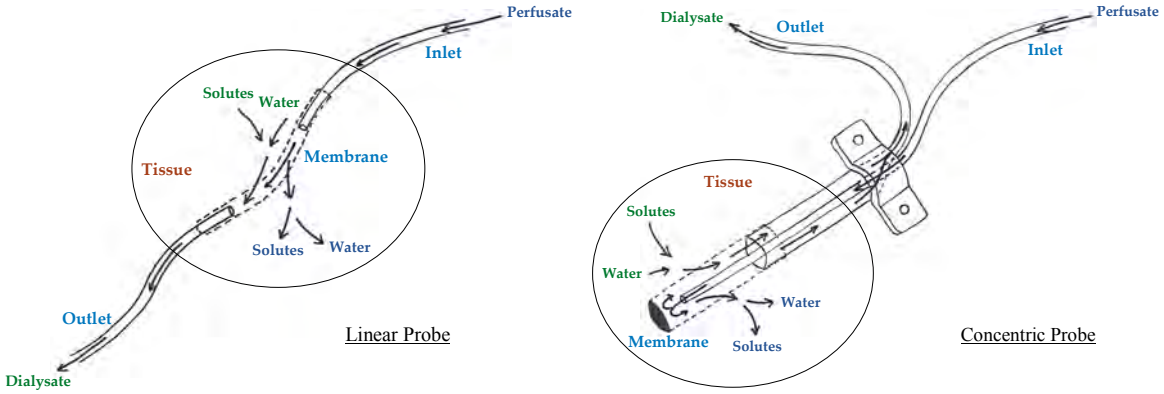
1 Literature review

1.1 Microdialysis – general aspects

Microdialysis is a method which allows tissue concentrations of endogenous and exogenous compounds to be determined at frequent intervals, without removing any tissue samples. For this purpose, a tubular, semipermeable dialysis membrane is introduced into the tissue of interest. The tubular membrane is connected to an inlet and an outlet tubing for perfusion. The only contact between the perfusion fluid and the tissue is the semipermeable membrane, through which an exchange of molecules can occur (Figure 1).

Figure 1: Fluid and solute movement in linear and concentric microdialysis probes

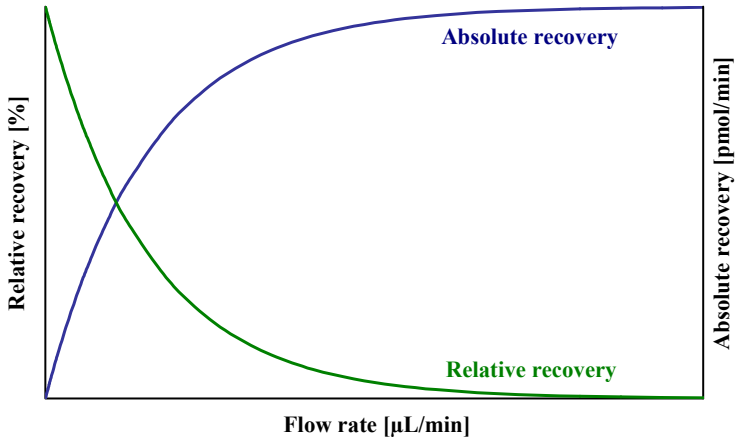


Molecules in the perfusate and in the surrounding tissue will diffuse down their concentration gradient, either into the perfusate (recovery) or into the tissue (delivery). If a physiological buffer is used as the perfusate (for example Ringer's solution), generally only recovery is observed. The size of the molecules that can diffuse through the membrane is limited by the pore size, as defined by the 'cut-off' of the membrane (CMA, 2005). Since this cut-off is determined at a static equilibrium, rather than at a dynamic equilibrium seen in a microdialysis probe which is constantly perfused, the true cut-off is considerably lower (Eliasson, 1991). As a rule of thumb, molecules not bigger than $1/10^{th}$ the size of the indicated cut-off can be sufficiently recovered by microdialysis. The two compounds used for this thesis (MW < 400 Da) have therefore no size restriction to recovery by any of the membranes tested (cut-off ≥ 6kDa).

1.1.1 Perfusion rate and recovery

In a dynamic microdialysis system, the recovery of a compound depends strongly on the perfusion rate used (Eliasson, 1991). A distinction is made between the absolute recovery (the total amount of compound recovered per unit time) and the relative recovery (the concentration of the dialysate in relation to the concentration in the surrounding medium). As the flow rate approaches zero, at near static conditions, the relative recovery reaches its maximum, and the absolute recovery becomes zero (no dialysate is obtained). As the flow rate approaches infinity, the relative recovery becomes zero, and the absolute recovery reaches a maximum (see Figure 2). Commonly, a flow rate between 1 and 2 µL/min is chosen (2 µL/min is adopted for this thesis), allowing for reasonable sample volumes to be collected (10-20 µL every 10 minutes), with sufficiently high concentrations for further analysis.

Figure 2: The relationship between relative and absolute recovery as a function of flow rate.



Generally, the relative, rather than the absolute recovery for a compound is determined, under a specific set of experimental conditions. Apart from the flow rate used, other conditions also influence the recovery *in vitro*: the temperature of the medium, stirring conditions, the type of microdialysis probe and tubing material and the type of medium and perfusate used. These factors are discussed briefly below. For a good overview of quantitative microdialysis *in vivo*, see Cano-Cebrián, Zornoza et al., 2005.

1.1.2 Temperature and recovery

The temperature of the medium influences the recovery, since molecules move faster in warmer solutions. Therefore the diffusion of the solutes and their recovery increases with increasing temperatures (Lindefors, Amberg et al., 1989). Usually, *in vitro* experiments are either carried out at ambient (room) temperature, or at human body temperature at 37°C. To simulate *in vivo* conditions as much as possible *in vitro*, a temperature of 37°C is adopted for this thesis.

1.1.3 Stirring and recovery

Stirring the medium maintains the supply of solutes at the dialysis membrane. In an unstirred medium, a depletion zone is formed in the unstirred aqueous layer around the membrane, resulting in a lower recovery, and in a time delay until a steady-state recovery is obtained (Stenken, 1990). For the purpose of this thesis, the medium is stirred at maximum setting (1500 rpm), to eliminated any depletion effects.

1.1.4 Materials and recovery

Finally, the materials used (tubing, probe and perfusate) are major factors influencing the observed recovery. Here, general aspects are discussed, more detailed studies on material/compound interactions are discussed below (Chapter 1.2).

1.1.4.1 Tubing

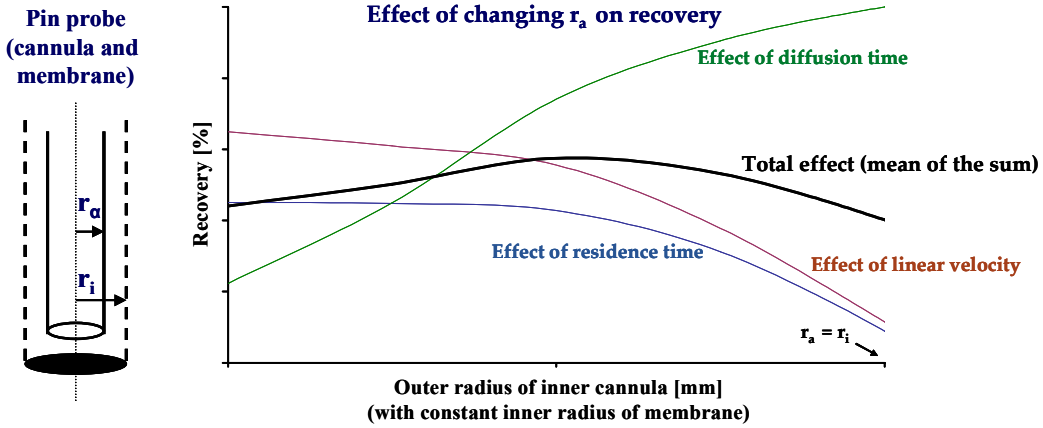
The tubing used *in vivo* is often very long (around 1 m), and may need to be chosen carefully in the preceding *in vitro* experiments. A tubing material should be used to which the compound of interest does not adhere. In addition, the inner diameter should be small enough to minimize the dead volume, but big enough to minimize back pressure, thereby preventing ultrafiltration of the perfusate out of the dialysis membrane (Wisniewski, 2001).

1.1.4.2 Microdialysis probe

The choice of microdialysis probe and especially the membrane is critical in any microdialysis experiment (for probe properties assessed in this thesis, see Table 6, page 14). As discussed above, the pore size of the membrane must be big enough for the compound of interest. For most small molecules of < 600 Da, even the smallest cut-off membranes of 6 kDa suffice. In addition, the membrane length is important; the larger the membrane surface area exposed to the surrounding medium, the greater the recovery will be. However, the membrane diameter and length must be suited for the intended *in vivo* use. The material of the membrane used can also affect the recovery, especially for lipophilic compounds, which may adhere to the membrane material (see chapter 1.2.2 below).

For concentric pin probes (Figure 1), a rarely considered property affecting the recovery is the ratio of the outer radius of the inner cannula (r_α) to the inner radius of the membrane (r_i) (Wisniewski and Torto, 2002, Figure 3). As the fluid layer between the membrane and the cannula decreases, less diffusion through the perfusate needs to take place, therefore increasing the recovery. Opposing this effect, reducing the fluid layer increases the effective flow rate past the membrane, therefore reducing the residence time of the perfusate at the membrane and reducing the recovery (Figure 3). Providing the membrane itself poses minimal resistance to diffusion, optimizing the ratio of r_α to r_i could improve the observed recovery.

Figure 3: Possible effect of changing the outer radius of the inner cannula of a pin probe on recovery (adapted from Torto and Wisniewski, 2002)



r_α = outer radius of inner cannula
r_i = inner radius of membrane

1.1.4.3 Perfusate

The perfusate used *in vitro* should be chosen according to the intended *in vivo* use. Ideally the perfusate is isotonic and isosmotic to the extracellular fluid of the tissue to be dialyzed. Commonly used are Ringer's solution, or an artificial cerebrospinal electrolyte solution. Sometimes glucose is added to prevent energy depletion of the tissue (McNay and Sherwin, 2004), which may however not be necessary (Ronne-Engström, Carlson et al., 1995). The perfusion fluid should be filtered before use to remove any small particles that could block the tubing or microdialysis probe. In addition, the perfusate should be degassed to prevent the formation of bubbles at the membrane, and should be sterile if metabolism of the compound of interest is to be

prevented. For *in vitro* experiments, the medium surrounding the probe is usually the same as the perfusate, and since Ringer's solution is most commonly used in the literature, this solution is also used for the experiments in this thesis.

1.2 Microdialysis of lipophilic compounds

Microdialysis of lipophilic compounds is considered highly problematic for pharmacokinetic purposes, mainly due to the low recoveries observed (sometimes < 2%, as found by Carneheim and Ståhle, 1991). This low recovery is thought to be due to compound adhesion to the materials used (Lindefors, Amberg et al., 1989). Considering the large surfaces within the membrane pores and along the tubing, any affinity of the compound to these materials will affect the recovery, and will result in a delay until equilibrium is reached. The low recoveries stated in the literature would however suggest that not only adhesion to the microdialysis membrane is an issue (adhesion is a saturable process, which would not result in a low recovery as such), but that in addition the actual membrane permeability is low. A possible explanation for this would be that poorly soluble compounds might aggregate within the pores, impairing further passage. Alternatively, the observed recoveries may have been determined while saturation of the available binding sites was still ongoing, and the higher, 'true' recovery at steady-state was not yet reached. It is interesting to note that in many articles citing a low recovery with lipophilic compounds, no experimental equilibration time is given, nor an indication of the times the samples were taken (for example Groth, 1996, or Müller, Schmid et al., 1995). Indeed, in one article which does show the recoveries to be determined at steady-state, recoveries for all compounds tested were reasonable (all $\geq$ 11%), even for the more lipophilic ones with a $LogP_{OW}$ of up to 2.14 (Zhao, Liang et al., 1995).

Overall, the time taken to reach steady-state conditions in *in vitro* microdialysis experiments is generally poorly documented. Yet for pharmacokinetic studies, where concentration changes are monitored, the response time of any sampling method must be shown to be at least faster than the speed at which the concentrations to be measured change (as indicated by the calculated half-life). Since the half-life of a compound in the animal and organ of interest is generally not known before the study, the sampling method (in this case microdialysis) should ideally respond immediately to concentration changes.

Also rarely discussed in the literature is the response of the microdialysis set up to decreasing (rather than increasing) concentrations, and therefore the validity of the method to determine pharmacokinetic parameters deduced from the elimination phase of a drug. Any 'bleeding' of compound from the materials used would result in erroneously long elimination half-lives being obtained by this method *in vivo*, and erroneously large areas under the tissue concentration-time curve.

Nevertheless, attempts have been made to improve the accessibility of microdialysis to lipophilic compounds. Different materials have been tested *in vitro* with a range of compounds, using the observed recoveries for comparison, and linking the findings to selected material properties. An overview of these studies is given below.

1.2.1 Compound adsorption to tubing materials

The standard tubing materials available for microdialysis are FEP (fluorinated ethylene propylene), FEP/Teflon[1] and PEEK (polyetheretherketone). In addition, fused silica (glass) has occasionally been used. With regards to physicochemical or adhesion properties of these materials very little is noted in the microdialysis literature. However, similar materials are also used for other pharmaceutical purposes, and a few studies with regards to adhesion of compounds to these materials (as well as to the catheter material silicone) have been carried out. A selection of data obtained in two of these studies is given in Table 1 below.

Table 1: Overview of selected findings with different tubing materials and compounds

Tubing material	Compound	$LogP_{OW}$*	Findings	Reference
Teflon® 1)	Methylparaben	1.96	No adsorption during 6 hour exposure to compound	Bahal and Romansky, 2001
	Propylparaben	3.04	≤ 2% adsorption after 6 hour exposure to compound	
Silicone	Methylparaben	1.96	> 20%adsorption after 6 hour exposure to compound, saturation not achieved	
	Propylparaben	3.04	>65% adsorption after 6 hour exposure to compound, saturation not achieved	
PEEK2), PTFE3), fused silica, nickel (all capillary tubes for gas chromatography)	Trifluoroethanol	0.41	Adsorption capacity fused silica << PEEK < PTFE << nickel (but capacity of first three very small))	Dallas and Carr, 1991
	Hexanenitrile	1.66	Adsorption capacity fused silica < PTFE << PEEK < nickel (but all >> trifluoroethanol)	
	Propylbenzene	3.69	Adsorption capacity fused silica << PTFE = PEEK < nickel (but all >> trifluoroethanol)	
	Butylbenzene	4.38	Adsorption capacity fused silica << PTFE = PEEK < nickel (but all > propylbenzene)	

1)Teflon® = fluorinated ethylene propylene (FEP)
2) PEEK = polyetheretherketone
3) PTFE = Polytetrafluoroethylene, an older Teflon material
* $LogP_{OW}$ values not given in the reference were taken from Syracuse Research Corporation, PhysProp online database

As can be seen, Teflon® has little adsorption capacity for the more hydrophilic compounds ($LogP_{OW} < 2$), but shows increasing adsorption of compound with increasing $LogP_{OW}$. The adsorption capacity of PEEK also varies, depending on the lipophilicity of the compound used. Silicone has a considerable adsorption capacity for either compound tested, and fused silica is the only material that does not adsorb any of the selected compounds. Seeing that the compounds only vary with regards to their $LogP_{OW}$ (polarity or acidity for example are not considered), the findings with the two non-polar compounds tested in this thesis can be expected to be similar.

[1] 'FEP' is possibly the same as 'FEP/Teflon', but is obtained from a different supplier (see Table 5). Since the manufacturer is not given by the supplier, these two materials will be treated separately in this thesis.

1.2.2 Influence of the membrane material on compound recovery

For microdialysis probes, the permeability of a membrane to a compound is generally determined by calculating the relative recovery. In order to compare these recoveries between probe types (with different membrane sizes, S), and between experiments (with different flow rates, Q), the use of a mass transfer coefficient K has been suggested (Sun and Stenken, 2003). Once the recovery has been determined at its steady-state *in vitro* ($REC_{in\ vitro}$), K can be calculated according to:

$$K = \frac{-Q \times \ln(1 - REC_{in\ vitro})}{S} \ (\mu L/min/mm^2)$$

This mass transfer coefficient K has been shown to generally be independent of flow rate (1, 1.5, 2 or 2.5 µL/min, Sun and Stenken, 2003), but Buttler et al. reported very different K-values at 4 compared to 1 µL/min. Indeed, the first author to introduce K (Jacobson, Sandberg et al., 1985), noted that the above equation is only valid at flow rates above 0.3 µL/min (and up to 2 µL/min, the highest flow rate tested by him). Thus, a flow rate between 0.3 and 2.5 µL/min should be maintained when K is to be calculated, and a flow rate of 2 µL/min is used for this thesis.

An overview of studies where K is calculated, or where K can be deduced from the data is given in Table 2 below.

Table 2: Overview of selected findings with different microdialysis probes and compounds

Membrane material	Compound	MW* [Da]	$LogP_{OW}$*	Experimental conditions	K [µL/min/mm^2]	Reference
PC, 2.26 mm^2	Glycerol	92	-1.76	Q = 1 µL/min, T = ?, N = ? (Compound concentration = ?)	0.11	Buttler, Nilsson et al., 1996
	2,3-Butanediol	90	-0.92		0.11	
	n-Propanol	60	0.25		0.15	
	Ethanol	46	-0.31		0.16	
PC, 3.14 mm^2	1 µM ACET	151	0.46	Ambient T, stirred, Q = 1 µL/min, N=4	0.14	Hsiao, Ball et al., 1990
	1 µM 5-HIAA	133	1.11		0.11	
PC, 4.52 mm^2	0.01 µM 5-HT	176	0.21	Ambient T, Q = 1.2 µL/min, N=4-6	0.09-0.11	Tao and Hjorth, 1992
	1 µM 5-HIAA	133	1.11		0.11	
PC, 6.28 mm^2	10 µM Leukotriene B_4	336	**3.58**	Ambient T, stirred, Q = 1.5.µL/min, N=3	**0.03**	Sun and Stenken, 2003
PC, 15.7 mm^2	4.6 µM 8-MOP	216	**1.93**	T = ?, Q = 2 µL/min, N=3	**0.05**	Mary, Muret et al., 1998
	4.6 µM 5-MOP	216	**2.00**		**0.02**	
PES, 6.28 mm^2	10 µM Leukotriene B_4	336	**3.58**	Ambient T, stirred, Q = 2.µL/min, N=3	**0.02**	Sun and Stenken, 2003
PES, 15.7 mm^2	4.6 µM 8-MOP	216	**1.93**	T = ?, Q = 2 µL/min, N=3	**0.06**	Mary, Muret et al., 1998
	4.6 µM 5-MOP	216	**2.00**		**0.03**	
PAN, 2.83 mm^2	1 µM ACET	151	0.46	Ambient T, stirred, Q = 1 µL/min, N=4	0.23	Tao and Hjorth, 1992
	1 µM 5-HIAA	133	1.11		0.17	
PAN, 4.02 mm^2	10 µM Leukotriene B_4	336	**3.58**	Ambient T, stirred, Q = 2.µL/min, N=3	**0.01**	Sun and Stenken, 2003
PAN, 5.84 mm^2	0.1 µM 5-HT	176	0.21	Ambient T, Q = 1.2 µL/min, N=4-6	0.14-0.16, steady-state only reached after 100 min.	Tao and Hjorth, 1992
Cup, 2.95 mm^2	1 µM ACET	151	0.46	Ambient T, stirred, Q = 1 µL/min, N=4	0.12	Tao and Hjorth, 1992
	1 µM 5-HIAA	133	1.11		0.07	
Cup, 3.01 mm^2	10 µM Leukotriene B_4	336	**3.58**	Ambient T, stirred, Q = 2.5.µL/min, N=3	**0.04**	Sun and Stenken, 2003
Cup, 4.18 mm^2	0.01 µM 5-HT	176	0.21	Ambient T, Q = 1.2 µL/min, N=4-6	0.10-0.12	Tao and Hjorth, 1992
	1 µM 5-HIAA	133	1.11		0.09	
Cup, 27.1 mm^2	5.2 mM Glucose	180	-3.24	T = ?, stirred, Q = 3 µL/min, N=3	0.15	Groth and Jørgensen, 1997
	600 µM Calcipotriol	412	**4.4**	T = ?, stirred, Q = 3 µL/min, N=11	**0.04**	

MW	Molecular weight	$LogP_{OW}$	Octanol/Water coefficient
T	Temperature	Q	Flow rate
PC	Polycarbonate	PES	Polyethylenesulfone
PAN	Polyacrylonitrile	Cup	Cuprophane
ACET	Acetaminophen	5-HIAA	5-Hydroxyindoleacetic acid
5-HT	5-Hydroxytryptamine	5-MOP	5-Methoxypsoralen
8-MOP	8-Methoxypsoralen	?	Not specified

* MW and/or $LogP_{OW}$ are taken from Syracuse Research Corporation, PhysProp online database, if not given in the reference

Bold $LogP_{OW}$ > 1.9 (lipophilic compounds)

As can be seen, K varies between membrane materials and compounds, and ranges from 0.07 to 0.23 μL/min/mm^2 for hydrophilic compounds with $LogP_{OW} \leq 1.11$, but is much lower at 0.02 – 0.06 μL/min/mm^2 for lipophilic compounds with $LogP_{OW} > 1.9$. However, the factor K only allows for a comparison of membrane properties at steady-state conditions. It does not give any indication regarding the time needed to reach this steady-state (for the purpose of this dissertation called 'responsiveness'), or of the reversible binding capacity of the membrane for the compound tested. Therefore, in order to determine the suitability of a membrane for the microdialysis of a particular compound for pharmacokinetic purposes, the responsiveness of a membrane to concentration changes needs to be further defined. In this dissertation, an approach is chosen which monitors the response to both increasing and decreasing concentration, with an observation period of 1 hour for each phase. With a collection interval of 6 minutes, the data allow to describe the responsiveness to concentration changes of the materials tested.

Finally, it is important to realize that the membranes used for microdialysis are the same as those used for blood dialysis of renal patients, and that these materials are continually optimized to enhance the removal of toxins from the blood, while maintaining good biocompatibility. In order to minimize a foreign body reaction by the patient, the removal of inflammatory mediators is therefore advantageous. Yet for dialysis purposes, increasing the binding affinity of the membrane to these mediators is actually desirable, if filtration alone is insufficient (as both processes remove the mediators from the blood, see Klinkmann and Vienken, 1995). As a general rule of thumb, the original cellulose materials have a low binding affinity to proteins, and the newer synthetic polymer membranes have a varyingly high adsorption potential by rendering them hydrophilic or hydrophobic. However, the exact physicochemical characteristics of microdialysis membrane materials are generally not given by the manufacturers (such as the molecular composition, surface charge, lipophilicity, or exposed surface area), and the same polymer produced by different manufacturers (for example "PES") is often blended differently, resulting in differences also with regards to their adhesion potential (Klinkmann and Vienken, 1995). Thus, for the purpose of this thesis, only the official material description and the known physical characteristics of each membrane are given (see Table 6).

1.2.3 Influence of perfusate additives on compound recovery

In order to improve the recovery of lipophilic compounds, several authors have studied the effect of adding proteins or solubility enhancers to the aqueous perfusate (bovine serum albumin (BSA) or cyclodextrins), or of substituting the perfusate by using a fatty emulsion (Intralipid®). A summary of these studies, with selected results, is given in Table 3.

Table 3: Overview of selected microdialysis findings with different perfusate additives

Perfusate	Compound	MW* [Da]	$LogP_{OW}$*	Experimental conditions	REC [%]	Reference
Water	Oleic acid	283	7.7	Q = 0.5 µL/min, T = ?, N = 4	**1.2**	Carneheim and Ståhle, 1991
20% Intralipid®					4.4	
4% BSA in Ringer					4.8	
Ringer	Methylparaben	152	1.96	T = ?°C, Q = 1 µL/min, N=4	**62.8**	Kurosaki, Nakamura et al., 1996
20% Intralipid®					127	
Ringer	Butylparaben	194	3.57	T = ?°C, Q = 1 µL/min, N=4	**1.7**	
20% Intralipid®					738	
0.9% Saline	Carbamazepine	236	2.45	T = 25°C, stirred, Q = 1 µL/min, N=3	**17.7**	Khramov and Stenken, 1999
2% ß-CD in saline					23.7	
5% HP-ß-CD in saline					28.9	
0.9% Saline	Leukotriene B_4	336	3.58	Ambient T, stirred, Q = 1.5.µL/min, N=3	**13.2**	Sun and Stenken, 2003
1% ß-CD in saline					26.3	
0.9% Saline	SB-265123	402	5.23	T = ?°C, Q = 1.5 µL/min, N=4-6	**1.69**	Ward, Medina et al., 2003
5% HP-ß-CD in saline					65.1	
20% Intralipid®					59.0	
aCSF	ZK 975	387	4.0	T = 37°C, stirred, Q = 2 µL/min, N=4. Data not obtained at steady-state!	**15.9**	Own data (unpublished)
4%BSA in aCSF					43.3	
20% HP-ß-CD in aCSF					130	
20% Intralipid®					21.1	

MW	Molecular weight	$LogP_{OW}$	Octanol/Water coefficient
T	Temperature	Q	Flow rate
BSA	Bovine serum albumin	ß-CD	ß-Cyclodextrin
HP-ß-CD	Hydroxypropyl-ß-CD	aCSF	Artificial cerebrospinal fluid

* MW and/or $LogP_{OW}$ are taken from Syracuse Research Corporation, PhysProp online database, if not given in the reference

Bold recovery in watery perfusate, without additive or substitution

As can be seen, any of the perfusate additives or substitute give an improvement of the recovery compared to the electrolyte solution alone (in bold). Of these, only BSA is commonly used and recommended for the *in vivo* microdialysis of lipophilic compounds (Müller, Schmid et al., 1995; Kehr, 1991). Yet more dramatic improvements in the recovery have been achieved with cyclodextrins and Intralipid® (note the recoveries of >100% obtained in some studies). However, all additives have serious drawbacks that should be considered before using them for the microdialysis of lipophilic compounds, the main problem with these additives being the limitation imposed by their particle size, as discussed below.

With BSA, adhesion of compound to the materials used is reduced, since the protein competes for binding sites. The large molecular weight (MW = 67 kDa) means that BSA can only alter compound adhesion to the inner microdialysis surfaces (mainly the tubing). As it cannot diffuse through the pores, adhesion to the exposed membrane surface within the pores is not prevented. This would explain the rather small improvements in recovery observed in the reported studies (Table 3).

Likewise, Intralipid® is an emulsion of different oils in water, and although the molecular weights of the individual constituents are small (MW = 92-760 Da), the lipids form droplets, which are too large to penetrate the membrane. Yet, as opposed to BSA, very high recoveries can be obtained (>100%), which can be explained by the improved solubility of the lipophilic compound in this perfusate, which therefore 'sucks' the compound out of the aqueous medium surrounding the probe. This high recovery however would make it extremely difficult to determine the recovery in pharmacokinetic studies *in vivo*. Also, with such high recoveries of the compound of interest, the recovery of endogenous compounds from the tissue is also likely to be increased, raising the question if the tissue surrounding the probe may become depleted of vital nutrients, or otherwise altered in their physiological constitution.

Similarly, recoveries of >100% can be obtained with cyclodextrins (see Table 3). These also improve the solubility of lipophilic compounds in the perfusate, and in addition are small enough to diffuse through the membrane (MW of HP-ß-CD = 1375 Da), therefore potentially 'catching' the compound before it can bind to the membrane. However, as cyclodextrins enter the tissue, they may also affect the local pharmacokinetic behavior of the compound, the very focus of the *in vivo* study!

Most importantly however, none of the reported studies address the effect of perfusate alterations on the responsiveness of the microdialysis set up. Indeed, in own studies using a lipophilic compound and a polycarbonate membrane (Table 3), the observation time from the moment the concentration was changed to the time of the last sample taken was always exactly 1 hour, with samples collected every 10 minutes. Steady-state was not achieved in this time period with any of the perfusates used, which would preclude the use of this microdialysis set up for any pharmacokinetic study, regardless of the excellent recoveries achieved!

Therefore this thesis concentrates on testing tubing and probe materials only, with an emphasis on selecting materials which respond immediately to concentration changes, and which can therefore be used to monitor rapid concentration changes *in vivo*. Only when a microdialysis set up with good responsiveness is selected, can further measures be taken to improve the recovery, if necessary.