3 Results

3.1 Results tubing materials

As a reminder, the relevant dimensions of the tubing materials tested are shown in Table 11. For the complete list, see Table 5, Chapter 2.1.2.

Table 11: Dimensions of the five tubing materials tested

Material	OD [mm]	ID [mm]	Dead volume [µL]	Inner surface area [cm^2]
FEP	0.80	0.12	12	3.9
FEP/Teflon	0.65	0.12	12	3.9
PEEK	0.65	0.12	12	3.9
Fused silica	0.36	0.10	7.9	3.1
Silicone	0.76	0.25	23	3.6

OD Outer diameter
ID Inner diameter

In Chapter 3.1.1 below, the individual '% of starting concentration' versus 'number of dead volume exchanges' curves, and the individual Ae are presented for each tubing material tested. In Chapter 3.1.2 the corresponding mean curves and Ae are compared between the five tubing materials, and between the two compounds tested.

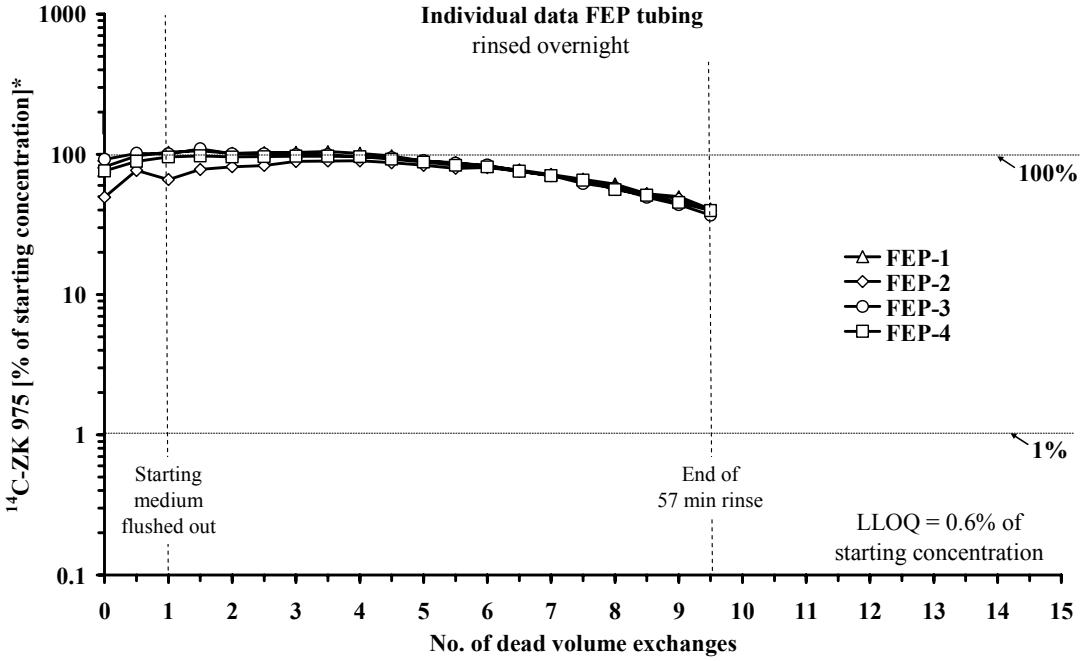
3.1.1 Individual graphical data and Ae for tubing materials

3.1.1.1 Results individual FEP tubes

Using FEP tubes and the lipophilic compound ZK 975 (see Figure 13), the starting concentration was not reached at the end of the priming phase (the individual concentrations of sample 0 ranged from 50 to 92% of starting concentration), indicating that saturation of binding sites on the tube surface was not complete. Compound concentrations remained high throughout the one hour rinsing phase. The $Ae_{1\text{-}5}$ ranged from 42 to 57 pmol/cm^2, but collection of eluted compound was not complete after the 5th dead volume exchange, and the total binding capacity of the FEP tubing for ZK 975 is therefore even higher.

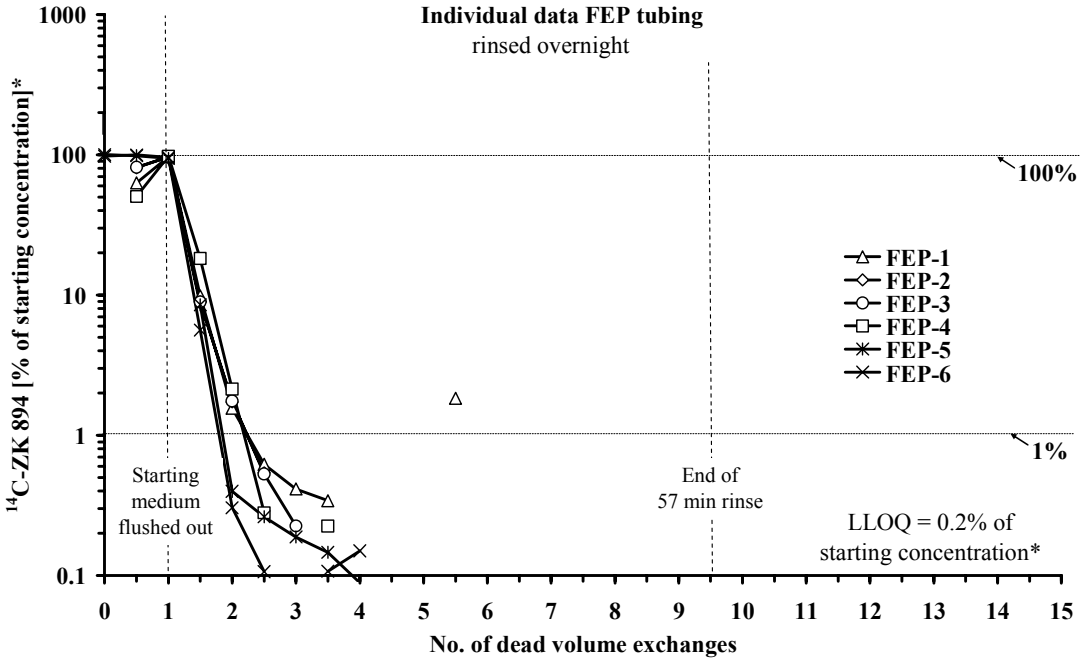
With the hydrophilic compound ZK 894 (see Figure 14), C_0 was 98-100% of the starting concentration (N = 2). Upon rinsing, concentrations of < 1% of the starting concentration were reached after two dead volume exchanges. The $Ae_{1\text{-}5}$ ranged from 1.6 to 3.0 pmol/cm^2, with all eluted compound being collected, indicating that compound binding was only minimal.

Figure 13: Time course of ZK 975 elution from FEP tubes after 1 hour incubation with ^{14}C-ZK 975, tubes rinsed overnight with Ringer's solution



* Starting concentration = 4.8 µM

Figure 14: Time course of ZK 894 elution from FEP tubes after 1 hour incubation with ^{14}C-ZK 894, tubes rinsed overnight with Ringer's solution



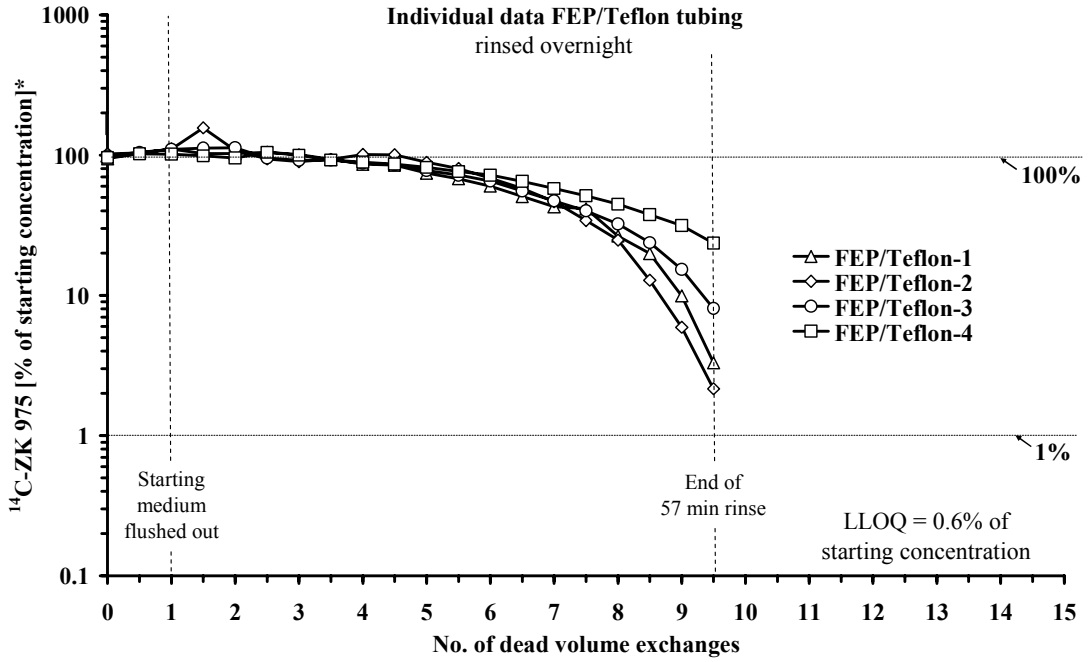
* Starting concentration = 10 µM for tubes 1-4, and 6.8 µM for tubes 5 and 6, samples <LLOQ were set to zero, and are not shown in this semi-log graph.
Note: the first two samples from tubes 1-4 are implausible (sample 0 is 0% and not shown), therefore N = 2 for these two samples, and N = 6 for samples 2 onwards.

3.1.1.2 Results individual FEP/Teflon tubes

Using FEP/Teflon tubes and the lipophilic compound ZK 975 (see Figure 15), the starting concentration was reached at the end of the priming phase (C_0 was 94-102% of the starting concentration). During the rinsing phase, compound concentrations in the eluate remained high for most of the one hour observation period. The Ae_{1-5} ranged from 52 to 61 pmol/cm^2, but collection of eluted compound was not complete, and the total binding capacity of the FEP/Teflon tubing for ZK 975 is therefore even higher.

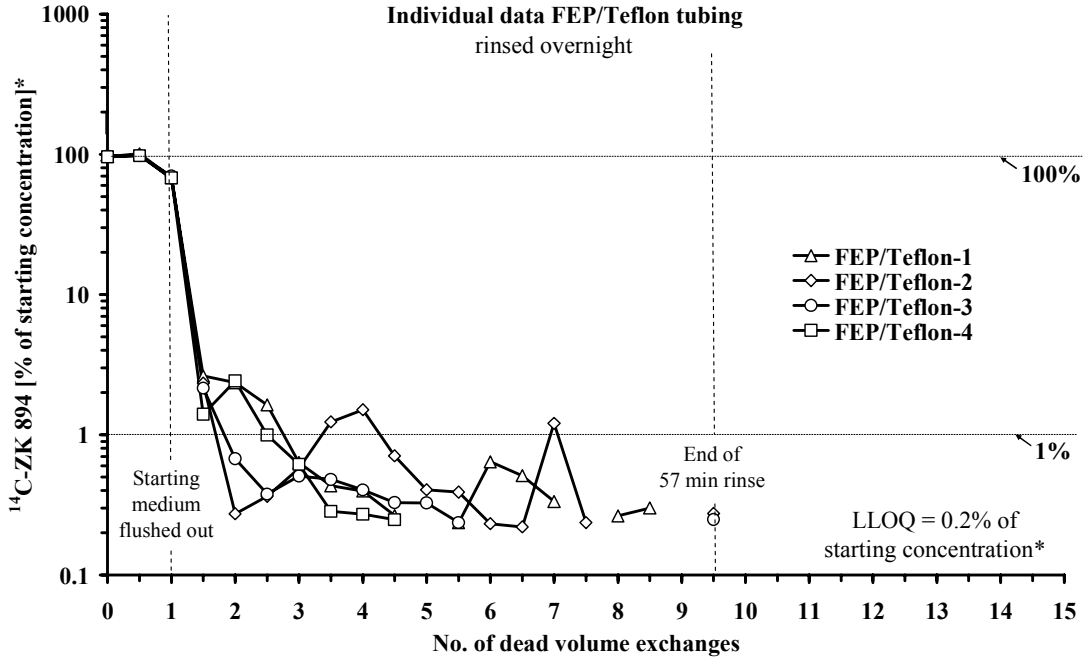
With the hydrophilic compound ZK 894 (see Figure 16), C_0 was 96-97% of the starting concentration. Upon rinsing, concentrations of < 1% of the starting concentration were reached after two dead volume exchanges. The Ae_{1-5} was always 0 pmol/cm^2, indicating that no binding of the compound had taken place.

Figure 15: Time course of ZK 975 elution from FEP/Teflon tubes after 1 hour incubation with ^{14}C-ZK 975, tubes rinsed overnight with Ringer's solution



* Starting concentration = 4.8 µM

Figure 16: Time course of ZK 894 elution from FEP/Teflon tubes after 1 hour incubation with ^{14}C-ZK 894, tubes rinsed overnight with Ringer's solution



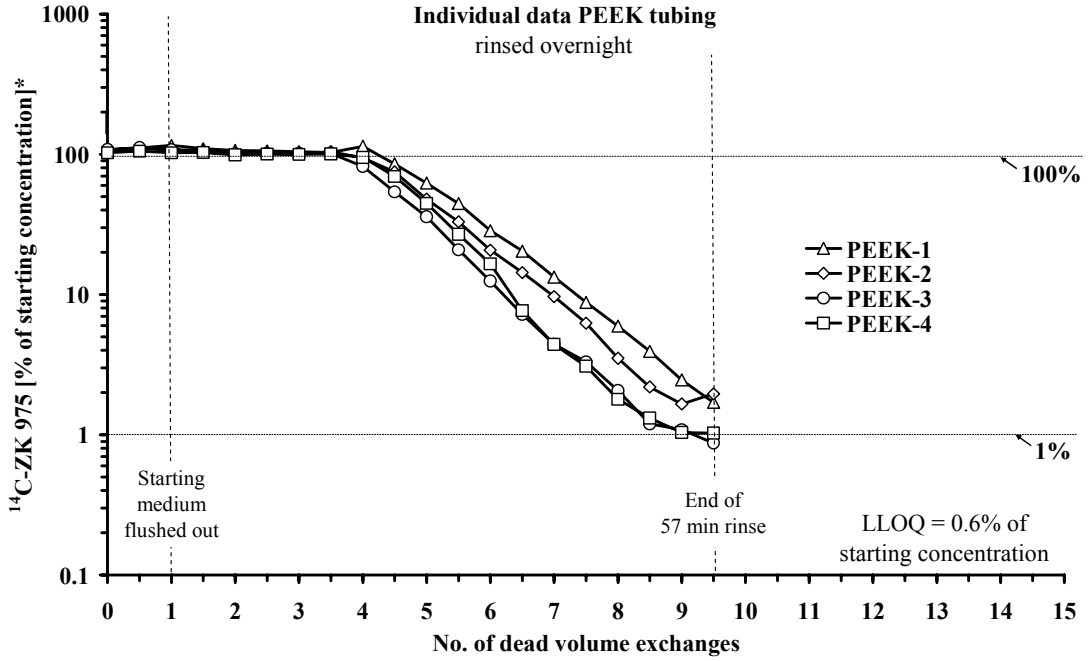
* Starting concentration = 10 µM, samples <LLOQ were set to zero, and are not shown in this semi-log graph (giving the appearance of a broken line)

3.1.1.3 Results individual PEEK tubes

Using PEEK tubes and the lipophilic compound ZK 975 (see Figure 17), the starting concentration was reached at the end of the priming phase(C_0 was 102-108% of the starting concentration). During the rinsing phase, compound concentrations remained near 100% of the starting concentration for the first 4 dead volume exchanges, then decreased slowly. The Ae_{1-5} ranged from 47 to 50 pmol/cm^2, but collection of eluted compound was not complete, and the total binding capacity of the PEEK tubing for ZK 975 is therefore even higher.

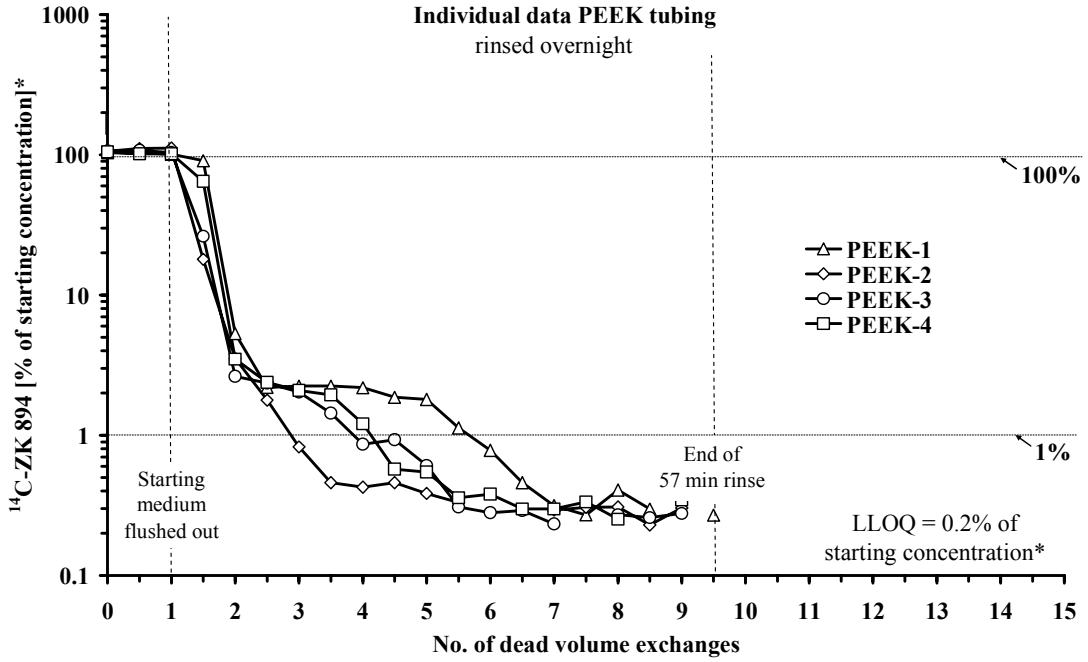
With the hydrophilic compound ZK 894 (see Figure 18), C_0 was 103-106% of the starting concentration. Upon rinsing, concentrations of < 1% of the starting concentration were reached only after 3 to 6 dead volume exchanges. Concentrations < 3% of the starting concentration however were already reached after two dead volume exchanges. The Ae_{1-5} ranged from 0.3 to 11 pmol/cm^2 (with all eluted compound being collected), indicating a variable binding capacity of PEEK for ZK 894. This variability might be due to batch differences of the tubing material. The PEEK tubes were supplied in precut 1-meter lengths, and batches and storage times of these individual tubes may have differed (the batch number and date of purchase of each tube could not be reconstructed retrospectively).

Figure 17: Time course of ZK 975 elution from PEEK tubes after 1 hour incubation with ^{14}C-ZK 975, tubes rinsed overnight with Ringer's solution



* Starting concentration = 4.8 µM

Figure 18: Time course of ZK 894 elution from PEEK tubes after 1 hour incubation with ^{14}C-ZK 894, tubes rinsed overnight with Ringer's solution



* Starting concentration = 10 µM, samples <LLOQ were set to zero, and are not shown in this semi-log graph

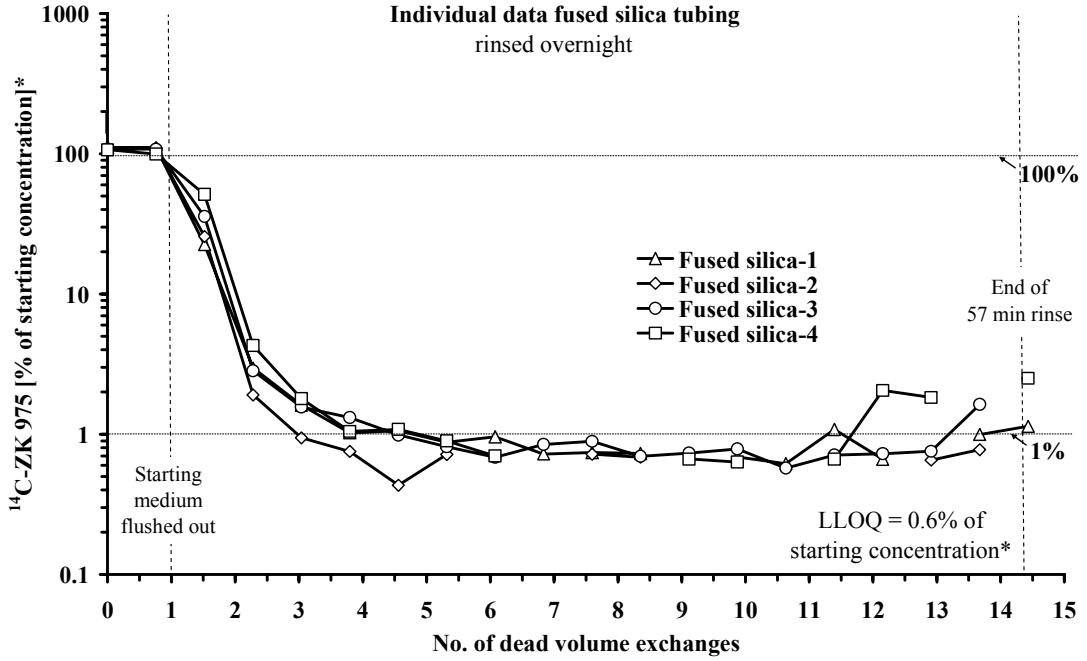
3.1.1.4 Results individual fused silica tubes

Using fused silica tubes and the lipophilic compound ZK 975 (see Figure 19), C_0 was 107-110% of the starting concentration. Upon rinsing, compound concentrations fell rapidly below 1% of starting concentration within 3 to 5 dead volume exchanges. The Ae_{1-5} ranged from 0 to 0.3 pmol/cm^2, indicating that no binding of the compound had taken place.

With the hydrophilic compound ZK 894 (see Figure 20), C_0 was 107-108% of the starting concentration. Upon rinsing, concentrations of < 1% of the starting concentration were also reached rapidly within 2 to 3 dead volume exchanges. The Ae_{1-5} was always 0 pmol/cm^2, indicating that no binding of compound had taken place.

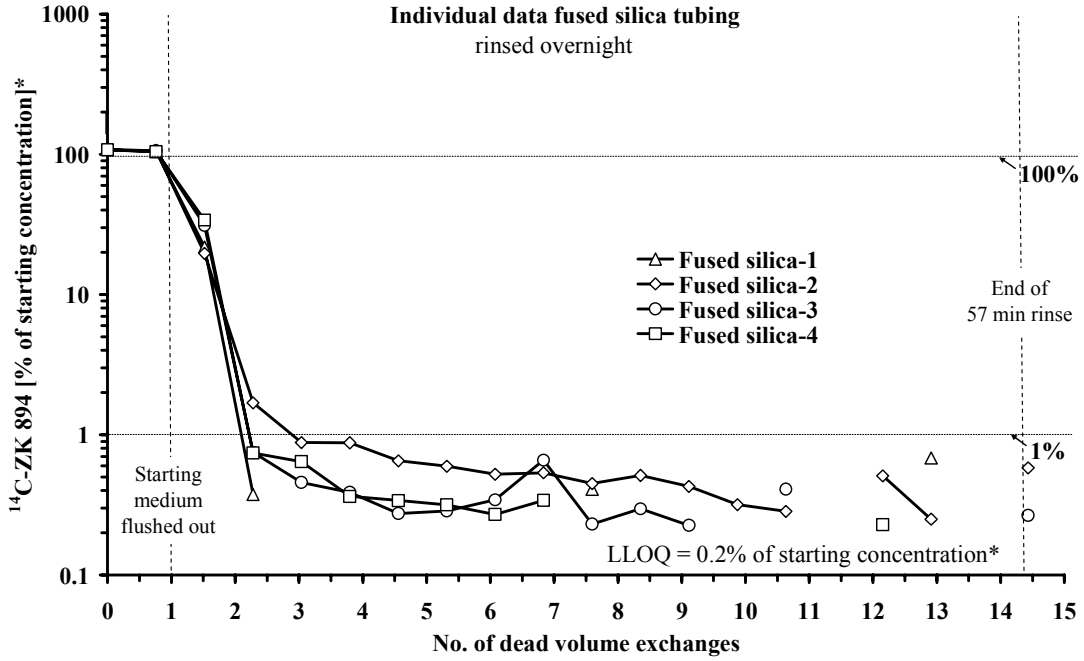
The minimal apparent bleeding observed with either compound below 1% of the starting concentration is quantitatively not relevant (as is evident from the 0 pmol/cm^2 Ae), and would have no consequence for any pharmacokinetic study.

Figure 19: Time course of ZK 975 elution from fused silica tubes after 1 hour incubation with ^{14}C-ZK 975, tubes rinsed overnight with Ringer's solution



* Starting concentration = 4.8 µM, samples <LLOQ were set to zero, and are not shown in this semi-log graph

Figure 20: Time course of ZK 894 elution from fused silica tubes after 1 hour incubation with ^{14}C-ZK 894, tubes rinsed overnight with Ringer's solution



* Starting concentration = 10 µM, samples <LLOQ were set to zero, and are not shown in this semi-log graph (giving the appearance of a broken line)

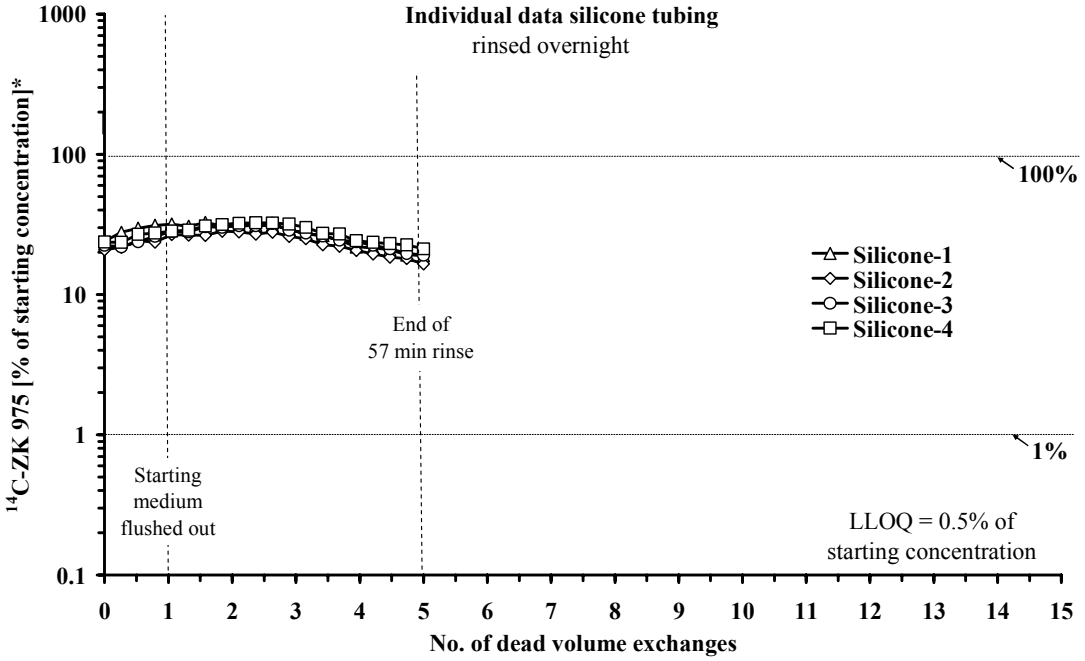
3.1.1.5 Results individual silicone tubes

Using silicone tubes and the lipophilic compound ZK 975 (see Figure 21), the starting concentration was not reached at the end of the priming phase (the mean concentration of sample 0 was barely 23% of the starting concentration), indicating that saturation of binding sites was not complete. Also, compound concentrations remained high throughout the one hour rinsing phase. The $Ae_{1\text{-}5}$ ranged from 17 to 22 pmol/cm^2, but collection of eluted compound was not complete, and the total binding capacity of the silicone tubing for ZK 975 is therefore even higher.

With the hydrophilic compound ZK 894 (see Figure 22), C_0 was 110-112% of the starting concentration. Upon rinsing, concentrations of < 1% of the starting concentration were reached only after four dead volume exchanges. The $Ae_{1\text{-}5}$ ranged from 3.7 to 5.5 pmol/cm^2, but collection of eluted compound was not complete, again indicating that the actual total binding capacity of the silicone tubing for ZK 894 is somewhat higher.

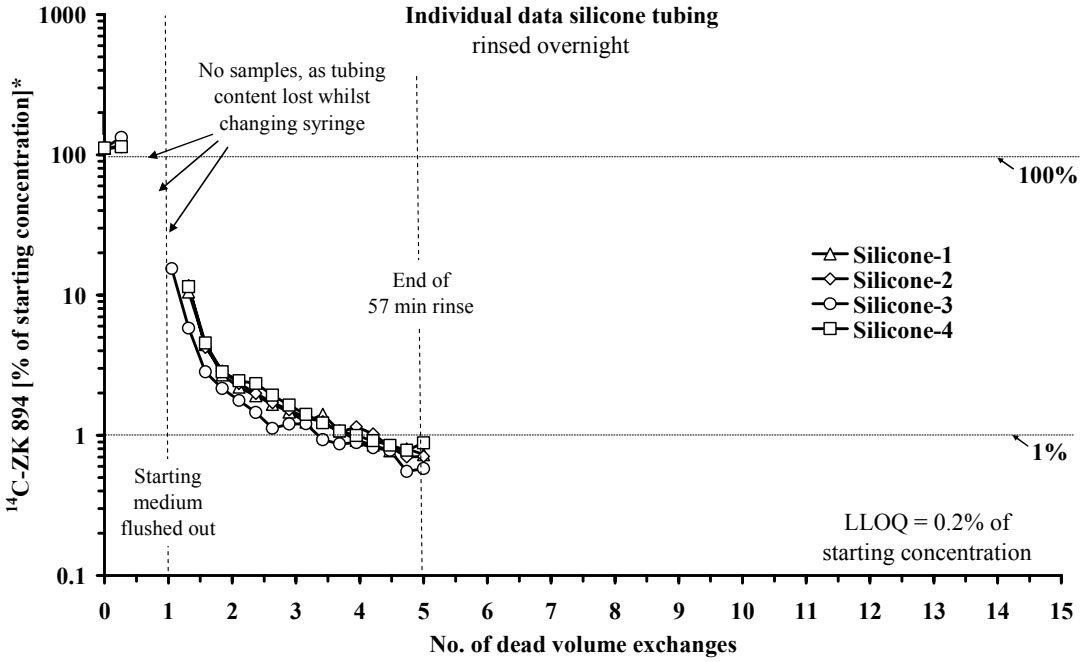
(With ZK 894, the content of the tubes was lost when the syringes were exchanged, due to the large internal diameter and lack of capillary action (the tubing content flowed out as the tubes were held upright). Hence no correction was made for the initial tubing content for the calculation of the $Ae_{1\text{-}5}$ (which will not affect the value).

Figure 21: Time course of ZK 975 elution from silicone tubes after 1 hour incubation with ^{14}C-ZK 975, tubes rinsed overnight with Ringer's solution



* Starting concentration = 4.5 µM

Figure 22: Time course of ZK 894 elution from silicone tubes after 1 hour incubation with ^{14}C-ZK 894, tubes rinsed overnight with Ringer's solution



* Starting concentration = 9.8 µM

Note: although a number of data are missing, a repeat experiment was not carried out, since the high binding of compound precludes this material for pharmacokinetic applications anyway.

3.1.2 Mean graphs and Ae for tubing materials

3.1.2.1 Comparison of mean results for different tubing materials tested

3.1.2.1.1 Mean results for tubing materials tested with ZK 975

The mean concentration-time curves for the tubing materials tested with ZK 975 are shown in Figure 23 below. The individual (●) and mean (—) Ae_{1-5} are graphically depicted in Figure 24.

All the 'conventional' microdialysis tubing materials FEP, FEP/Teflon and PEEK showed extensive adhesion of the lipophilic compound ZK 975, with prolonged bleeding during the rinsing phase. This binding was reflected by a high mean Ae_{1-5} of 52 ± 6.8 pmol/cm^2 (FEP), 55 ± 4.2 pmol/cm^2 (FEP/Teflon), and 48 ± 1.6 pmol/cm^2 (PEEK).

Silicone also showed profound adhesion of the lipophilic compound ZK 975, with the binding sites not being saturated even after 1 hour priming with a 10 µM solution, and collected concentrations remaining high throughout the rinsing period. The calculated mean Ae_{1-5} was still high at 20 ± 2.6 pmol/cm^2, but collection of eluted compound was far from complete.

Only fused silica performed well with ZK 975, with mean concentrations of < 5% of starting concentration being reached within 2 dead volume exchanges, and mean concentrations of < 1% of starting concentration being reached within 5 dead volume exchanges. The low mean Ae_{1-5} of 0.007 ± 0.014 pmol/cm^2 confirmed that no binding of the compound to the tubing material had occurred.

Thus, of the five tubing materials tested, only fused silica is suitable for pharmacokinetic studies with ZK 975.

Figure 23: Mean time courses of ZK 975 elution from all tubing materials after 1 hour incubation with ^{14}C-ZK 975, tubes rinsed overnight with Ringer's solution

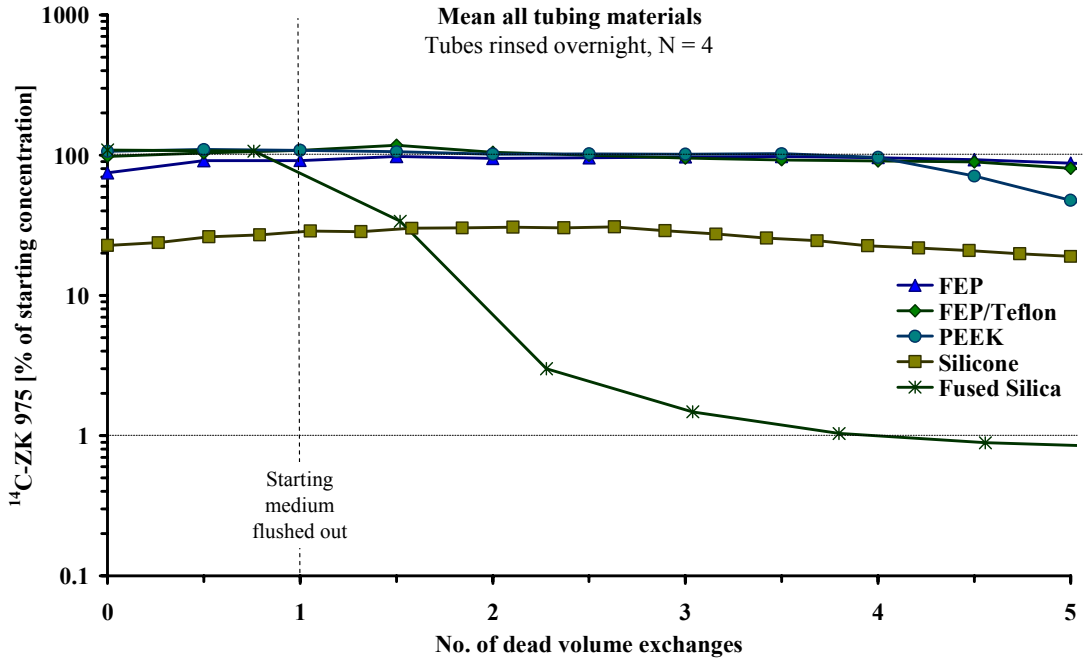
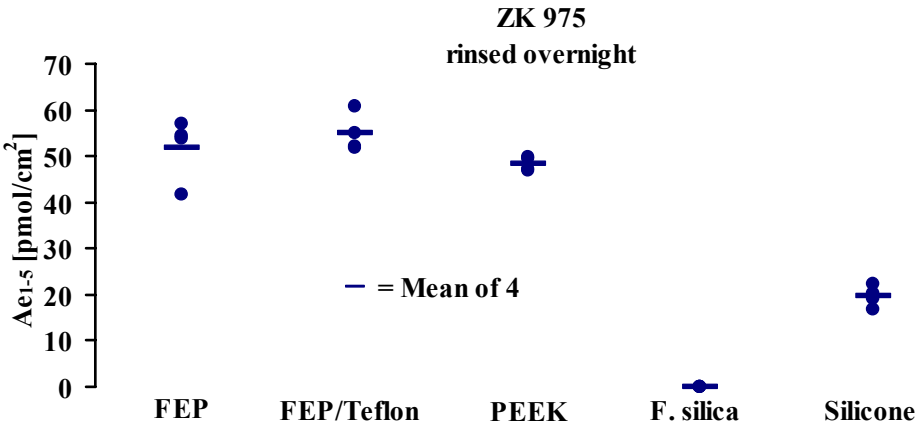


Figure 24: Individual and mean Ae_{1-5} for all tubing materials tested with ZK 975



3.1.2.1.2 Mean results of tubing materials tested with ZK 894

The mean concentration-time curves for the tubing materials tested with ZK 975 are shown in Figure 25 below. The individual (○) and mean (—) Ae_{1-5} are graphically depicted in Figure 26.

Of the 'conventional' microdialysis tubing materials, FEP and FEP/Teflon showed little or no adhesion of the hydrophilic compound ZK 894: concentration of < 1% were reached within 3 dead volume exchanges, and the mean Ae_{1-5} was very low at 2.2 ± 0.6 pmol/cm^2 (FEP, N = 6) and 0 ± 0 pmol/cm^2 (FEP/Teflon).

The only other tubing material performing consistently well was fused silica, with concentrations of < 1% of starting concentration being reached within 2 dead volume exchanges. The calculated Ae_{1-5} was always 0 pmol/cm^2, indication that no adhesion of the compound ZK 894 occurred.

The microdialysis tubing PEEK on the other hand showed variable adhesion of the hydrophilic compound ZK 894, with concentration of < 1% of starting concentrations being reached on average only after 4.5 dead volume exchanges, but ranging from 3 to 6 dead volume exchanges. Likewise, the mean Ae_{1-5} was 5.1 ± 4.4 pmol/cm^2, ranging from a 'good' 0.3 to a 'mediocre' 11 pmol/cm^2. This variation could be due to batch differences, as discussed in Chapter 3.1.1.3 above.

Also Silicone showed evidence of slight adhesion of the hydrophilic compound ZK 894, with concentration of < 1% of starting concentrations being reached on average only after 4 dead volume exchanges. The Ae_{1-5} was 4.9 ± 0.8 pmol/cm^2, but collection of eluted compound was not entirely complete at the end of the 1 hour rinsing phase.

Overall, based on the findings with the hydrophilic compound ZK 894, the three tubing materials FEP, FEP/Teflon and fused silica can be recommended without reservations for pharmacokinetic applications of this hydrophilic compound, whereas the usefulness of PEEK and silicone should be assessed according to requirements.

Figure 25: Mean time courses of ZK 894 elution from all tubing materials after 1 hour incubation with ^{14}C-ZK 894, tubes rinsed overnight with Ringer's solution

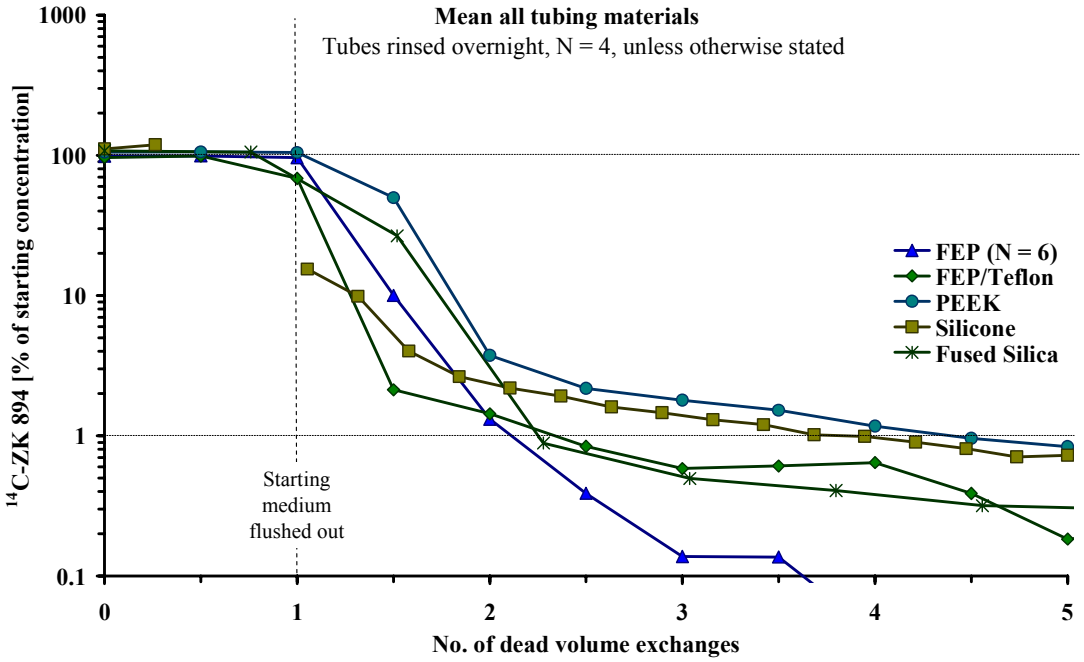
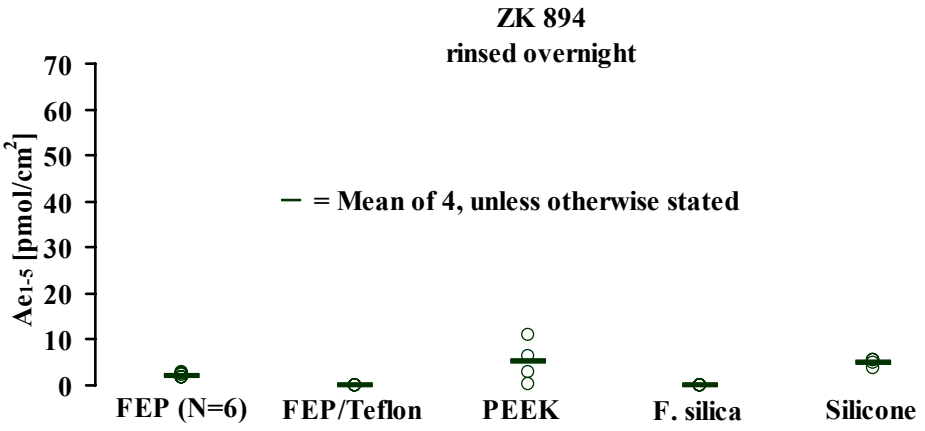


Figure 26: Individual and mean Ae_{1-5} for all tubing materials tested with ZK 894



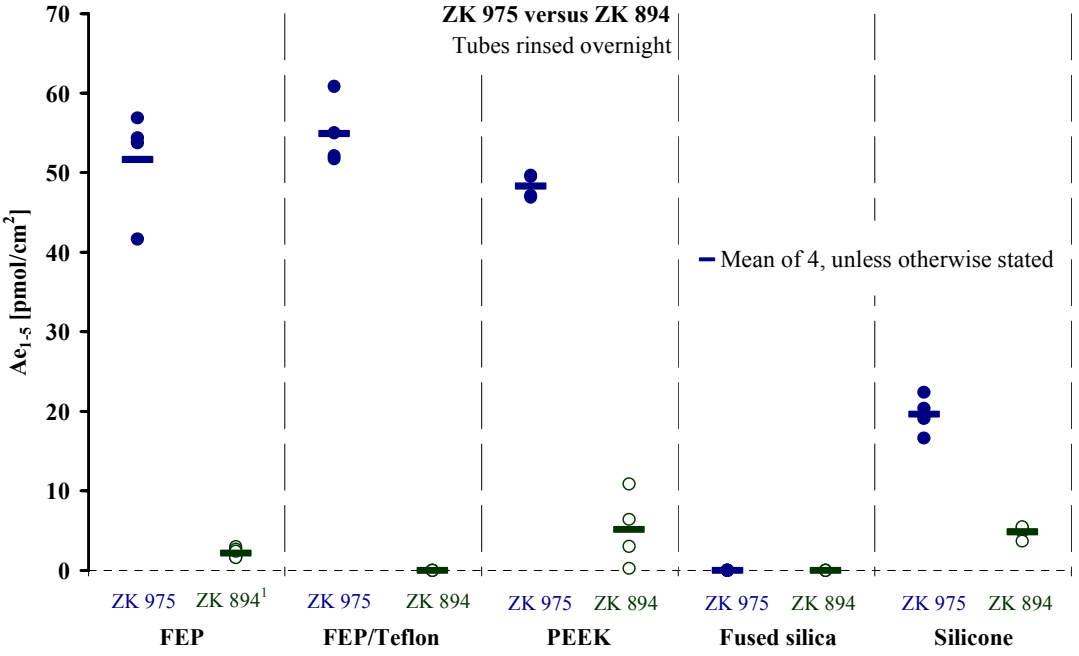
3.1.2.2 Comparison of mean tubing results for ZK 975 versus ZK 894

The individual (● for ZK 975 and ○ for ZK 894) and mean (▬) Ae_{1-5} for all materials and both compounds tested are shown graphically in Figure 27 below.

The adhesion properties of the tubing materials differed considerably between the two compounds tested, except for fused silica. Fused silica performed equally well with both compounds, with no adhesion being observed. For all other materials (FEP, FEP/Teflon, PEEK and silicone), extensive binding was observed with the lipophilic compound ZK 975, but only little or no binding was observed with the hydrophilic compound ZK 894.

Therefore, based on these data, fused silica should be the first tubing material tested with a new compound for pharmacokinetic applications.

Figure 27: Individual and mean Ae_{1-5} for all tubing materials tested with the lipophilic compound ZK 975 and the hydrophilic compound ZK 894



[1] N = 6

3.1.3 Stability of ZK 975 and ZK 894 under tubing test conditions

The percentage of the parent compound in each of the samples analyzed is given in Table 12 (individual chromatograms are given in Appendix III):

Table 12: Percentage of parent compound in the medium before, and after 1.5 hours storage at room temperature

	^{14}C-ZK 975		^{14}C-ZK 894	
Sample number	**Before 10 µM**	**After 1.5 hrs, 10 µM**	**Before 10 µM**	**After 1.5 hrs, 10 µM**
1	95.2	93.3	88.6	89.5
2	95.6	93.8	89.8	90.7
mean [% RAD]	95.4	93.6	89.2	90.1
SD	0.29	0.36	0.83	0.84
	Remaining*	**98.1**	**Remaining***	**101**

RAD = total ^{14}C-radioactivity

* Remaining = %RAD afterwards / %RAD before × 100

For ^{14}C-ZK 975, the percentage of parent compound in the solution was 95% immediately after making the 10 µM solution, and was 94% when kept at room temperature for 1.5 hours. For ^{14}C-ZK 894, the percentage of parent compound was 89% before storage, and stayed 90% when kept at room temperature for 1.5 hours.

For both compounds the total amount of parent compound remained well above 90% of the starting concentration, and therefore both compounds were stable at room temperature for the one hour priming phase used for the tubing tests.

The low percentage of parent compound ZK 894 of 89% in freshly made solution was also observed with samples analyzed before each experiment (data not shown), and is likely due to ^{14}C-impurities in the solution. Since no elution of radioactivity was observed for either FEP or FEP/Teflon tested with ZK 894, two materials which showed high affinity for the lipophilic compound ZK 975, it can be concluded that these 10% radioactive breakdown products did not affect the conclusions drawn from the tubing results for ZK 894.

3.2 Results microdialysis probes

As a reminder, the relevant properties of the microdialysis probes tested are shown in Table 13, in the order in which the data are presented. For the complete list of dimensions and suppliers, see Table 6, Chapter 2.1.3.

Table 13: Selected properties of the eleven microdialysis probe materials tested

Probe description	Membrane material	Pore size [kDa]	S [1] [mm^2]	V [2] [mm^3]	Membrane thickness [µm]	Fluid layer [3] [µm]	Outlet material
CMA/12 (PES)	PES	**100**	6.28	0.283	50	75	14 mm Steel
MAB 2.14.4	**PES**	**35**	7.54	0.248	35	150	14 mm PEEK
MAB 6.14.4	PES	**15**	7.54	0.248	35	150	14 mm PEEK
MAB 9.14.4	PES	**6**	**7.54**	0.248	35	150	14 mm PEEK
MAB 8.4.4	PES	6	**3.02**	0.079	30	28	**4 mm Steel**
MAB 4.15.4.PES	PES	6	3.02	0.079	30	28	**15 mm PEEK**
MAB 4.15.4.Cu	**Cu**	6	3.02	0.079	**30**	28	15 mm PEEK
CMA/11	Cu	6	3.02	0.055	**20**	25	14 mm Steel
MBR-4-10	**Cell**	38	2.76	0.016	**5**	52	12 mm PEEK
CMA/12 (PC)	**PC**	20	6.28	0.091	15	110	14 mm Steel
BR-4	**PAN**	30	4.02	0.141	40	45	15 mm PEEK

PC = Polycarbonate
PES = Polyethylenesulfone
PAN = Polyacrylonitrile
Cu = Cuprophane
Cell = Cellulosic
[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

First, the mean $REC_{A/B}$ and $\%iAUD_{A/B}$ are presented for each probe tested, and for each phase separately, as well as the individual recovery-time curves (chapter 3.2.1). In chapter 3.2.2 the mean numerical values (K, %iAUD and Ae) obtained from the different materials tested are presented and compared.

3.2.1 Graphical data, REC and %iAUD for microdialysis probes

3.2.1.1 Results individual CMA/12 probes (20 kDa PC-membrane)

With the lipophilic compound ZK 975 (see Figure 28), the responsiveness of the CMA/12 (PC) probe was very poor during the concentration phases, with steady-state not being reached within the one hour observation period. The calculated recovery was 28 ± 1.5% at Phase A and 28 ± 1.2% at Phase B. The %iAUD was 67 ± 1.0% at Phase A and slightly higher at 73 ± 1.3% at Phase B. Extensive bleeding was observed during both rinsing phases.

With the hydrophilic compound ZK 894 (see Figure 29), the responsiveness of the CMA/12 (PC) probe was reasonable during the concentration phases. The recovery was 50 ± 2.6% at Phase A and 45 ± 4.5% at Phase B. The %iAUD was 92 ± 1.5% at Phase A and slightly higher at 98 ± 0.7% at Phase B. Slight bleeding was observed during both rinsing phases, especially after the higher concentration phase B.

For a graphical presentation of the individual and mean REC and %iAUD at Phase A and Phase B for the CMA/12 (PC) probe with either compound, see Appendix I, F 1.

Figure 28: Individual recovery-time profiles for CMA/12 (PC) probe with ZK 975

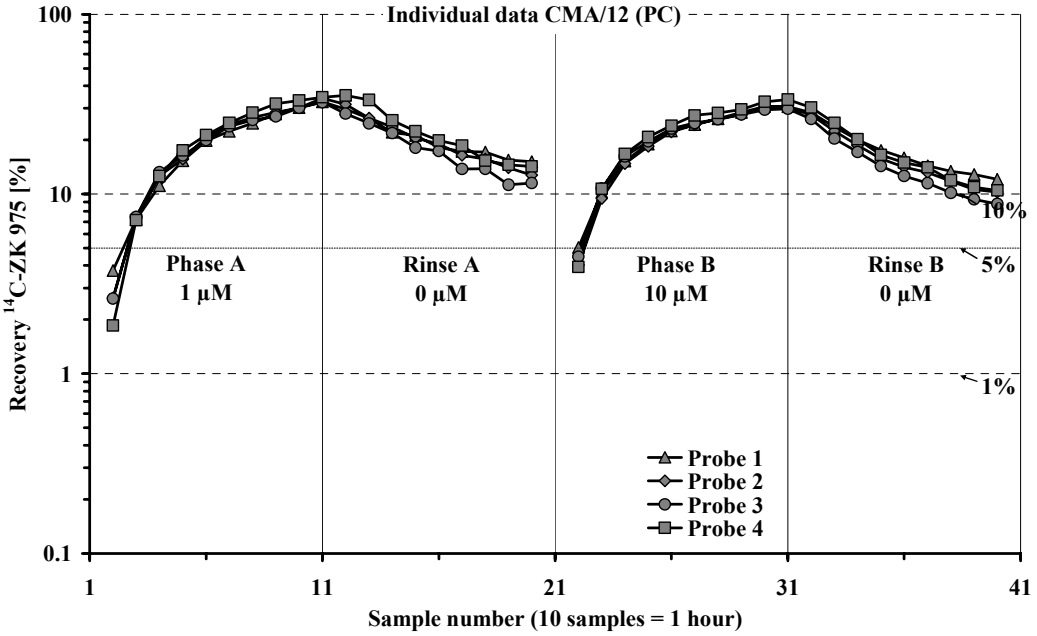
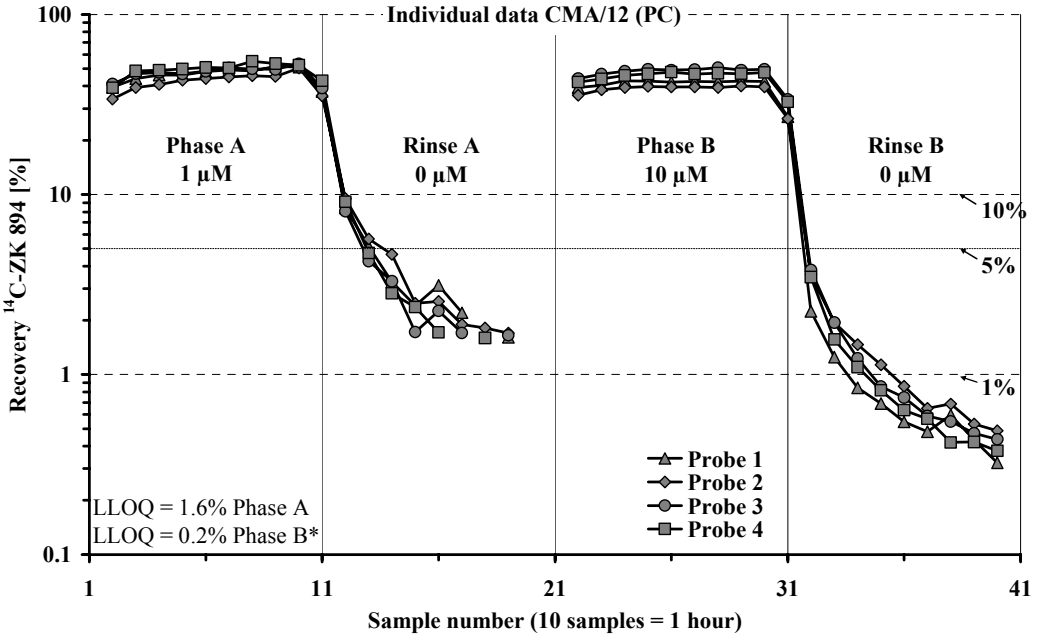


Figure 29: Individual recovery-time profiles for CMA/12 (PC) probe with ZK 894



* Data missing from the graph have recoveries below the detection limit (LLOQ), and are not depicted on this semi-logarithmic scale (REC<LLOQ are set to 0%).

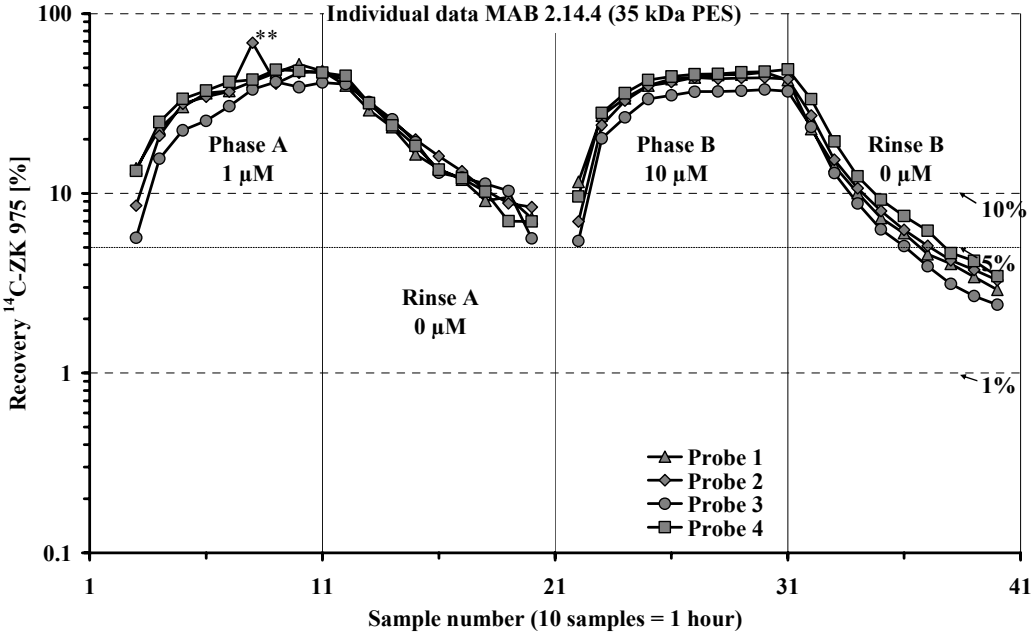
3.2.1.2 Results individual MAB 2.14.4 probes (35 kDa PES-membrane)

With the lipophilic compound ZK 975 (see Figure 30), the responsiveness of the MAB 2.14.4 probe was quite poor during the concentration phases, with steady-state not being reached during the lower concentration Phase A. Steady-state was however reached at the higher concentration Phase B. The calculated recovery was 42 ± 3.6% at Phase A and 43 ± 4.3% at Phase B. The %iAUD was 67 ± 2.5% at Phase A and slightly higher at 82 ± 0.7% at Phase B. Extensive bleeding was observed during both rinsing phases.

With the hydrophilic compound ZK 894(see Figure 31), the responsiveness of the MAB 2.14.4 probe was good during the concentration phases. The recovery was 48 ± 3.5% at Phase A and 43 ± 6.9% at Phase B. The %iAUD was 95 ± 0.9% at Phase A and slightly higher at 99 ± 0.5% at Phase B. Only minimal bleeding was evident during the second rinsing phases following the higher concentration Phase B.

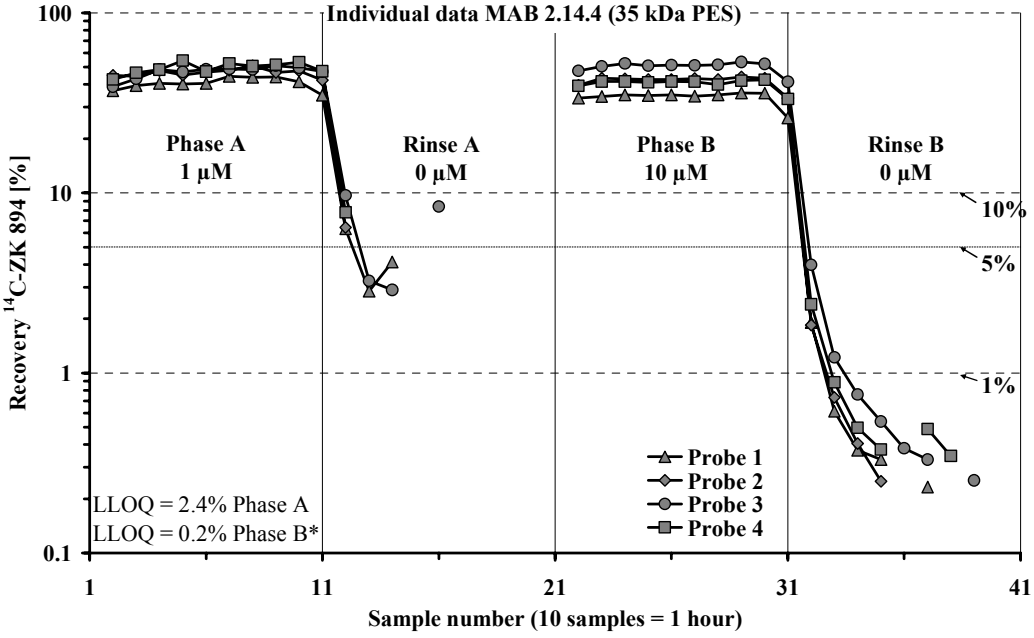
For a graphical presentation of the individual and mean REC and %iAUD at Phase A and Phase B for the MAB 2.14.4 probe with either compound, see Appendix I, F 2.

Figure 30: Individual recovery-time profiles for MAB 2.14.4 probe with ZK 975



** Individual datum excluded from all further calculations.

Figure 31: Individual recovery-time profiles for MAB 2.14.4 probe with ZK 894



* Data missing from the graph have recoveries below the detection limit (LLOQ), and are not depicted on this semi-logarithmic scale (REC<LLOQ are set to 0%).

3.2.1.3 Results individual BR-4 probes (20 kDa PAN-membrane)

With the lipophilic compound ZK 975 (see Figure 32), the responsiveness of the BR-4 probe was quite poor during the concentration phases, with steady-state not being reached during the lower concentration Phase A. Steady-state was however reached at the higher concentration Phase B. The calculated recovery was 20 ± 3.9% at Phase A and 18 ± 3.0% at Phase B. The %iAUD was 83 ± 1.7% at Phase A and slightly higher at 88 ± 3.0% at Phase B. Extensive bleeding was observed during both rinsing phases.

With the hydrophilic compound ZK 894 (see Figure 33), the responsiveness of the BR-4 probe was good during the first concentration Phase A, but was clearly slower during the second concentration Phase B, although steady-state was reached at both phases. The recovery was 28 ± 4.4% at Phase A, but was obviously higher at 36 ± 5.1% at Phase B. The %iAUD was good at 97 ± 1.7% at Phase A, but considerably lower at 86 ± 2.1% at Phase B. Also, bleeding was clearly evident during the second rinsing phase following the higher concentration Phase B, but not during the first rinsing phase following the lower concentration Phase A.

For a graphical presentation of the individual and mean REC and %iAUD at Phase A and Phase B for the BR-4 probe with either compound, see Appendix I, F 3.

Overall, the BR-4 probe behaves differently between the two concentrations tested with the hydrophilic compound ZK 894. At the lower concentration of 1 µM, the responsiveness is comparable to that seen with the other membrane materials tested, but at the higher concentration of 10 µM, the response is delayed, and the final observed recovery much higher than expected. Also, bleeding was observed for the second rinsing phase B, but not for the first rinsing phase A. A similar result was found by Tao and Hjorth, 1992, testing the PAN membrane with a 10 µM solution of serotonin in artificial cerebrospinal fluid (an otherwise 'unproblematic' hydrophilic compound). Since only one concentration was tested, the authors explained this finding by presuming an interaction between the compound and the membrane. If this were the case however, both concentrations tested in this thesis should have shown this behavior, as observed with the lipophilic compound ZK 975. A better explanation is given by Snyder, Nathan et al., 2001, who suggested that additional diffusion through the solid polymeric phase of the membrane might also occur. This theory would explain the findings in this thesis, whereby the route of least resistance (through the water filled pores) is favored by the analyte, as seen by the 'normal' microdialysis behavior of the probe at the lower concentration tested. When diffusion by this route becomes rate limiting however, the slower additional diffusion through the polymeric phase becomes apparent, as seen at the higher concentration tested.

Figure 32: Individual recovery-time profiles for BR-4 probe with ZK 975

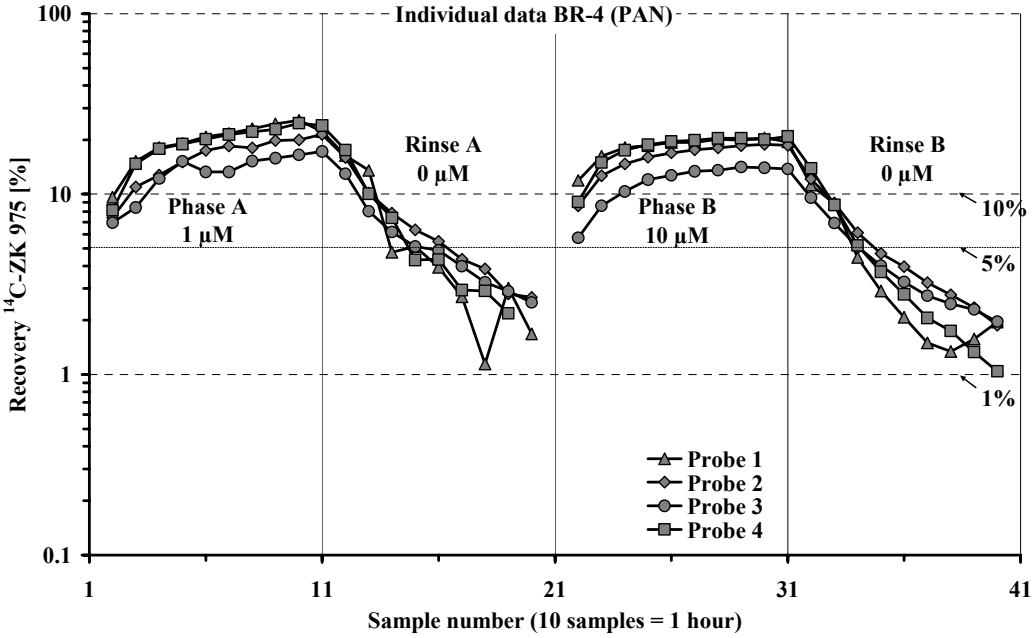
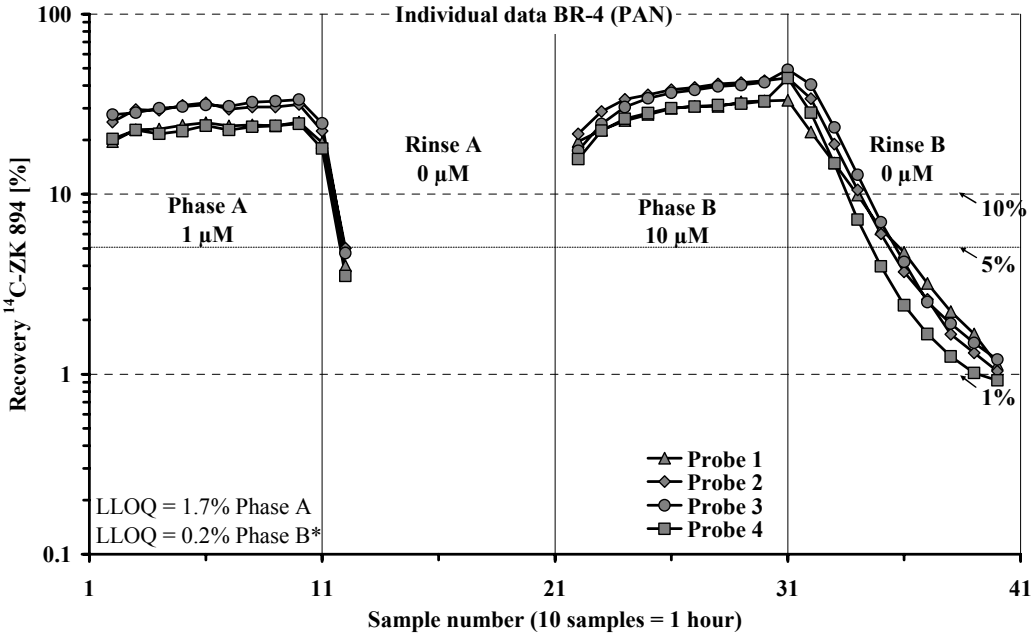


Figure 33: Individual recovery-time profiles for BR-4 probe with ZK 894



* Data missing from the graph have recoveries below the detection limit (LLOQ), and are not depicted on this semi-logarithmic scale (REC<LLOQ are set to 0%).

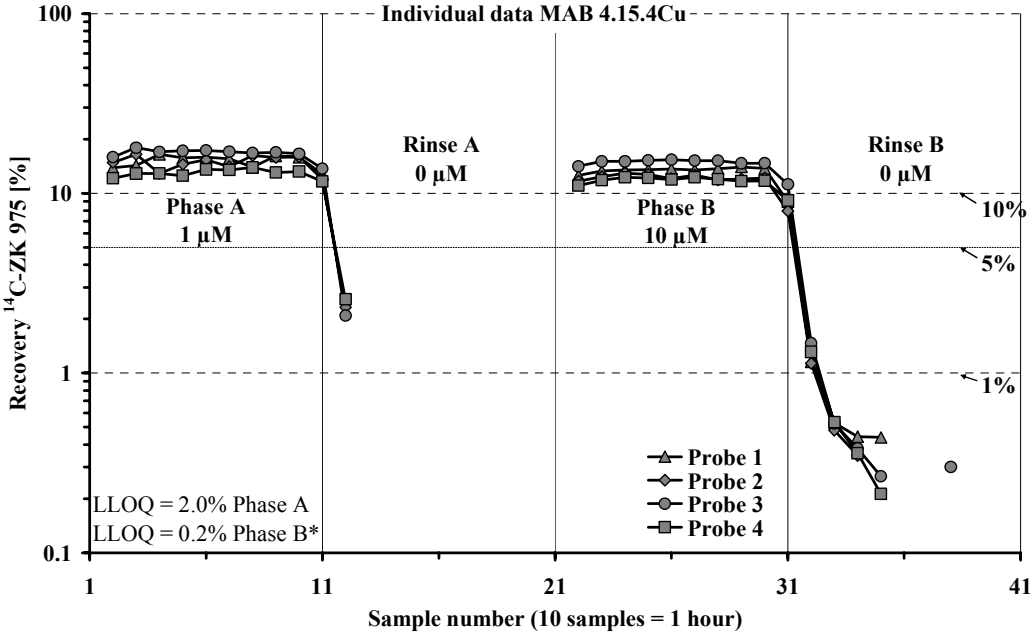
3.2.1.4 Results individual MAB 4.15.4.Cu probes (6 kDa Cu-membrane)

With the lipophilic compound ZK 975 (see Figure 34), the responsiveness of the MAB 4.15.4.Cu probe was very good for both concentration phases, with steady-state being reached immediately. The recovery was 15 ± 1.4% at Phase A and 13 ± 1.4% at Phase B. The %iAUD was 99 ± 2.0% at Phase A and 100 ± 1.2% at Phase B. Only minimal bleeding was observed during the rinsing Phase B.

With the hydrophilic compound ZK 894 (see Figure 35), the responsiveness of the MAB 4.15.4.Cu probe was also excellent for both concentration phases. The recovery was 14 ± 1.7% at Phase A and 16 ± 1.2% at Phase B. The %iAUD was calculated as 103 ± 3.9% at Phase A and as 100 ± 0.5% at Phase B. No bleeding could be observed during either rinsing phase.

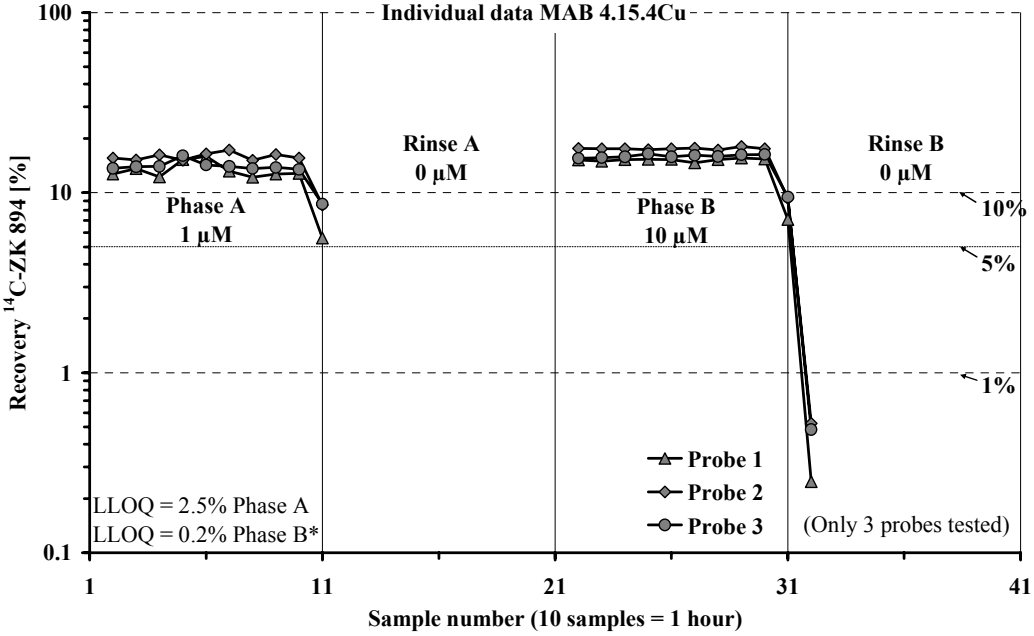
For a graphical presentation of the individual and mean REC and %iAUD at Phase A and Phase B for the MAB 4.15.4.Cu probe with either compound, see App. I, F 4.

Figure 34: Individual recovery-time profiles for MAB 4.15.4.Cu probe with ZK 975



* Data missing from the graph have recoveries below the detection limit (LLOQ), and are not depicted on this semi-logarithmic scale (REC<LLOQ are set to 0%).

Figure 35: Individual recovery-time profiles for MAB 4.15.4.Cu probe with ZK 894



* Data missing from the graph have recoveries below the detection limit (LLOQ), and are not depicted on this semi-logarithmic scale (REC<LLOQ are set to 0%).

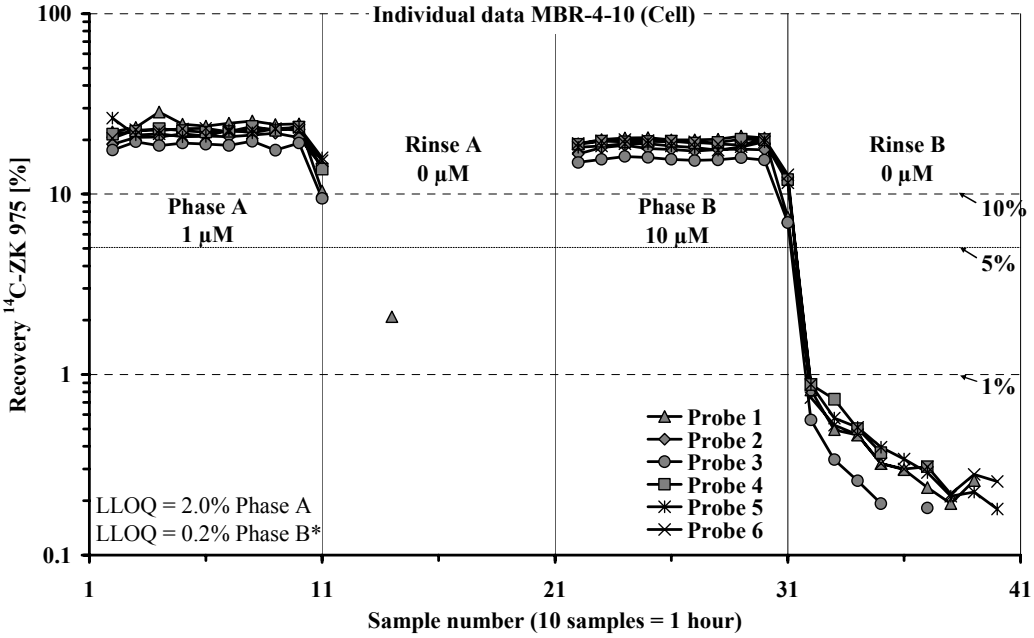
3.2.1.5 Results individual MBR-4-10 probes (38 kDa Cell-membrane)

With the lipophilic compound ZK 975 (see Figure 36), the responsiveness of the MBR-4-10 probe was very good for both concentration phases, with steady-state being reached immediately. The recovery was 22 ± 2.0% at Phase A and 19 ± 1.8% at Phase B. The %iAUD was 99 ± 1.4% at Phase A and 100 ± 0.9% at Phase B. Only minimal bleeding was observed during the rinsing Phase B. Note that six MBR-4-10 probes were tested with ZK 975.

With the hydrophilic compound ZK 894 (see Figure 37), the responsiveness of the MBR-4-10 probe was also excellent for both concentration phases. The recovery was 23 ± 3.5% at Phase A (one probe had a somewhat lower recovery for unknown reasons) and 23 ± 1.5% at Phase B. The %iAUD was calculated as 98 ± 1.5% at Phase A and as 100 ± 0.2% at Phase B. No bleeding could be observed during either rinsing phase.

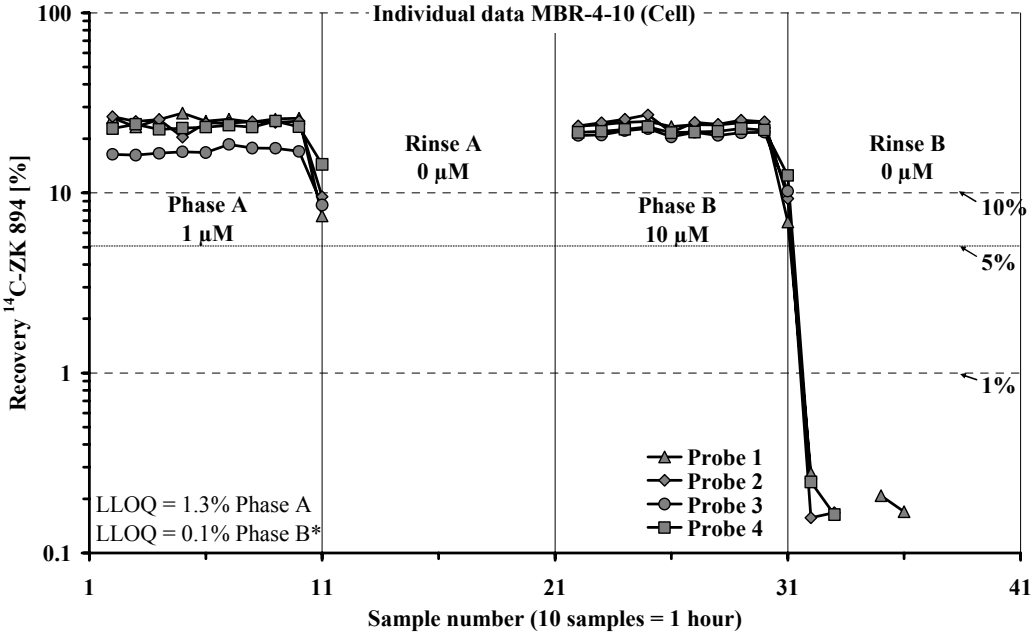
For a graphical presentation of the individual and mean REC and %iAUD at Phase A and Phase B for the MBR-4-10 probe with either compound, see Appendix I, F 5.

Figure 36: Individual recovery-time profiles for MBR-4-10 probe with ZK 975



* Data missing from the graph have recoveries below the detection limit (LLOQ), and are not depicted on this semi-logarithmic scale (REC<LLOQ are set to 0%).

Figure 37: Individual recovery-time profiles for MBR-4-10 probe with ZK 894



* Data missing from the graph have recoveries below the detection limit (LLOQ), and are not depicted on this semi-logarithmic scale (REC<LLOQ are set to 0%).

3.2.1.6 Results individual CMA/12 probes (100 kDa PES-membrane)

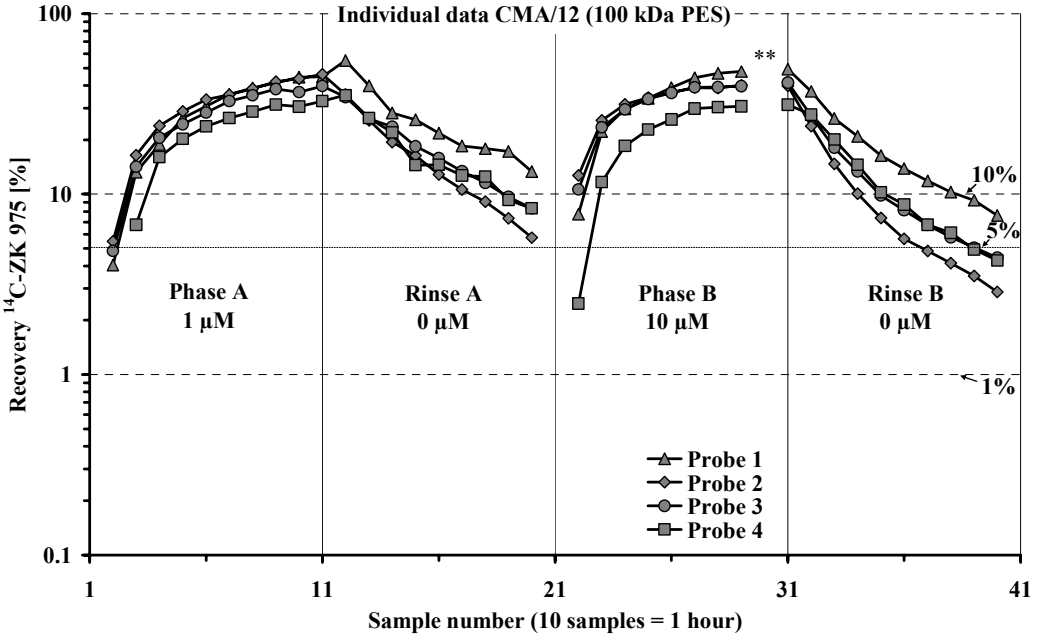
With the lipophilic compound ZK 975 (see Figure 38), the responsiveness of the CMA/12 (PES) probe was quite poor during the concentration phases, with steady-state not being reached during the lower concentration Phase A. Steady-state was however reached at the higher concentration Phase B. The calculated recovery was 36 ± 5.1% at Phase A and 39 ± 6.6% at Phase B. The %iAUD was 71 ± 2.2% at Phase A and slightly higher at 79 ± 4.7% at Phase B. Extensive bleeding was observed during both rinsing phases.

With the hydrophilic compound ZK 894 (see Figure 39), the responsiveness of the CMA/12 (PES) probe was reasonable during the concentration phases. The recovery was 50 ± 4.0% at Phase A and 46 ± 3.5% at Phase B. The %iAUD was 86 ± 3.1% at Phase A and slightly higher at 96 ± 1.7% at Phase B. Slight bleeding was evident during the rinsing phases, especially after the higher concentration phase B.

Using the CMA/12 probe with the 100 kDa PES membrane, varying degrees of ultrafiltration were observed, due to back pressure from the narrow fused silica tubing. Flow rates ranged from 0.8 to 1.5 µL/min for ZK 975 and from 0.7 to 0.9 µL/min for ZK 894, varying not only between probes, but between phases as well. Interestingly enough, an effect of actual flow rate on recovery was not observed, suggesting that although a reduction in the set flow rate will increase recovery (see Chapter 1.1.1), increased ultrafiltration resulting in low observed flow rates will not. The only effect of flow rate on the graphical data was generally a horizontal shift to the right as the observed flow rate decreased, corresponding to a delay in the time taken for the dialysate to reach the sample vial from the membrane.

For a graphical presentation of the individual and mean REC and %iAUD at Phase A and Phase B for the CMA/12 (PES) probe with either compound, see App. I, F 6.

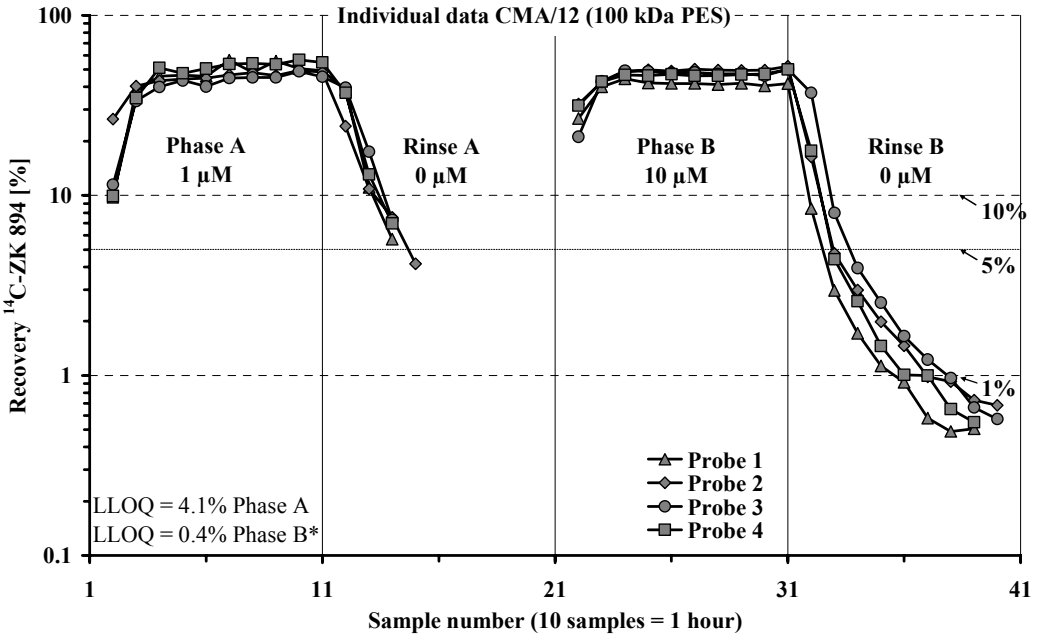
Figure 38: Individual recovery-time profiles for CMA/12 (PES) probe with ZK 975



* Data missing from the graph have recoveries below the detection limit (LLOQ), and are not depicted on this semi-logarithmic scale (REC<LLOQ are set to 0%)

** The probes were moved to the 0 µM medium one sample early, hence no data are available for sample 30.

Figure 39: Individual recovery-time profiles for CMA/12 (PES) probe with ZK 894



* Data missing from the graph have recoveries below the detection limit (LLOQ), and are not depicted on this semi-logarithmic scale (REC<LLOQ are set to 0%)

3.2.1.7 Results individual MAB 6.14.4 probes (15 kDa-membrane)

With the lipophilic compound ZK 975 (see Figure 40), the responsiveness of the MAB 6.14.4 probe was quite poor during the concentration phases, with steady-state not being reached during the lower concentration Phase A. Steady-state was however reached at the higher concentration Phase B. The calculated recovery was 37 ± 3.9% at Phase A and 40 ± 2.2% at Phase B. The %iAUD was 75 ± 3.6% at Phase A and slightly higher at 83 ± 2.6% at Phase B. Extensive bleeding was observed during both rinsing phases.

With the hydrophilic compound ZK 894 (see Figure 41), the responsiveness of the MAB 6.14.4 probe was reasonable for Phase A, and quite good for Phase B. The recovery was 23 ± 5.8% at Phase A and 24 ± 7.2% at Phase B. The %iAUD was 93 ± 4.7% at Phase A and slightly 97 ± 2.9% at Phase B. Only minimal bleeding was observed after the higher concentration phase B.

For a graphical presentation of the individual and mean REC and %iAUD at Phase A and Phase B for the MAB 6.14.4 probe with either compound, see Appendix I, F 7.

Figure 40: Individual recovery-time profiles for MAB 6.14.4 probe with ZK 975

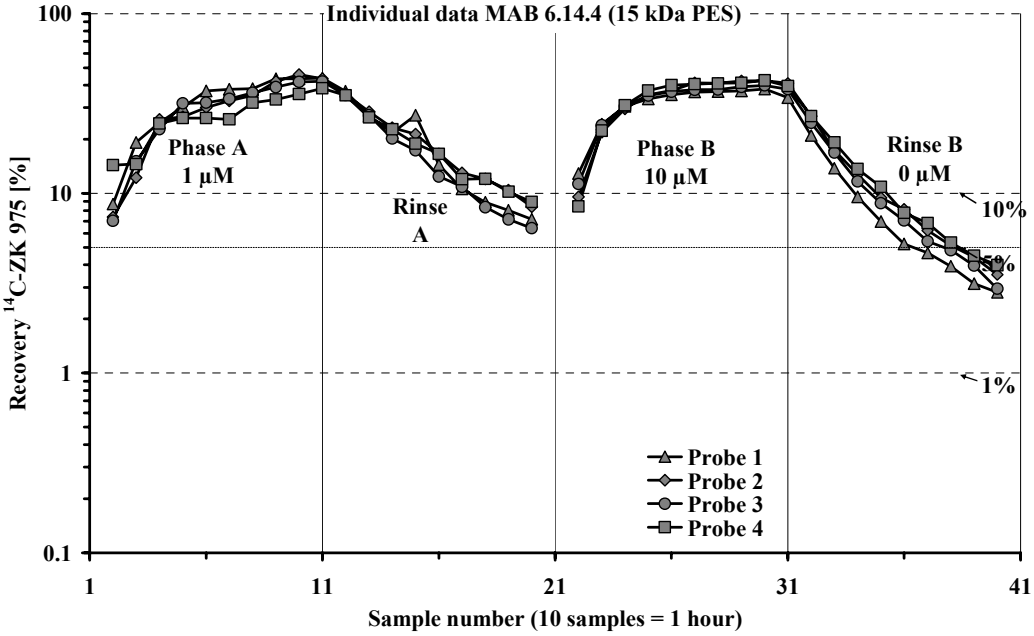
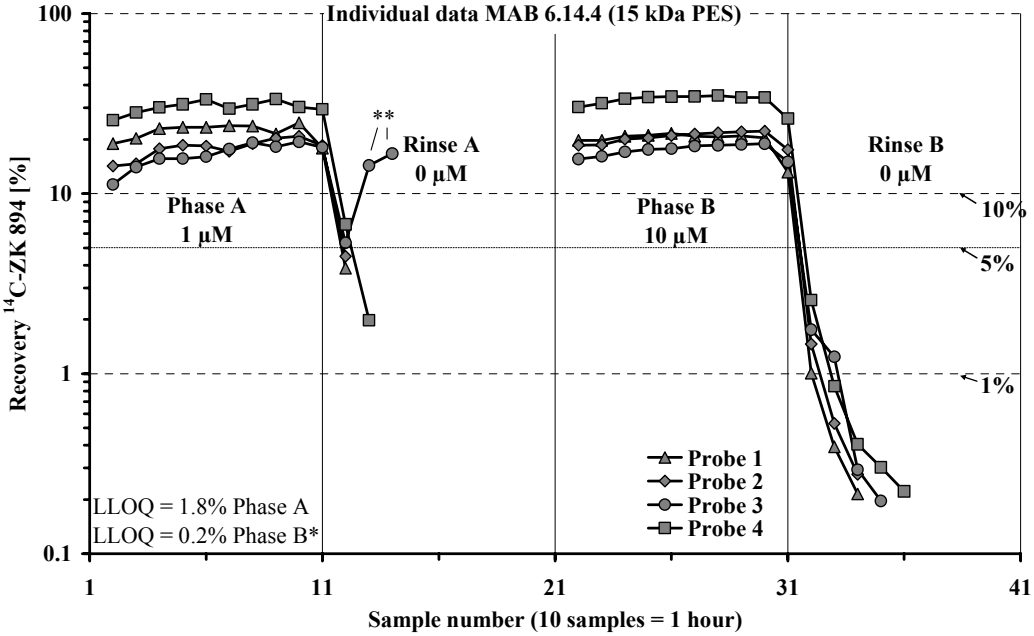


Figure 41: Individual recovery-time profiles for MAB 6.14.4 probe with ZK 894



* Data missing from the graph have recoveries below the detection limit (LLOQ), and are not depicted on this semi-logarithmic scale (REC<LLOQ are set to 0%).

** Individual data excluded from all further calculations.

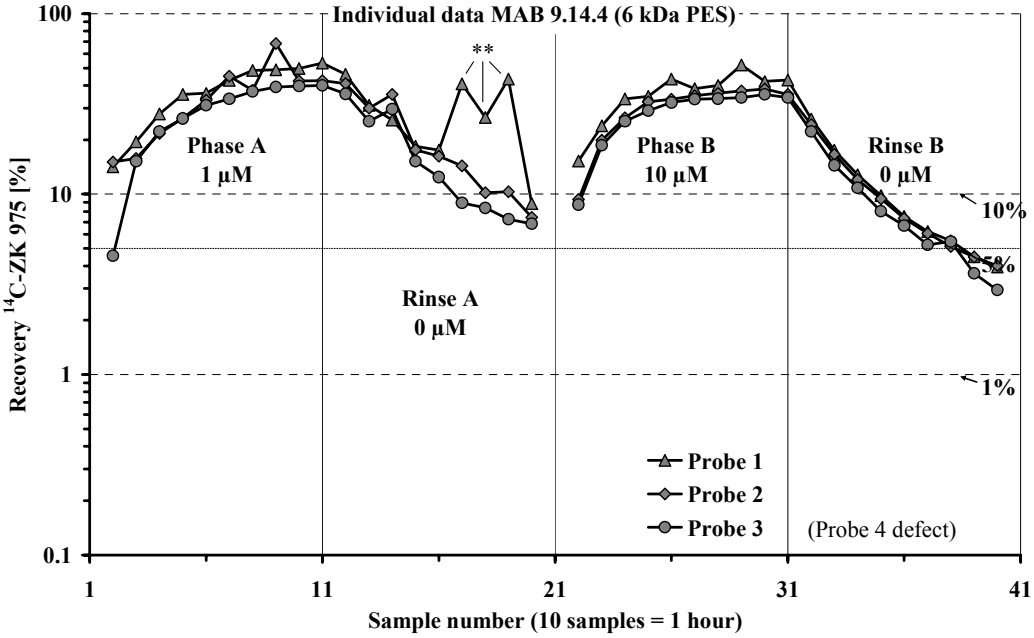
3.2.1.8 Results individual MAB 9.14.4 probes (6 kDa PES-membrane)

With the lipophilic compound ZK 975 (see Figure 42), the responsiveness of the MAB 9.14.4 probe was quite poor during the concentration phases, with steady-state not being reached during the lower concentration Phase A. Generally, steady-state was however reached at the higher concentration Phase B. The calculated recovery was 44 ± 6.0% at Phase A and 38 ± 4.5% at Phase B. The %iAUD was 71 ± 3.5% at Phase A and slightly higher at 82 ± 0.9% at Phase B. Extensive bleeding was observed during both rinsing phases.

With the hydrophilic compound ZK 894 (see Figure 43), the responsiveness of the MAB 9.14.4 probe was quite good for both concentration phases. The recovery was 42 ± 3.4% at Phase A and 43 ± 3.7% at Phase B. The %iAUD was 98 ± 2.8% at Phase A and 98 ± 0.5% at Phase B. Slight bleeding was evident only after the higher concentration phase B.

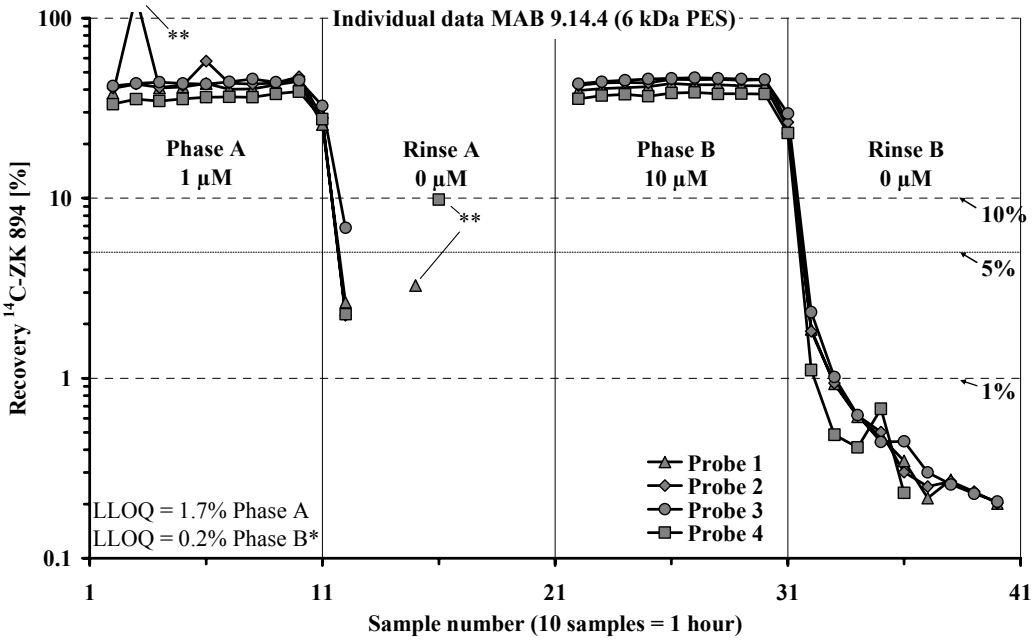
For a graphical presentation of the individual and mean REC and %iAUD at Phase A and Phase B for the MAB 9.14.4 probe with either compound, see Appendix I, F 8.

Figure 42: Individual recovery-time profiles for MAB 9.14.4 probe with ZK 975



** Individual data excluded from all further calculations.

Figure 43: Individual recovery-time profiles for MAB 9.14.4 probe with ZK 894



* Data missing from the graph have recoveries below the detection limit (LLOQ), and are not depicted on this semi-logarithmic scale (REC<LLOQ are set to 0%).

** Individual data excluded from all further calculations.

3.2.1.9 Results individual MAB 8.4.4 probes (6 kDa PES-membrane)

With the lipophilic compound ZK 975 (see Figure 44), the responsiveness of the MAB 8.4.4 probe was reasonable, with steady-state being reached for both concentration phases. The recovery was 23 ± 1.5% at Phase A and 21 ± 0.7% at Phase B. The %iAUD was 83 ± 2.2% at Phase A and 91 ± 0.4% at Phase B. Extensive bleeding was observed during both rinsing phases.

With the hydrophilic compound ZK 894 (see Figure 45), the responsiveness of the MAB 8.4.4 probe was quite good for both concentration phases. The recovery was 26 ± 3.8% at Phase A and 26 ± 3.2% at Phase B. The %iAUD was 99 ± 1.5% at Phase A and 98 ± 1.4% at Phase B. Only minimal bleeding was observed after the higher concentration phase B.

For a graphical presentation of the individual and mean REC and %iAUD at Phase A and Phase B for the MAB 8.4.4 probe with either compound, see Appendix I, F 9.

Figure 44: Individual recovery-time profiles for MAB 8.4.4 probe with ZK 975

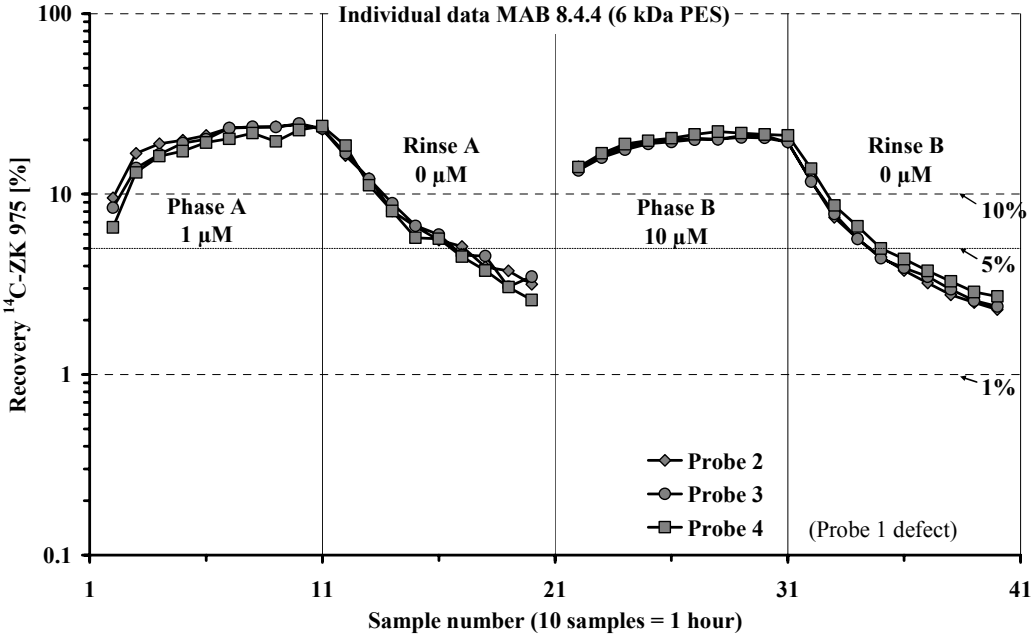
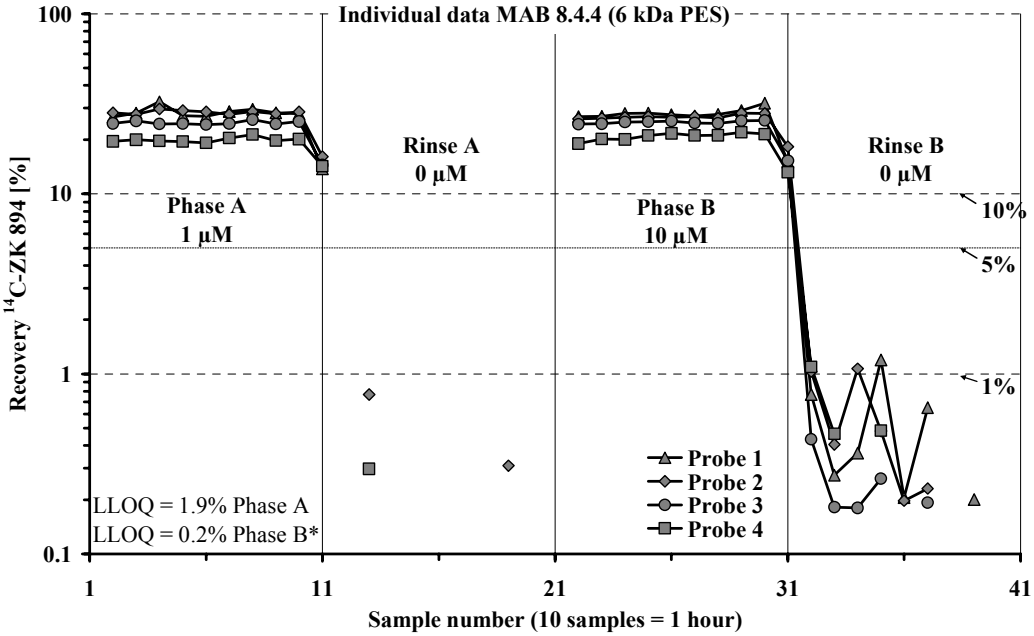


Figure 45: Individual recovery-time profiles for MAB 8.4.4 probe with ZK 894



* Data missing from the graph have recoveries below the detection limit (LLOQ), and are not depicted on this semi-logarithmic scale (REC<LLOQ are set to 0%).

3.2.1.10 Results individual MAB 4.15.4.PES probes (6 kDa PES-membrane)

With the lipophilic compound ZK 975 (see Figure 46), the responsiveness of the MAB 4.15.4.PES probe was reasonable, with steady-state being reached for both concentration phases. The recovery was 32 ± 3.1% at Phase A and 28 ± 1.5% at Phase B. The %iAUD was 84 ± 2.3% at Phase A and 91 ± 1.8% at Phase B. Extensive bleeding was observed during both rinsing phases.

With the hydrophilic compound ZK 894 (see Figure 47), the responsiveness of the MAB 4.15.4.PES probe was quite good for both concentration phases. The recovery was 24 ± 8.4% at Phase A and 22 ± 8.1% at Phase B. The %iAUD was 98 ± 0.8% at Phase A and 101 ± 1.1% at Phase B. Only minimal bleeding was observed after the higher concentration phase B.

With the MAB 4.15.4PES probe, the data obtained with three of the four probes were very consistent with ZK 894, whereas one probe showed a much lower recovery. A likely explanation would be that an air bubble was trapped at the membrane. Attempts were made to check for air bubbles before the start of each experiment (using a hand-held magnifying glass), and any air bubbles detected were removed by tapping the holder sharply and/or by increasing the flow rate up to 10 µL/min. However, the PES membranes were surrounded by a white protective layer, making the detection of bubbles quite difficult. These probes were all tapped before being used, even if air bubbles could not be seen. Nonetheless it is possible that an undetected bubble may not have been dislodged. Since no technical deviations could be identified, the data from this probe were included in the mean calculations, increasing the variability of this data set.

For a graphical presentation of the individual and mean REC and %iAUD at Phase A and Phase B for the MAB 4.15.4.PES probe with either compound, see App. I, F 10.

Figure 46: Individual recovery-time profiles for MAB 4.15.4.PES probe with ZK 975

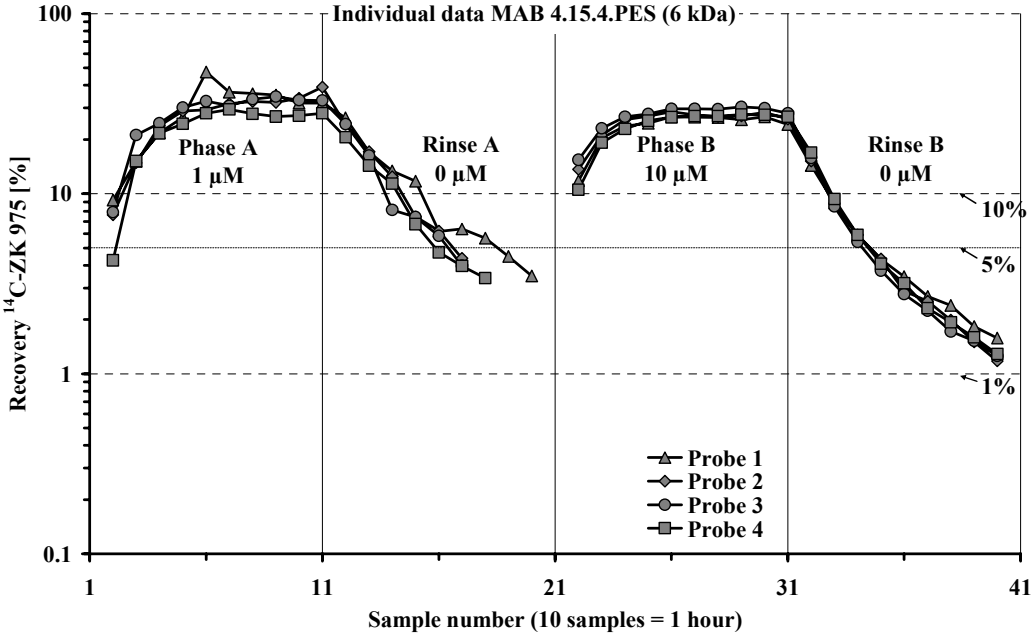
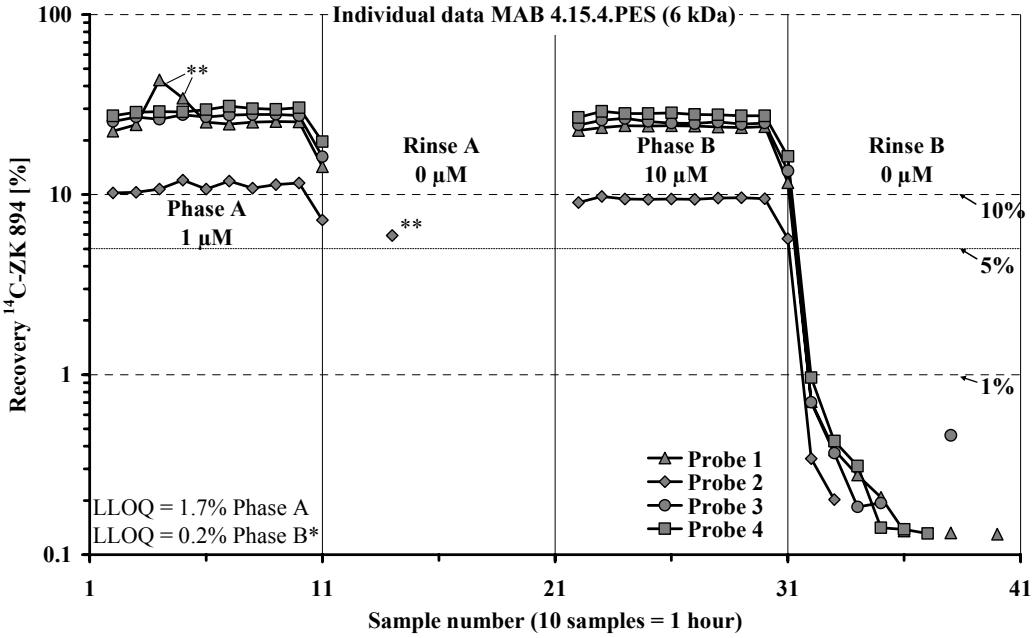


Figure 47: Individual recovery-time profiles for MAB 4.15.4.PES probe with ZK 894



* Data missing from the graph have recoveries below the detection limit (LLOQ), and are not depicted on this semi-logarithmic scale (REC<LLOQ are set to 0%).

** Individual data excluded from all further calculations.

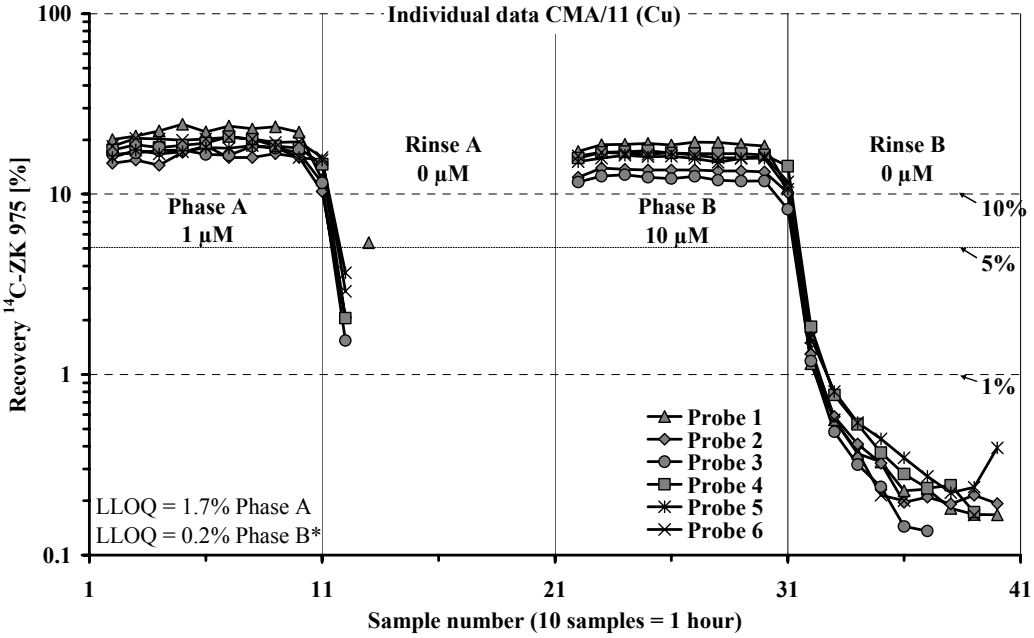
3.2.1.11 Results individual CMA/11 probes (6 kDa Cu-membrane)

With the lipophilic compound ZK 975 (see Figure 48), the responsiveness of the CMA/11 probe was very good for both concentration phases, with steady-state being reached immediately. The recovery was 19 ± 2.5% at Phase A and 16 ± 2.5% at Phase B. The %iAUD was very good at 99 ± 1.1% at Phase A and at 101 ± 1.2% at Phase B. Only minimal bleeding was observed during the rinsing Phase B.

With the hydrophilic compound ZK 894 (see Figure 49), the responsiveness of the CMA/11 probe was also excellent for both concentration phases. The recovery was 22 ± 2.4% at Phase A and 21 ± 2.0% at Phase B. The %iAUD was calculated as 98 ± 2.1% at Phase A and as 100 ± 0.6% at Phase B. Only minimal bleeding was observed for one probe during the rinsing Phase B. No bleeding could be observed during either rinsing phase for the other three probes tested.

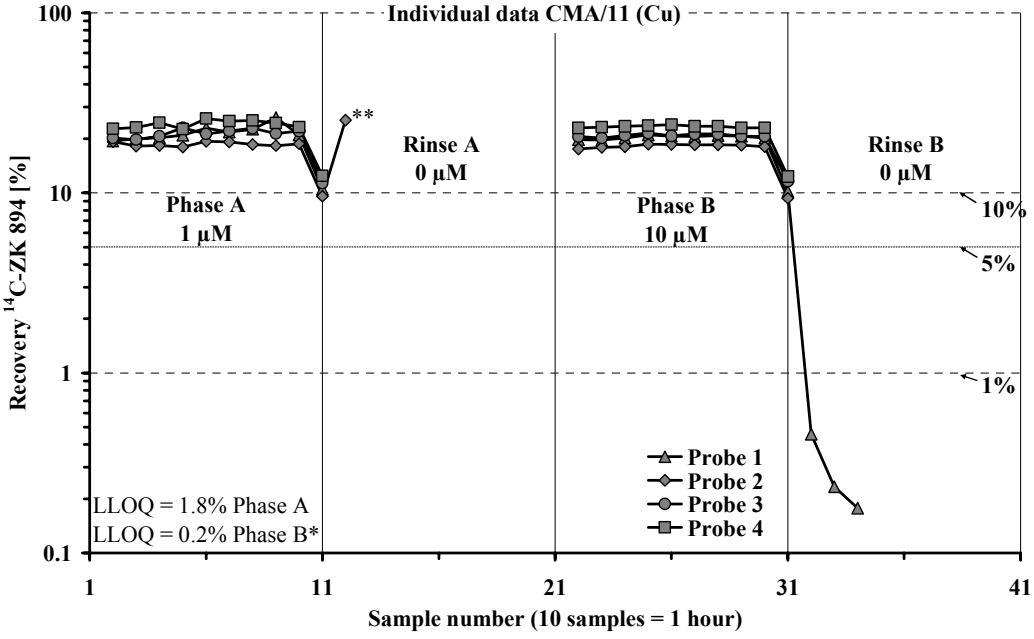
For a graphical presentation of the individual and mean REC and %iAUD at Phase A and Phase B for the CMA/11 probe with either compound, see Appendix I, F 11.

Figure 48: Individual recovery-time profiles for CMA/11 probe with ZK 975



* Data missing from the graph have recoveries below the detection limit (LLOQ), and are not depicted on this semi-logarithmic scale (REC<LLOQ are set to 0%).

Figure 49: Individual recovery-time profiles for CMA/11 probe with ZK 894



* Data missing from the graph have recoveries below the detection limit (LLOQ), and are not depicted on this semi-logarithmic scale (REC<LLOQ are set to 0%).

** Individual datum excluded from all further calculations.

3.2.2 Mean graphs, %iAUD, Ae and K of microdialysis probes tested

In order to determine the effect of different probe properties on probe behavior, the eleven probes tested are grouped for comparison as described in Chapter 2.1.3. For graphical comparison, the mean K versus time curves are presented, and for the numerical description of the responsiveness, the mean %iAUD and Ae (± standard deviation) are compared. For completeness, and to allow comparison with data from the literature, the mean overall K of each probe type is also given. First, the data for ZK 975 are presented (Chapter 3.2.2.1), followed by the data for ZK 894 (Chapter 3.2.2.2). Finally, the mean %iAUD, Ae and K are compared between the two compounds for all materials tested (Chapter 3.2.2.3).

3.2.2.1 Mean results for microdialysis probes tested with ZK 975

3.2.2.1.1 Comparison of membrane materials tested with ZK 975[1]

A graphical presentation of the mean K versus time for the five different materials tested (PC, PES, PAN, Cu and Cell) is given in Figure 50. A plot of the individual (○) and the mean (—) Ae is presented in Figure 51. Additional plots of the overall K and %iAUD can be found in Appendix I, F 12 and F 13.

The two membrane materials cellulosic and cuprophane behaved very similarly with ZK 975. Both had an excellent responsiveness, as reflected by a high overall %iAUD of > 99% and a low Ae of < 1 pmol/mm^3. Thus, both cuprophane and cellulosic are suitable for pharmacokinetic studies with ZK 975. The two materials differed only in their K of 0.17 ± 0.02 µL/min/mm^2 for the MBR-4 probe and 0.10 ± 0.01 µL/min/mm^2 for the MAB 4 probe.

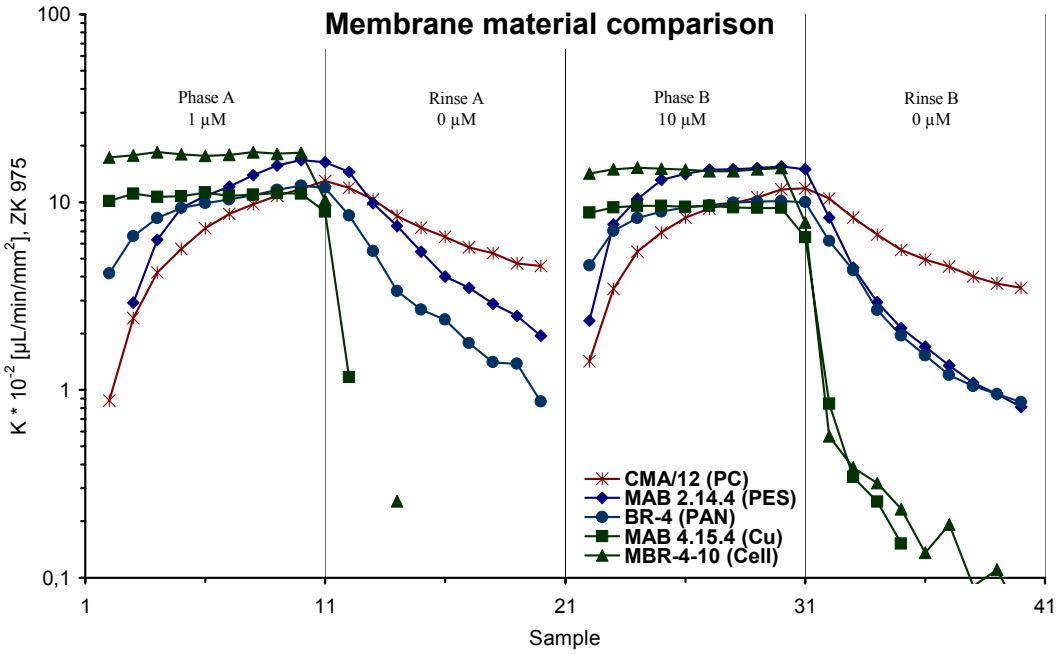
The three membrane materials PAN, PES (35 kDa) and PC all responded poorly to concentration changes, as reflected by a low overall %iAUD of < 90% and a high mean Ae ranging from 6.6 to 35 pmol/mm^3 (Figure 51). These calculated Ae-values clearly demonstrated elution of compound during the rinsing phases, even though collection of compound was not complete, and therefore not all conditions for a valid calculation of Ae were met (resulting in an underestimation of the true Ae). Thus, neither of these three materials are suitable for pharmacokinetic studies with ZK 975. The mean K were all in the same order of magnitude at 0.11 ± 0.02 µL/min/mm^2 for the BR-4 probe, 0.15 ± 0.02 µL/min/mm^2 for the MAB 2 probe, and 0.10 ± 0.05 µL/min/mm^2 for the CMA/12 (PC) probe. Note that these K-values were not determined at steady-state, and are therefore slightly underestimated.

The differences in the %iAUD and the Ae observed obviously reflect differences in the binding capacity of the membranes for ZK 975, with cellulosic and cuprophane not binding the compound at all. The differences in K however (Appendix I, F 12) may have a variety of causes, other than membrane composition. The difference in pore size does not appear to affect K (see below), but the difference in membrane thickness might certainly explain the different K found for Cell and Cu (see Chapter 3.2.2.1.2 below). For PAN, PES and PC; membrane thickness alone does not explain the difference in K seen (K of PES>PAN>PC, but thickness of PAN<<PC<PES, see Table 13), but the probes also differ in their probe geometry (fluid layer

[1] The data presented in Chapter 3.2.2.1.1 have been published in part as poster abstracts (Meier-Ince and Günther, 2006a; Meier-Ince and Günther, 2006b)

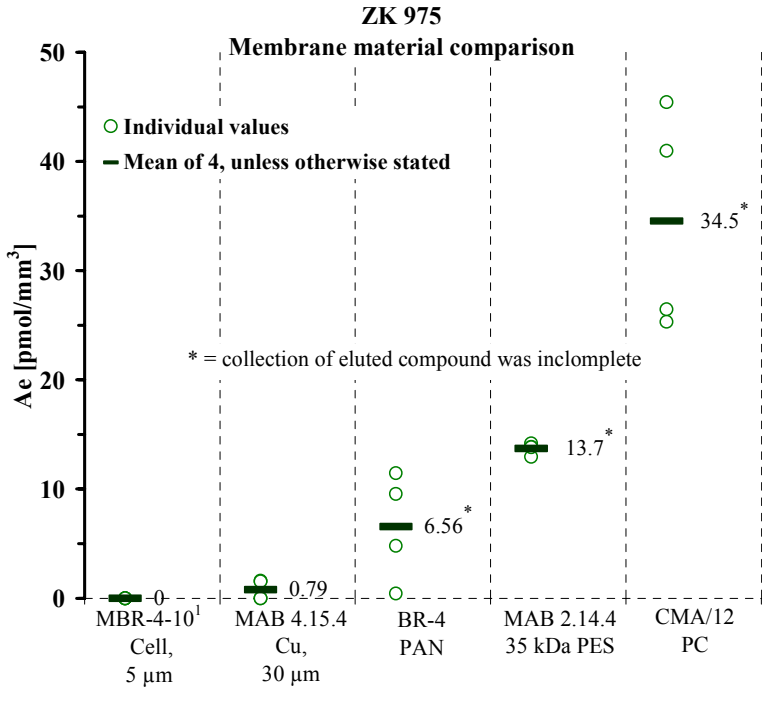
PES>PC>>PAN, see Table 6 and Chapter 1.1.4.2). Thus, a direct comparison of K for the different membrane materials is misleading, as the probes differ in other aspects too. Some of these aspects are therefore compared more selectively below.

Figure 50: Mean K versus time profiles for probes with different membrane materials tested with ZK 975



Data missing from the graph have recoveries below the detection limit (LLOQ), and are not depicted on this semi-logarithmic scale (REC<LLOQ are set to 0%).

Figure 51: Overall Ae of different membrane materials tested with ZK 975



1 N = 5

3.2.2.1.2 Comparison of pore sizes tested with ZK 975

A graphical presentation of the mean K versus time for the four different pore sizes tested (6, 15, 35 and 100 kDa PES-membranes) is given in Figure 52. A plot of the individual (○) and the mean (—) Ae is presented in Figure 53. Additional plots of the overall K and %iAUD can be found in App. I, F 14 and F 15.

The four PES-pore sizes tested all behaved very similarly with ZK 975. All had a poor responsiveness, with steady-state only being reached at the higher concentration Phase B. This poor responsiveness was reflected in a mean %iAUD of only 75 to 80%, and in a high Ae of 8.3 to 14 pmol/mm^3 (Figure 53). As with the membrane comparison, these calculated Ae-values clearly demonstrated elution of compound during the rinsing phases, even though collection of compound was not complete (especially for the 100 kDa PES membrane, with a lower effective flow rate of < 1.5 µL/min), and therefore not all conditions for a valid calculation of Ae were met (resulting in an underestimation of the true Ae). The overall mean K were similar, between 0.13 and 0.15 µL/min/mm^2, but note that these K-values were not determined at steady-state, and are therefore slightly underestimated. Thus, pore size does not affect the recovery or K of this small molecule, neither does it alter the binding capacity of the membrane.

Figure 52: Mean K versus time profiles for probes with different membrane pore sizes (PES-membranes only) tested with ZK 975

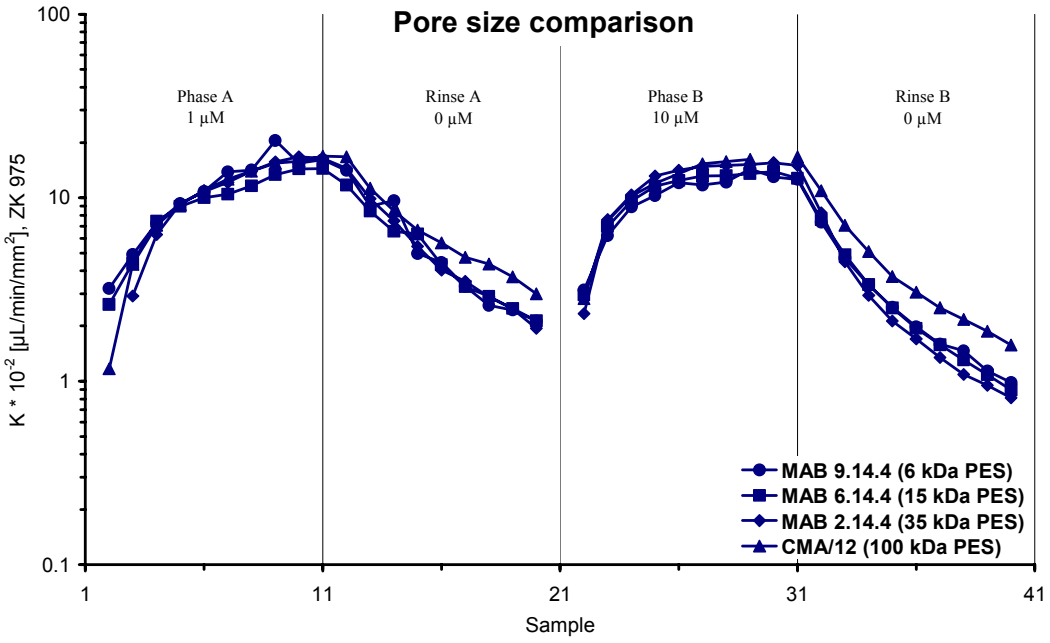
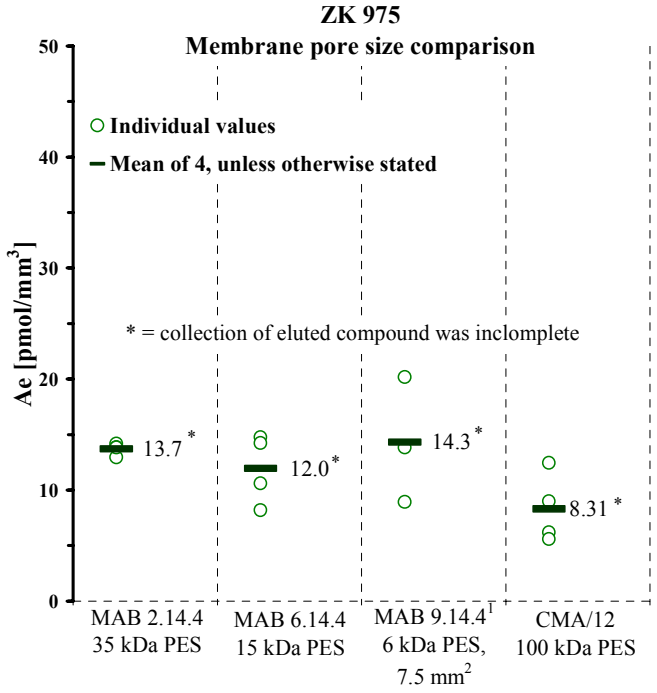


Figure 53: Overall Ae of membranes with different pore sizes (PES-membranes) tested with ZK 975



1 N = 3

3.2.2.1.3 Comparison of membrane surface areas tested with ZK 975

A graphical presentation of the mean K versus time for the two different membrane surface areas tested (3.0 and 7.5 mm^2, both 6 kDa PES-membranes) is given in Figure 54. A plot of the individual (○) and the mean (—) Ae is presented in Figure 55. Additional plots of the overall K and %iAUD can be found in Appendix I, F 16 and F 17.

The two membrane sizes tested behaved slightly differently to each other with ZK 975. Although the rinsing profile was similar for both probes, with similar mean Ae of 16 and 14 $pmol/mm^3$, the smaller MAB 8 probe appeared to respond more rapidly during the concentration phases. Steady-state was reached at both phases for this smaller probe, whereas steady-state was only reached at the higher concentration Phase B for the larger MAB 9 probe. This difference was reflected in the varying %iAUD of 87 ± 4.7% for the MAB 8 probe and of only 76 ± 6.4% for the MAB 9 probe. The K of 0.16 ± 0.01 $\mu L/min/mm^2$ from the MAB 8 probe was slightly higher compared to 0.14 ± 0.03 $\mu L/min/mm^2$ for the MAB 9 probe, but note that the latter K-value was not determined at steady-state, and is therefore slightly underestimated.

Thus, although the binding capacity per mm^3 remains the same, the total binding capacity is reduced with a smaller membrane, resulting in an improved responsiveness. Therefore, if a test compound adheres only slightly to the available material, the smallest possible membrane size should be chosen for pharmacokinetic applications.

Figure 54: Mean K versus time profiles for probes with different membrane surface areas (6 kDa PES-membranes only) tested with ZK 975

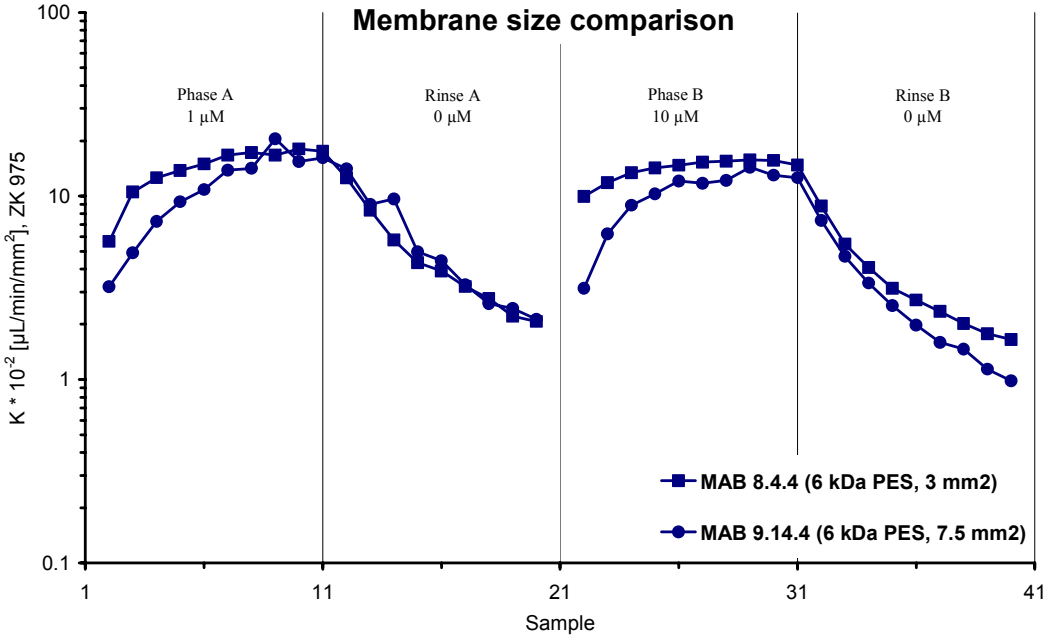
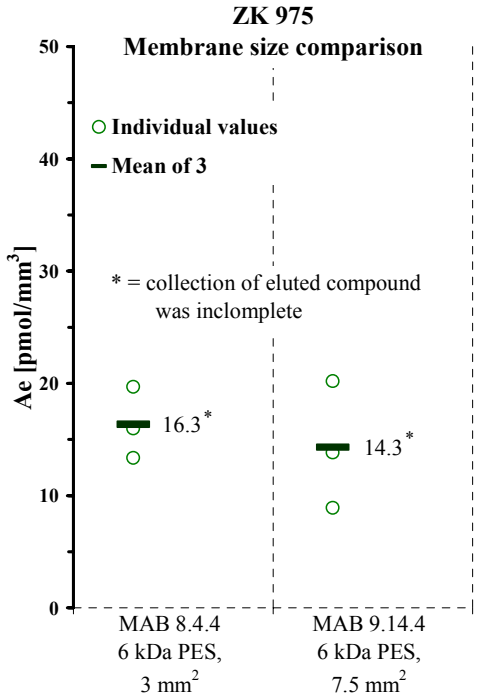


Figure 55: Overall Ae of different membrane surface areas (6 kDa PES -membranes) tested with ZK 975



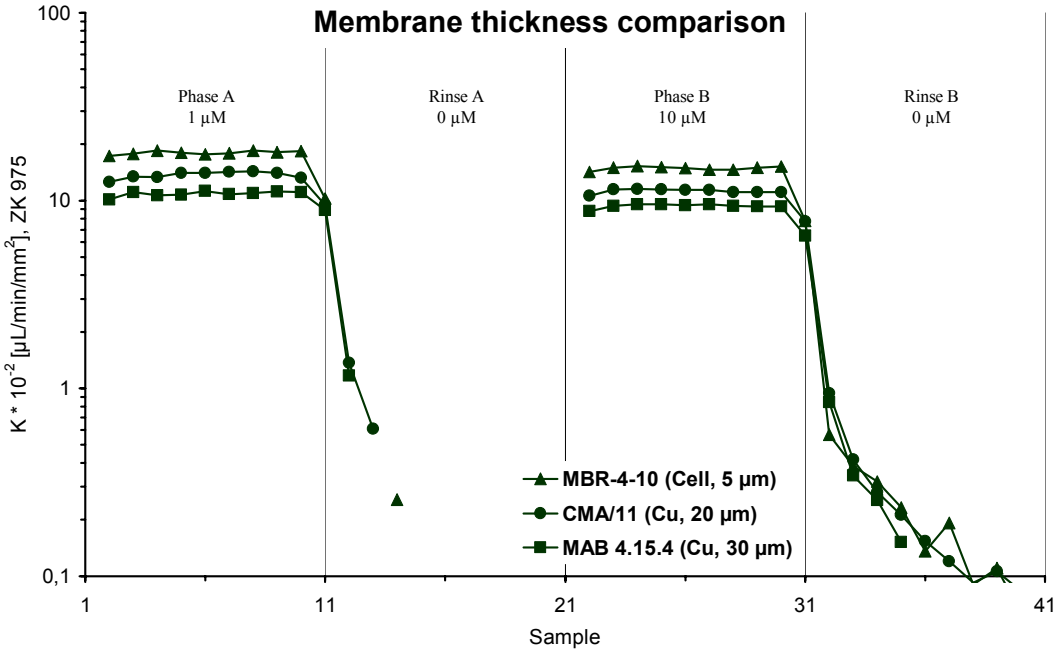
3.2.2.1.4 Comparison of membrane thicknesses tested with ZK 975

A graphical presentation of the mean K versus time for the three different membrane thicknesses tested (5, 20 or 30 µm, all cellulose based membranes) is given in Figure 56. A plot of the individual (○) and the mean (—) Ae is presented in Figure 57. Additional plots of the overall K and %iAUD can be found in Appendix I, F 18 and F 19.

The three cellulose type membranes behaved similarly well, regardless of membrane thickness. The mean %iAUD was ≥ 99% and the mean Ae ≤ 2 pmol/mm^3 for all three materials. The only discernable difference was K, which was 0.17 ± 0.02 µL/min/mm^2 for the 5 µm MBR-4 probe, 0.13 ± 0.02 µL/min/mm^2 for the 20 µm CMA/11 probe, and 0.10 ± 0.01 µL/min/mm^2 for the 30 µm MAB 4 probe. In other words, with no potential adhesion of the compound tested (ZK 975), the mass transfer coefficient seemed to increase with decreasing membrane thickness, as was to be expected.

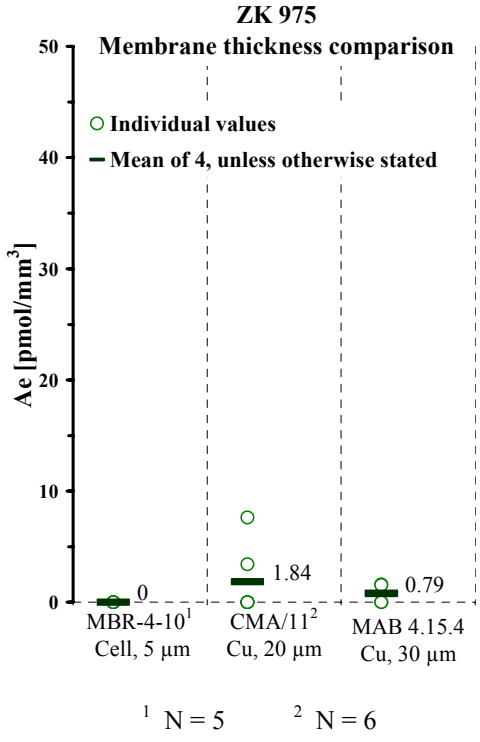
Therefore, if a high recovery is required (in order for sample concentrations to be above the detection limit of the available analytical method), the thinnest possible membrane should be chosen.

Figure 56: Mean K versus time profiles for probes with different membrane thicknesses (cellulose based membranes only) tested with ZK 975



Data missing from the graph have recoveries below the detection limit (LLOQ), and are not depicted on this semi-logarithmic scale (REC<LLOQ are set to 0%).

Figure 57: Overall Ae of different membrane thicknesses (cellulose based membranes) tested with ZK 975



3.2.2.1.5 Comparison of outlet material with ZK 975

A graphical presentation of the mean K versus time for the two different outlet materials tested (steel or PEEK, both 3.0 mm^2 6 kDa PES-membranes) is given in Figure 58. A plot of the individual (○) and the mean (▬) Ae is presented in Figure 59. Additional plots of the overall K and %iAUD can be found in Appendix I, F 20 and F 21.

The two probes with the different outlet material behaved quite similarly with ZK 975. The %iAUD was 87 ± 4.7% for the MAB 8 steel probe and 87 ± 4.2% for the MAB 4 PEEK probe, and the Ae was 16 ± 2.6 $pmol/mm^3$ for the steel probe and 16 ± 6.5% for the PEEK probe. Only K differed clearly, at 0.16 ± 0.01 $\mu L/min/mm^2$ for the MAB 8 steel probe and 0.24 ± 0.03 $\mu L/min/mm^2$ for the MAB 4 PEEK probe. There was no obvious reason for this discrepancy, but the K of 0.24 $\mu L/min/mm^2$ was by far the highest value found for any membrane-compound combination tested for this dissertation (see Chapter 3.2.2.3.3). The only discrepancy found in the protocol is that the concentrations of the media determined at the end of each concentration phase were higher than they were before the start of the experiment. For all other experiments the concentration of the media afterwards was slightly lower than before (if at all), as would be expected when compound is removed by microdialysis. This suggests that either the starting concentration was determined wrongly, which is unlikely with 3 samples taken of either concentration, or that the temperature was set too high, resulting in accelerated evaporation of the media. Since the temperature was only set at 37°C at the start of the experiment and not further monitored during the experiment, this might be a possible explanation for the high recoveries found.

Thus, although the effect of outlet material on K could not be assessed for ZK 975, there appeared to be no effect on the binding capacity of the probe, based on the similar %iAUD and Ae found. In other words, if binding of compound to the membrane is observed, any additional affinity for the outlet material is negligible.

Figure 58: Mean K versus time profiles for probes with different outlet materials (3 mm^2 6 kDa PES-membranes only) tested with ZK 975

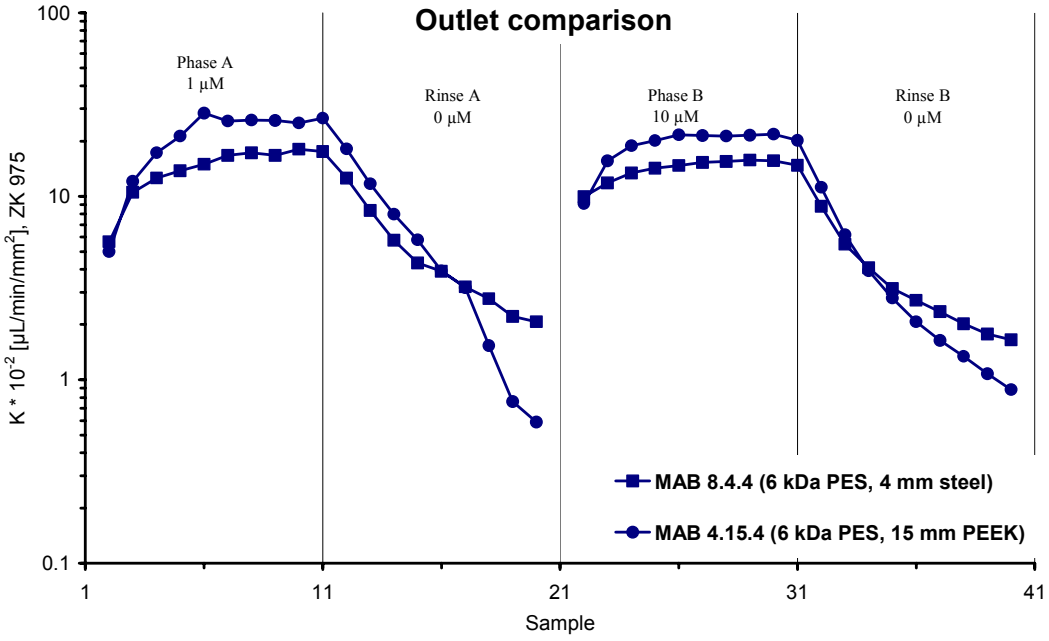
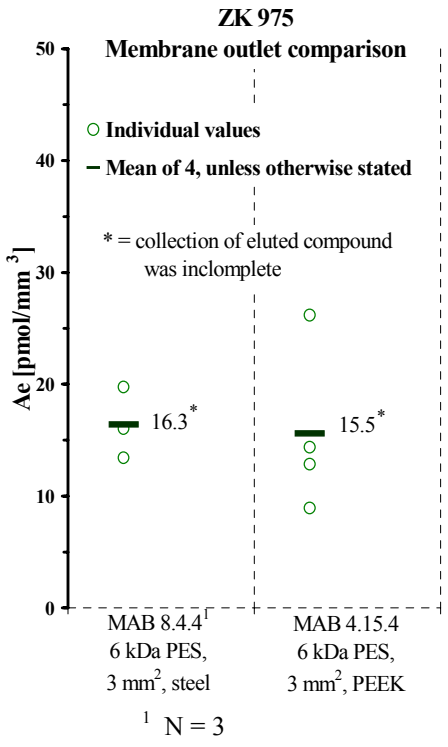


Figure 59: Overall Ae of probes with different outlet materials (3 mm^2 6 kDa PES-membranes) tested with ZK 975



3.2.2.2 Mean results for microdialysis probes tested with ZK 894

3.2.2.2.1 Comparison of membrane materials tested with ZK 894[1]

A graphical presentation of the mean K versus time for the five different materials tested (PC, PES, PAN, Cu and Cell) is given in Figure 60. A plot of the individual (○) and the mean (▬) Ae is presented in Figure 61. Additional plots of the overall K and %iAUD can be found in Appendix. I, F 22 and F 23.

As with ZK 975, the two membrane materials cellulosic and cuprophane behaved very similarly with ZK 894. Both had an excellent responsiveness, as reflected by a high overall %iAUD of ≥ 99% and a low Ae of < 1 pmol/mm^3 (Figure 61). The two materials differed only in their K of 0.19 ± 0.02 µL/min/mm^2 for the MBR-4 probe and 0.11 ± 0.01 µL/min/mm^2 for the MAB 4 probe.

The membrane material PES (35 kDa) performed equally well, with a %iAUD of 97 ± 2.1%, an Ae of 2.2 ± 1.6 pmol/mm^3, and a K of 0.16 ± 0.03 µL/min/mm^2.

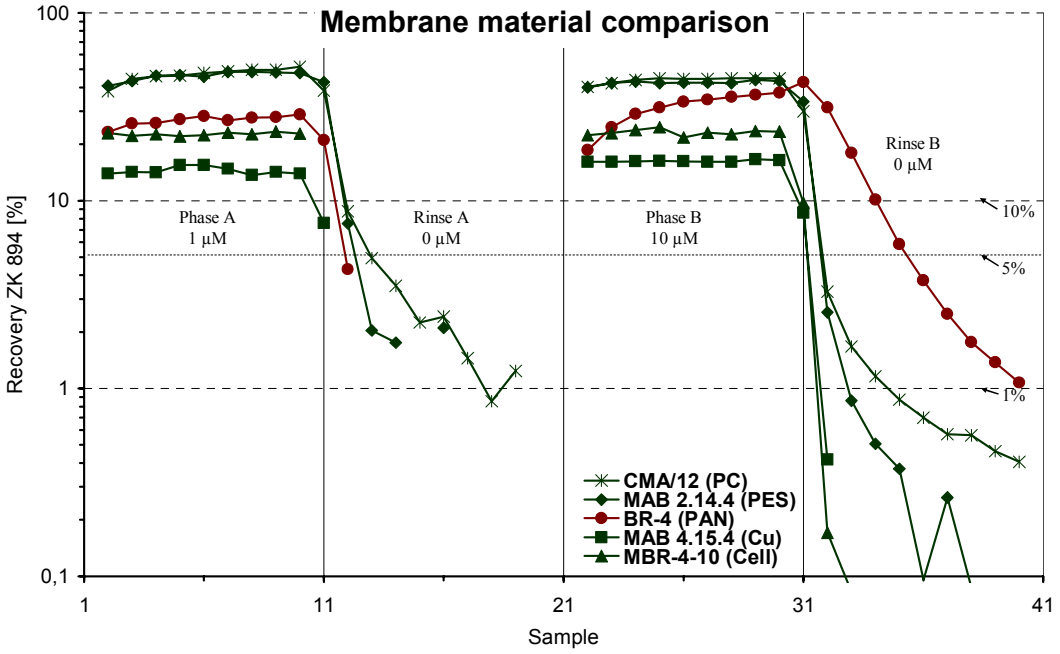
The PC-membrane also responded well to increasing concentration changes, with a %iAUD of 95 ± 3.2% and a K of 0.21 ± 0.03 µL/min/mm^2. However, considerable bleeding was observed during the rinsing phases, as reflected by a high Ae of 31 ± 5.1 pmol/mm^3. Although collection of compound was not entirely complete, and Ae is therefore underestimated, the calculated Ae clearly demonstrated elution of compound during the rinsing phases.

The PAN-membrane performed well at the lower concentration tested, with a %iAUD of 97 ± 1.7%. At the higher concentration however, steady-state was reached later (%iAUD = 86 ± 2.1%), and at a higher recovery (see chapter 3.2.1.3). Also the elution observed differed between the two rinsing phases. Therefore, the mean K, %iAUD and Ae could not be calculated, and a numerical comparison with the other membrane materials tested was not possible.

Overall, of the five materials tested, only Cu, Cell & PES can be recommended without reservations for pharmacokinetic studies with ZK 894. The PC-membrane would only be suitable for determining steady-state concentrations, and the PAN-membrane is not suitable for pharmacokinetic applications at all.

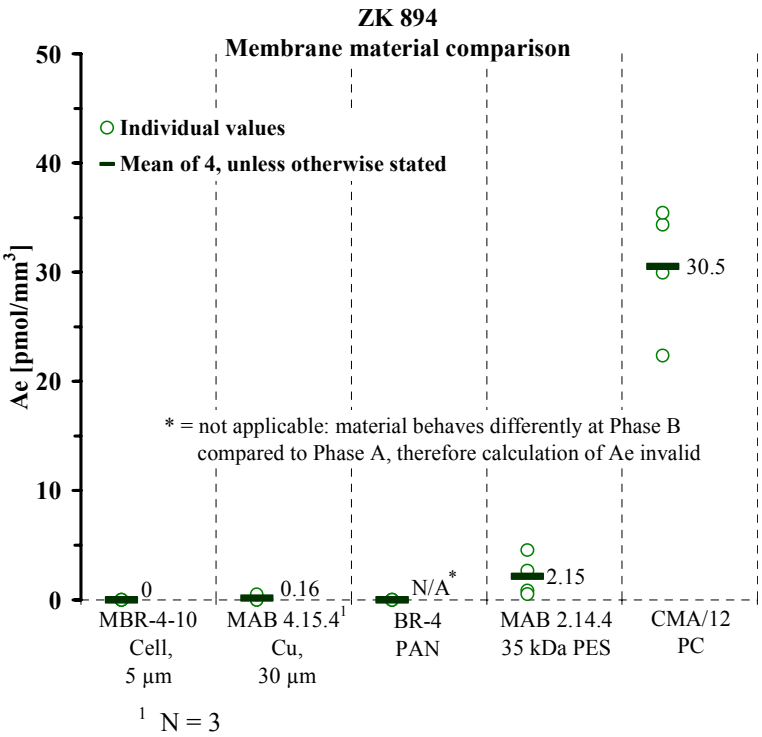
[1] The data presented in Chapter 3.2.2.2.1 have been published in part as poster abstracts (Meier-Ince and Günther, 2006a; Meier-Ince and Günther, 2006b)

Figure 60: Mean K versus time profiles for probes with different membrane materials tested with ZK 894



Data missing from the graph have recoveries below the detection limit (LLOQ), and are not depicted on this semi-logarithmic scale (REC<LLOQ are set to 0%).

Figure 61: Overall Ae of different membrane materials tested with ZK 894



3.2.2.2.2 Comparison of pore sizes tested with ZK 894

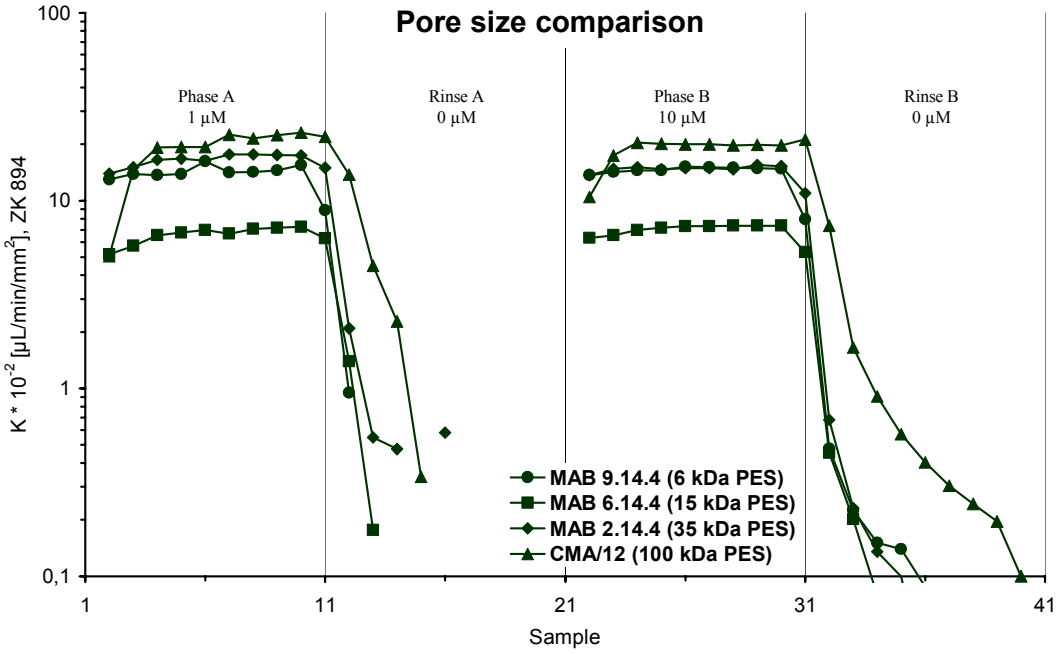
A graphical presentation of the mean K versus time for the four different pore sizes tested (6, 15, 35 and 100 kDa PES-membranes) is given in Figure 62. A plot of the individual (○) and the mean (—) Ae is presented in Figure 63. Additional plots of the overall K and %iAUD can be found in Appendix I, F 24 and F 25.

The three PES-membranes with pore sizes of 6, 15 or 35 kDa all behaved very similarly with ZK 894. All had a good responsiveness, with a mean %iAUD of 95 to 98%, and a low Ae of 1.1 to 2.2 pmol/mm^3. Only the overall mean K differed, at 0.15 ± 0.02 µL/min/mm^2 for the MAB 9 probe (6 kDa) and 0.16 ± 0.03 µL/min/mm^2 for the MAB 2 probe (35 kDa), but considerably lower at 0.07 ± 0.02 µL/min/mm^2 for the MAB 6 probe (15 kDa). This K of 0.07 µL/min/mm^2 was by far the lowest value found for any membrane-compound combination tested for this dissertation (the next lowest value found was 0.10 µL/min/mm^2 for the MAB 4 probe with ZK 975), and therefore seems implausible. No experimental deviations were noted in the protocol, and the results were consistent for all four probes tested (K = 0.05 to 0.07 µL/min/mm^2 for three probes and K = 0.11 µL/min/mm^2 for the fourth probe). It is possible that the temperature was overall slightly lower that day, as it was not monitored after setting it to 37°C. Alternatively, it is possible that all probes had air bubbles trapped under the membrane. Since the PES-membranes were protected by a white layer, detecting air bubbles was not always possible, and although all probes were tapped sharply at the holder before use, not all air bubbles may have been dislodged.

The CMA/12 probe with the 100 kDa PES-membrane appeared to respond more slowly overall (%iAUD = 91 ± 6.1%), and from a graphical appraisal appeared to also bleed for longer. However, due to ultrafiltration, the actual flow rate was only 0.8 µL/min, and any response at the membrane would therefore be monitored in the samples later compared to the other probes with a higher actual flow rate. Indeed, the calculated amount eluted was only 2.0 ± 1.7 pmol/mm^3, similarly low to that found with the other three pore sizes, and K was similar to that seen with the 6 and 35 kDa membranes, at 0.21 ± 0.03 µL/min/mm^2.

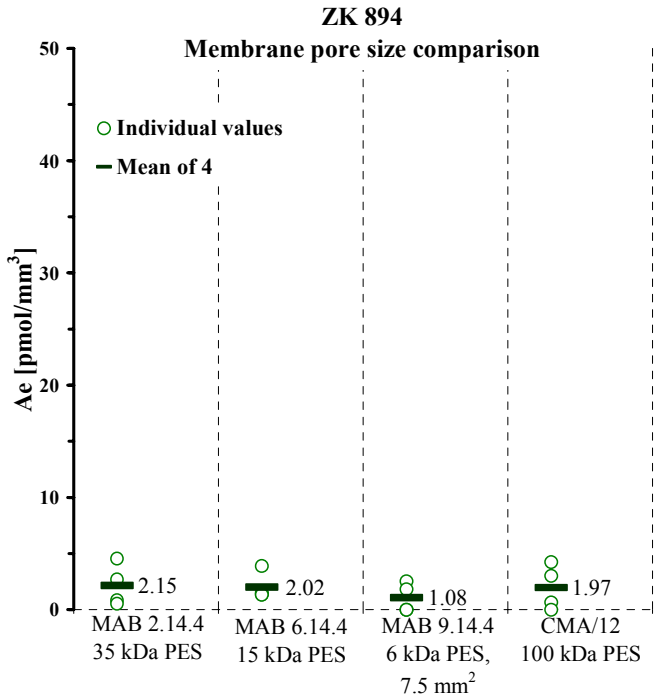
Overall, membrane behavior and K were similar between the different pore sizes tested (with one outlier), and an effect of pore size on recovery could not be found.

Figure 62: Mean K versus time profiles for probes with different membrane pore sizes (PES-membranes only) tested with ZK 894



Data missing from the graph have recoveries below the detection limit (LLOQ), and are not depicted on this semi-logarithmic scale (REC<LLOQ are set to 0%).

Figure 63: Overall Ae of membranes with different pore sizes (PES-membranes) tested with ZK 894



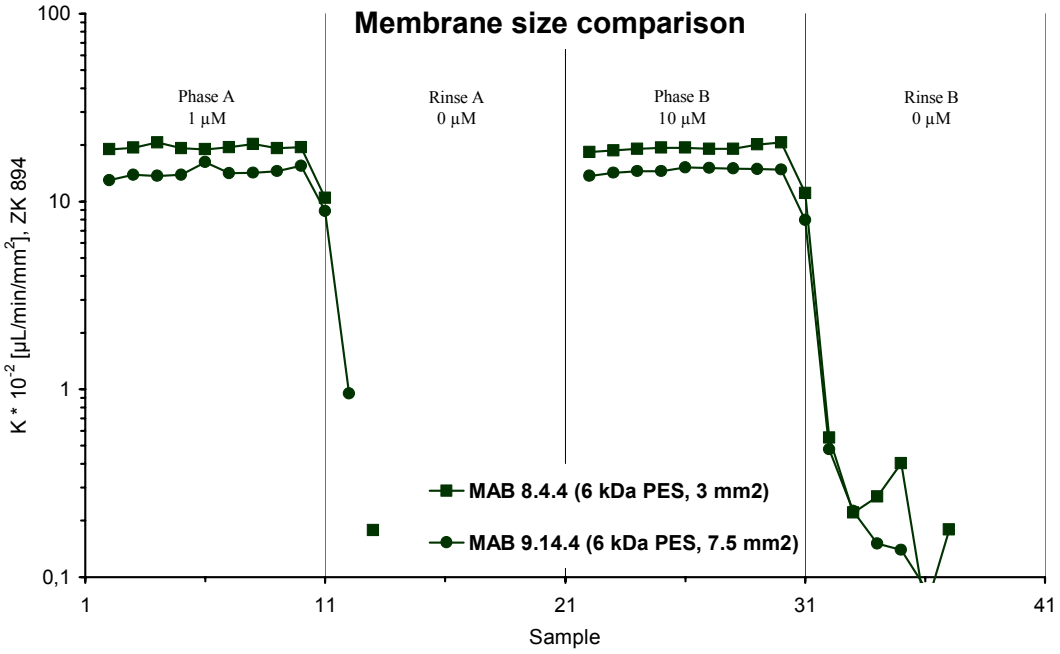
3.2.2.2.3 Comparison of membrane surface areas tested with ZK 894

A graphical presentation of the mean K versus time for the two different membrane surface areas tested (3.0 and 7.5 mm^2, both 6 kDa PES-membranes) is given in Figure 64. A plot of the individual (○) and the mean (—) Ae is presented in Figure 65. Additional plots of the overall K and %iAUD can be found in Appendix I, F 26 and F 27.

The two membrane sizes tested behaved very similarly to each other with ZK 894. The %iAUD were both high at 99 ± 1.6% for the MAB 8 probe and 98 ± 1.9% for the MAB 9 probe, and the Ae were both low at <3 $pmol/mm^3$. The K of 0.20 ± 0.03 $\mu L/min/mm^2$ for the MAB 8 probe was slightly higher compared to 0.15 ± 0.02 $\mu L/min/mm^2$ for the MAB 9 probe, which suggests that the mass transfer coefficient decreases with increasing membrane size. However, the two probes also differ in their probe geometry: the ratio of the outer radius of the inner cannula (r_α) to the inner radius of the membrane (r_i) is smaller for the MAB 9 probe ($r_\alpha / r_i = 0.4$) compared to the MAB 8 probe ($r_\alpha / r_i = 0.7$). Therefore the fluid layer is thicker for the MAB 9 probe (see also Table 6), the linear velocity of the perfusate along the membrane lower and the recovery higher (see also Figure 3 in Chapter 1.1.4.2), which is the more likely explanation for the differences in K seen.

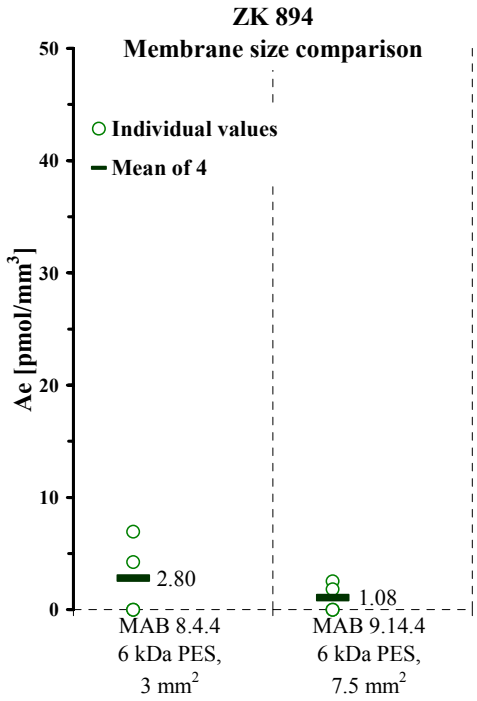
Thus, the higher K observed for the smaller MAB 8 probe is likely due to a more optimal probe design, compared to the larger MAB 9 probe, rather than a direct effect of membrane size. Therefore, if a maximization of the recovery is important, and the possibility of constructing own probes exists, changing the probe geometry should be considered.

Figure 64: Mean K versus time profiles for probes with different membrane surface areas (6 kDa PES-membranes only) tested with ZK 894



Data missing from the graph have recoveries below the detection limit (LLOQ), and are not depicted on this semi-logarithmic scale (REC<LLOQ are set to 0%).

Figure 65: Overall Ae of different membrane surface areas (6 kDa PES -membranes) tested with ZK 894

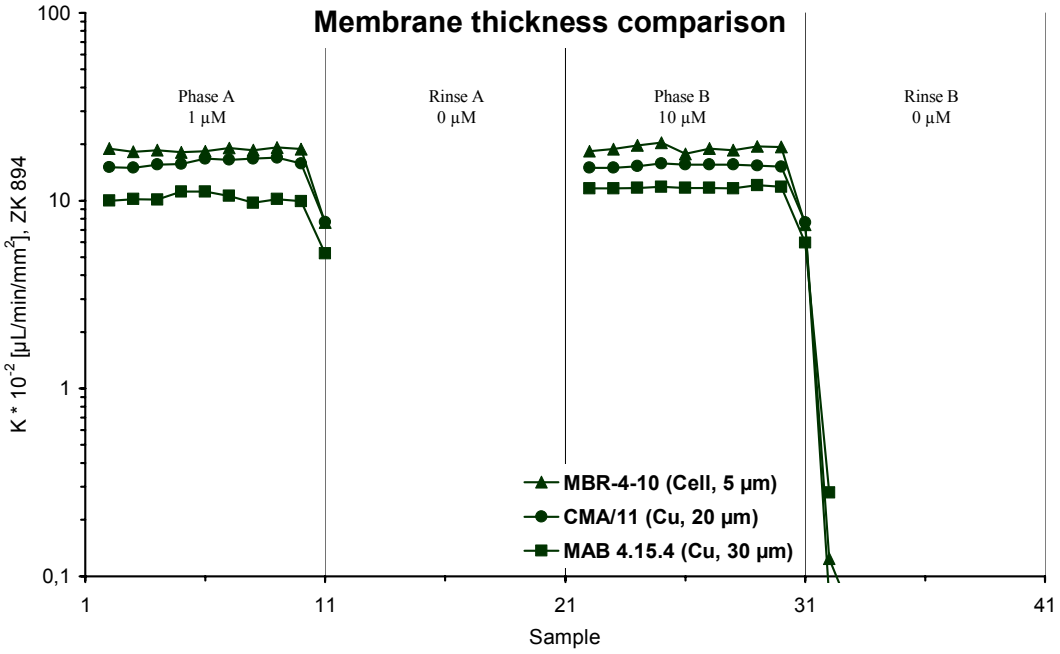


3.2.2.2.4 Comparison of membrane thicknesses tested with ZK 894

A graphical presentation of the mean K versus time for the three different membrane thicknesses tested (5, 20 or 30 µm, all cellulose based membranes) is given in Figure 66. A plot of the individual (○) and the mean (▬) Ae is presented in Figure 67. Additional plots of the overall K and %iAUD can be found in Appendix I, F 28 and F 29.

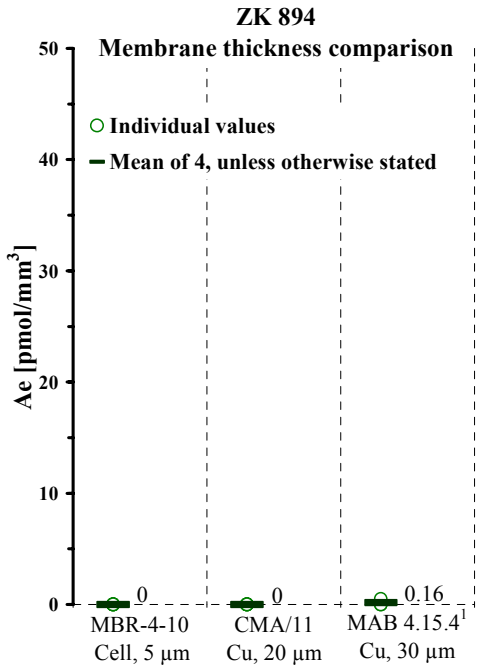
The three cellulose type membranes behaved similarly well, with a mean %iAUD of ≥ 99% and a mean Ae of < 1 pmol/mm^3 for all three materials. The only discernable difference was K, which was 0.19 ± 0.02 µL/min/mm^2 for the 5 µm MBR-4 probe, 0.16 ± 0.02 µL/min/mm^2 for the 20 µm CMA/11 probe, and 0.11 ± 0.01 µL/min/mm^2 for the 30 µm MAB 4 probe. Therefore, as was also found with ZK 975 (Chapter 3.2.2.1.4), the mass transfer coefficient seemed to increase with decreasing membrane thickness, and if a high recovery is important, a thin membrane should be chosen.

Figure 66: Mean K versus time profiles for probes with different membrane thicknesses (cellulose based membranes only) tested with ZK 894



Data missing from the graph have recoveries below the detection limit (LLOQ), and are not depicted on this semi-logarithmic scale (REC<LLOQ are set to 0%).

Figure 67: Overall Ae of different membrane thicknesses (cellulose based membranes) tested with ZK 894



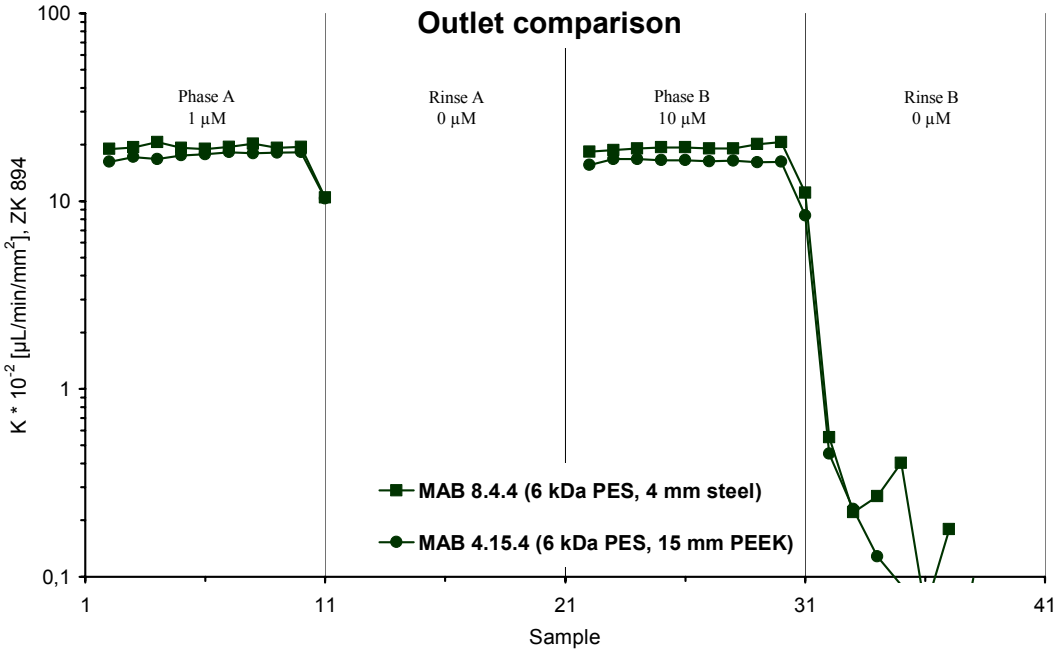
[1] N = 3

3.2.2.2.5 Comparison of outlet materials tested with ZK 894

A graphical presentation of the mean K versus time for the two different outlet materials tested (steel or PEEK, both 3.0 mm^2 6 kDa PES-membranes) is given in Figure 68. A plot of the individual (○) and the mean (—) Ae is presented in Figure 69. Additional plots of the overall K and %iAUD can be found in Appendix I, F 30 and F 31.

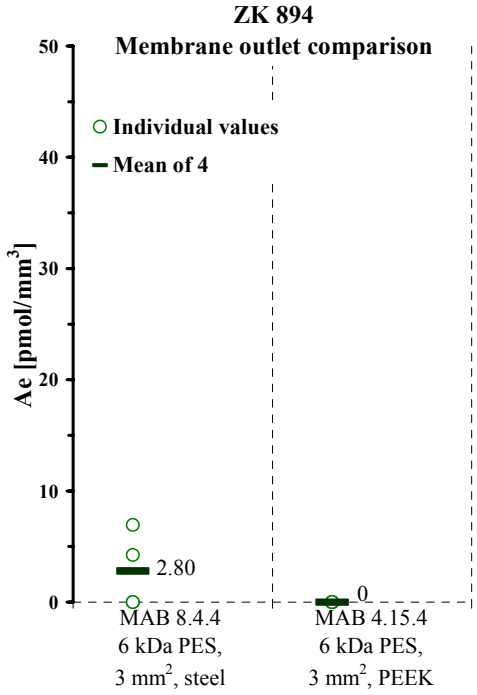
The two probes with the different outlet material behaved very similarly with ZK 894. The mean %iAUD was 99% and the mean Ae <3 pmol/mm^3 for both probes. In contrast to ZK 975, K was also similar at 0.20 ± 0.03 µL/min/mm^2 for the MAB 8 steel probe and 0.17 ± 0.06 µL/min/mm^2 for the MAB 4 PEEK probe. Note that for the MAB 8 probe one overall low outlier was included in this mean (see Figure 47). If only the three higher probes were considered, the mean K would also be 0.21 µL/min/mm^2. Therefore, no influence of outlet material on probe performance was found with ZK 894.

Figure 68: Mean K versus time profiles for probes with different outlet materials (3 mm^2 6 kDa PES-membranes only) tested with ZK 894



Data missing from the graph have recoveries below the detection limit (LLOQ), and are not depicted on this semi-logarithmic scale (REC<LLOQ are set to 0%).

Figure 69: Overall Ae of probes with different outlet materials (3 mm² 6 kDa PES - membranes) tested with ZK 894

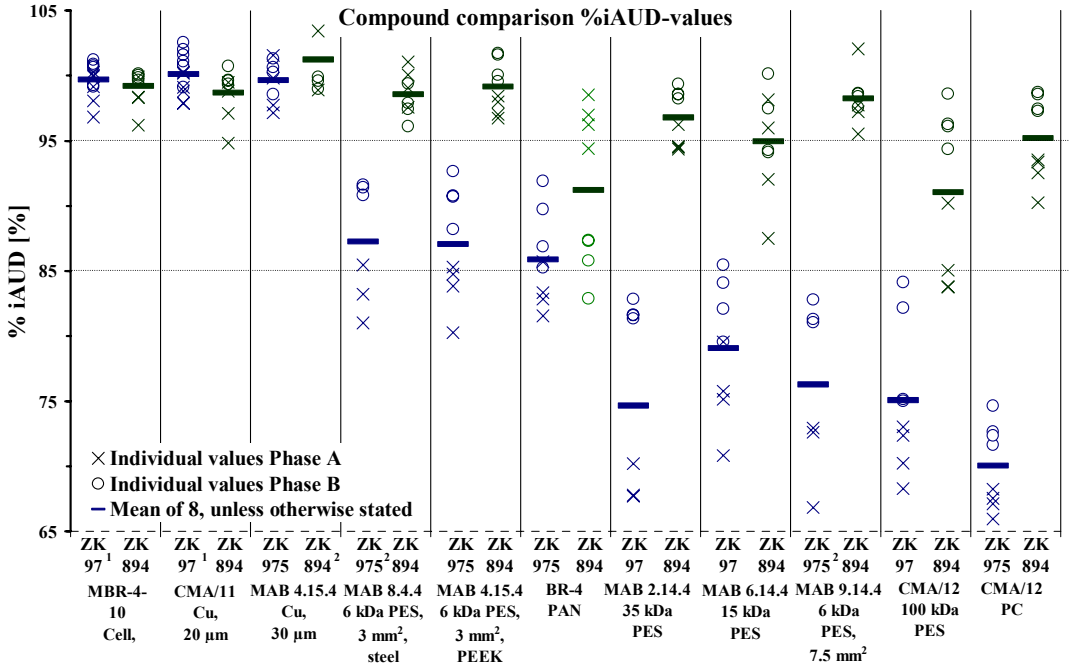


3.2.2.3 Comparison of mean microdialysis results for ZK 975 versus ZK 894

3.2.2.3.1 Comparison of mean %iAUD obtained with either compound

The %iAUD differed greatly between the two compounds (Figure 70), except when probes performed well with either compound (the Cell and Cu probes). Generally, %iAUD > 95% for probes with a rapid response to both increasing and decreasing concentrations (= suitable for pharmacokinetic applications). For probes which responded more slowly, but still reached steady-state within the 1 hour observation period (= suitable for determination of steady-state concentrations only), 85% < %iAUD < 95%. For probes which did not reach steady-state and showed extensive elution (= not suitable for pharmacokinetic applications), %iAUD < 85%.

Figure 70: Overview of all individual and mean %iAUD obtained with ZK 894 and ZK 975

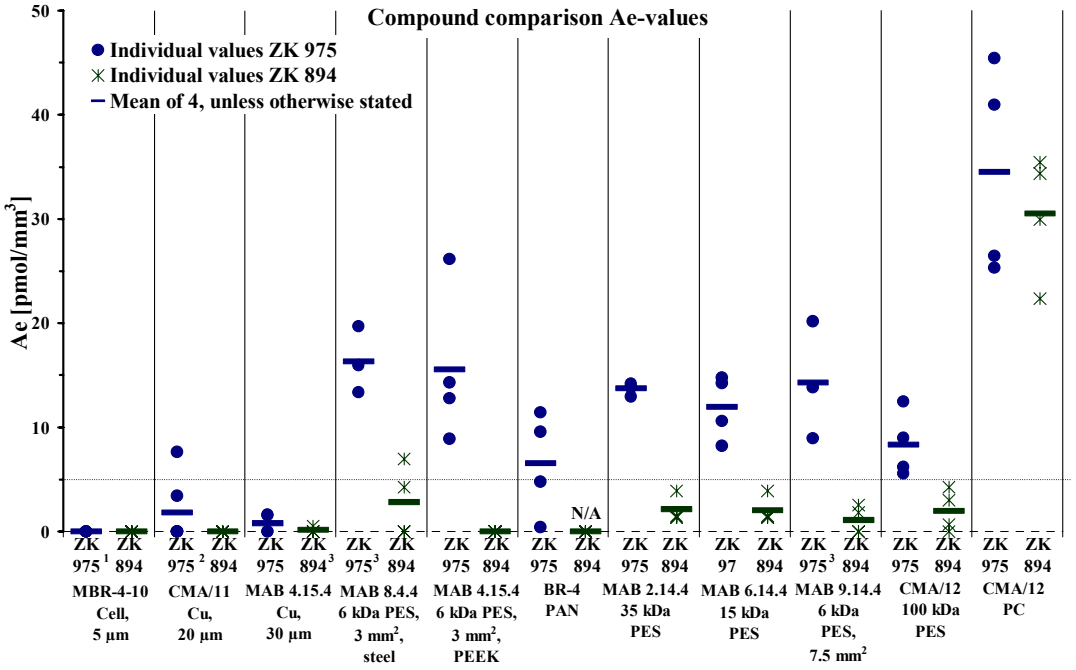


[1] N = 6, thus mean of 12 values [2] N = 3, thus mean of 6 values

3.2.2.3.2 Comparison of mean Ae obtained with either compound

Like the %iAUD, the Ae differed greatly between the two compounds (Figure 71), except for those probes which performed well with either compound (the Cell and Cu probes). Generally, for probes which graphically showed little elution (= suitable for pharmacokinetic applications), Ae < 5 pmol/mm^3. For probes which graphically clearly showed prolonged elution of compound (= not suitable for pharmacokinetic applications), Ae > 5 pmol/mm^3, even if collection of compound was not complete, and therefore not all criteria for a valid calculation of the Ae were met, resulting in an underestimation of Ae.

Figure 71: Overview of all individual and mean Ae obtained with ZK 894 and ZK 975

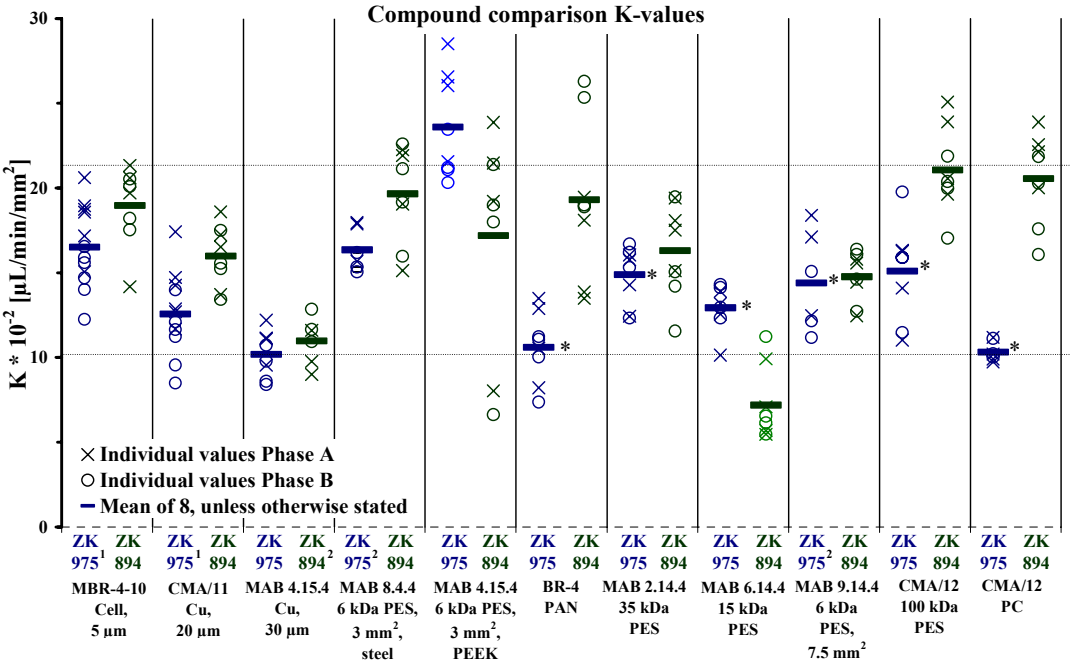


N/A = not applicable, since behavior different between the two concentrations tested.
[1] N = 5 [2] N = 6 [3] N = 3

3.2.2.3.3 Comparison of mean K obtained with either compound

With the exception of the two outlying datasets with the MAB 4 (PES) and the MAB 6 probes, the mean K was always within the range of 0.10 to 0.21 µL/min/mm^2 (Figure 72), and no clear trend could be observed. Generally, K obtained with the lipophilic compound ZK 975 appeared slightly lower than K obtained with the hydrophilic compound ZK 894. A clear difference however was only evident with the 100 kDa PES- and the PC-membrane, the two materials with the poorest responsiveness for ZK 975 (as reflected by low %iAUDs, see Figure 70), and therefore the most underestimated K (since steady-state not reached at either concentration phase).

Figure 72: Overview of all individual and mean K obtained with ZK 894 and ZK 975



[1] N = 6, thus mean of 12 values [2] N = 3, thus mean of 6 values

* steady-state not reached therefore K is underestimated

3.2.3 Stability of ZK 975 and ZK 894 under microdialysis test conditions

The radioactivity in the microdialysis samples analyzed by thin layer chromatography (TLC) was too little to evaluate the scanned imaging plates, even after 3 weeks of exposure time. Therefore, an additional one-off stability test was carried out under the same experimental conditions as the microdialysis probe tests, but samples for TLC-analysis were taken from the medium, instead of the dialysate. The percentage of the parent compound in each of the samples analyzed is given in Table 14 (individual chromatograms are given in Appendix III):

Table 14: Percentage of parent compound in the medium before, after 1.5 hours and after 3.5 hours storage under experimental conditions as for the probe tests

	^{14}C-ZK 975			
	Before		After 1.5 hrs	After 3.5 hrs
Sample number	1 µM	10 µM	1 µM	10 µM
1	91.8	95.2	85.4	91.4
2	90.7	95.6	85.9	91.7
mean [% RAD]	91.8	95.5	84.6	91.8
SD	0.64	0.23	0.04	0.09
		Remaining*	**92.2**	**96.0**

	^{14}C-ZK 894			
	Before		After 1.5 hrs	After 3.5 hrs
Sample number	1 µM	10 µM	1 µM	10 µM
1	85.5	88.6	86.4	87.7
2	86.2	89.8	87.7	87.6
mean [% RAD]	85.6	88.7	87.0	87.0
SD	0.45	0.18	1.27	0.17
		Remaining*	**102**	**98.1**

RAD = total ^{14}C-radioactivity

* Remaining = %RAD afterwards / %RAD before × 100

For ^{14}C-ZK 975, the percentage of parent compound was 92% in the 1 µM solution, and 96% in the 10 µM solution at the start of the test. The total amount of parent compound remained well above 90% of the starting concentration, throughout the experimental time (1.5 hours for Phase A and 3.5 hours for Phase B).

For ^{14}C-ZK 894, the percentage of parent compound was 86% in the 1 µM solution, and 89% in the 10 µM solution at the start of the test. The total amount of parent compound remained above 98% of the starting concentration throughout the experimental time. Therefore, both compounds were stable under the experimental conditions of the microdialysis probe tests.

The low percentage of parent compound ZK 894 of 86-89% in freshly made solution was also observed with samples analyzed before each experiment (data not shown), and is likely due to ^{14}C-impurities in the solution. Since no elution of radioactivity was observed for any PES-membrane tested with ZK 894, a materials which showed high affinity for the lipophilic compound ZK 975, it can be concluded that these 10-15% radioactive impurities did not affect the conclusions drawn from the microdialysis results for ZK 894.