Protocol Note

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Title: Lipid-PAMPA with the MultiScreen® Filer Plates

Abstract

Assays that predict passive absorption of orally administered drugs have become increasingly important in the drug discovery process. As previously described by Faller[1] and Kansy[2] such assays provide rapid, low cost and automation friendly methods to measure a compound's passive permeability. The Lipid-PAMPA method 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 (MAIPN4510) and measures the ability of compounds to diffuse from a Donor to an Acceptor compartment separated by a PVDF membrane filter pretreated with a lipid-containing organic solvent. This protocol note details the steps required to determine compound permeability rates across the artificial membrane.

Materials and Methods

Experimental Materials

Reagents

- Lipid or lipid mixture such as L-a-phosphatidylcholine (lecithin) (for example: # P3556; Sigma Chemical Co., St. Louis, MO)
- Dodecane (for example: # 112403; Sigma)
- Phosphate buffered saline (for example: # P-3813; Sigma)

Equipment

- MultiScreen filter plate for PAMPA assay with underdrain removed (# MAIPN45XX; Millipore Corporation, Billerica, MA)
- PTFE Acceptor plate (# MSSACCEPTOR; Millipore)
- Spectramax® Plus microtiter plate reader (Molecular Devices, Sonnyvale, CA) or similar UV-Vis device
- SoftMax® Pro software (Molecular Devices) (optional)
- UV compatible quartz plate (for example: # R8024; Molecular Devices) or 96-well Costar® with UV transparent bottom (for example: #3635 ; Corning Inc., Corning, NY) or Greiner UV-Star Plates—96 well flat bottom #655801 (Greiner Bio-One, Inc., Longwood, FL).
- Polypropylene reagent reservoirs (for example: # 175-RBAS-000; ELKay laboratory consumables Shrewsbury, MA)
- Multichannel pipette such as:
  - Finnpipette® electronic pipettor (# 21377232; Thermolab Systems, Helsinki, Finland)
  - Biohit™ 8 channel electronic pipettor with polypropylene tips (# W67-710-800, W16-160045, Vanguard International, Neptune, NJ)

Method

1. Prepare a 1 to 4% solution (w/v) of lecithin in dodecane (~500 µL/plate) and sonicate the mixture to ensure complete dissolution. See **Notes 1, 2**

2. Carefully pipette 5 µL of the lecithin/dodecane mixture into each Donor plate well, avoiding pipette tip contact with the membrane. See **Note 3**.

3. Immediately after the application of the artificial membrane (within 10 minutes maximum), add 150 µL of drug-containing donor solutions (drugs dissolved in 5% DMSO, PBS) to each well of the Donor plate.

4. Add 300 µL of aqueous buffer to each well of the PTFE Acceptor plate (MSACCEPTOR).

5. Slowly and carefully place the drug-filled Donor plate into the Acceptor plate, making sure the underside of the membrane is in contact with the buffer in all wells. See **Note 4**.

6. Replace the plate lid and incubate at room temperature for 16 hours. See **Note 5**.

7. After incubation, analyze the acceptor plate for compound concentration. (See **Note 6**)

8. Make up drug solutions at the theoretical equilibrium (i.e., the resulting concentration if the Donor and Acceptor solutions were simply combined) and similarly analyze.

9. The equation used to determine permeability rates (P_e) is displayed in **Figure 1** and calculates log P_e. Variables for the equation are defined in **Table 1.**

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

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

Table 1: Description of variables used to calculate Log P_e

Term	Definition	Notes
V_D	Volume of donor compartment	Expressed in cm^3, 150 µL = 0.15 cm^3
V_A	Volume of acceptor compartment	Expressed in cm^3, 300 µL = 0.30 cm^3
Area	Active surface area of membrane	Defined as membrane area x porosity. For the membrane in MultiScreen Permeability Filter Plate, area = .24 cm^2 x 100%; or **0.24 cm^2**
Time	Incubation time for the assay	Expressed in seconds, 1 hr = 3600 s
$[drug]_{acceptor}$	Concentration of compound in the acceptor compartment at the completion of the assay	The absorbance of the sample as recorded by the SoftMax Pro Software
$[drug]_{equilibrium}$	Concentration of compound at theoretical equilibrium from step 9 above	The absorbance of the equilibrium sample as recorded by the SoftMax Pro Software

Note 1 Although this protocol specifically recommends a lecithin solution in dodecane, many alternative lipid mixtures are also compatible with the membrane and protocol. For a particular target barrier, it is critical to choose the appropriate lipid mixture and lipid concentration to ensure accurate permeability ranking and correlation with human absorption data.[3]

Note 2: Typically it is necessary to sonicate the lipid mixture until the solution approaches the clarity of water (using a probe typically used for cell lysis takes about 2 minutes). The lecithin solution will clarify greatly but will never be completely clear. This solution should be used immediately. The lecithin will begin to aggregate and become turbid again after a few minutes.

Note 3: It is best to add the lecithin/dodecane solution to the membrane while the donor plate is sitting in a single well tray so that the underside of the membrane does not make contact with any surfaces. When the mixture is ejected from the pipette tip it will form a pendant drop suspended from the tip. The pendant drop should be placed close enough to the membrane so that it will wick off, while insuring that the plastic pipette tip itself does not make contact with the membrane.

Note 4: The donor plate must be carefully placed into the acceptor plate. Any excess downward pressure will cause the buffer in the acceptor plate to be squeezed up and out of the wells resulting in cross talk.

Note 5: To avoid evaporation, the plate should be placed in humidity controlled environment such as a sealed container with wet paper towels during incubation.

Note 6: Generally sample analysis using a 96 well UV/Vis plate reader is recommended. Sample quantification techniques such as scanning over a broad absorbance range (e.g. 250-500 nm), a single wavelength (λ_{max}) or a summation of pre-selected fixed wavelengths are all suitable for analysis. HPLC-UV or LC-MS/MS are also alternative means of detection.

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[1] Wohnsland, F.; Faller, B. High-throughput Permeability pH Profile and High-throughput Alkane/Water Log P With Artificial Membranes, J. Med. Chem., 2001; 44, p. 923–930.

[2] Kansy, M.; Senner, F.; Gubernator, K. Physicochemical High Throughput Screening: Parallel Artificial Membrane Permeation Assay in the Description of Passive Absorption Processes, J. Med. Chem., 1998; 41, p. 1007–1010.

[3]MultiScreen Filter Plates for PAMPA: Evaluation of the reproducibility of Parallel Artificial Membrane Permeation Assays (PAMPA); Application Note Millipore Lit. No. AN1728EN00

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