Electronic Supplementary Material (ESI) for Analytical Methods.

Supporting Information

In-situ setup for screening of drug permeation by NMR spectroscopy

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Table of Contents

1 NMR spectroscopic analysis

1.1 Slice-selective NMR spectroscopy

Selective excitation of defined localized slices of the NMR tube enables separate measurement of our donor and acceptor compartment. Different pulse sequences can be employed to achieve slice selection.

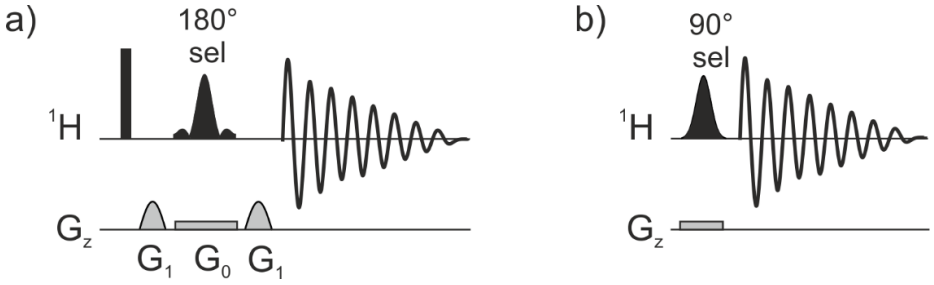

Figure S1. Schematic representation of the (a) spatially-encoded pulsed filed gradient spin-echo (PFGSE) and (b) spatially-encoded pulse-acquired pulse sequence. The narrow rectangle represents a hard 90° radiofrequency pulse. The 180° and 90° shaped pulses denote a frequency-selective refocusing and excitation pulse, respectively. Pulsed field gradients are represented in the Gz channel. Gradient pulses G_1 are used to enforce the coherence transfer pathway and G_0 is a weak spatial-encoding gradient.

To excite only a small slice of the NMR tube a frequency-selective, long, shaped pulse and a weak pulsed filed gradient have to be simultaneously applied. The z position of the selectively measured horizontal slice depends on the offset frequency v_z of the selective pulse and can be calculated according to equation (1) as is shown in **Figure S2**:

$$v_z = \frac{\gamma G_0 z}{2\pi} \qquad (1)$$

where γ is the gyromagnetic ratio of the observed nucleus and G_0 is the spatial-encoding gradient field applied along the z-axis (the axis of the tube) and z is the position of the frequency relative to the center of the gradient coil. The thickness of the slice Δz can be calculated according to equation (2):[1]

$$\Delta z = \frac{2\pi}{\gamma G_s} \Delta v \qquad (2)$$

where Δv is the bandwidth of the selective pulse. Δz and z must be set with a sufficient safety margin, as imperfections of the gradient and the shaped pulse occur at the edges of the top-hat excitation profile and therefore may excite part of the other slide.[1]

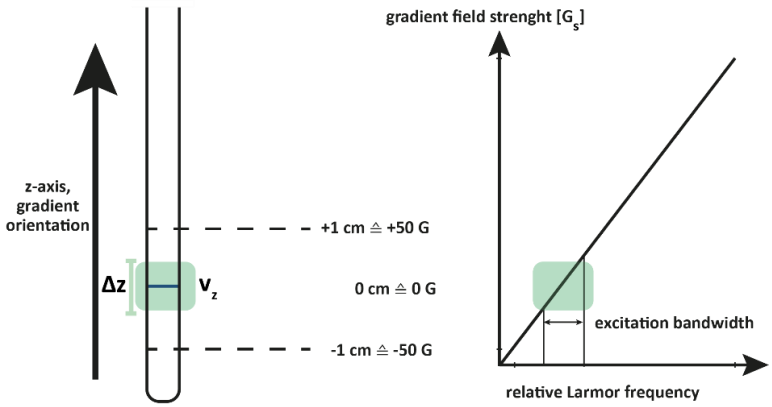

Figure S2: Schematic diagrams showing (left) the position of the selected slice (in light green) along the NMR tube and (right) the effect of the gradient field strength on the relative Larmor frequency and the excitation bandwidth.

1.2 Determination of the T_1 relaxation times

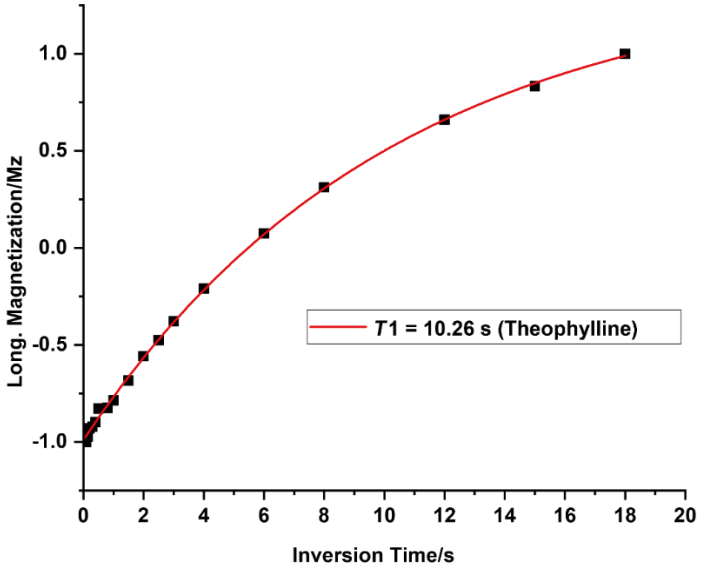

Figure S3: Exemplary normalized integral intensity of the aromatic signal of theophylline at 7.89 ppm as a function of the inversion time in seconds, which is the incremented delay in the "t1ir" inversion-recovery pulse sequence and resulting in a T_1 relaxation time of 10.26 ± 0.38 s. Fitting was carried out using Origin.

Table S1. T_1 relaxation times of the resonances of interest of the different, investigated drug molecules.

Drug	Signal of interest for quantification [ppm]	T_1 [s]
Theophyllin	7.89	10.26
Paracetamol	6.80	3.64
Metoprolol	6.89	1.58
Testosterone	5.87	0.7

1.3 Phasing difficulties with the initial pulse sequence

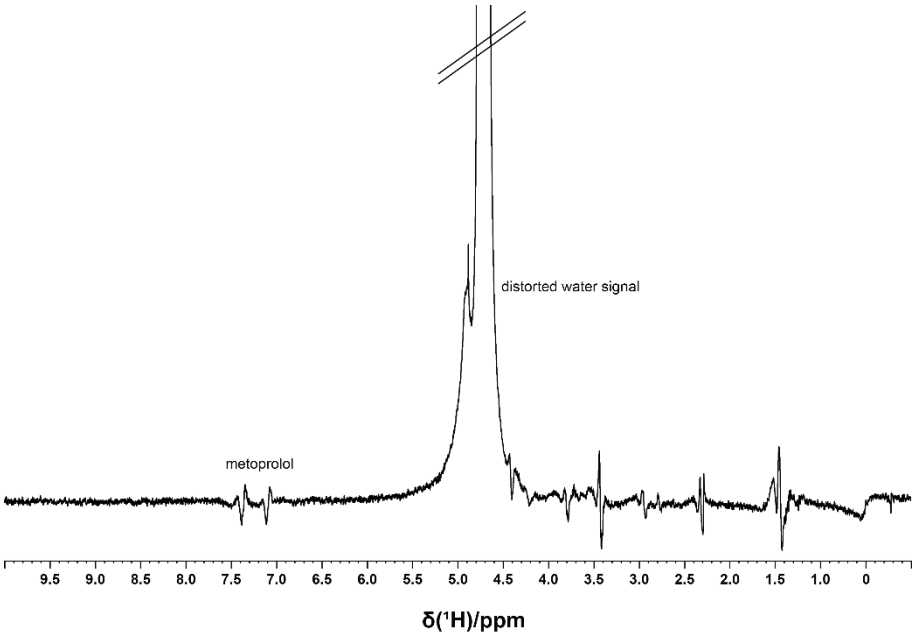

Figure S4. Example of a slice-selective ^{1}H NMR spectrum of metoprolol in deuterated PBS with uncorrectable signal distortions measured with a selzg based pulse program.

1.4 Spatially-encoded pulsed field gradient spin-echo pulse program

The optimized pulse sequence for Bruker Avance NEO consoles is provided below:

```
;lc_selgpse_euromar (Laura Castanar, 13/07/2022)
;selgpse
;1D spatial-encoded spin echo with gradients
;   using Zangger-Sterk element as active spin refocussing element
;WaveMaker compatible (type "wvm -a" to calculate and set up parameters)
;
;Avance NEO Version
;Topspin.4x
;
;
;$CLASS=HighRes
;$DIM=1D
;$TYPE=
;$SUBTYPE=
;$COMMENT=
#include <Avance.incl>
#include <Grad.incl>

;%%%%%%%%%%%%% WaveMaker Parameters %%%%%%%%%%%%%
;sp12:wvm:lc_rsnob:f1 rsnob(cnst13 Hz, cnst12 ppm; NP=10000) ss=1 us;
;%%%%%%%%%%%%% Delay Calculations %%%%%%%%%%%%%
"d16=1ms"
"d11=30m+1s/(cnst12)-1s/(cnst12)"
"d11=30m+1s/(cnst13)-1s/(cnst13)"

;%%%%%%%%%%%%% Pulse Calculations %%%%%%%%%%%%%
"p16=1ms"
```

```
;%%%%%%%%%%%% Selective Pulse %%%%%%%%%%%%%
"spoff12=0"
"acqt0=0"
baseopt_echo
1 ze
2 30m
  50u BLKGRAMP      ;PFG amp blanked
  50u LOCKH_OFF     ;sample lock signal
  d1 pl1:f1
  50u LOCKH_ON      ;lock sampling off (lock-hold)
  50u UNBLKGRAMP    ;PFG amp unblanked
  p1 ph1
    d10
  p16:gp1
  d16 pl0:f1
  10u
  10u gron0
  (p12:sp12 ph2):f1
  10u
  10u groff
  p16:gp1
  d16 BLKGRAMP
  d10
  go=2 ph31
  30m mc #0 to 2 F0(zd)
exit
50u LOCKH_OFF

ph1= 0 0 0 0 2 2 2 2
ph2= 0 1 2 3
ph31=0 2 0 2 2 0 2 0

;POWER LEVEL
;pl0   : 0W
;pl1   : f1 channel - power level for pulse (default)
;spw12 : RF power of selective 180 degree pulse

;PULSE DURATION
;p1 : f1 channel -  90 degree high power pulse
;p12: f1 channel - 180 degree shaped pulse
;p16: homospoil/gradient pulse [1 ms]

;DELAY
;d1  : relaxation delay; 1-5 * T1
;d10 : extra time either side of the selective spin element [0 s by default]
;d16 : delay for homospoil/gradient recovery [1 ms]

;PULSE SHAPE
;spnam12: shape of the selective 180 pulse

;GRADIENT STRENGTH
;gpz0: spatial encoding gradient [0.1-3%]
;gpz1: CTP gradient for selective 180 pulse [17%]

;GRADIENT SHAPE
;gpnam1: SMSQ10.100

;OTHERS
;ns: 2 * n, total number of scans: NS * TD0
;ds: 2
;cnst12: chemical shift for selective refocussing pulse (in ppm)
;cnst13: bandwidth for selective refocussing pulse (in Hz)
;set in the center of the spectral window to be excited
;choose p12 according to desired selectivity
```

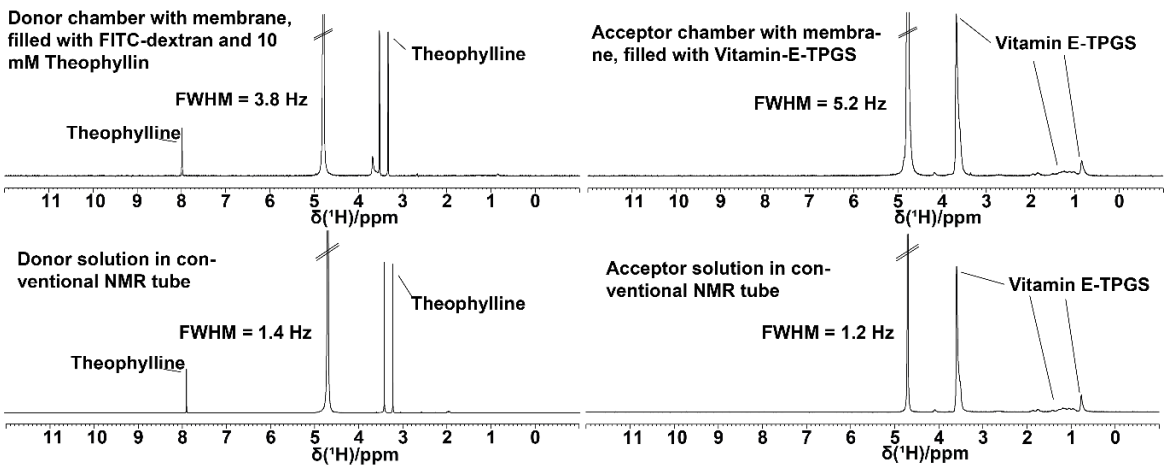

Figure S5. Slice-selective ^{1}H NMR spectra of the donor (left) and acceptor (right) compartment solutions using an NMR tube with a membrane attached (top) and in a conventional NMR tube (bottom). At the beginning of the experiment, the donor compartment contained 10 mM theophylline and 10 mg mL^{-1} FITC-Dextran in PBS and the acceptor compartment 2% vitamin E-TPGS in PBS. The FWHM of the water signal is given. For the measurements, two slices were acquired using a gradient strength of 0.25 G mm^{-1} simultaneously applied with a 463 µs 180 ° Rsnob pulse (747.30 Hz bandwidth) and a slice thickness of 0.69 mm centered approximately 7.4 mm (7000 Hz) below and above the gradient center.

2 Experimental section

2.1 Drug substances and exemplary NMR spectra

Metoprolol tartrate, paracetamol, and theophylline were purchased from Merck KGaA, Darmstadt, Germany. For each of the drug substances, time-resolved NMR spectra showing the aryl region are depicted below.

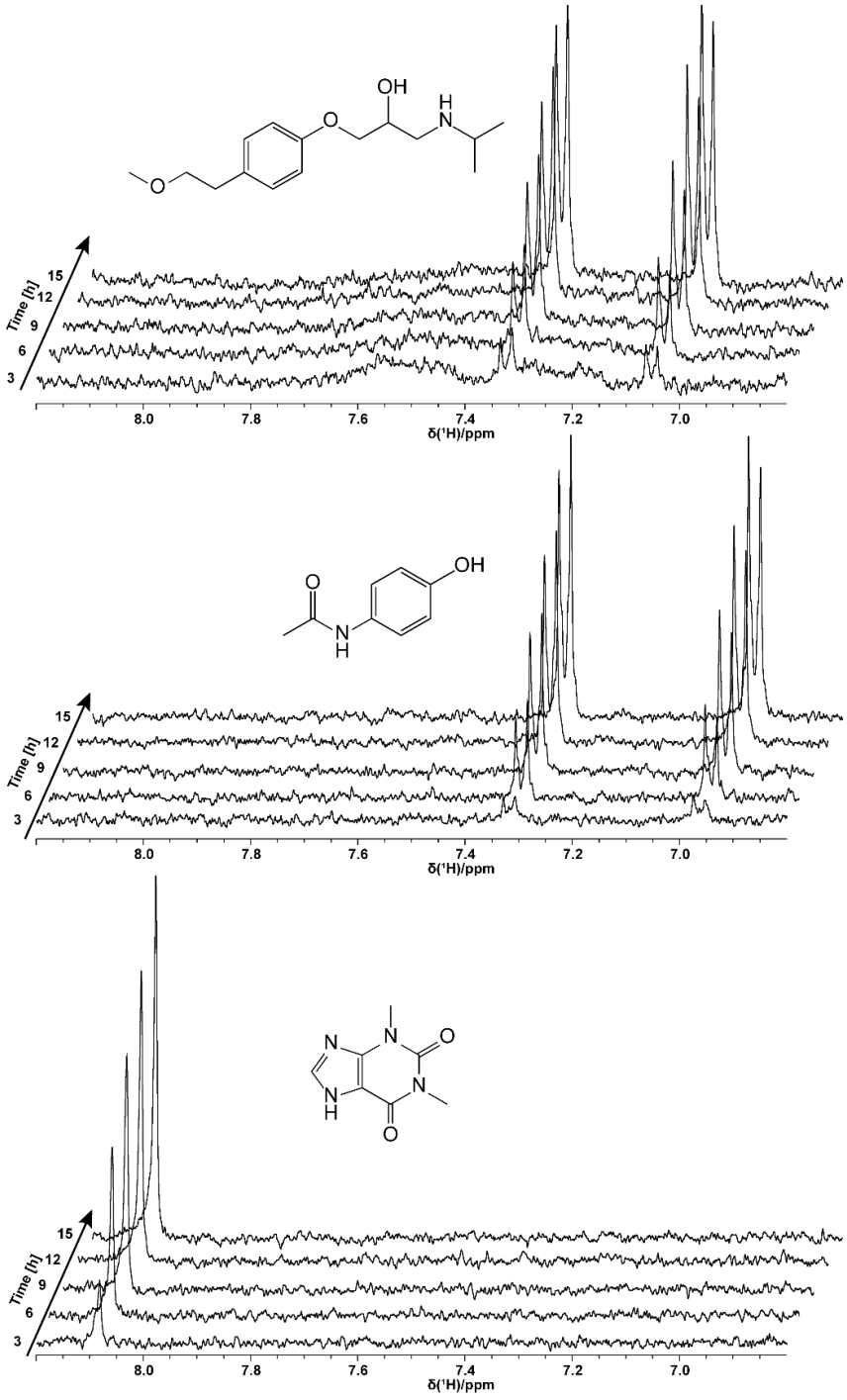

Figure S6. Exemplary comparison of the aryl signal intensity with the membrane in position 1d (see main manuscript Figure 1) of metoprolol (top), paracetamol (center), and theophylline (bottom) over time.

2.2 Diffusion measurement

Diffusion NMR data of testosterone (Merck KGaA, Darmstadt, Germany) was acquired with a BBFO probe (maximum gradient strength 50 G cm^{-1}) using the *ledbpgp2s* Bruker pulse program without rotation. The diffusion gradient strength was linearly incremented in 32 steps from 2 to 98%. The diffusion time (d20) was set to 50 ms and the length of the gradient pulses (p30) was adjusted to 1.3 ms to achieve signal attenuation below 1%. The diffusion coefficient was determined by fitting the attenuation curves of well-separated signals in Origin using a mono-exponential fit. An exemplary attenuation curve and the corresponding fit is shown below.

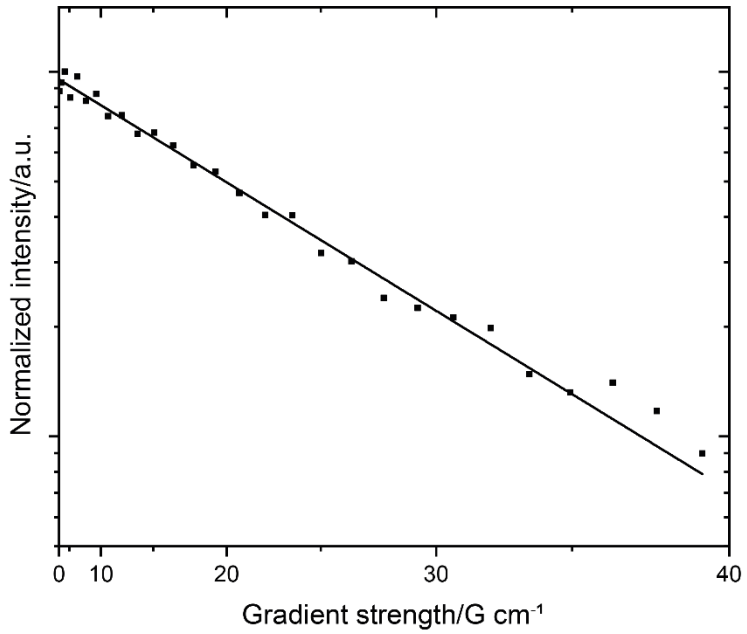

Figure S7. Exemplary normalized and linearized diffusion attenuation curve of the testosterone signal at 5.87 ppm in PBS without vitamin-E-TPGS.

2.3 Media preparation

Modified phosphate buffered saline (PBS) with a pH value of 6.5 was prepared according to the protocol of Biorelevant.com. PBS contained 106 mM sodium chloride and 29 mM anhydrous sodium monobasic phosphate. The pH value was adjusted to 6.50 ± 0.05 with sodium hydroxide or hydrochloride acid using a daily calibrated pH electrode “phenomenal” (Mettler Toledo, Greifensee, Switzerland).

2.4 Equilibrium solubility determination by shake flask method

The equilibrium solubility of each drug was determined by the shake flask method. Ca. 10 mg of the drug substance were weighed into a 2.0 mL amber reaction vial. PBS was added achieving a nominal drug concentration of 100 mM. Samples were shaken for 24 hours at a temperature of 37 °C and a shaking speed of 750 rpm on a Thermomixer F1.5 (Eppendorf). Then, the samples were centrifuged at 13400 rpm for 10 min with a MiniSpin centrifuge

(Eppendorf). Supernatans were diluted with acetonitrile containing 0.1% TFA. Measurements were performed in triplicate.

2.5 Integrity test

The membrane integrity was assessed measuring the fluorescence of a 40 kDa dextran labelled with fluorescein isothiocyanate (FITC) (Merck KGgA) on a Tecan Reader Infinite 200 pro plate reader (Tecan Group, Maennedorf, Switzerland) at an excitation and emission wavelength of 489 nm and 520 nm, respectively. The donor concentration of the dextran was 10 mg ml^{-1}. Quantification of permeated dextran was done based on a calibration curve. The limit of detection (LOD) was determined to be 0.57 ng mL^{-1} following the manufacturer's manual. The membrane was defined as intact if less than 1% of the donor concentration of the dextran was found in the acceptor chamber at the end of the experiment, which ranged from 14 to 16 hours. An approximate, yet straightforward visual inspection also helps to assess membrane integrity (time points 0, 7 and 24 hours):

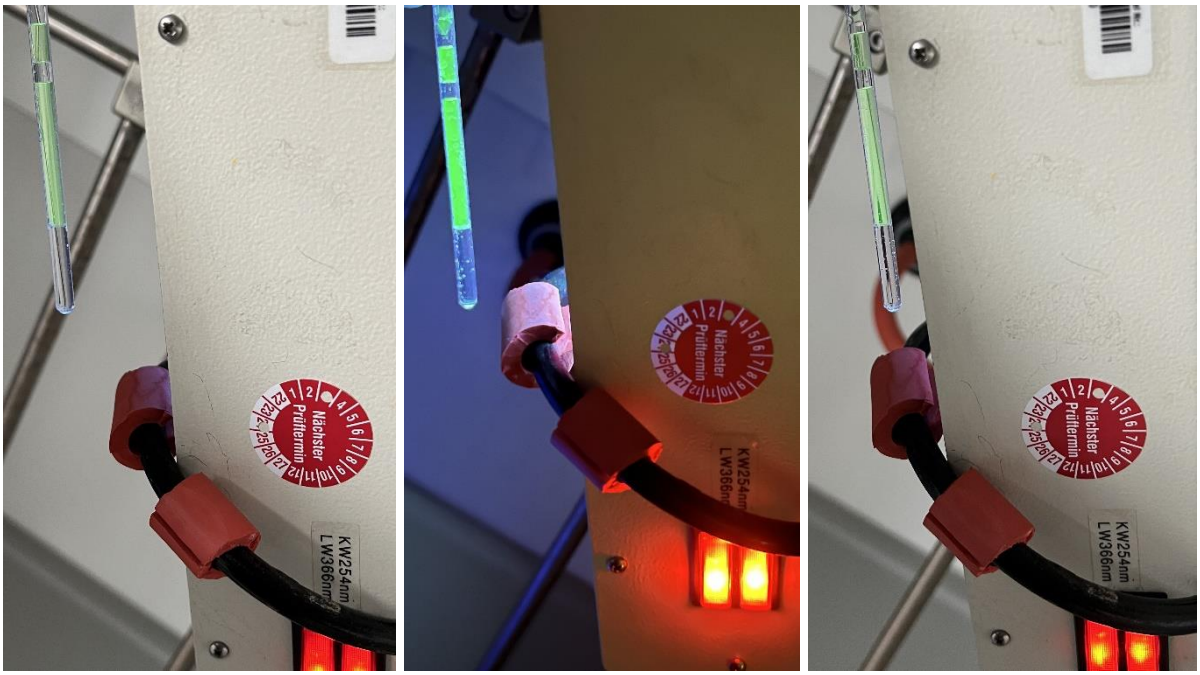

Figure S8. Visual inspection of a permeation experiment through a cellulose membrane in the presence of 40 kDa dextran labelled with fluorescein isothiocyanate using UV-light after zero- (left), seven- (middle) and 24- (right) hours.

3 HPLC analysis

Samples were analyzed with an Agilent 1260 infinity II HPLC (Agilent Technologies Inc., Waldbronn, Germany) equipped with a wavelength detector G7114A, an automatic vial sampler G7129C, a flexible pump G7104C, and a multicolumn oven G7116A. The HPLC methods are listed from **Table S2** to **Table S4**, exemplary chromatograms are given in **Figure S9** to **Figure S11,** and calibration curves in **Figure S13** to **Figure S15**.

3.1 HPLC methods

Table S2. HPLC method for metoprolol.

Drug substance	**Metoprolol**		
Column	Zorbax Eclipse XDB-C18, 4,6 * 150mm, 5 µm		
Flow rate [mL min^{-1}]	1		
Injection volume [µL]	5		
Detection wavelength	275		
Mobile phase	A:	Water with 0.1 % (v/v) trifluoroacetic acid	
	B:	Acetonitrile with 0.1 % (v/v) trifluoroacetic acid	
Gradient	Time [min]	% of mobile phase A	% of mobile phase B
	0	95	5
	1	95	5
	9	55	45
	10	0	100
	13	0	100
	15	95	5
	19	95	5
Retention time [min]	8.165		
Limit of detection [µM]	6		
Limit of quantification [µM]	19		
Calibration range [µM]	19 – 1000		

Table S3. HPLC method for paracetamol.

Drug substance	**Paracetamol**		
Column	Zorbax Eclipse XDB-C18, 4,6* 150mm, 5 µm		
Flow rate [mL min^{-1}]	1		
Injection volume [µL]	5		
Detection wavelength	273		
Mobile phase	A:	Water with 0.1 % (v/v) trifluoroacetic acid	
	B:	Acetonitrile with 0.1 % (v/v) trifluoroacetic acid	
Gradient	Time [min]	% of mobile phase A	% of mobile phase B
	0	95	5
	6	80	20
	6.5	0	100
	10	0	100
	10.3	95	5
	14	95	5
Retention time [min]	4.7		
Limit of detection [µM]	29		
Limit of quantification [µM]	89		
Calibration range [µM]	89 – 1500		

Table S4. HPLC method for theophylline.

Drug substance	**Theophylline**		
Column	Zorbax Eclipse XDB-C18, 4,6 * 150mm, 5 µm		
Flow rate [mL min^{-1}]	1		
Injection volume [µL]	5		
Detection wavelength	273		
Mobile phase	A:	Water with 0.1 % (v/v) trifluoroacetic acid	
	B:	Acetonitrile with 0.1 % (v/v) trifluoroacetic acid	
Gradient	Time [min]	% of mobile phase A	% of mobile phase B
	0	95	5
	6	80	20
	6.5	0	100
	10	0	100
	10.3	95	5
	14	95	5
Retention time [min]	5.1		
Limit of detection [µM]	22		
Limit of quantification [µM]	68		
Calibration range [µM]	68 – 1500		

Table S5. HPLC method for testosterone.

Drug substance	**Testosterone**		
Column	Zorbax Eclipse XDB-C18, 4,6 * 150mm, 5 µm		
Flow rate [mL min^{-1}]	1		
Injection volume [µL]	50		
Detection wavelength	234		
Mobile phase	A:	Water with 0.1 % (v/v) trifluoroacetic acid	
	B:	Acetonitrile with 0.1 % (v/v) trifluoroacetic acid	
Gradient	Time [min]	% of mobile phase A	% of mobile phase B
	0	80	20
	6	0	100
	10	0	100
	11	80	20
	15	80	20
Retention time [min]	5.6		
Limit of detection [µM]	2		
Limit of quantification [µM]	15		
Calibration range [µM]	15 – 1000		

3.2 HPLC - exemplary chromatograms

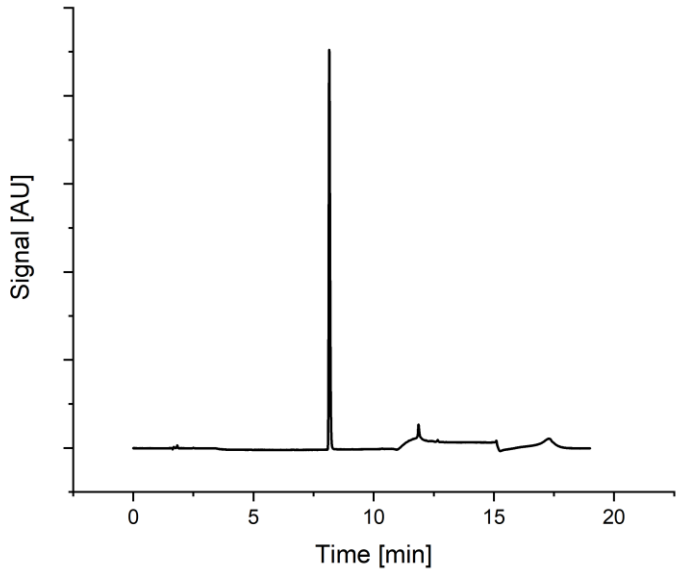

Figure S9. Exemplary chromatogram of metoprolol with a retention time of 8.2 min.

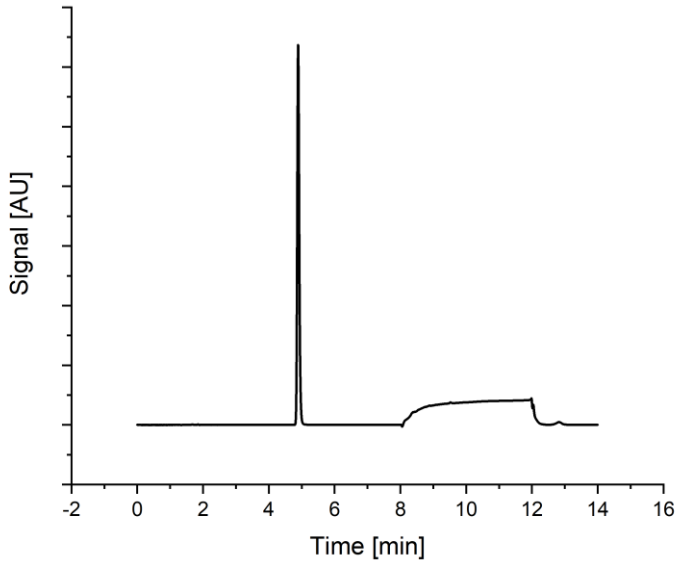

Figure S10. Exemplary chromatogram of paracetamol with a retention time of 4.7 min.

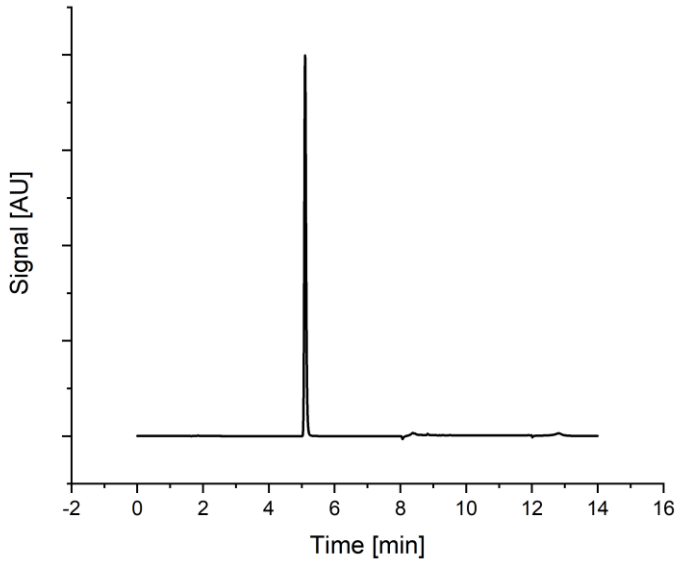

Figure S11. Exemplary chromatogram of theophylline with a retention time of 5.1 min.

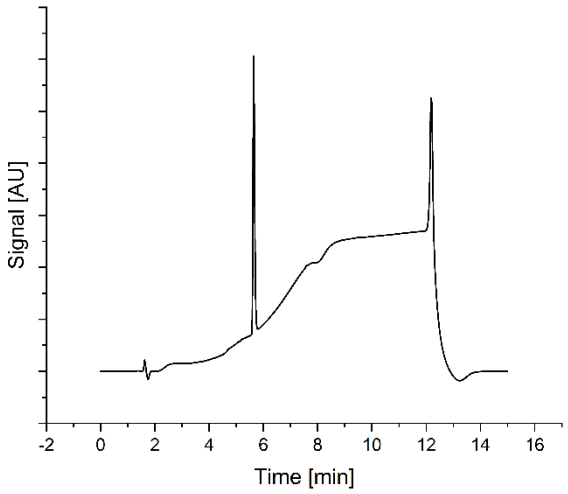

Figure S12. Exemplary chromatogram of testosterone with a retention time of 5.6 min.

3.3 HPLC calibration curves

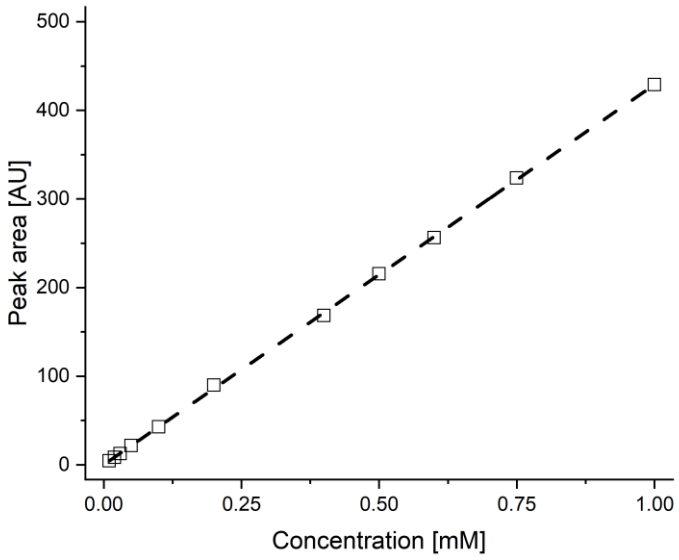

Figure S13. HPLC calibration curve of metoprolol. Data shown as unfilled squares, linear fit as dashed line (Pearson's r: 0.999).

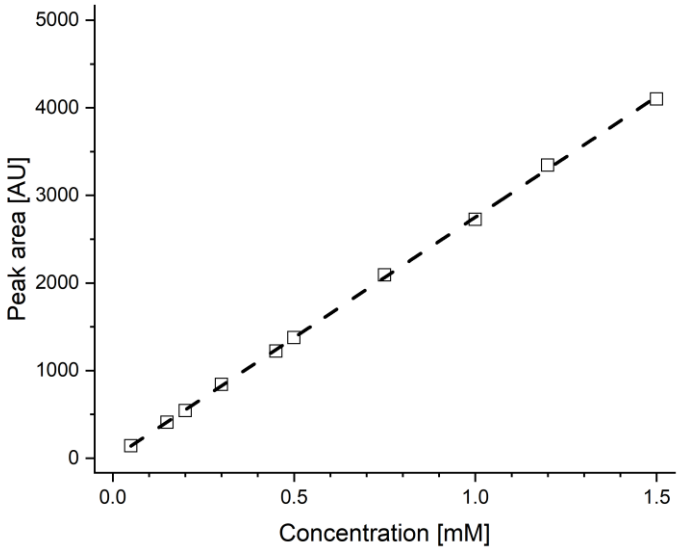

Figure S14. HPLC calibration curve of paracetamol. Data shown as unfilled squares, linear fit as dashed line (Pearson's r: 0.999).

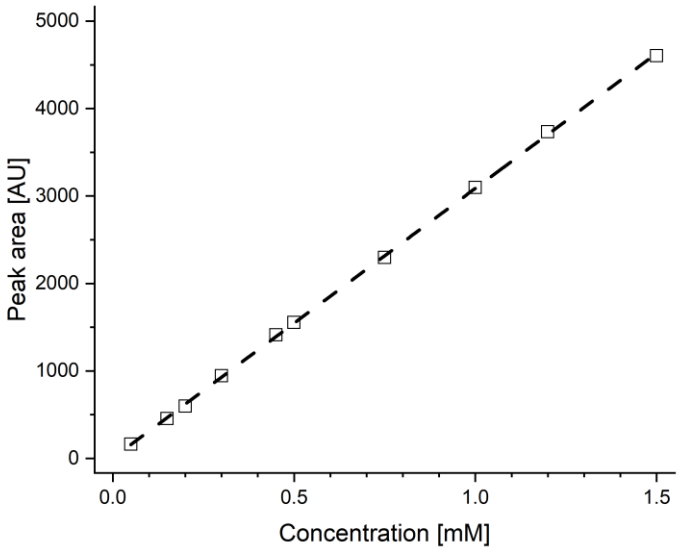

Figure S15. HPLC calibration curve of theophylline. Data shown as unfilled squares, linear fit as dashed line (Pearson's r: 0.999).

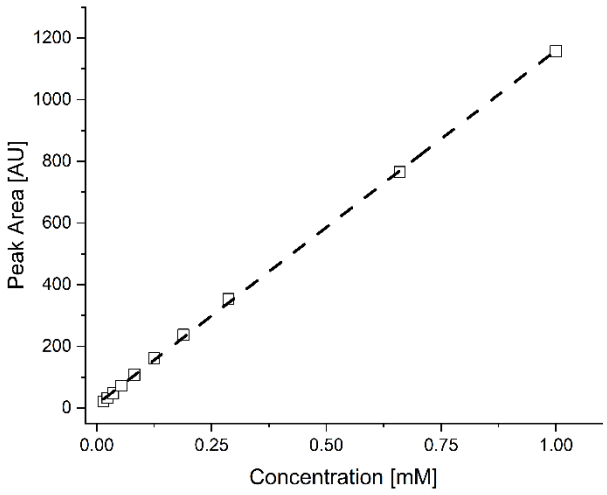

Figure S16. HPLC calibration curve of testosterone. Data shown as unfilled squares, linear fit as dashed line (Pearson's r: 0.999).

3.4 Flux by side-by-side diffusion cells

A side-by-side diffusion cell (PermeGear Inc., Hellertown, PA, USA) was used to determine the permeability. The donor and receiver compartments were separated by a cellulose membrane (innoME GmbH, Espelkamp, Germany) with a pore size and a surface area of 33 nm and 1.77 cm², respectively. While the donor compartment contained 10 mL of donor solution, the receiver compartment was filled with 10 mL of PBS containing 2% (w/v) vitamin-E-TPGS. The temperature was kept constant at 310 K using a Haake Fisons C1 water circulator (Thermo Fisher Scientific Inc., Karlsruhe, Germany) with a DLK 1002 cooling unit (FRYKA GmbH, Esslingen, Germany). The fluids were stirred at 500 rpm on a H9-CB-02 stirring apparatus (SES GmbH, Bechenheim, Germany). At the beginning of the experiment, 100 µL of a 1 mol L^{-1} metoprolol or paracetamol stock solution, 100 µL of a 100 mmol L^{-1} testosterone stock solution in DMSO or 1000 µL of a 0.1 mol L^{-1} theophyllin stock solution in PBS were added to the donor compartment filled with 9.9 or 9 mL of PBS, respectively. After 15, 30, 60, 120, and 180 min aliquots of 100 µL were taken from the acceptor compartment followed by replacement with fresh medium. The samples were diluted with acetonitrile (ACN) with 0.1% (v/v) trifluoroacetic acid (TFA), vortexed for at least 30 s on a VTX-3000 L (LMSCO. LTD., Tokyo, Japan) and centrifuged with a MiniSpin centrifuge (Eppendorf, Hamburg, Germany) at 13.000 rpm for 10 min. Experiments were carried out in triplicate. The flux was calculated from the slope of the resulting concentration, as obtained from HPLC, versus time profile using linear regression per permeated area.

3.5 Concentration-time profiles using the side-by-side cell setup

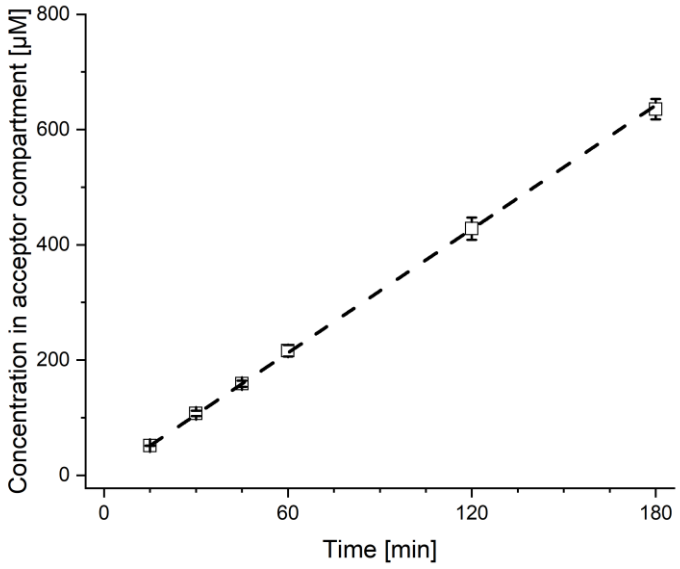

Figure S17. Metoprolol concentration in the flux acceptor compartment over time, measured in the standard side-by-sidel cell setup. Permeability was 3.34 ± 0.11 x 10^{-5} cm s^{-1}. Data shown as mean ± standard deviation, linear fit as dashed line.

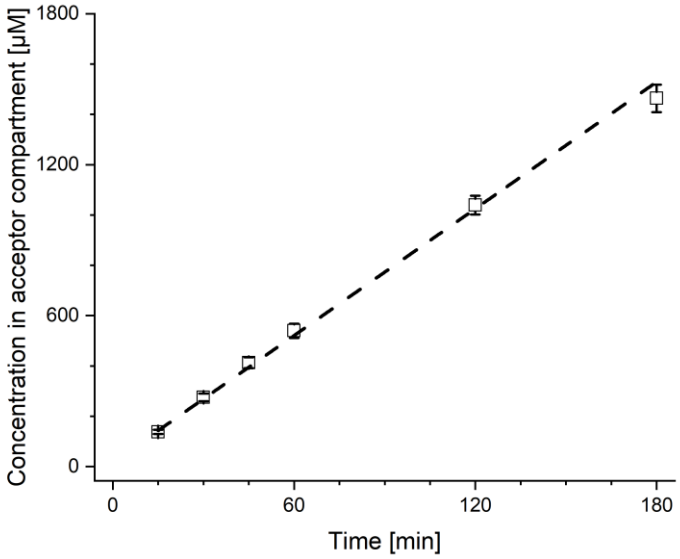

Figure S18. Paracetamol concentration in the flux acceptor compartment over time, measured in the standard side-by-side cell setup. Permeability was 8.06 ± 0.25 x 10^{-5} cm s^{-1}. Data shown as mean ± standard deviation, linear fit as dashed line.

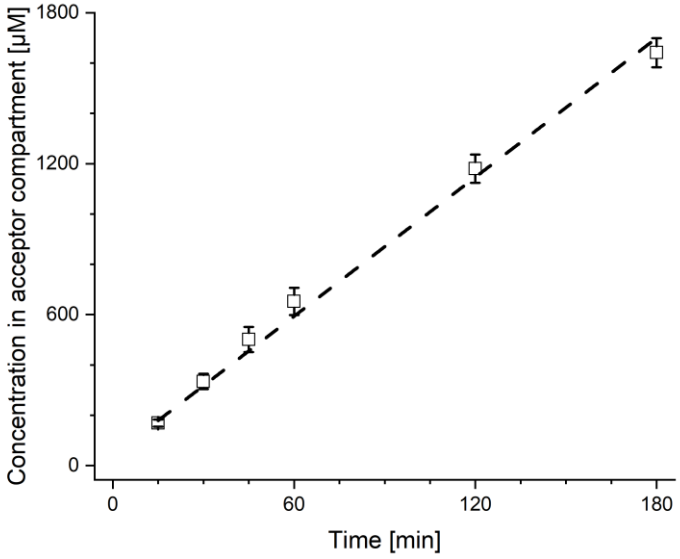

Figure S19. Theophylline concentration in the flux acceptor compartment over time, measured in the standard side-by-side cell setup. Permeability was 9.32 ± 0.81 x 10^{-5} cm s^{-1}. Data shown as mean ± standard deviation, linear fit as dashed line.

4 Determination of the height of the Unstirred Water Layer (UWL)

The height h of the UWL was determined with the following equation and the drug testosterone, according to Korjamo *et al*.[2]

$$h = \frac{D}{P_{UWL}} \tag{3}$$

with the diffusion coefficient D, and the permeability across the UWL P_{UWL}. Together with the previously determined D of 6.84 x 10^{-6}cm^2 s^{-1}, and a P_{app} of 1.088 x 10^{-5}cm s^{-1} this resulted in a height of 6290 µm for our setup. For the side-by-side diffusion cell, a P_{app} of 5.568 x 10^{-5}cm s^{-1} yields a height of the UWL of 1230 µm.

5 Images of alternative NMR tube setups

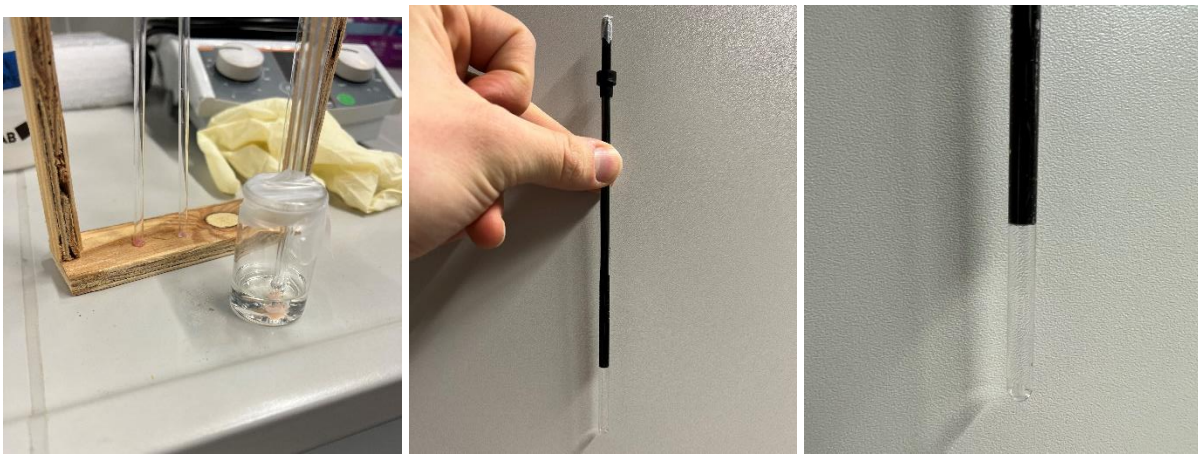

Figure S20. Images of alternative NMR tube setups including a pig intestine as alternative barrier (left) as well as 3D-printed inserts (mid and right) to substitute the glass tubes.

6 References

1. A.-C. Pöppler, S. Frischkorn, D. Stalke and M. John, *Chemphyschem*, 2013, **14**, 3103-3107.
2. T. Korjamo, A. T. Heikkinen, J. Mönkkönen, J. Pharm. Sci. 2009, **98**, 4469-4479.