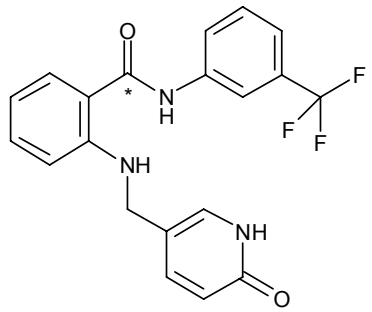
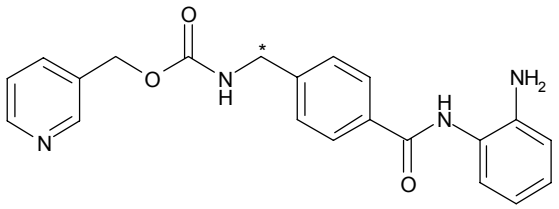
2 Materials and methods

2.1 Materials

2.1.1 Compounds

In order to characterize tubing materials and microdialysis probes with regards to their adhesion properties, a model lipophilic compound (ZK 894) and a model hydrophilic compound (ZK 975) are used. ZK 975 was previously found to strongly adhere to the microdialysis membranes so far used in our lab, and similarly ZK 894 was selected as an unproblematic compound. Pharmacokinetic data were available for both these compounds, facilitating the choice of the concentration to be used *in vitro* (1 and 10 µM, at the low and high end of the concentration range observed *in vivo*), and both compounds were available with a ^{14}C-radiolabel, allowing analogous and rapid analysis of the samples by radioactivity counting (the stability of the compounds throughout the *in vitro* experiments is tested separately by thin layer chromatography, see chapter 2.2.1.2). In addition, both compounds have very similar molecular weights (384 and 376 kDa) and the same solubility at the pH tested (43 µM), allowing specific comparison of the effect of lipophilicity versus hydrophilicity on the suitability of materials for microdialysis. An overview of these and other selected physicochemical properties is given in Table 4.

Table 4: Selected properties of the model lipophilic compound (ZK 975) and the model hydrophilic compound (ZK 894)

^{14}C-ZK 975 (Lipophilic compound)	**^{14}C-ZK 894** (Hydrophilic compound)
	
* Position of the ^{14}C isotope	* Position of the ^{14}C isotope
Specific activity = 2.15 MBq/µmol MW = 387.4 Da Log P_{OW} = 4.0 (calculated) S_{20} (pH 7) = 43 µM pK_a (25°C) = 2.6, 11.5 (neutral at pH 7)	Specific activity = 2.09 MBq/µmol MW = 376.4 Da Log P_{OW} (pH 7) = 1.5 S_{20} (pH 7) = 43 µM pK_a (25°C) = 2.9, 3.4 (neutral at pH 7)
MW = molecular weight S_{20} = aqueous solubility at 20°C	Log P_{OW} = octanol : water coefficient pK_a = acid dissociation constant

2.1.2 Tubing materials

In order to identify tubing materials suitable for pharmacokinetic applications, as many materials are tested as possible. Table 5 shows the five tubing materials obtainable with suitably small internal diameters for microdialysis studies: fluorinated ethylene propylene (FEP), FEP/Teflon, polyetheretherketone (PEEK), fused silica and silicone. (Since the manufacturer of these materials is not given by the suppliers, FEP and FEP/Teflon are tested separately, even though the material may well be the same.) The tubing materials FEP, FEP/Teflon and PEEK are standard microdialysis tubes and generally obtainable from microdialysis suppliers. Another tubing material easily obtainable and occasionally used for microdialysis is fused silica, which is however not a standard microdialysis material. Finally, to assess a material not normally used for microdialysis, silicone is included. The material is very soft however, making the attachment of the tubing adapters difficult, and due to the larger outer diameter of 0.8 mm, the fraction collectors cannot be used, meaning that the samples have to be collected manually.

The length of tubing used is chosen to be as long as possible to maximize the chance of observing any binding effects, and is limited by the supplied lengths of one meter for the FEP, FEP/Teflon and PEEK tubes. For fused silica, the same length is used. Only for silicone, with such obviously different inner dimensions (0.25 mm inner diameter, compared to the 0.12 mm of the standard microdialysis tubes), a different length of 45 cm is chosen to provide a similar inner surface area to the other materials. As the binding capacity of a tubing material is directly proportional to the exposed surface area, using lengths that provide similar inner surface areas allows for a better graphical comparison of the data.

Table 5: Selected characteristics of the five tubing materials tested

Material	OD [mm]	ID [mm]	Length used [cm]	Dead volume [µL]	Inner surface area [cm^2]	Description	Supplier
FEP	0.6	0.12	100	12	3.9	FEP Tubing	Axel Semrau
FEP/Teflon	0.65	0.12	100	12	3.9	FEP Teflon Tubing	BASi
PEEK	0.65	0.12	100	12	3.9	PEEK Tubing	BASi
Fused silica	0.36	0.10	100	7.9	3.1	Fused Silica Tubing	GAT Gamma
Silicone	0.76	0.25	45	23	3.6	Tygon® S-54-HL	H. Riesbeck

OD Outer diameter
ID Inner diameter
Axel Semrau Axel Semrau GmbH & Co, Sprockhövel, Germany (CMA supplier)
BASi Bioanalytical Systems, Inc, West Lafayette, USA
GAT Gamma Analysen Technik GmbH, Bremerhaven, Germany
H. Riesbeck Biebergemünd, Germany

One further material considered for testing was electroformed nickel tubing from Vici AG, Valco International. The outer diameter is slightly larger at 0.8 mm, but the tubing can still be fitted to the pump with the tubing adapters. The tubing can also be used with the fraction collectors, once the guiding needles are removed. However, the material is very stiff, and during preliminary testing the tubing became completely obstructed within a few hours, possibly due to corrosion. Therefore no data are available for this tubing material.

2.1.3 Microdialysis probes

As with the tubing material, the primary criterion for the choice of microdialysis probes was the commercial availability. Furthermore, the probes had to be comparable with regards to their probe geometry and dimensions. Therefore only intracerebral microdialysis probes are tested, and only those available from the three suppliers used (CMA, BASi and Microbiotech) , all with a membrane length of 4 mm. Eleven different probe types were available, and to allow comparison of like with like, an attempt is made to group the probes in such a way that the influence of only one property at a time can be assessed. For an overview of the dimensions and suppliers of these eleven microdialysis probes, see Table 6.

So, firstly and most importantly, the different membrane materials are compared: polyethylenesulfone (PES), cuprophane (Cu), cellulose (Cell), polycarbonate (PC) and polyacrylonitrile (PAN). However, all these materials differ in their pore size, so the influence of pore size (6, 15, 35 and 100 kDa) on probe performance is tested, using the only material available with different pore sizes (PES). Among these PES membranes, the outer membrane surface area differs slightly between the probes from the different manufactures (6.28 mm^2 for the PES 100 kDa membrane versus 7.54 mm^2 for the 6, 15 and 35 kDa PES membranes). Therefore the influence of membrane size on probe performance is assessed, using two 6 kDa PES membranes. For the cuprophane membrane, two probes with different membrane thicknesses (20 and 30 µm), but otherwise similar probe geometries were available, thus allowing good comparison of this property. Since cuprophane is based on a cellulosic material, the cellulosic membrane with a thickness of only 5 µm is included in this comparison. Finally, the influence of probe outlet material is tested (PEEK versus steel), using otherwise identical 6 kDa PES probes (both from Microbiotech).

Table 6: Selected characteristics of the 11 concentric microdialysis probes tested

Probe description	Membrane material	Pore size [kDa]	OD [µm]	ID [µm]	S [1)] [mm^2]	V [2)] [mm^3]	Dead Volume [µL]	Membrane thickness [µm]
CMA/12 (PES)	PES	**100**	500	400	6.28	0.283	3.153	50
MAB 2.14.4	**PES**	**35**	600	530	7.54	0.248	0.63	35
MAB 6.14.4	PES	**15**	600	530	7.54	0.248	0.63	35
MAB 9.14.4	PES	**6**	600	530	**7.54**	0.248	0.63	35
MAB 8.4.4	PES	6	240	180	**3.02**	0.079	1.36	30
MAB 4.15.4.PES	PES	6	240	180	3.02	0.079	0.92	30
MAB 4.15.4.Cu	**Cu**	6	240	180	3.02	0.079	0.83	**30**
CMA/11	Cu	6	240	200	3.02	0.055	1.027	**20**
MBR-4-10	**Cell**	38	220	208	2.76	0.016	0.051	**5**
CMA/12 (PC)	**PC**	20	500	470	6.28	0.091	3.249	15
BR-4	**PAN**	30	320	240	4.02	0.141	0.055	40

For legend, see below

Table 6 cont: Selected characteristics of the 11 microdialysis probes tested

Probe description	Membrane material	Inner cannula OD [μm]	Fluid layer [3] [μm]	Inner cannula material	Outlet material	Manufacturer
CMA/12 (PES)	PES	250	75	Stainless steel	14 mm Steel	CMA
MAB 2.14.4	**PES**	230	150	Polyimide	14 mm PEEK	Microbiotech*
MAB 6.14.4	PES	230	150	Polyimide	14 mm PEEK	Microbiotech*
MAB 9.14.4	PES	230	150	Polyimide	14 mm PEEK	Microbiotech*
MAB 8.4.4	PES	125	28	Fused Silica	**4 mm Steel**	Microbiotech*
MAB 4.15.4.PES	PES	125	28	Fused Silica	**15 mm PEEK**	Microbiotech*
MAB 4.15.4.Cu	**Cu**	125	28	Fused Silica	15 mm PEEK	Microbiotech*
CMA/11	Cu	150	25	Fused Silica	14 mm Steel	CMA
MBR-4-10	**Cell**	105	52	Fused Silica	12 mm PEEK	BASi
CMA/12 (PC)	**PC**	250	110	Stainless steel	14 mm Steel	CMA
BR-4	**PAN**	150	45	Fused Silica	15 mm PEEK	BASi

OD = Outer diameter
ID = Inner diameter
PES = Polyethylenesulfone
Cu = Cuprophane
PC = Polycarbonate
PAN = Polyacrylonitrile
Cell = Cellulosic
CMA = CMA Microdialysis AB, Solna, Sweden
BASi = Bioanalytical Systems, Inc, West Lafayette, USA
* Microbiotech/se AB, Stockholm, Sweden
[1] S = Membrane surface area = $\pi \times$ Membrane OD $\times$ membrane length of 4 mm
[2] V = Membrane volume = $\pi \times (OD^2 - ID^2)/4 \times$ membrane length of 4 mm
[3] Fluid layer = (Membrane ID – Inner cannula OD) / 2
For each probe, the relevant property for comparison is printed bold – membrane material, pore size, membrane surface area, membrane thickness or non-membranous outlet material

2.1.4 Equipment and miscellaneous materials

A list of all the equipment used and the suppliers, including the make or suppliers description, is given in Table 7. All the solvents and solutions used are listed in Table 8, and all the miscellaneous materials used in Table 9.

Table 7: List of equipment used

Equipment	Make/Description	Manufacturer or supplier
Microdialysis pump	CMA/400	Axel Semrau GmbH, Sprockhövel, Germany
Microfraction collector	CMA/142	Axel Semrau GmbH, Sprockhövel, Germany
Scales	AX205 Delta Range® scales	Mettler Toledo GmbH, Giessen, Germany
Rotor	Heidolph overhead mixer	Heidolph Instruments, Schwabach, Germany
Liquid Scintillation Analyzer	Tri-Carb 2900TR Liquid Scintillation Analyzer	PerkinElmer® LAS (Germany) GmbH, Rodgau-Jügesheim, Germany
In vitro stand for microdialysis probes	CMA/130	Axel Semrau GmbH, Sprockhövel, Germany
Magnetic stirrer	IKA® RET basic C	IKA®-Werke GmbH & Co.KG, Staufen, Germany
Fuzzy temperature sensor	IKA® ETS-D4	IKA®-Werke GmbH & Co.KG, Staufen, Germany
Bio-Imaging Analyzer	Fujix BAS 2000	raytest, Isotopenmeßgeräte GmbH, Germany

Table 8: List of solvents and solutions used

Solvent/Solution	Make/Description	Manufacturer or supplier
Ringer's solution	"Ringer-Infusionslösung"	B Braun Melsungen AG, Germany
Water for chromatography	LiChrosolv®	Merck KGaA, Darmstadt, Germany
Ethanol	Absolute GR for analysis	Merck KGaA, Darmstadt, Germany
Dichloromethane	GR for analysis	Merck KGaA, Darmstadt, Germany
Methanol	GR for analysis	Merck KGaA, Darmstadt, Germany
Triethylamine (TEA)	$C_6H_{15}N$	Riedel-de Haën, Seelze, Germany
Scintillator	Atomlight™	PerkinElmer® LAS (Germany) GmbH, Rodgau-Jügesheim, Germany

Table 9: List of miscellaneous materials used

Item	Make/Description	Manufacturer or supplier
Microdialysis probe clips	CMA/11+12 Clip	Axel Semrau GmbH, Sprockhövel, Germany
Tubing connectors	Red tubing adapters	Microbiotech/se AB, Stockholm, Sweden
PEEK tubing sleeves	MicroTight, 380 µm	GAT Gamma Bremerhaven, Germany
Glue for sleeves	Vetbond™ tissue adhesive	3M Deutschland GmbH, Neuss, Germany
Perfusion syringes	1 and 5 mL Hamilton syringes	Axel Semrau GmbH, Sprockhövel, Germany
Microvials	300 µL, 31.5 × 5.5 mm	VWR International GmbH, Darmstadt, Germany
Imaging plates	Fujix BAS-III	raytest, Isotopenmeßgeräte GmbH, Germany
Eppendorf vials	Safe-Lock Tubes 2.0 mL	Eppendorf Vertrieb Deutschland GmbH, Wesseling-Berzdorf, Germany
Polyethylene vials	Miniature 6 mL PE Vials	PerkinElmer® LAS (Germany) GmbH, Rodgau-Jügesheim, Germany
Crystallization dish	Schott, Ø 12 cm, H 6.5 cm	Schering AG Berlin, in-house magazine
Floating microcentrifuge rack	Round, for 1L beaker, PP 1-5mL	VWR International GmbH, Darmstadt, Germany
Triangular stirring magnets	For 1 mL Eppendorf vials	Neolab®, Heidelberg, Germany
TLC plates	Silica gel 60 F254, 10 × 10 cm	Merck KGaA, Darmstadt, Germany
Magnifying glass	Enlargement 4×	Schering AG Berlin, in-house magazine

TLC = Thin layer chromatography

2.2 Methods

2.2.1 Testing of tubing materials

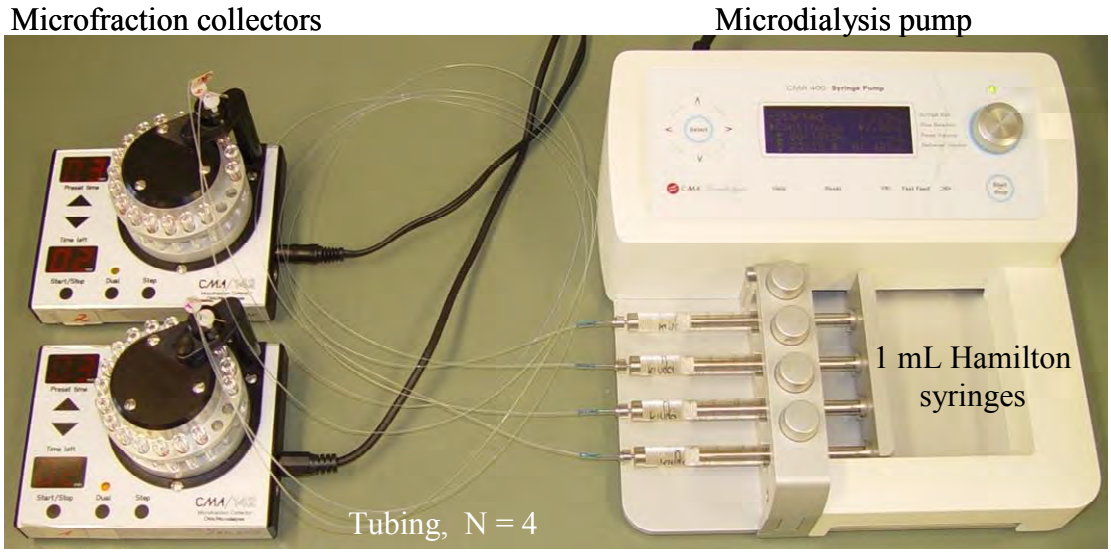
Aim: selection of a tubing material with minimal adhesion of the test compound.

2.2.1.1 Experimental method tubing test

In order to select a suitable tubing material, a quick and easy *in vitro* test is used. The main criterion is to identify those materials that show no binding whatsoever, considering that for later examinations of compound adhesion to microdialysis probes, a tubing material is needed that does not superimpose any own binding kinetics. Therefore an approach is taken that saturates all potential binding sites with a suitably large concentration of compound (5-10 µM in Ringer's solution), for a suitably long period of time (about one hour). Upon rinsing of the tubing material, any 'bleeding' observed would indicate that compound binding had occurred. A collection interval is chosen that allows sufficient material to be collected in each sample for analysis, yet gives a good time resolution of the rinsing phase (3 minutes).

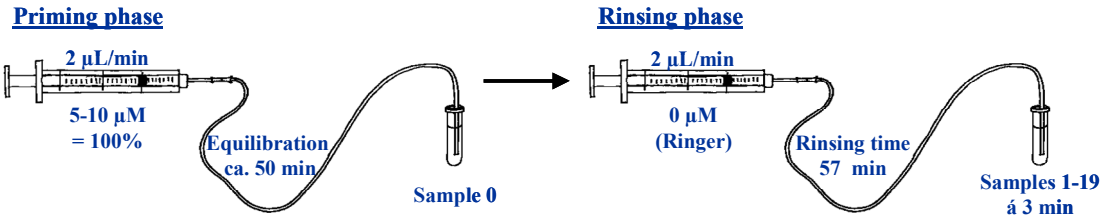
The experimental set up for testing the tubing materials is shown below in Figure 4. Standard microdialysis equipment is used, but the inlet tubing and microdialysis probes are omitted from the normal microdialysis set up.

Figure 4: Experimental set up tubing test



Of each material, four pieces of tubing are tested in parallel. Before the start of the experiment, each tube is preconditioned by rinsing with Ringer's solution at 2 µL/min overnight (see discussion, Chapter 4.1.2). The testing procedure for the tubing materials, as devised for the purpose of this dissertation, is shown in Figure 5.

Figure 5: Experimental procedure tubing test



After being rinsed overnight, each tube is perfused at 2 µL/min with 5-10 µM compound solution for approximately 50 minutes (priming phase), after which one 3-minute-sample is collected (sample 0). Subsequently, the tubes are rinsed at 2 µL/min with Ringer's solution for 57 minutes, with samples collected every 3 minutes, 19 samples in total (rinsing phase).

2.2.1.2 Sample analysis tubing test

The glass microvials are weighed (gross minus tara) to determine the exact net volume of the collected sample (1 mg ≡ 1 µL). The entire vials are then placed in polyethylene (PE) vials, filled up with circa 4.5 mL scintillator, and rotated overhead for at least 30 min. Then the polyethylene vials are placed in a liquid scintillation analyzer for radioactivity counting (4 minutes maximum per sample, or 1% accuracy if reached sooner). Quench control and correction are performed according to the external standard channel ratio method. To determine the background radiation, six empty microvials are also prepared with scintillator and counted accordingly. The highest and lowest of these six counts are ignored, and the mean of the remaining four used to set the lower limit of quantification (LLOQ) at twice the background radiation in decays per minute (DPM) per sample. Samples containing radiolabel concentrations below the LLOQ have their concentration set to zero for further evaluation.

The stability of the test compounds under experimental conditions is tested in a parallel experiment. A separate batch of 10 µM solution of either compound is analyzed by thin layer chromatography (TLC) immediately after the dilution is made, and after being left for 1.5 hrs at room temperature (1.5 hours correspond to preparation time plus 50 minutes priming phase). The TLC is run on silica gel plates, with Dichloromethane / Ethanol 10:1 (v:v) for ZK 975, and with Dichloromethane / Methanol / TEA 19:1:1 (v:v:v) for ZK 894. Both systems are routinely used for the detection of degradation products from these compounds by the isotope chemistry department in house. The TLC-plates are exposed on imaging plates in a lead cupboard for 3 weeks, after which the imaging plates are read by a BAS 2000 bio-imaging analyzer and evaluated with the computer program TINA (version 2.08), according to the manufacturer's manual.

2.2.2 Testing of microdialysis probes

Aim: selection of a microdialysis probe with minimal adhesion of the test compound.

2.2.2.1 Experimental method microdialysis test

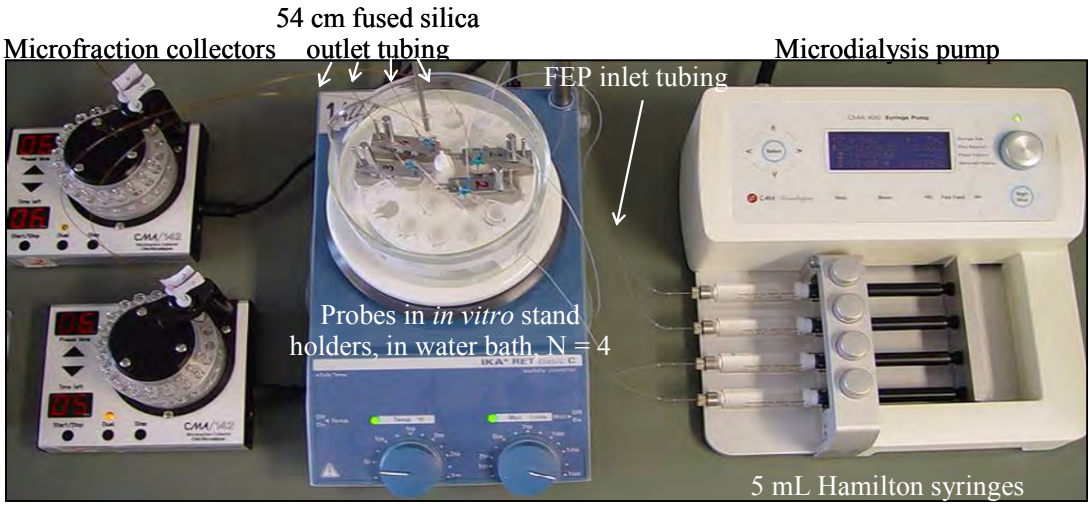
In order to select a microdialysis probe suitable for pharmacokinetic applications with a given compound, an *in vitro* experiment is required that tests the necessary probe properties under realistic pharmacokinetic conditions: a rapid response to rapid concentration changes and a constant recovery at different concentrations. A microdialysis probe to which the test compound adheres will show a delayed response (see Chapter 1.2). A microdialysis probe with no binding, and therefore with good responsiveness, still needs to show a constant recovery at different concentrations in order to make quantitative measurement *in vivo*.

Therefore a simple approach is chosen to test a probe at two different concentrations, which are deemed realistic high and low unbound concentrations as expected *in vivo*. Two concentrations are used to ensure that the recovery and probe performance are concentration independent. The lower concentration is tested first, to minimize the carry over of compound to the next concentration phase. Rinsing phases are included to test the responsiveness of the probe to decreasing concentrations, which is important when monitoring the elimination phase of a compound *in vivo*.

The sampling interval is chosen as short as possible to get a good time resolution, but long enough to have enough radioactivity in the sample for analysis, and is set at 6 minutes for the purpose of this thesis. The observation period of 1 hour is chosen to include one discarded mixed sample, a short equilibration time (if any), and four samples at steady-state to determine the recovery.

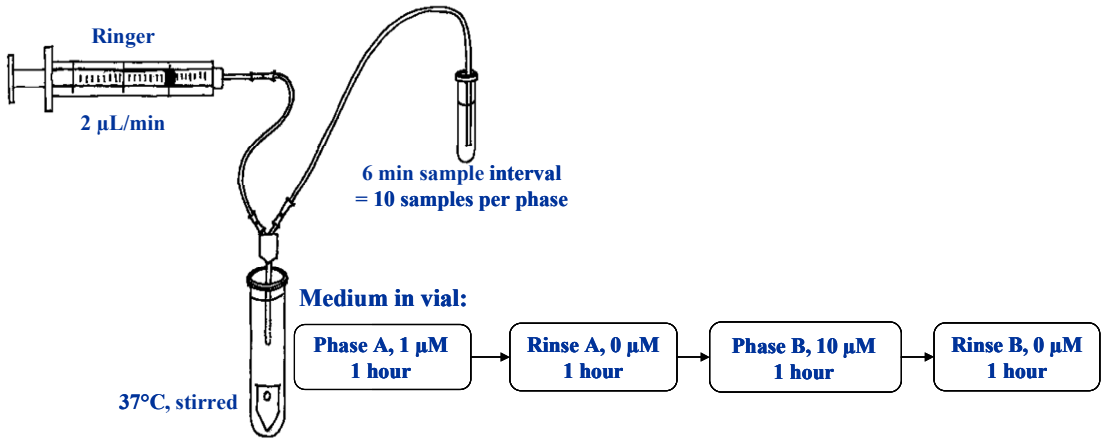
The experimental set up for testing the probes is shown below in Figure 6. Although not described explicitly in the literature, similar set ups may have been used for other *in vitro* microdialysis studies under stirred and/or heated conditions.

Figure 6: Experimental set up microdialysis probe test



Of each probe type, four probes are tested in parallel. Before the start of the actual experiment, each probe is prepared by flushing with Ringer's solution, with the probe submerged in ethanol or in water, according to the manufacturer's instruction. Each membrane is then checked with a hand-held magnifying lens, and any air bubbles are removed by increasing the flow rate to 10 µL/min and/or by tapping the holder sharply. The probes are then submerged in Ringer's solution and perfused with Ringer at 2 µL/min overnight (see discussion, Chapter 4.2.1). The testing procedure for the microdialysis probes, as devised for the purpose of this dissertation, is shown in Figure 7.

Figure 7: Experimental procedure microdialysis probe test



After being rinsed overnight, the probe is placed in a 2 mL Eppendorf vial with 1 µM compound dissolved in Ringer for one hour (Phase A), then in 2 mL pure Ringer's solution for one hour (Rinse A), then in 2 mL 10 µM compound/Ringer's solution for one hour (Phase B) and finally in 2 mL Ringer's again for one hour (Rinse B). The flow rate is set at 2 µL/min, the temperature at 37°C, and the stirring is maintained at maximum setting (1500 rpm). Samples are collected every 6 min, 10 samples per phase, 40 samples in total. At the end of Phase A and Phase B, an additional sample is taken for the analysis of compound stability by thin layer chromatography (TLC).

2.2.2.2 Sample analysis microdialysis test

The microdialysis samples are analyzed for total radioactivity, and the two additional samples taken at the end of Phase A and Phase B are analyzed for stability by TLC, both as described above for the tubing samples (see chapter 2.2.1.2).

2.3 Data evaluation

2.3.1 Evaluation of tubing data

2.3.1.1 Evaluation of individual sample data – tubing materials

2.3.1.1.1 Calculation of the sample concentration

For each sample the total amount of compound (A_c) is calculated by correcting the decays per minute (DPM) for the background radiation and the specific activity (see Table 4, Chapter 2.1.1) of the compound used:

$$A_c = \frac{\text{DPM} - \text{background DPM}}{\text{specific activity}} \ [\text{pmol}] \qquad [\ 1\ ]$$

Dividing A_c by the sample volume (determined by weighing) then gives the sample concentration (C_{sample}):

$$C_{sample} = \frac{A_c}{\text{Volume}} \ [\mu\text{M}] \qquad [\ 2\ ]$$

For samples with DPM below the LLOQ of twice the background radiation, the concentration is set to zero (see Chapter 2.2.1.2)

2.3.1.1.2 Calculation of the percentage of starting concentration

In order to better compare the tubing data, the C_{sample} is related to the concentration of the medium used in the priming phase (C_{medium}) to give the '% of starting concentration' (in analogy to the probe recovery, as described in Chapter 2.3.2.1.2):

$$\%\ \text{of starting concentration} = \frac{C_{sample}}{C_{medium}} \times 100 \ [\%] \qquad [\ 3\ ]$$

The '% of starting concentration' is depicted graphically on the y-axis.

2.3.1.1.3 Calculation of the number of dead volume exchanges

To allow comparison of the tubing materials with different inner diameters (ID), the difference in the ID (see Table 5, page 13) is taken into account by translating the time the samples are taken into 'number of dead volume exchanges':

$$\text{No. of dead volume exchanges} = \frac{\text{sample time} \times \text{flowrate}}{\text{dead volume}} \ , \qquad [\ 4\ ]$$

where the flow rate is always 2 µL/min. For example, for the FEP tubing with a dead volume of 12 µL (= $\pi \times (0.12\ \text{mm ID} / 2)^2 \times 1000$ mm length), each 3-minute sample of 6 µL (= 3 min × 2 µL/min) corresponds to half a dead volume exchange. For the entire observation period of 57 min, the same tubing has its dead volume exchanged 9.5 times. For silicone, with the largest dead volume of 22.8 µL, the dead volume is exchanged only 0.26 times per 3-minute sample, in total 5 times within the 57 minutes rinsing phase.

2.3.1.2 Evaluation of individual data sets – tubing materials

2.3.1.2.1 Deduction of the amount eluted

To describe the adhesion properties of each material, the amount eluted (Ae) from the exposed inner surface of the tubing is calculated (see also Figure 8). First, the total amount eluted is calculated as the sum of the amount of compound (A_c) in all samples collected within the first five dead volume exchanges (see T 1 to T 10 in Appendix two for the sampling times, and the corresponding number of dead volume exchanges for each material tested).

The $Ae_{0\text{-}5}$ however also includes the initial amount of compound that is still present in the tubing at the start of the rinsing phase ($A_{c,\ dead\ volume}$). In order to calculate $A_{c,\ dead\ volume}$, the concentration in the tubing at the start of the rinsing is multiplied by the dead volume. If all binding sites are saturated at the end of the priming phase (or if no binding occurs), then the concentration throughout the tube is the same as the concentration of the perfused medium (C_{medium}), which then corresponds to the concentration measured in sample 0 taken at the end of the priming phase (C_0). If however saturation is not complete ($C_0 < C_{medium}$), then the average concentration throughout the tube can be considered to be the mean of the C_{medium} and C_0, and therefore:

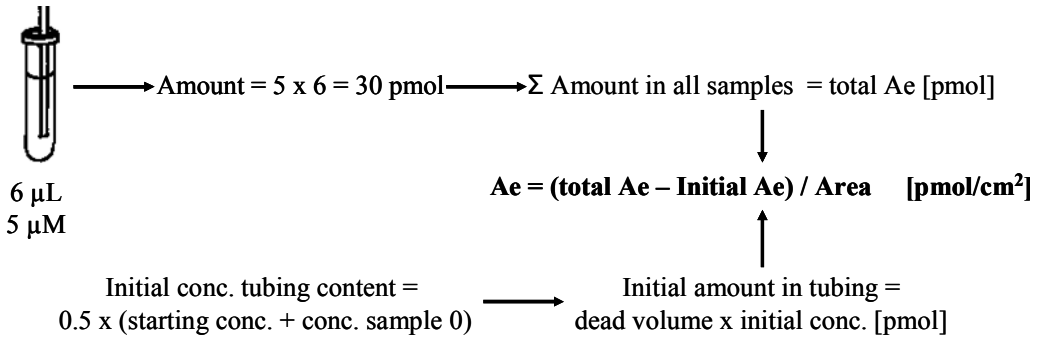
$$A_{c,\ dead\ volume} = 0.5(C_{medium} + C_0) \times \text{dead volume}\,[\text{pmol}] \quad \textbf{[5]}$$

To obtain the amount eluted from the tubing material itself within the first five dead volume exchanges ($Ae_{1\text{-}5}$), the $A_{c,\ dead\ volume}$ is subtracted from the $Ae_{0\text{-}5}$:

$$Ae_{1-5} = \frac{Ae_{0-5} - A_{c,\ dead\ volume}}{\text{inner surface area}} \ [\text{pmol/cm}^2] \quad \textbf{[6]}$$

where the correction for the inner surface area (see Table 5, page 13) is made to give a comparable parameter, regardless of the diameter or length of the tubing used.

Figure 8: Schematic presentation for the calculation of the amount eluted (Ae)



2.3.1.3 Descriptive statistical evaluation of the tubing data

2.3.1.3.1 Graphical and numerical mean and standard deviation

For each tubing material tested (FEP, FEP/Teflon, PEEK, fused silica and silicone), the individual graphical and numerical data are given. For comparison of the different tubing materials a descriptive statistical evaluation is carried out, generally with N = 4, unless otherwise stated. For the graphical comparison, the mean of the '% of starting concentration' at each data point is determined for each material. For a numerical comparison, the mean and standard deviation (SD) of the individual $Ae_{1\text{-}5}$ are determined for each material. The correspondence of the calculated $Ae_{1\text{-}5}$ to the visual graphical appraisal will be assessed.

2.3.1.3.2 Comparison of tubing materials

For both compounds (ZK 975 and ZK 894), the mean graphical data and the mean $Ae_{1\text{-}5}$ of the different tubing materials are compared.

2.3.1.3.3 Compound comparisons

For each material, a comparison of the mean graphical data and the mean $Ae_{1\text{-}5}$ will be made between the two compounds tested (ZK 975 and ZK 894).

2.3.1.3.4 Deduction of suitability criteria

In the discussion (Chapter 4.1.5), cut-off values for Ae will be suggested as decision criteria to classify a tubing material as suitable or unsuitable for pharmacokinetic applications with the tested compound.

2.3.2 Evaluation of microdialysis data

2.3.2.1 Evaluation of individual sample data – microdialysis probes

2.3.2.1.1 Calculation of the sample concentration

The concentration of each sample taken is calculated in the same way as for the tubing materials (see chapter 2.3.1.1.1). Samples which are taken under different experimental conditions (as noted in the experimental protocol, for example if a leakage is observed at the membrane or at a connection point) are defined as outliers. These samples are excluded from any further calculations.

2.3.2.1.2 Calculation of the probe recovery

To convert the concentration of the samples collected with microdialysis to actual tissues concentrations *in vivo*, the recovery of the probe must be determined. *In vitro*, the concentration of the medium surrounding the probe (C_{medium}) is known, and the apparent recovery for each sample (REC_{sample}) can be calculated by relating the concentration of each sample (C_{sample}) to the C_{medium}:

$$REC_{sample} = \frac{C_{sample}}{C_{medium}} \times 100 \ [\%] \qquad [7]$$

For the two rinsing phases (Rinse A and Rinse B, where $C_{medium} = 0$), the REC_{sample} is calculated using the C_{medium} of the preceding concentration phase (Phase A or Phase B). Graphically, the REC_{sample} is depicted on the y-axis.

2.3.2.1.3 Calculation of the membrane mass transfer coefficient

In order to better compare the microdialysis materials, the REC_{sample} is adjusted for flow rate (Q, in this case always 2 µL/min) and the membrane surface area (S, see Table 6) to give the apparent mass transfer coefficient K_{sample} (see Chapter 1.2.2):

$$K_{sample} = \frac{-Q \times \ln(1 - REC_{sample})}{S} \; [\mu L/min/mm^2] \qquad [8]$$

The K_{sample} is depicted graphically on the y-axis, where data from different probe tests are shown in one diagram.

2.3.2.2 Evaluation of individual data sets – microdialysis probes

2.3.2.2.1 Deduction of the overall REC and K

In order to obtain one descriptive numerical value for each microdialysis probe, the average recovery at either concentration phase ($REC_{A/B}$) is determined by taking the mean of the REC_{sample} from the last four samples of the corresponding phase A or B, i.e. for REC_A from samples 7-10, and for REC_B from samples 27-30 (for the sample numbers, see Table 10). The overall recovery (REC) for each probe is calculated as the mean of the REC_{sample} from samples 7-10 and 27-30. Likewise, the average mass transfer coefficient at either concentration ($K_{A/B}$) is determined by taking the mean of the K_{sample} from the last four samples of either phase, i.e. from samples 7-10 for K_A and from samples 27-30 for K_B. The overall mass transfer coefficient (K) is calculated as the mean of the K_{sample} from samples 7-10 and 27-30 (see Table 10).

Table 10: Example of a data set with the calculation of K_A, K_B and K (mean ± standard deviation)

Phase A			Phase B		
Sample number	**K * 10^{-2} [µL/min/mm²]**		**Sample number**	**K * 10^{-2} [µL/min/mm²]**	
1	N/V		21	N/V	
2	16.8		22	18.5	
3	20.1		23	20.0	
4	20.7		24	21.1	
5	20.1		25	21.8	
6	21.0		26	21.5	
7	**22.4**		**27**	**21.6**	
8	**22.0**	→ **K_A = 22.6 ± 1.08**	**28**	**22.4**	→ **K_B = 21.9 ± 0.41**
9	**21.8**		**29**	**21.5**	
10	**24.1**		**30**	**21.9**	

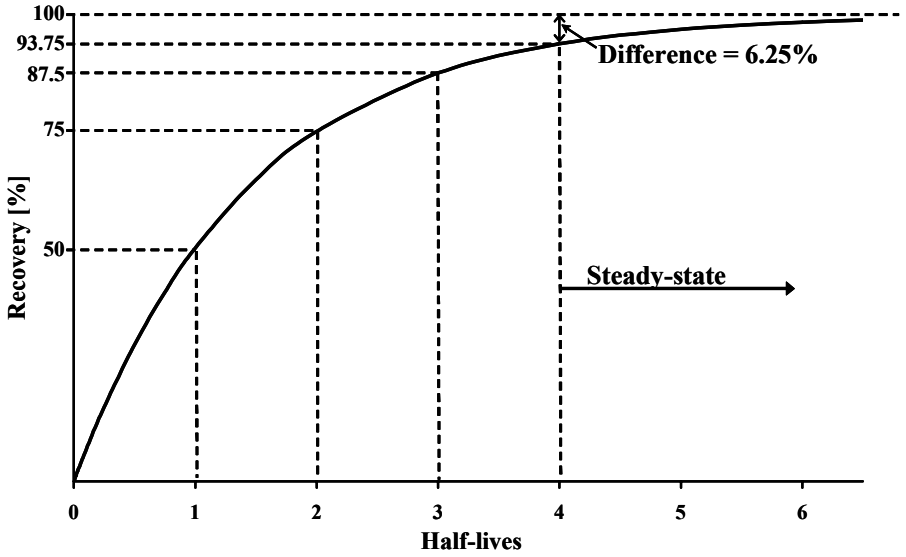
→ **K = 22.2 ± 0.85**

2.3.2.2.2 Description of steady-state

To determine if the overall REC and K are deduced from steady-state conditions, 'steady-state' needs to be defined. For the purpose of this dissertation, steady-state is considered achieved after four half-lives, when 94% of the maximum obtainable level has been reached (see Figure 9). In other words, once steady-state is reached, only a 6.25% margin remains to the 100% asymptote. This would correspond to a difference of ≤ 6.25% between any two data points taken at steady-state. Thus, steady-state is considered achieved when the calculated coefficient of variance (CV)

between the last 4 REC-values at either phase is ≤ 6.25%. If steady-state is not reached, the $REC_{A/B}$ and $K_{A/B}$ for that probe are marked with an asterix (*), as an indication that the calculated means are only apparent values, rather that true mean values at steady-state.

Figure 9: Graphical representation of steady-state after 4 half-lives



2.3.2.2.3 Percentage of the ideal Area Under the Data achieved

In an attempt to numerically describe the shape of the curve, with relevance to pharmacokinetic implications, a pharmacokinetic evaluation of the recovery data was initially carried out to determine the half-life of a probe's response to concentration changes. However, different compartmental models had to be used for different data sets, and often also for the different phases recorded within each data set. This made comparison of the obtained half-lives difficult and highly subjective.

Another approach attempted was to determine the time needed to reach steady-state (for the concentration phases), or to reach concentrations below 5 or 1% of the starting concentration (for the rinsing phases), as was done by Tao and Hjorth, 1992. This resulted in exact values given in minutes, giving the false impression that the method allows a 1-minute resolution of the data obtained. Since the resolution of the data depends on the collection interval chosen for the experiment, this approach proved also unsatisfactory.

Therefore, to allow a description of the curve that gives a single numerical parameter, that reflects the graphical data regardless of the collection interval, the observation time or the size of membrane used, and that is a good indicator of the responsiveness of a probe to concentration changes, the new parameters %iAUD (see below) and Ae (see Chapter 2.3.2.2.4) are introduced instead.

An 'ideal' probe for pharmacokinetic use has a negligible equilibration time, so that steady-state is reached at the first sample taken (see Figure 10). Considering however that the first sample taken at each phase is a mixed sample, due to the dead volume in the outlet tubing, the area under the data for this ideal microdialysis probe (iAUD) is calculated from the second sample onwards by multiplying the $K_{A/B}$ at either concentration with the number of sample intervals (= 8 for samples 2 to 9 and for

samples 22 to 29):

$$iAUD = K_{A/B} * 8 \quad [\mu L/min/mm^2] \qquad [9]$$

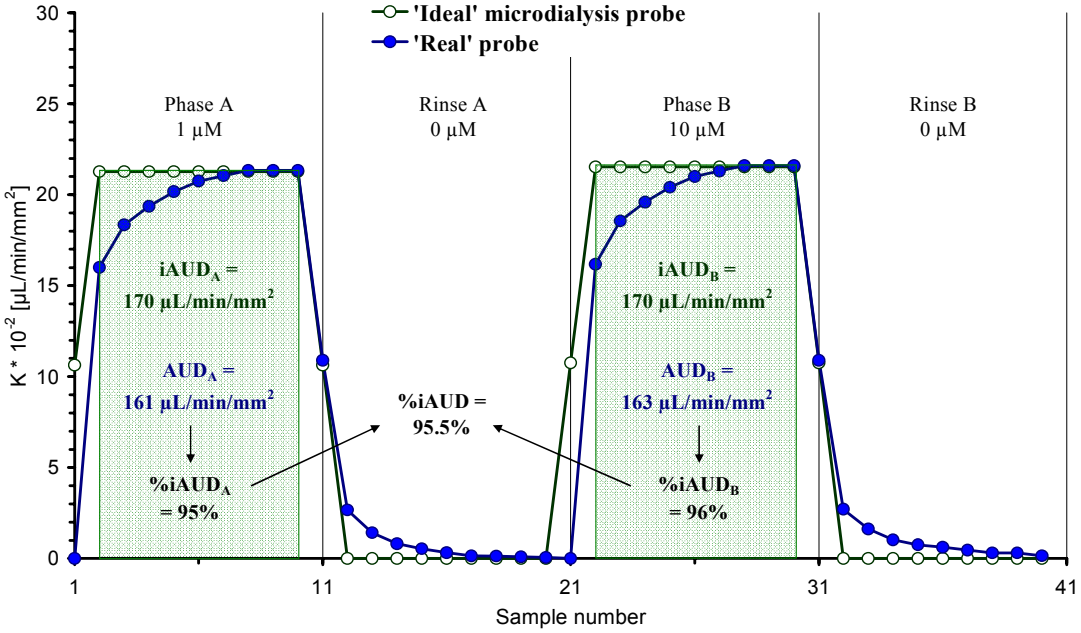
Having determined the ideal AUD, the true AUD of the collected data set is compared to this iAUD to give the percentage of the ideal AUD achieved (%iAUD):

$$\%iAUD_{A/B} = \frac{AUD_{A/B}}{iAUD_{A/B}} x100 \qquad [10]$$

This %iAUD is determined for Phase A and Phase B separately, then combined for both phases:

$$total\ \%iAUD = \frac{AUD_A + AUD_B}{iAUD_A + iAUD_B} x100 \qquad [11]$$

Figure 10: Example data from an imaginary probe, and calculation of the %iAUD



AUD = Area under the data iAUD = ideal AUD

2.3.2.2.4 Calculation of the amount eluted

As with the concentration phases, an evaluation of the rinsing phases with calculation of a half-life was not possible for many data sets, even when using compartmental modeling. To use a comparable parameters for all data sets, the amount eluted (Ae) from the total surface of the membrane is calculated instead (see equation [17]). To derive this parameter from the available data, first the amount eluted is calculated for each rinsing phase separately, in analogy to the tubing data (Chapter 2.3.1.2.1):

$$Ae_{first\ hour,\ A/B} = Ae_{total,\ A/B} - A_{c,\ dead\ volume} \quad [pmol] \ , \qquad [12]$$

with the dead volume being the sum of the dead volumes of the tubing material (54 cm fused silica = 4.24 µL), and the dead volume of the microdialysis probe (see Table 6, Chapter 2.1.3). The concentration in the dead volume equals that of the last sample taken for the preceding rinsing phase (sample 10 or sample 30).

Since the exact exposed surface area of the microdialysis membrane is not known, the total amount eluted is related to the total membrane volume (see Table 6), rather than to the internal surface area, as for the tubing materials:

$$Ae_{A/B} = \frac{Ae_{total,\,A/B} - A_{c,\,dead\ volume}}{membrane\ volume} \quad [pmol/mm^3] \qquad [\,13\,]$$

Also, the amount eluted from the membrane includes not only compound reversibly bound to the exposed membrane material (A_b), but also unbound compound still present in the fluid filled pores (A_u, see ▲ and Δ in Figure 12). Thus the collected amount eluted at either rinsing phase also includes an unknown unbound fraction:

$$Ae_A = A_{b,A} + A_{u,A} \quad \wedge \quad Ae_B = A_{b,B} + A_{u,B} \qquad [\,14\,]$$

The only aspect known about this unbound fraction is that the concentration at Phase B is ten times the concentration at Phase A, i.e. $A_{u,A}$:$A_{u,B}$ = 10:1, or more precisely:

$$\frac{A_{u,B}}{A_{u,A}} \equiv \frac{C_B}{C_A} \quad \rightarrow \quad A_{u,B} = \frac{C_B}{C_A} A_{u,A} \quad , \qquad [\,15\,]$$

where C_A is the concentration of the last sample collected for Phase A (= sample 10, representing the free concentration of compound within the pores at that time) and the C_B is the corresponding concentration of sample 30 at Phase B (for the allocation of sample numbers, see tables in Appendix II).

If binding does occur however, it can be presumed that the number of available binding sites is constant, regardless of the concentration used, and therefore:

$$A_{b,B} = A_{b,A} = A_{bound} \qquad [\,16\,]$$

Thus, provided that saturation at the end of the concentration phases is complete, and that all the membrane bound compound is eluted within the one hour observation period, then the true amount eluted from the actual membrane material (Ae) can be calculated by combining [14], [15] and [16], and solving the resulting equations (see also Figure 11 and Figure 12):

$$A_{u,A} = Ae_A - A_{bound} \quad \wedge \quad A_{bound} = Ae_B - A_{u,B}$$

$$\downarrow$$

$$A_{u,A} = Ae_A - (Ae_B - A_{u,B}) \quad \wedge \quad A_{u,B} = \frac{C_B}{C_A} A_{u,A}$$

$$\downarrow$$

$$A_{u,A} = Ae_A - Ae_B + \frac{C_B}{C_A} A_{u,A}$$

$$\downarrow$$

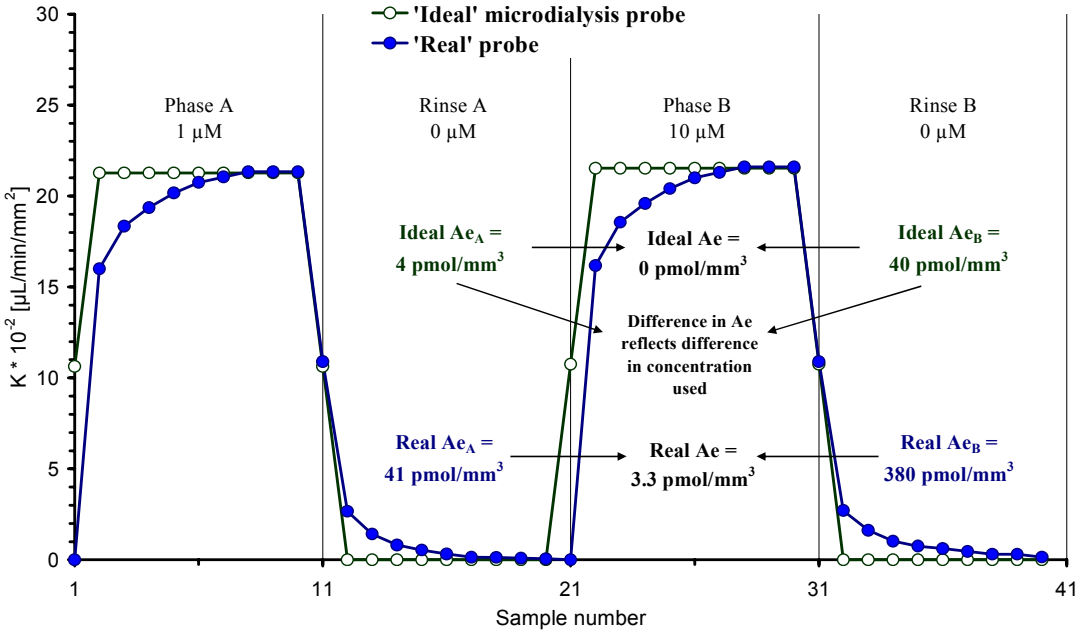
$$A_{u,A} = \frac{Ae_B - Ae_A}{\frac{C_B}{C_A} - 1} \quad \wedge \quad A_{bound} = Ae_A - A_{u,A}$$

$$\downarrow$$

$$\boxed{A_{bound} = Ae_A - \frac{Ae_B - Ae_A}{\frac{C_B}{C_A} - 1} = Ae \quad [pmol/mm^3]} \qquad [17]$$

Figure 11 shows an example of a K versus time curve for an imaginary probe and the corresponding ideal curve, giving possible Ae_A and Ae_B values, to demonstrate how the numerical values compare for different elution patterns during the rinsing phases (in this case no elution versus some elution observed).

Figure 11: Example data from an imaginary probe, and calculation of the amount eluted (Ae)

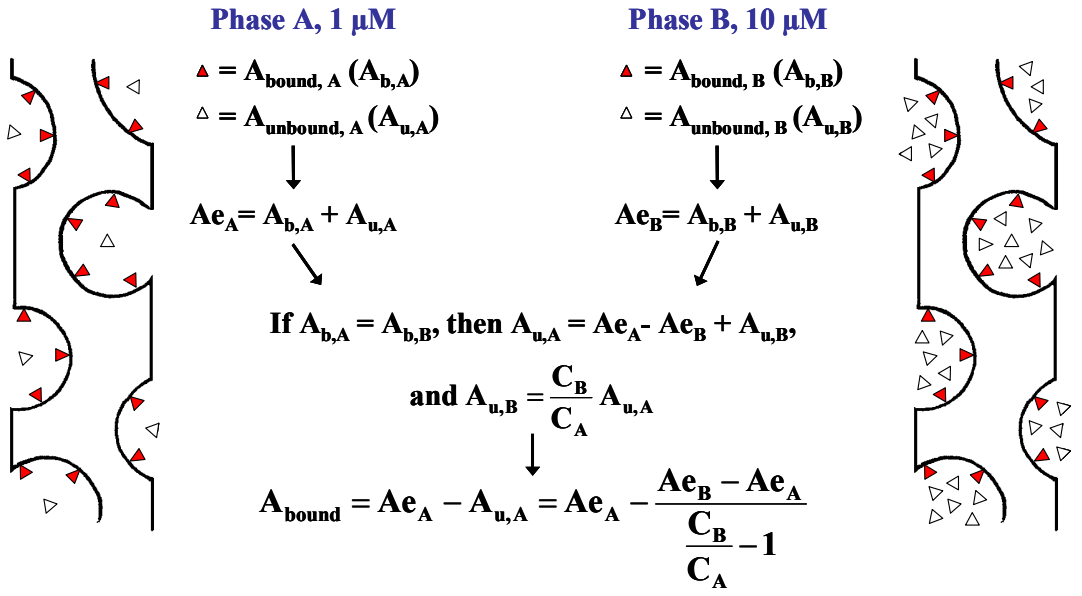


So, for the example given in Figure 11:

$$Ae = Ae_A - \frac{Ae_B - Ae_A}{C_B/C_A - 1} = 41 - \frac{380 - 41}{10/1 - 1} = 3.3 \text{ pmol/mm}^3 \qquad [18]$$

Note that for suitable materials with fast responsiveness all presumptions and conditions apply (binding capacity is constant, saturation of binding sites is complete, and all amount eluted is collected). For materials with such a slow responsiveness that any of the conditions do not apply, the concentration ratio C_B/C_A will be weighted more heavily compared to the membrane bound fraction, and the Ae will be underestimated. Nonetheless, Ae should still be sufficiently large to show that extensive binding is taking place.

Figure 12: Schematic presentation of compound distribution within the microdialysis membrane at the end of each concentration phase, and calculation of A_{bound}



$A_{A/B}$ = Amount at phase A or B
$Ae_{A/B}$ = Amount eluted at phase A or B
$C_{A/B}$ = Concentration at phase A or B

2.3.2.3 Descriptive statistical evaluation of microdialysis data

2.3.2.3.1 Graphical and numerical mean and standard deviation

For each material tested, the mean recovery and mass transfer coefficient K at each data point are determined for the graphical representation. For a numerical description, the mean and standard deviation of the overall K, the %iAUD and the Ae are determined for each material. The overall mean K is calculated as the mean of the 8 individual K_A and K_B for the material/compound combination tested (two concentration phases per probe, and generally N = 4, unless otherwise stated). Likewise, the overall mean %iAUD is calculated as the mean of the 8 individual $\%iAUD_A$ and $\%iAUD_B$. Since Ae is calculated using the data from both rinsing phases, the overall mean Ae is the mean of only 4 individual values. The correspondence of the calculated numerical values to the visual graphical appraisal will be assessed.

2.3.2.3.2 Comparison of microdialysis probe materials

For both compounds (ZK 975 and ZK 894), the mean data of the different tubing materials are compared, graphically and numerically. For the graphical comparison, the mean K versus time (= sample number) is shown, for the numerical comparison the parameters K, %iAUD and Ae are used.

For either compounds separately, the different materials studied are compared with regards to their suitability for pharmacokinetic applications (see also Chapter 2.3.2.3.4 below). First, the different membrane materials tested are compared (20 kDa PC, 30 kDa PAN, 35 kDa PES, 38 kDa Cell and 6 kDa Cu). For the microdialysis probes with PES membranes, the influence of pore size on probe performance is evaluated (6, 15, 35 and 100 kDa). In addition, for the 6 kDa PES membranes, the effect of different probe outlet materials is studied (steel or PEEK), as well as the effect of membrane size (3.02 mm^2 or 7.54 mm^2). Finally, for the cellulose type membranes (Cell and Cu), the influence of membrane thickness on probe performance is determined (5, 20 or 30 µm).

2.3.2.3.3 Compound comparisons

For all material used, a numerical comparison of the mean K, %iAUD and Ae will be made between the two compounds tested (ZK 975 and ZK 894), in order to evaluate the usefulness of these three parameters as descriptors of the suitability of a microdialysis probe for pharmacokinetic applications.

2.3.2.3.4 Deduction of suitability criteria

According to the data collected, cut-off values for the %iAUD and the Ae are suggested, that would classify a microdialysis probe as:

- 'suitable for pharmacokinetic applications',
- 'suitable for determination of steady-state concentrations only', or
- 'not suitable for pharmacokinetic applications' with the tested compound.

An optimal membrane material, 'suitable for pharmacokinetic applications', has no adhesion of the compound tested, and would therefore reach steady-state immediately upon changing the concentration in the surrounding medium (%iAUD approximates 100%, Ae approximates 0 $pmol/mm^3$). This membrane can be used for obtaining pharmacokinetic profiles with rapid concentration changes.

As ' suitable for determination of steady-state concentrations only' can be defined a membrane with slight adhesion of the dialyzed compound, but which does reach steady-state within 30 min of changing the surrounding concentration, as reflected by a reasonable high %iAUD, and a reasonably low Ae. This membrane can still be used for monitoring concentrations that change only slowly, for example to determine steady-state concentrations during an infusion.

An unsuitable membrane material suffers strong adherence of the compound of interest, with steady-state conditions not being reached within one hour of changing the surrounding concentration (low %iAUD and high Ae). This material cannot be used for quantitative microdialysis.

Since K is a parameter obtained at steady-state, K gives no information on a probe's responsiveness to concentration changes and is therefore not useful as a suitability criterion for pharmacokinetic applications. Since K is directly related to the recovery, K must be sufficiently high however to allow analysis of the samples obtained with the available analytical method. Therefore, K is also compared between the tested materials and compounds.

All calculations are carried out using Microsoft® Office Excel 2003.