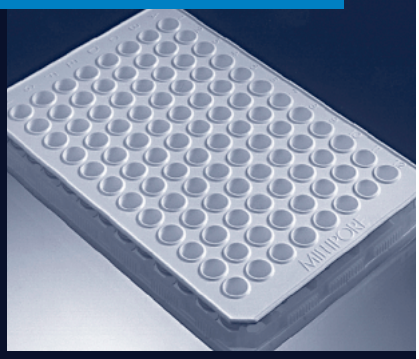
application note



MultiScreen™ Permeability Plates

The evaluation of the reproducibility of passive, transcellular drug permeability assays

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Abstract

The reproducibility and precision of a high throughput method based on a published method[1] using a Millipore plate (MultiScreen Cat. MAPBMN310) for predicting passive, transcellular compound permeability was assessed. The permeability of six drugs (propranolol, methotrexate, warfarin, carbamazepine, furosemide, and testosterone) was measured on different days using five different lots of (MultiScreen) plates. In addition several protocol variations (incubation times, volumes, temperature, etc.) were tested to simulate the analytical variability that might be encountered from lab to lab and from operator to operator. Our results show that under typical conditions, this permeability assay is robust and generates reproducible data. We also observed that small protocol variations can have an effect on the apparent permeability rates of some drugs but in general the rank order (by log P_e) of the compounds is unaffected.

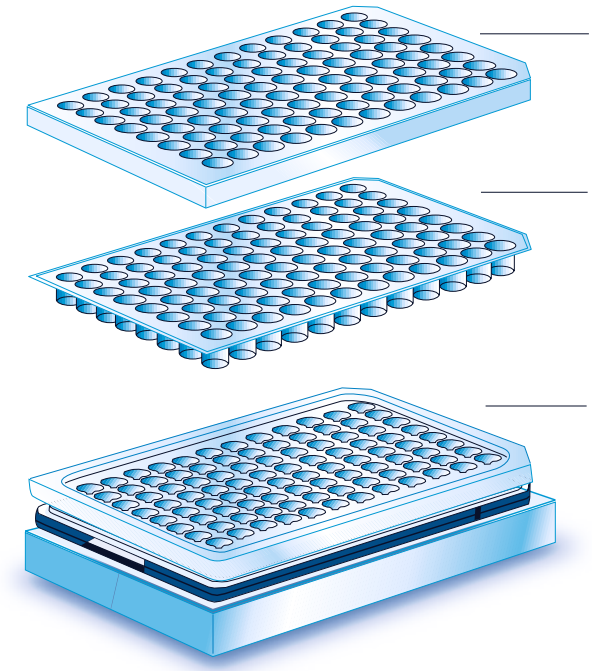
Introduction

The permeability assay is a non-cell based assay designed to predict passive, transcellular permeability of drugs in early drug discovery. The assay is carried out in a 96-well MultiScreen Permeability plate (MAPBMN310) and measures the ability of compounds to diffuse from a Donor to an Acceptor compartment separated by a hexadecane liquid layer on a polycarbonate membrane support.

After the artificial membrane has been applied to the polycarbonate membrane in the filter plate (known as the Donor plate), the 96-well Donor plate is filled with buffer solutions containing the compounds to be tested. The Donor plate is then placed upon a 96-well Acceptor plate filled with sufficient buffer so that there is liquid contact between the liquid in the Acceptor plate and the polycarbonate membrane. The Acceptor plate can be either the plate provided with this device or an equivalent such as MSSACCEPTOR (a PTFE 96-well plate). The Donor and Acceptor plates are incubated together for 5 – 7 hours after which time the Donor plate is removed from the Acceptor plate. The 96 wells in the Acceptor plate can then be analyzed by LC/MS or transferred to a UV compatible 96-well plate and analyzed immediately in a UV/Vis spectrophotometer. At the end of the incubation time, the integrity of the artificial membrane layer can be measured using electrical resistance. The permeability method can also be used to determine the effect of pH on compound permeability by adjusting the pH of the solutions used in the analysis. These plates are particularly recommended for use in pre-ADME or Discovery programs requiring compound rank ordering or profiling.

MILLIPORE

System Components



General Protocol Considerations

In addition to the MultiScreen Permeability plate, a UV/Vis spectrophotometer capable of analyzing 96-well plates and UV compatible, 96-well sample plates are also required to run the assay. Preparation of the hexadecane layer in a fume hood is recommended.

Experimental Materials

Methotrexate (cat. A-7019), propranolol (cat. P-0884), warfarin (cat. A-2250), carbamazepine (cat. C-8981), furosemide (cat. F-4381), testosterone (cat. T-1500), hexadecane (cat. H670-3), hexane (cat. 27050-4), dimethyl sulfoxide (cat. D-8779) and phosphate buffered saline (cat. P-3813) were purchased from Sigma Chemical Co. (St. Louis, MO). MultiScreen Permability assay plates (cat. MAPBMN310) and PTFE Acceptor plate (cat. MSSACCEPTOR) are available from Millipore Corporation (Bedford, MA). Spectramax® Plus microtiter plate reader, SoftMax® Pro and UV compatible quartz plate (part no. R8024) were purchased from Molecular Devices (Sunnyvale, CA). Polypropylene reagent reservoirs (cat. 175-RBAS-000) were purchased from ELKay laboratory consumables (Shrewsbury, MA). Finnpipette® electronic pipettor (cat. 21377232) was purchased from Thermolab Systems (Helsinki, Finland). Biohit™ 8 channel electronic pipettor and polypropylene tips (cat. W67-710-800 and W16-160045) were purchased from Vangard International (Neptune, NJ). An ohm meter and 96 well Trans Epithelial Electrical Resistance (TEER) testing tray (model #'s EVOMX-G and MULTI-96) were purchased from World Precision Instruments (Sarasota, FL).

Electrical Resistance Testing

To ensure that hexadecane layers were intact, electrical resistance measurements were made both before and after permeability assays were conducted. Intact hexadecane layers exhibit extremely high electrical resistance (normally exceeding 25 kΩ). Data from wells with electrical resistance measurements below 5 kΩ were excluded.

Methods

The following protocol was used to determine log P_e for methotrexate, propranolol, warfarin, carbamazepine, furosemide and testosterone.

a) Prepare a 5% solution (v/v) of hexadecane in hexane (~3 mL/plate).

b) Pipette 15 µL of the hexadecane/hexane mixture each well carefully, avoiding pipette tip contact with the membrane. *Note: use polypropylene reservoir.*

c) Allow the plates to dry for 1 hour in a fume hood to ensure complete evaporation of the hexane resulting in a uniform layer of hexadecane.

d) Add 300 µL of buffer (5% DMSO in phosphate buffered saline pH 7.4) to each well of the PTFE Acceptor plate (MSSACCEPTOR).

e) Place the hexadecane treated Donor plate into the Acceptor plate making sure the underside of the membrane is in contact with the buffer.

f) Dissolve drugs of interest in 5% DMSO, PBS to the desired concentration and add 150 µL to each well in the Donor plate (for the following experiments testosterone Donor concentration equals 100 µM, all other drugs at 500 µM).

g) Replace plate lid and incubate at room temperature for 5 hours.

h) After incubation, measure UV/Vis absorption from 250 to 500 nm for 100 µL/well of the Donor solution and 250 µL/well of the Acceptor solution.

i) Make up drug solutions at the theoretical equilibrium (i.e., the resulting concentration if the Donor and Acceptor solutions were simply combined) and measure UV/Vis absorption from 250 to 500 nm for 250 µL/well of each.

j) Calculate log P_e using the equations provided (see below).

Drug Standard Curves

Standard curves were prepared to determine limits of quantification for each drug when using UV/Vis spectroscopy as the detection method. The limit of quantification is defined as the concentration of compound whose absorbance (optical density, OD) is five times greater than the standard deviation of the background (noise) absorbance. Two-fold serial dilutions starting at 100 µM of each drug were made in 5% DMSO/PBS. UV/Vis absorbance for each dilution was measured across 250 – 500 nm in 10 µm steps using the Spectramax Plus plate reader and quartz sample plate. Peak maximum and area under the curve data were then plotted for each drug (see Table 1 and Figures 1a – e).

Results of Log P_e Studies

To assess the analytical variability (precision) within a plate, the permeability of testosterone and propranolol were measured in all 96 wells of multiple plates (n = 1 plate for testosterone, n = 3 plates for propranolol). Each value in Table 2 is the average of the log P_e calculated from 96 readings (wells).

The five drugs listed in Table 3 were assayed for permeability using four different plates over two days to measure the reproducibility of assay performance from plate to plate. Each value in the table is the average of 16 wells per drug for each plate.

To determine the assay reproducibility from day to day, the six drugs in Table 4 were assayed for permeability on eight different days, in two different plates using 16 wells per plate. *[Note: Testosterone data on days 1, 2 and 3 were not included because of problems with solubility. Beginning on day 4 the Donor concentration was dropped to 100 µM.]*

Five different lots of membrane were tested to determine lot to lot reproducibility. All assays were performed using the standard protocol described. These results are listed in Table 5. *[Note: Lot 2 was tested before the solubility issue with testosterone was resolved.]*

Summarized in Table 6 are the results of several small protocol changes that were tested to determine the impact of protocol variability. The impact of changes in how the hexadecane layer is formed was a major focus of the tested changes. The average and standard deviation for the standard protocol includes data from at least 5 lots, 8 days and 2 plates with 16 wells for each drug per plate (i.e., at least 1280 assays). For each of the protocol changes, 1 plate was evaluated with 16 wells for each drug. Diffusion of the tested compounds from the Donor to Acceptor compartments was monitored over the course of 48 hours. Drugs were added to successive rows of a plate for a set time (48, 32, 28, 24, 8, 6, 4, 2 hours) prior to plate separation and analysis by UV/Vis. Each row of the plate had 2 replicates for each of the 6 drugs and two plates were assayed resulting in a total of 4 replicates of each drug per time point. The average of these replicates is plotted in Figures 2 and 3 for the Donor and Acceptor plates, respectively. The log P_e calculated for each time point is listed in Table 7.

Equations

Log P_e can be calculated from the following equation as reported by Faller *et al.*[1]

$$\log P_e = \log\{C \bullet -1n(1 - \frac{[drug]_{Acceptor}}{[drug]_{equilibrium}})\} \text{ where } C = (\frac{V_D \bullet V_A}{(V_D + V_A)\ Area \bullet time})$$

Table 1: UV/Vis Limits of Quantification (LOQ)

Drug	LOQ Concentration	Typical Acceptor Concentration After Incubation
Propranolol	5.0 µM	82.5 µM
Warfarin	2.89 µM	12.3 µM
Methotrexate	1.36 µM	0.07 µM
Carbamazepine	4.51 µM	59.8 µM
Furosemide	1.6 µM	0.09 µM
Testosterone	0.91 µM	24.1 µM

Figure 1a: Propranolol Standard Curve

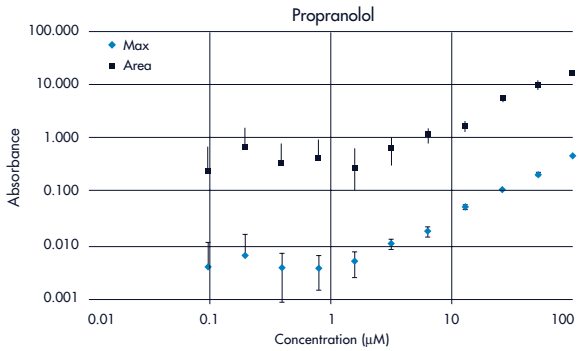


Figure 1b: Warfarin Standard Curve

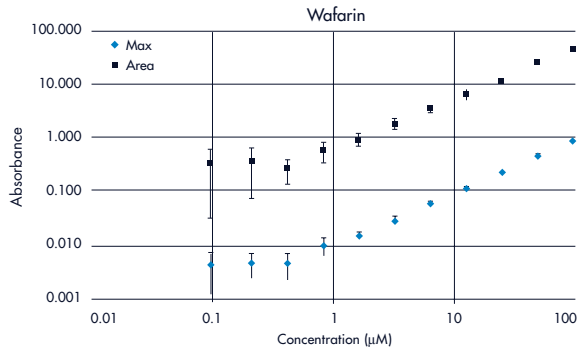


Figure 1c: Methotrexate Standard Curve

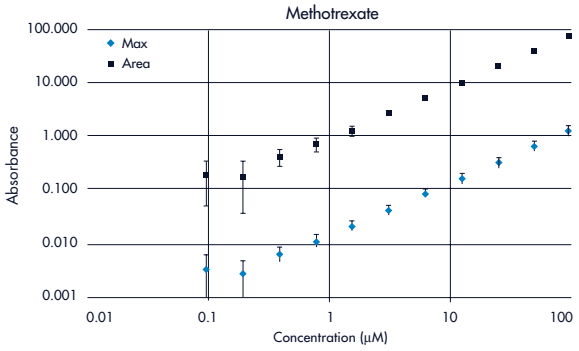


Figure 1d: Carbamazepine Standard Curve

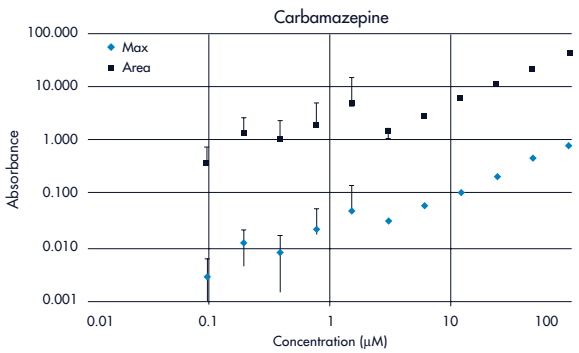


Figure 1e: Furosemide Standard Curve

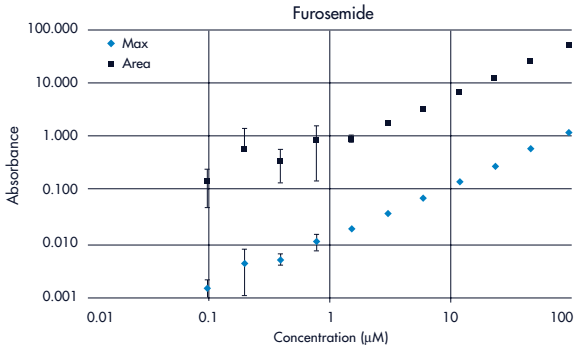
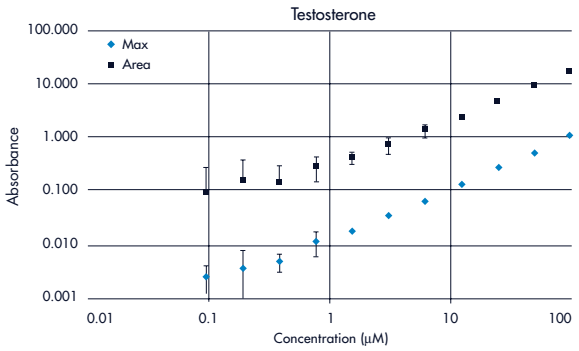


Figure 1f: Testosterone Standard Curve



Discussion

The permeability of the 6 drugs was determined using a Donor plate concentration of 500 µM. For testosterone, the Donor plate concentration was reduced to 100 µM after initial testing revealed its marginal solubility at the higher concentration. Overall, there were no significant (< 0.2 log units) variations due to well-to-well, plate-to-plate, day-to-day and lot-to-lot changes for the medium and high permeability drugs. Log P_e values obtained for furosemide and methotrexate were more variable than for the other four drugs because of analytical limitations in detecting the small amounts present in the Acceptor plate (using the standard, 5 to 7 hour incubation period). The results obtained here agree well with the ranking reported by Faller *et al.*[1] with the exception of warfarin. This variation is probably due to the difference in pH (7.4 versus 6.8) and the fact that warfarin contains an ionizable group with a pKa in this range.

In addition to testing assay reproducibility, several small protocol changes were tested to determine the effect that typical protocol variability might have on the calculated log P_e. A table of the tested protocol changes can be found in the results section (Table 6). By and large, the impact of any of these protocol modifications is quite small and does not result in any

Table 2: Well-to-well Reproducibility

Drug	Log P_e ± 1 S.D.	Log P_e ± 1 S.D.	Log P_e ± 1 S.D.
Testosterone	-3.7 ± 0.09	———	———
Propranolol	-4.3 ± 0.07	-4.1 ± 0.10	-4.0 ± 0.09

Table 3: Plate-to-plate Reproducibility

Day	Plate	Data	Drug Carbamazepine	Furosemide	Methotrexate	Warfarin	Propranolol
1	1	Ave.	-4.2 ± 0.00	-6.8 ± 0.48	-6.9 ± 0.52	-4.9 ± 0.03	-4.1 ± 0.06
	2	Ave.	-4.1 ± 0.03	-6.9 ± 0.62	-6.7 ± 0.56	-4.8 ± 0.00	-4.0 ± 0.05
2	1	Ave.	-4.3 ± 0.00	-6.6 ± 0.24	-6.7 ± 0.30	-5.1 ± 0.03	-4.1 ± 0.05
	2	Ave.	-4.3 ± 0.03	-6.5 ± 0.43	-7.0 ± 0.36	-5.1 ± 0.04	-4.0 ± 0.04

Table 4: Day-to-day Reproducibility

Day	Data	Carbamazepine	Furosemide	Methotrexate	Testosterone	Warfarin	Propranolol
1	Ave.	-4.1 ± 0.05	-6.3 ± 0.78	-6.3 ± 0.61	Sol. Issue	-4.8 ± 0.09	-4.0 ± 0.07
2	Ave.	-4.2 ± 0.05	-6.8 ± 0.55	-6.8 ± .054	Sol. Issue	-4.8 ± 0.05	-4.1 ± 0.06
3	Ave.	-4.3 ± 0.02	-6.5 ± 0.34	-6.9 ± 0.35	Sol. Issue	-5.1 ± 0.04	-4.0 ± 0.05
4	Ave.	-4.1 ± 0.00	-7.2 ± 0.63	-7.6 ± 0.56	-3.7 ± 0.06	-4.9 ± 0.00	-3.9 ± 0.05
5	Ave.	-4.1 ± 0.04	-6.8 ± 0.42	-7.1 ± 0.51	-3.7 ± 0.05	-4.9 ± 0.00	-4.0 ± 0.05
6	Ave.	-4.2 ± 0.05	-7.0 ± 0.37	-7.4 ± 0.42	-3.8 ± 0.07	-5.0 ± 0.03	-4.1 ± 0.05
7	Ave.	-4.2 ± 0.04	-7.0 ± 0.42	-7.3 ± 0.85	-3.8 ± 0.05	-5.0 ± 0.06	-4.0 ± 0.02
Total Ave.		-4.2 ± 0.07	-6.8 ± 0.59	-7.0 ± 0.69	-3.7 ± 0.12	-4.9 ± 0.11	-4.0 ± 0.07

Table 5: Lot-to-lot Reproducibility

Lot	Data	Drug Carbamazepine	Furosemide	Methotrexate	Testosterone	Warfarin	Propranolol
1	Ave.	-4.2 ± 0.08	-6.7 ± 0.49	-6.8 ± 0.45	-3.7 ± 0.15	-5.0 ± 0.13	-4.1 ± 0.06
2	Ave.	-4.1 ± 0.05	-6.3 ± 0.78	-6.3 ± 0.61	Sol. Issue	-4.8 ± 0.09	-4.0 ± 0.09
3	Ave.	-4.1 ± 0.05	-7.1 ± 0.58	-7.4 ± 0.60	-3.7 ± 0.09	-4.9 ± 0.05	-4.0 ± 0.05
4	Ave.	-4.2 ± 0.08	-7.0 ± 0.41	-7.4 ± 0.69	-3.7 ± 0.10	-5.0 ± 0.06	-4.0 ± 0.07
5	Ave.	-4.2 ± 0.05	-6.9 ± 0.40	-7.2 ± 0.56	-3.7 ± 0.09	-4.9 ± 0.05	-4.0 ± 0.06
Total Ave.		-4.2 ± 0.07	-6.8 ± 0.59	-7.0 ± 0.69	-3.7 ± 0.12	-4.9 ± 0.11	-4.0 ± 0.09

change in the rank ordering of compound permeability.

As described, the typical orientation of the assay is top down (starting with drug on the top), although the assay should perform comparably if the format is inverted (i.e., by adding the compounds to the bottom and analyzing the solution on top of the artificial membrane). Included in Table 6 are the results of performing the assay in the inverted orientation. The values for high-permeable compounds are reduced while low permeable compounds are unaffected and this is because the unstirred water layer is thicker in the inverted orientation. It is not recommended to use this orientation because it reduces the dynamic range of the assay. In general the rank order was unaffected.

A 48-hour time course experiment was performed to monitor the concentration changes of the Donor and Acceptor compartments for all six drugs. Graphs of the OD readings for each drug can be seen in the results section (see figures 2 & 3). As can be seen in the data, permeability rates are not constant and are governed by many factors. Short incubation times (e.g., 2 hours) result in observed permeability rates that are elevated relative to the standard protocol (5 hours). This observation may be due to concentration gradients driving the initial rate. At the end of the 48 hour incubation some drugs are approaching equilibrium, indicated by the flattening out of the curve.

As with any assay, there is an initial period of learning and trial and error before reproducible results are obtained. Experience and familiarity with an assay helps in spotting potential problems and making adjustments accordingly. Some potential pitfalls are not obvious, such as a drug's ability to be assayed by UV/Vis spectroscopy. Many drugs are not good UV/Vis chromophores so the proper analytical techniques must be chosen accordingly. Refer to Table 1 for limits of detection determined from the stan-

Table 6: Tested Protocol Variations

Assay Changes	Propranolol	Methotrexate	Warfarin	Carbamazepine	Furosemide	Testosterone
Standard Protocol	-4.1 ± 0.02	-7.3 ± 0.53	-5.0 ± 0.02	-4.3 ± 0.02	-7.0 ± 0.32	-3.8 ± 0.05
Inverted assay	-4.5 ± 0.03	-6.8 ± 0.34	-5.4 ± 0.04	-4.7 ± 0.02	-6.1 ± 0.19	-4.4 ± 0.03
Hex. side of well	-4.1 ± 0.03	-7.2 ± 0.59	-5.2 ± 0.03	-4.3 ± 0.03	-6.4 ± 0.16	-3.8 ± 0.02
4 °C incubation	-4.6 ± 0.04	-7.1 ± 0.61	-5.6 ± 0.14	-4.9 ± 0.04	-6.1 ± 0.22	-4.2 ± 0.04
37 °C incubation	-3.9 ± 0.02	-7.3 ± 0.36	-4.5 ± 0.12	-4.0 ± 0.05	-6.2 ± 0.06	-3.5 ± 0.10
2hr incubation	-4.0 ± 0.02	-6.7 ± 0.31	-5.2 ± 0.04	-4.3 ± 0.03	-6.0 ± 0.05	-3.7 ± 0.05
16hr incubation	-4.2 ± 0.03	-7.6 ± 0.51	-5.1 ± 0.01	-4.3 ± 0.02	-6.6 ± 0.26	-3.8 ± 0.10
10 µL Hex. mixture (5%)	-4.0 ± 0.03	-7.0 ± 0.30	-5.1 ± 0.01	-4.2 ± 0.02	-6.1 ± 0.23	-3.8 ± 0.08
20 µL Hex. mixture (5%)	-4.0 ± 0.01	-7.7 ± 0.40	-5.1 ± 0.02	-4.2 ± 0.01	-6.2 ± 0.11	-3.7 ± 0.02
3% Hex. (15 µL applied)	-3.8 ± 0.04	-4.9 ± 0.61	-4.5 ± 0.25	-4.0 ± 0.11	-4.4 ± 0.50	-3.6 ± 0.05
7% Hex. (15 µL applied)	-3.9 ± 0.02	-7.2 ± 0.46	-5.0 ± 0.01	-4.2 ± 0.01	-6.1 ± 0.08	-3.6 ± 0.06

Table 7: Average log P_e for Different Incubation Times

Time (hrs)	Drug Carbamazepine	Furosemide	Methotrexate	Propranolol	Testosterone	Warfarin
2	-4.2	-5.5	-5.3	-3.9	-3.7	-4.9
4	-4.2	-6.0	-6.3	-4.0	-3.7	-5.0
6	-4.2	-6.1	-6.2	-4.0	-3.7	-5.0
8	-4.3	-6.2	-6.3	-4.1	-3.8	-5.0
24	-4.3	-6.7	-7.0	-4.3	-3.8	-5.0
28	-4.3	-6.9	-7.2	-4.3	-4.0	-5.0
32	-4.3	-6.8	-6.9	-4.4	-4.0	-5.1
48	-4.4	-7.1	-5.5	-4.3	-4.2	-4.9

dard curves of the six drugs assayed. Carbamazepine, for example, exhibits a comparatively high limit of detection by UV/Vis, however due to its relatively high permeability; sufficient quantities are present in the Acceptor compartment to permit the use of this detection method. Methotrexate, on the other hand, is a good UV/Vis chromophore and exhibits a much lower limit of detection, however due to its slow permeability rate, very little compound is present in the Acceptor volume to be measured. Consequently, greater variability in the calculated log P_e of methotrexate is observed.

Another significant source of method error may be related to compound solubility. Initial attempts to measure testosterone permeability were conducted using a Donor solution concentration of 500 µM – a concentration at which testosterone is only marginally soluble. This resulted in the drug partitioning onto the plate and membrane surfaces – thereby rendering accurate log P_e calculations impossible.

Lastly, environmental factors such as the temperature in the lab can affect the reproducibility of permeability experiments. The permeability assay protocol calls for room temperature incubations. If the ambient temperature in the lab tends to fluctuate it could affect the measured permeability rates of some drugs. The melting temperature of hexadecane is 18 °C and even small temperature changes have the potential to affect the viscosity/permeability of the artificial membrane layer.

Figure 2: Ratio of OD Donor/Initial Donor Concentration

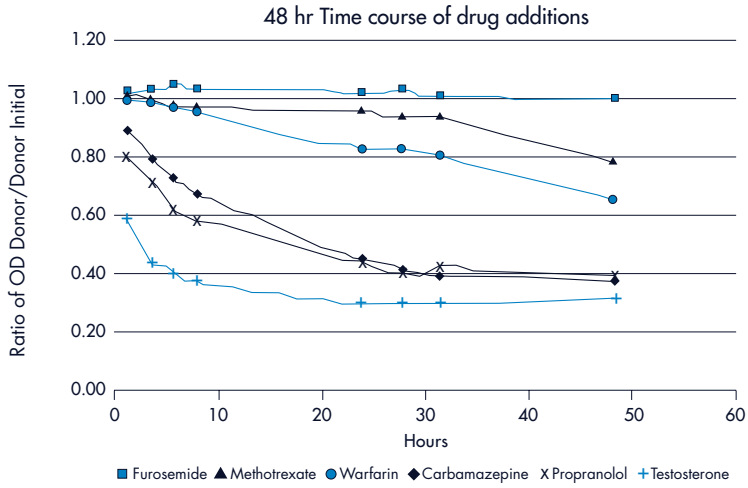
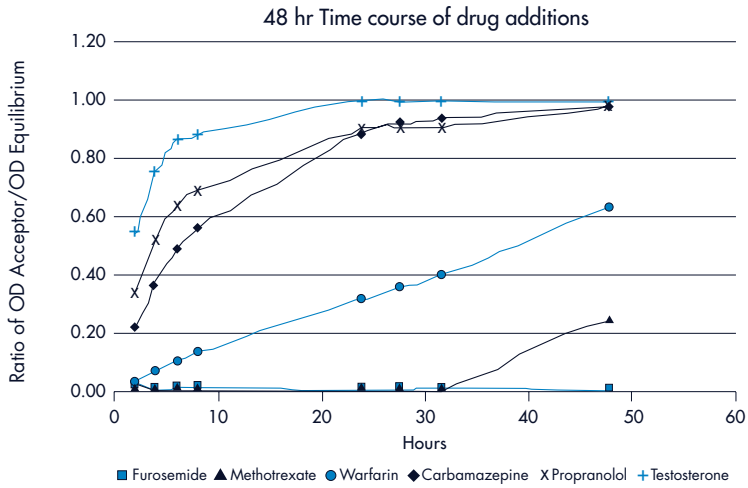


Figure 3: Ratio of OD Acceptor/Equilibrium OD



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1. (a) Faller, B.; Wohnsland, F. "Physicochemical Parameters as Tools in Drug Discovery and Lead Optimization." in: Testa, B., van de Waterbeemd, H.,Folkers, G., Guy, R. (Eds.), Pharmacokinetic Optimization in Drug Research, Verlag Helvetica Chimica Acta: Zürich and Wiley – VCH: Weinheim, (2001) pp. 257 – 274.

   (b) Wohnsland, F.; Faller, B. "High-throughput Permability pH profile and High-throughput alkane/water log P with artificial membranes." *J. Med. Chem.* (2001), 44, pp. 923 – 930.

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