift to values lower than 0.5. Thus, a drug with a first phase elimination half-life of 10 min, a second phase elimination half-life of 30 min and a sampling interval of 10 min the values of the sampling midpoint $T/\Delta t$ will be 0.42 and 0.47 in the first and second elimination phase respectively

2.6 Conclusions

Microdialysis is a very powerful technique to obtain quantitative data on concentration–time profiles of unbound drugs in virtually any tissue in animals and human. One issue is the need for calibration when using microdialysis probe perfusion flow rates by which equal concentrations between periprobe extracellular fluids will not be reached. In such conditions, many calibration techniques and approaches have been proposed, with different levels of practicality, usefulness, and assumptions, to obtain quantitative information on extracellular tissue

concentrations of the drug as a function of time. The recently developed modified ultraslow microdialysis approach seems to set aside many limitations of the calibration techniques as concentrations obtained in the dialysate only have to be corrected for dilution by the carrier flow.

Thus, today we have build up enough experience to know how to obtain quantitative data using the microdialysis technique. With that, it is the only technique that provides data on the unbound concentration of compounds in extracellular fluid spaces in the body, being highly important information in drug development as the unbound concentrations in target tissues are the drivers for the effect (De Lange et al. 2005).

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