Review Article

Permeability assessment of drug substances using *in vitro* and *ex vivo* screening techniques

P.V. Ravikanth[1], K.V. Ramanamurthy[2]

[1]Department of Pharmaceutics, University of Utah, Salt Lake City, UT 84112, USA, [2]Department of Pharmaceutics, University College of Pharmaceutical Sciences, Andhra University, Visakhapatnam, Andhra Pradesh, India.

Correspondence:
P.V. Ravikanth, Department of Pharmaceutics, University of Utah, Salt Lake City, UT 84112, USA.
E-mail: pv_rk.9@rediffmail.com

How to cite this article:
Ravikanth PV, Ramanamurthy KV, Permeability assessment of drug substances using *in vitro* and *ex vivo* screening techniques. Innov Pharm Pharmacother 2018;6(2):17-20.

Source of Support: Nil,
Conflict of Interest: None declared.

ABSTRACT

Solubility and permeability are two important parameters used for the evaluation of drug substance to assess its bioavailability. Although dissolution can be studied using official dissolution apparatus, there are no official techniques to check permeability of drug substances. Apparent permeability index (Papp) is the index used to assess the degree of permeability of drug substances. Papp of any drug substance can be calculated using *in vitro, ex vivo, in situ,* and *in vivo* techniques. Parallel artificial membrane permeability assessment and human epithelial colorectal adenocarcinoma cells (Caco-2) are two *in vitro* models used for high throughput screening and ranking drugs permeability. *Ex vivo* studies include isolated tissue models and averted intestinal sacs where *in situ* model includes a segment of the intestine, cannulated, and filled with a probe solution. Ussing chamber study is *ex vivo* study used to investigate permeability and metabolism where alternative methods such as Caco-2 or intestinal microsomes are useful to study either permeability or metabolism, but not both. *In vivo* models range from animal gavage in rats to oral dosing to humans in clinical trials. Selection of proper choice depends on degree of screening we are interested in.

Keywords: Colorectal adenocarcinoma cells-2 study, permeability assessment, Ussing chamber study

Introduction

Parallel artificial membrane permeability assessment (PAMPA) was first demonstrated by Kansy *et al.* as a high-throughput screening tool for predicting absorption. It contained artificial membrane coated with organic solvent such as dodecane containing mixture of lecithin/phospholipids. Since then, several variants of PAMPA have been introduced. Marketed among them the first model was based on 2% w/v dioleoylphosphatidylcholine dissolved in n-dodecane. PAMPA has so many limitations such as it underestimates the permeability of drugs that are actively absorbed from the intestine. The compositions of phospholipid bilayers in different variants are different and cause major variation in Papp.[1,2] On the other hand, colorectal adenocarcinoma cells (Caco-2) exhibit polarized cell layers and intercellular tight junctions on incubation for 21 days. They also contain a host of CYP enzymes as well as human P-gp protein. Caco-2 study produces sufficient data in spite of the fact that these cells are different from the human intestinal cells.[3,4]

Access this article online	
Website: www.innpharmacotherapy.com	**e-ISSN:** 2321-323X **p-ISSN:** 2395-0781

In situ model involves permeability results performed in an anesthetized animal through a laparotomy and gently pulling the desired portion of the intestinal tract out of the abdomen for cannulation. Once the dose is injected, the desired region of the intestine can be tied off at one or both ends of the tract for the duration of the study. This method has the advantage of controlled concentrations in the intestinal lumen but is limited in duration and throughput.[5]

Ex vivo study carried using isolated intestinal tissue. A small piece of human intestinal epithelium will give reliable apparent permeability data since it maintained in an "organotypic" environment with all resident cell populations, i.e., include entrecotes, calciform cell, lymphocytes, and mucus layers.[6] The tissue mounted may be rats, humans, or monolayer of cells. Large libraries of pharmaceuticals have been tested in Ussing chamber using human isolated tissue and found a strong correlation between fraction absorbed and apparent permeability (Papp). One can carry multiple studies with single animal and reduce number of animals required. Ussing chambers are used to investigate the drug absorption, transportation, and metabolism in an intact tissue, to explore the role of ion channels in cell membrane, and to find drug-mediated changes within the tissue (intracellular signaling).[7] Since paracellular permeability and metabolism characteristics of the rat and human are similar, it is one

of the frequently used models when compared with other animals. In spite of these advantages, rats have disadvantages as different pH and dose volume. Caco-2 permeability assay and Ussing chamber experiments are two studies frequently employed for the assessment of oral bioavailability of drugs. In the present review, these two techniques are discussed in detail.

Caco-2 and Ussing chamber studies

Caco-2 and Ussing chamber studies are *in vitro* and *ex vivo* techniques, respectively. These two techniques are useful for screening drugs apparent permeability and to some extent their transportation pathways. Apparent permeability coefficient is used to ascertain degree of permeability to rank various drug substances and to understand transportation pathways. The detailed protocols of these two techniques are discussed in this article [Table 1].

Pre-experimental set up of Ussing chamber

With respect to drug permeability and metabolism, Ussing chamber was recognized as the gold standard since it allows investigator to work on tissues of interest while retaining drug transporters and metabolizing enzymes of tissue. Permeability and metabolism are two key factors for the estimation of oral bioavailability of any drug substance. Typical experiment involves addition of substances to the apical side of the tissue, followed by periodic sampling from both the apical and basolateral compartments. Epithelium will be separated by dissecting the ileum or colon along the mesenteric border and separating by seromuscular stripping. The separated epithelium will be mounted to the tissue mounting unit in the Ussing chamber such that the mucosal side of the membrane (apical or luminal side membrane) facing one chamber half, whereas the serosal membrane (basolateral) facing the other half-chamber. The area of exposed mounted tissue will be 0.3 cm^2–5 ml of transportation buffer. Chambers are water jacketed to maintain body temperature, and the transportation buffer will be injected with carbogen (95% O2 and 5% CO_2) to maintain oxygen and carbon dioxide tensions, respectively. Typical set up of Ussing chamber will be seen as shown in Figure 1. To understand the type of transportation pathway, paracellular and transcellular markers such as FITC-Dextran 4000 (FD-4) and (horseradish peroxidase Type VI) at 400 µg/ml and 200 µg/ml concentrations are added to the transportation buffer. The detailed procedure for carrying out Ussing chamber study is given below.

Step 1: Preparation of Krebs-Henseleit buffer (KH buffer)

Krebs-Henseleit buffer will be prepared using the ingredients given in Table 2. These ingredients will be added to deionized water one by one, and the final volume will be adjusted and filtered with 0.22 µm PVDF filter. The pH will be adjusted to 7.4 finally.

Step 2: Preparation of KCL-agar bridge electrodes

1 L agar solution containing 3 M KCl will be prepared by the addition of 223.68 g of KCl to WFI containing 2–5% agar slowly while heating for few minutes on water bath at temperature 70–80°C. The prepared KCL-agar solution will be filled in plastic cones while it is hot since it solidifies and becomes useless to use. The filling will be carried using syringe avoiding introduction of any air bubbles. KCL-agar bridge electrodes ready to insert into Ussing chamber are shown in Figure 2.

Table 1: Apparent permeability coefficient index

$Papp < 2 \times 10^{-6}$ cm/s	Low permeability
2×10^{-6} cm/s $< Papp < 20\times10^{-6}$ cm/s	Medium permeability
$Papp > 20\times10^{-6}$ cm/s	High permeability

Table 2: Composition of KREBBS

Component	Amount per L (g)
$MgSO_4 7H_2O$	29.6
KH_2PO_4	16.3
$CaCl_2$	36.76
KCl	35
NaCl	6.58
$NaHCO_3$	2.10
D-Glucose	2.16

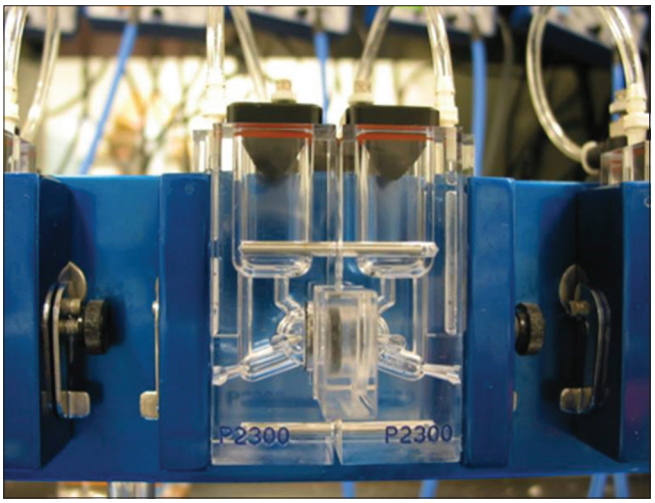

Figure 1: Ussing chamber

Figure 2: Electrodes used for the preparation of KCL-agar electrodes

Table 3: Experimental conditions of Caco-2 study

Test substance	100 µM
Passage number	10–20
Period of cell culture	21 days
Number of replicates	2
Incubation time	2 h
Temperature	37°C
Marker for integrity	Lucifer yellow
Control compounds	Atenolol and propranolol
Analysis method	LC–MS/MS quantification

LC–MS/MS: Liquid chromatography–mass spectrometry

Figure 3: 96- Well plate

Step 3: Seromuscular stripping

Seromuscular stripping is an act of separation of serosa, longitudinal and circular smooth muscles from the intestine to isolate epithelium. Tissue will be dissected along the mesenteric border and pinned all around making stripping easier. Stripping done by making a sharp cut in the end with a razor blade to get a clean edge that makes stripping easier. The separated epithelium will be kept in KREBS solution until it gets mounted and study initiated. The jejunum will not be used in the Ussing chamber since it is difficult to strip muscle from this portion. Timer is set to record the time taken from the rat sacrificed to tissue mounted. The time maintained equal <15 min for each experiment.[7] Since variability incurred due to animals and process is more, the study will always be performed taking at least four tissues from four animals.

Experimental protocol

From the Ussing chamber study, one can acquire permeability coefficient (*Papp*) of treatment and marker, transepithelial electrical resistance (TEER), and histopathology of tissue. TEER will be recorded throughout the experiment at predetermined time intervals. Papp will be obtained by measuring the concentration of treatments and calculating the Papp using permeability coefficient equation. On completion of the study, the histopathology of the tissue will be studied. The details of each of the study are given hereunder step by step.

Measuring apparent permeability (*Papp*) of drug substance

20 µL of samples collected from apical side at time 0 and 120 min where 200 µL from basal at time 0, 20, 40, 60, 80, 100, and 120 min in 96-well plates as shown in Figure 3. Same volumes are replaced with fresh/new KREBS. 20 µL of apical samples collected only at 0 and 120 min will be diluted in 1:10 using KREBS solution before reading (20 µL of apical sample and 180 µL of KREBS). The concentration of treatments can be measured using suitable analytical methods.[7,8]

The apparent permeability coefficient (*Papp*) calculated using Equation 1

$$Papp = \frac{dQ}{dt} X \frac{1}{A\ C_0} \quad (1)$$

Where, d*Q*/d*t* is the slope of the cumulative fraction absorbed versus time (in seconds), A is the area of the filter (cm^2), and C_0 is the initial concentration in the apical chamber. Linear rates of permeability with R^2 value >0.9 were used for calculating permeability. Statistical analysis of the *P*app data was compared using ANOVA ($P<0.05$), with Tukey's post-analysis in GraphPad® Prism (Version 7.0 C, CA, USA).

^{14}C-Mannitol permeability of PFOFs

200 µL of basal samples transferred to scintillation vials and diluted to 3 mL with scintillation fluids (Apical samples do not need to be diluted due to the sufficient concentration of ^{14}C-mannitol or other radioactive materials).

Measuring TEER

Tranepithelial/transendothelial electrical resistance (TEER) is a widely accepted quantitative technique to measure the integrity of tight junction dynamics in cell culture models of endothelial and epithelial monolayers. TEER values are strong indicators of the integrity of the cellular barriers before they are evaluated for transport of drugs or chemicals. The voltage of the tissue is usually range from 3 to −10 mV. The TEER cutoff values for *colon* and ileum are 70 Ω*cm^2 and 30 Ω*cm^2, respectively. Tissues with TEER cutoff value below these values are not useful for the experiment. TEER was measured at 15, 10, and 0 min before the addition of treatments followed by every 5 min for the initial 20 min thereafter every 20 min for an additional 2 h. TEER measurements can be performed in real time without cell damage and generally are based on measuring ohmic resistance or measuring impedance across a wide spectrum of frequencies.[7-9] EVOM2 as shown in Figure 4 is widely used instrument to measure TEER. Some of the barrier models that have been widely characterized using TEER include the blood–brain barrier, gastrointestinal tract, and pulmonary models. Variations in these values can arise due to factors such as temperature, medium formulation, and passage number of cells.

Histopathology study

Hematoxylin and eosin (H and E) staining is the most common staining technique in histopathology. H and E evaluation will be carried over the tissues studied in the Ussing chamber study. After the completion of the experiment, the tissue will be removed from the chambers and

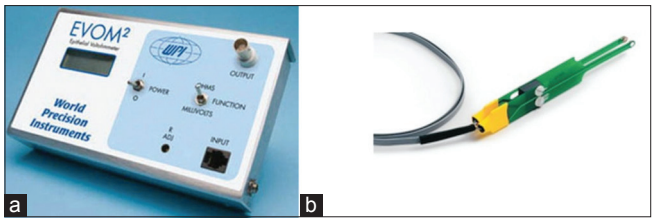

Figure 4: (a) Transepithelial electrical resistance measuring instrument (b) electrodes

Figure: 5: 24-well Transwell plate

fixed in 10% neutral buffered formalin for 48 h followed by storing it in 70% ethanol in water till the tissue is stained with H and E. Tissues will be mounted in paraffin blocks and stained using H and E for morphological evaluation.

Caco-2 study

Caco-2 cell line is a continuous line of heterogeneous human epithelial colorectal adenocarcinoma cells. Caco-2 cells differentiate into matured cells and develop tight junctions thus serve as a model to study paracellular permeability. Caco-2 cells also express transportation proteins, efflux pumps, and Phase II conjugation enzymes having role in transcellular permeability and hence useful for assessing permeability of drugs pass through using these membrane proteins. In addition to these advantages, Caco-2 has the disadvantage of the absence of CYP3A4, an isozyme of P450 family that is present abundantly in the intestine. Caco-2 cell monolayers are cultures on semi-permeable plastic supports called as transwells that are available commercially. Most frequently used 24-well Transwell plate is given in Figure 5. Transwells are available in the range of pore size, membrane types, and pore sizes to meet various research requirements. These transwells are comparable to the Ussing chamber containing apical and basolateral sides of the monolayer. An excellent review on Caco-2 protocol is given in nature journal.[10]

Caco-2 study protocol

Basolateral side of the Transwell will be filled with 650 µL of pre-warmed, sterile Hanks balanced stock solution (HBSS). Transportation study will be initiated by transferring and dissolving test substances in HBSS at apical side (250 µL) of the Transwell. Transwell plates will be placed on orbital shaker enclosed in incubator with 37°C temperature and 5% CO_2. These conditions assure that the cells are live during the study period. Studies are performed at 75 oscillations per minutes. Samples are taken from the receiver side of the monolayer at 30, 60, 90, and 120 min post-dosing and transferred them to the corresponding 96-well sample plate as it is performed in case of Ussing chamber study. The sample taken will be replaced with an equivalent volume of pre-warmed buffer. At the end of the experiment, both sides of the monolayers were replaced with fresh, drug-free, pre-warmed HBSS, and equilibrate for 10 min. At the end of the experiment, both sides of the monolayers were replaced with fresh, drug-free, pre-warmed HBSS, and equilibrate for 30 mins and finally TEER was measured. The summary of experimental protocol is given in Table 3.

Conclusion

Caco-2 study is useful for preliminary screening of drugs for assessing Papp. On the other hand, Ussing chamber technique can ascertain both active and passive transportation pathways reasonably. This *ex vivo* using chamber study compared to *in vitro* PAMPA and Caco-2 techniques provides the data closely comparable to *in vivo* techniques. Since this technique makes the use of animal tissues, it may provide some variability which will be reduced by conducting the study with $n = 4$ or more. Properly conducted experiment with perfect protocol will provide more reliable information.

References

1. Li C, Wainhaus S, Uss AS, Cheng KC. High-throughput screening using Caco-2 Cell and PAMPA systems. In: Drug Absorption Studies. Boston, MA: Springer; 2008. p. 418-29.
2. Avdeef A. The rise of PAMPA. Expert Opin Drug Metab Toxicol 2005;1:325-42.
3. Hilgers AR, Conradi RA, Burton PS. Caco-2 cell monolayers as a model for drug transport across the intestinal mucosa. Pharm Res 1990;7:902-10.
4. Hidalgo IJ, Raub TJ, Borchardt RT. Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. Gastroenterology 1989;96:736-49.
5. Ten Cate JM. *In situ* models, physico-chemical aspects. Adv Dent Res 1994;8:125-33.
6. Lennernäs H. Animal data: The contributions of the using chamber and perfusion systems to predicting human oral drug delivery *in vivo*. Adv Drug Deliv Rev 2007;59:1103-20.
7. Clarke LL. A guide to using chamber studies of mouse intestine. Am J Physiol Gastroin Liver Physiol 2009;296:G1151-66.
8. Ungell AL, Nylander S, Bergstrand S, Sjöberg Å, Lennernäs H. Membrane transport of drugs in different regions of the intestinal tract of the rat. J Pharm Sci 1998;87:360-6.
9. Maher S, Kennelly R, Bzik VA, Baird AW, Wang X, Winter D, *et al*. Evaluation of intestinal absorption enhancement and local mucosal toxicity of two promoters. I. Studies in isolated rat and human colonic mucosae. Eur J Pharm Sci 2009;38:291-300.
10. Kerns EH, Di L, Petusky S, Farris M, Ley R, Jupp P. Combined application of parallel artificial membrane permeability assay and Caco-2 permeability assays in drug discovery. J Pharm Sci 2004;93:1440-53.