4 Discussion

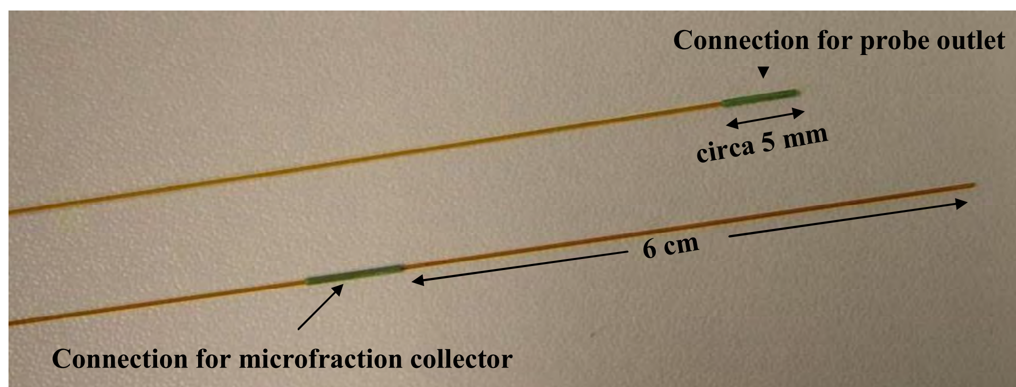
The *in vitro* approach to test tubing and probe materials for use with microdialysis as described in thesis enables the adhesion of compound to these materials to be described and quantified, and thus allows materials that are suitable for pharmacokinetic applications to be identified. A few important aspects of the materials and experiments are discussed below, including an assessment of the descriptive parameters used in this thesis (REC, K, %iAUD and Ae). The relevance of the findings for pharmacokinetic applications will be discussed, and recommendations will be made for further research.

4.1 Discussion of the tubing experiments

4.1.1 Handling of fused silica tubing

Of the tubing materials tested, fused silica is the only material not showing any adhesion of either compound tested. However, since fused silica is not a standard microdialysis material, the tubing had to be obtained from a different supplier (Table 5), and could at the time only be obtained with an inner diameter of 0.1 mm, slightly smaller than the microdialysis tubing. The outer diameter is also considerably smaller at 0.36 mm. Therefore the tubing has to be fitted with sleeves (see Figure 73), to make it compatible with the standard microdialysis equipment.

Figure 73: Fused silica tubing ends fitted with PEEK sleeves

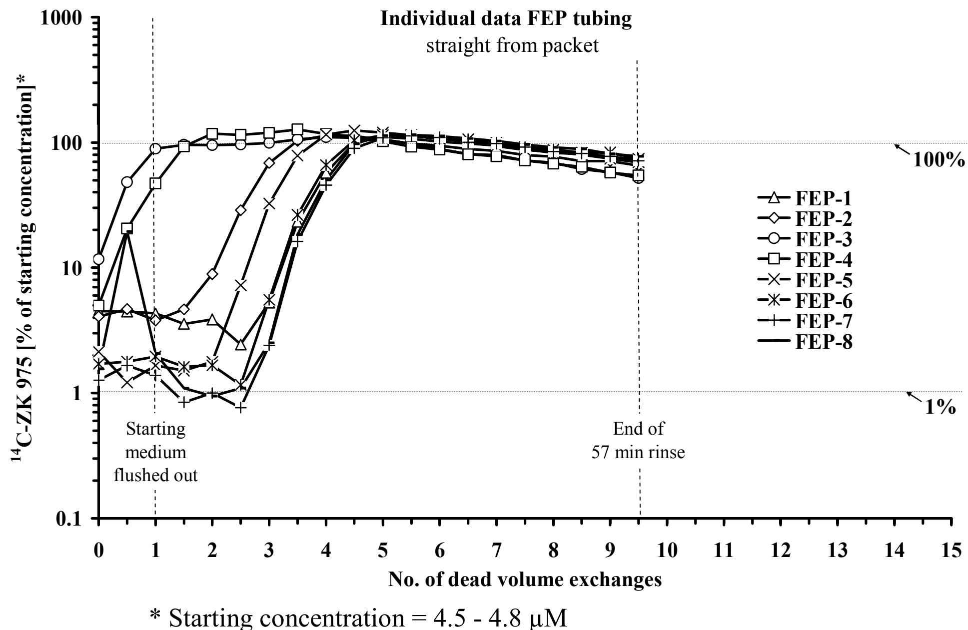


Due to the smaller inner diameter, fused silica caused some problems when used as outlet tubing for the microdialysis probes, with increased back pressure resulting in ultrafiltration, especially with the PES-membranes with larger pore sizes (35 kDa and 100 kDa). However, fused silica is now also available with an ID of 0.12 mm, which should eliminate this problem. Also, fused silica is quite a stiff material, compared to the softer PEEK and FEP. However, fused silica is still flexible enough to be used *in vivo*. With intracerebral probes, if the tubing is fitted with an extra loop before the swivel, the animal can still move freely, while the stiffness of the material reduces the chance of the animal reaching the tubing and sabotaging it. In addition, fused silica has been used for intravenous (Yang, Wang et al., 1997) and hepatic probes (Davies and Lunte, 1995), with part of the tubing tunneled under the skin. Handling properties and tissue response were favorable in these studies, but the animal tolerance of this rather stiff material compared to more flexible tubing materials remains to be examined.

4.1.2 Prerinsing of tubes with Ringer's solution

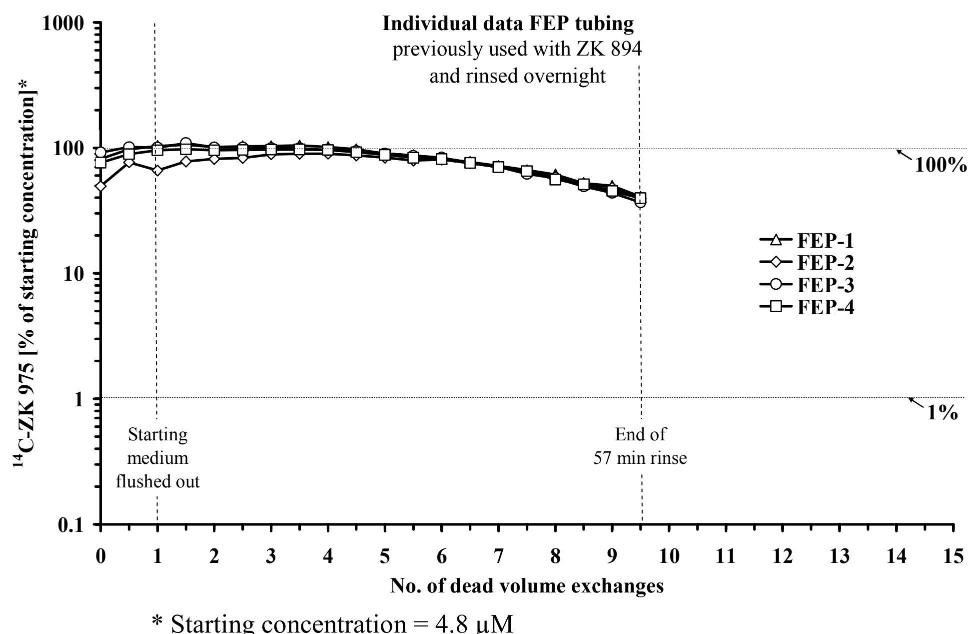
For the tubing experiments, new tubes were originally used for the tests, taken straight from the packet. The data obtained scattered widely for some materials (mainly FEP and FEP/Teflon), and seemed partly implausible, with very low initial concentrations observed (as an example, see Figure 74).

Figure 74: Time course of ZK 975 elution from FEP tubes after 1 hour incubation with ^{14}C-ZK 975, using new tubes taken straight from the packet



Therefore the test was repeated with the same tubes as used before with the hydrophilic compound ZK 894, after having been rinsed overnight and after ensuring that no more radioactivity was being eluted. The data thus obtained were far more consistent and plausible (Figure 75).

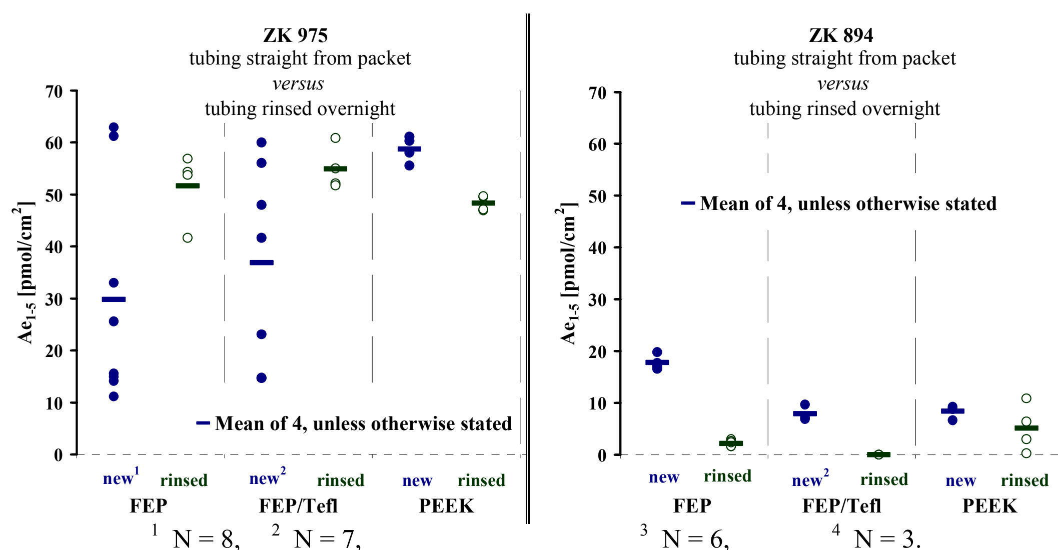
Figure 75: Time course of ZK 975 elution from FEP tubes after 1 hour incubation with ^{14}C-ZK 975, using tubes previously tested with ZK 894, and rinsed with Ringer's solution over night



* Starting concentration = 4.8 µM

However, it could not be determined whether the improvement was due to the previous treatment with the compound, or due to the perfusion with Ringer's solution. A further test was then done with the hydrophilic compound ZK 894 with new tubes, rinsed with Ringer's solution overnight. The data were almost identical to those obtained with the previously used tubes: the FEP, FEP/Teflon and PEEK tubing were more consistent and clearly different from those obtained with unrinsed tubes straight from the packet (see Figure 76). (The data for fused silica and silicone remained unchanged and are not shown here.) Therefore the observed effect was due to the overnight perfusion with Ringer's solution, rather than to the potential saturation of binding sites with the compound previously used.

Figure 76: Comparison of Ae obtained from FEP, FEP/Teflon and PEEK tubes used straight from the packet and from tubes rinsed overnight with Ringer's solution



[1] N = 8, [2] N = 7, [3] N = 6, [4] N = 3.

Thus, only data obtained from tubes rinsed overnight with Ringer's solution were considered in this thesis. For practical reasons this meant that the experiments with the hydrophilic ZK 894 were carried out first, with new and prerinsed tubes on the first day. Then the tubes were rinsed overnight again and reused with the lipophilic ZK 975, after ensuring that no more radioactivity was being eluted from the tubes.

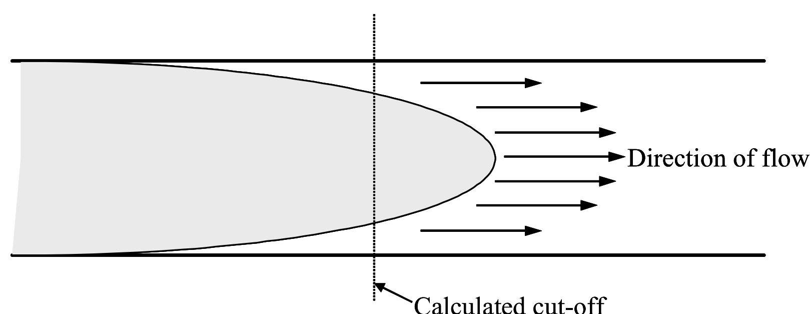
4.1.3 Comments on the length of the saturation phase

In retrospect, the one hour priming phase with the tubing experiments is too short for some materials to saturate all binding sites (especially silicone), as was evident from finding less than 100% of starting concentration at the end of the priming phase. However, since a tubing material that needs more than an hour to have its binding sites saturated would be of no interest for pharmacokinetic applications, a one hour priming phase is more than enough to test the suitability of a tubing material.

4.1.4 Graphical representation of the tubing data

The graphical representation of the tubing data using the '% of starting concentration' versus 'number of dead volume exchanges' allows an easy evaluation of the responsiveness of each material tested (see for example Figure 75). Because of the curved flow profile within the tubing however, the demarcation at 'one dead volume exchange' is not a sharp cut-off, even if the calculated first volume exchange corresponds exactly to the sampling time (Figure 77). Therefore, the first datum after this demarcation must still be considered a mixed sample for all materials shown.

Figure 77: Curved fluid flow through a tube



4.1.5 Assessment of the parameter Ae for tubing materials

The advantage of determining the amount eluted from the tubing material, rather than the time taken to reach a certain concentration (for example < 5% of starting concentration) is that the data from all samples can be used, even those from any mixed samples. By collecting all the eluted compound and subtracting the dead volume, the calculated value remains valid, and independent of the tubing length or the collection interval used. This only holds however when all binding sites are saturated at the beginning of the rinsing phase, and when all the eluted material is collected, i.e. the concentration of the last sample collected is zero (otherwise Ae is underestimated). In the described tubing experiments, these conditions were almost met for all materials that did not show any bleeding of the compound tested, and which are therefore suitable for pharmacokinetic applications (Ae < 3 pmol/cm^2). For those materials with obvious bleeding, which are therefore not suitable for pharmacokinetic applications, this condition was not always met, but the Ae_{1-5} was large enough (> 4 pmol/cm^2 or obviously higher) to recognize that adhesion of

compound to the tubing material had occurred. A graphical representation of '% of starting concentration' over 'number of dead volume exchanges', as suggested in this thesis (e.g. Figure 18) would allow the operator to decide whether borderline materials would still be suitable for the intended pharmacokinetic study.

4.1.6 Reproducibility of the tubing experiments

As discussed under Chapter 4.1.2, it is important to rinse the tubing materials overnight, to ensure consistent results. As such, scattering of the data is reduced, and therefore, based on the data presented here, it would even be possible to test tubing materials with new compounds with N = 1, to make a rapid yes/no decision (is the material suitable for the intended pharmacokinetic study or not). For the selected material, or if it is necessary to differentiate more closely between two or more materials, further repeat experiments should be carried out to confirm the findings.

4.1.7 Relevance of the tubing results for pharmacokinetic applications

Both the graphical and the numerical description (Ae_{1-5}) presented in this thesis provide an accurate indication of compound adhesion to the tubing material, and allow a good comparison of tubing materials and compounds. The graphical appraisal permits a rapid, but only rough evaluation of the time needed to reach concentrations below a certain level, for example < 5% of starting concentration. The Ae permits a quantitative description of the reversible binding capacity of the material tested, and therefore of the amount of compound that will bleed from the tubing material upon rinsing. Both assessments are important to decide if a material is suitable for the intended use: if measurements are only to be taken at high concentrations for example, a small amount of adhesion may well be tolerable. Also, a short delay in response to concentration changes may be acceptable, if the concentrations to be measured are expected to change only slowly. Generally however, a tubing material intended for the collection of samples for *in vivo* pharmacokinetic studies should not bind the compound of interest, so that even rapid concentration changes can be monitored accurately.

4.2 Discussion of the microdialysis experiments

4.2.1 Prerinsing of probes with Ringer's solution

As with the tubing tests, the microdialysis experiments were set up the day before, and left to perfuse with Ringer's solution overnight. No tests were done to examine if prolonged rinsing has any effect on probe performance. However, prerinsing with a NaCl-containing solution has been shown to neutralize the polarity of some membranes (Nakajima, Miyasaka et al., 2001), and would therefore reduce any charge interactions with the tested compound. Also, overnight rinsing corresponds to the *in vivo* setup, whereby an animal has a probe placed the day before the start of the experiment to allow the animal to recover from anesthesia, for the acute tissue response to the probe placement to settle (up to 1 day, see Lange, Boer et al., 2000), and to allow the animal to adapt to the additional restraint (swivel or Raturn system).

4.2.2 Comment on the microdialysis experimental method

The microdialysis *in vitro* set up was constructed to reflect the *in vivo* situation during pharmacokinetic studies as much as possible, while at the same time isolating the effect of the materials used from all other influences (such as the surrounding

medium or tissue). Thus, the medium is warmed to body temperature (approximately 37°C) and stirred, to prevent any possible rate limiting diffusion of compound through a quiescent solution (as might be observed *in vivo* in tissues with low local clearance). Both rising and falling tissue concentrations are simulated by including rinsing phases as well as concentration phases (the only other account of such an approach is given by Tao and Hjorth, 1992). To capture any potential concentration dependence of material responsiveness, two different concentrations are tested, at the high and low end of the concentration range that can realistically be expected *in vivo*. Short sampling intervals are used, to be able to describe the material responsiveness as precisely as possible, and to demonstrate the suitability of the setup to monitor even rapid concentration changes, as is seen *in vivo* for example during the distribution phase of a compound after (intravenous) administration. As such, the experimental approach followed in this thesis permits a good differentiation between microdialysis materials suitable or unsuitable for pharmacokinetic applications.

4.2.3 Graphical interpretation of the microdialysis data

The graphical representation of the microdialysis data using either REC or K versus 'sample number' (= time) both allow a rapid (but subjective) evaluation of the response to changing concentrations of each material tested (see also Tao and Hjorth, 1992). For the concentration phases A and B, a delay in response to concentration increases is indicated by a gently upward sloping curve (e.g. Figure 28), and it can be roughly assessed whether steady-state (= plateau) is reached or not. For the rinsing phases, the curves give a rough estimate of the time taken to reach recoveries below 1, 5 or 10% (e.g. Figure 29), and if the recovery reached 0% within the 1 hour observation period, collection of compound was complete (e.g. Figure 31). However, to allow comparison between different experiments, and with literary values, a better defined numerical description is necessary.

4.2.4 Assessment of the parameter REC for microdialysis probes

The recovery is the standard microdialysis probe property, as always mentioned in the literature. It remains an important value, as it gives the best impression as to the usefulness of a particular probe for the desired compound and the available analytical method (sample concentrations need to be quantifiable). Although the recovery is not used as a criteria for the suitability of a probe for pharmacokinetic applications in this dissertation, as it does not indicate responsiveness or adhesion potential, the sensitivity of the available analytical method will determine what recovery is needed for the intended *in vivo* study. For comparison between different probes and with literary value, the parameter K is more relevant, which adjusts the recovery for membrane surface area and flow rate.

4.2.5 Assessment of the parameter K for microdialysis probes

The parameter K is calculated for the microdialysis experiments, to allow comparison between materials and compounds tested, also with the literature. K generally ranged from 0.07 to 0.24 $\mu l/min/mm^2$ for the compound/material combinations tested in this thesis. Within this range, K varied between materials tested, indicating that K is indeed membrane specific (see Chapter 1.2.2). Also, for each probe tested, K was consistently lower for the lipophilic compound ZK 975 than for the hydrophilic compound ZK 894, confirming that K is also compound specific (Sun and Stenken, 2003). Being corrected for flow rate and membrane size,

K therefore indicates a potential recovery for a particular material/compound combination. The higher K, the higher the recovery that can be achieved by optimizing all probe geometries during probe design (highest workable membrane size, thinnest possible membrane and optimal fluid layer). Optimizing K is particularly important when sample concentrations close to the limit of detection of the available analytical method are expected, and the probe recovery is therefore aimed to be as high as possible. However, like the recovery, K is a steady-state parameter, and gives no indication of the time needed to reach this steady-state. Therefore K cannot be describe the suitability of a membrane to monitor rapid concentration changes within the setting of a pharmacokinetic study. Thus, the parameters %iAUD and Ae are introduced to describe membrane properties relevant to pharmacokinetic applications.

4.2.6 Assessment of the parameter %iAUD for microdialysis probes

The %iAUD is derived from the concept that for pharmacokinetic applications, a microdialysis probe should responds immediately to increasing concentration changes. Thus, as soon as the dead volume is flushed out, the recovery would be at steady-state, and the area under the data would give an 'ideal' AUD (see Figure 10). If a tested probe matches this property, it would have the same AUD as the iAUD of a corresponding ideal probe with the same recovery, therefore the %iAUD = 100%. Any delay in response would result in a reduction of the actual AUD, reducing the %iAUD. The obtained %iAUDs indeed reflect the observed graphical shapes very well, with %iAUD $> 95\%$ for quickly responding probes, and %iAUD $< 85\%$ for poorly responding probes (Chapter 3.2.2.3.1). Thus, the %iAUD is a good indicator of probe responsiveness, and of a probe’s suitability for pharmacokinetic studies.

4.2.7 Assessment of the parameter Ae for microdialysis probes

As with the tubing materials, the advantage of calculating the amount eluted from the microdialysis probe materials is that the data of all samples from the rinsing phases can be used, even those from any mixed samples. By combining the data from two different starting concentrations, the effect of the free amount of compound within the fluid filled pores (not accounted for by subtracting the calculated dead volume) is eliminated (see Chapter 2.3.1.2.1). This approach assumes that the binding capacity is constant, regardless of the concentration used, that all available binding sites are occupied at the start of the rinsing phases, and that all eluted compound is collected during the observation period. Even though the last two conditions were not always met in this thesis, the calculated Ae still reflects the extend of bleeding observed in the graphical representation (Chapter 3.2.2.3.2). For probes that rapidly reached 0% recovery when rinsed, Ae is less than 5 pmol/mm^3. For materials which clearly showed bleeding of compound during the rinsing phases, Ae > 5 pmol/mm^3 or obviously higher, indicating that adhesion of compound to the microdialysis probes had occurred. The additional graphical representation of the data allows the operator to decide whether materials with minimal adhesion (e.g. Figure 44) are still suitable for the intended use (for infusion kinetics for example, or if measurements are only made at high concentrations), and whether unexpected individual values are representative of the material tested (see for example Figure 48, with only minimal bleeding observed graphically for all probes, yet Ae ranging from 0-8 pmol/mm^3).

4.2.8 Reproducibility of the microdialysis experiments

For practical reasons, all probe tests were initially carried out in 4-fold, to demonstrate the reproducibility of the results obtained. If an experimental error occurred, the data were not shown, and were not included in any mean calculations. Generally, scattering of the data was limited (CV for %iAUD < 10%, see Figure 70), and therefore, based on the data presented here, it would even be possible to test microdialysis probes with new compounds with N = 1, to decide if the material is suitable for the intended pharmacokinetic study or not. If unexpected results are obtained (especially unusually high or low recoveries), and for the chosen material, further repeat experiments should be carried out to confirm the original findings.

4.2.9 Relevance of microdialysis results for pharmacokinetic applications

The range of different profiles obtained with the microdialysis probes tested in this thesis demonstrates the importance of choosing an experimental set up *in vitro* that is representative of the pharmacokinetic study to be carried out *in vivo*. In other words, when concentration changes are to be monitored *in vivo*, it is vital to demonstrate that the chosen equipment is capable of monitoring these changes accurately *in vitro* first. At least two concentrations must be tested to demonstrate a concentration independent recovery, and the transition period from the change in concentration to steady-state must be monitored to detect any delays in response. Also, rinsing phases must be included to uncover any potential bleeding of compound from the microdialysis materials that would also occur during the elimination phase of that compound *in vivo*.

The parameters %iAUD and Ae describe the observed behavior accurately, and can be used to define the suitability of a microdialysis probe for pharmacokinetic applications. The results for the hydrophilic compound ZK 894 (Figure 60) show that even with a theoretically 'unproblematic' compound, not all materials are suitable, and that therefore an *in vitro* validation must be carried out for all new compounds, before microdialysis data are collected for *in vivo* studies. The results for the lipophilic compound ZK 975 (Figure 50) show that even for theoretically 'sticky', and classically problematic compounds, testing different materials may well provide tubing / probe combinations suitable for pharmacokinetic application.

4.3 Further recommended research

The research for this thesis is limited to only one lipophilic compound and one hydrophilic compound. Further research is needed with a larger range of compounds (with a larger LogP-range), to test if the findings presented can be generalized. Likewise, other compound properties, such as polarity or acidity, also need to be studied with regards to their material interaction. Only if a large database of compound / material interactions is available, can a more general recommendation be made with regards to materials of choice for a compound of interest for pharmacokinetic applications.

Another limitation of the presented research is the limited availability of tubing materials and especially of microdialysis probes. For study groups capable of assembling their own probes, the effect of probe geometry on the recovery, or the recovery of different membrane materials with the same probe geometry should be tested in more detail, using compound/material combinations that do not show any

binding. That way, recommendations can be made with regards to optimal probe parameters to maximize K. Only if K is maximized, can microdialysis become accessible for the pharmacokinetic study of compounds with low unbound concentrations in the region of interest *in vivo*, e.g. due to high protein binding.

Finally, in order to validate microdialysis for pharmacokinetic applications further, the delays in response to concentration changes ('transients') often observed *in vivo* must be studied more closely. Transients occur when the tissue clearance of the compound is lower than the microdialysis clearance of the compound from the tissue, resulting in the formation of a depletion zone around the probe, which takes time to establish (similar to performing microdialysis in unstirred solution *in vitro*). Detailed mathematic models have been proposed to describe these transients (Bungay, Dedrick et al., 2001), but so far very little has been done to provide empirical data to support these models. Only when materials are used that are shown not to interact with the test compound (by using the approach suggested in this thesis), can transients observed *in vivo* be described and quantified accurately, providing an opportunity to account for these effects for pharmacokinetic studies.