Transport Across Caco-2 Monolayer: Biological, Pharmaceutical and Analytical Considerations

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INTRODUCTION:

- A major objective in the pharmaceutical industry is to develop new drugs with **Good Oral Bioavailability** that is a highly desirable property for molecules under investigation in the drug- discovery process because it opens up a variety of formulation possibilities, dosing conditions and leads to better patient compliance.
- Good oral bioavailability occurs when the drug has **Maximum Solubility** and **Maximum Permeability** at the site of absorption.
- Hence the extent of absorption of the drug in-vivo could be predicted based on permeability and solubility measurements In-vitro.
- Thus the **Assessment of Intestinal Permeability** represents one essential part in the prediction of oral any new drug candidate.
- Moreover, permeability information provides the formulation scientist with both **Biopharmaceutical** as well as **Regulatory Insight** during a prototype formulation.
- The intestinal permeability data have been also used in **preformulation studies** to evaluate the effects of various pharmaceutical excipients as co-solvents or absorption enhancers on drug permeation.
- So far as a number of in-vitro methods for assessing the intestinal permeability of a given drug candidate have been developed and recently reviewed.
- In the last decade, the use of **Caco-2 cell monolayer** has gained in popularity as an in-vitro primary absorption screening tool in several pharmaceutical companies and several examples of successful correlation with human absorption have been reported.

(1) Caco – 2: BIOLOGICAL CONSIDERATION

Origin: Human colon Adenocarcinoma that differentiates spontaneously into enterocyte- like cells.
Caco-2 cell monolayers are used as an in-vitro model to study intestinal absorption and for high throughput screening of drugs and excipients.

CHARACTERISTICS OF PARENTAL Caco-2 CELLS

Origin	Human colorectal adenocarcinoma
Growth in culture	Monolayer epithelial cells
Differentiation	14-21 days in standard culture medium
Morphology	Polarized cells, with tight junctions, apical, brush border
Electrical parameters	High electrical resistance
Digestive enzymes	Typical membranous peptidases and disaccharidases of the small intestine
Active transport	Amino acids, sugars, vitamins, hormones
Membrane ionic transport	Na+/K+ ATPase, H+/K+ ATPase, Na+/H+ exchange, NaI/K+/Cl- co-transport, apical Cl- channels
Membrane non-ionic transporters	Permeability-glycoprotein, multidrug resistant associated protein (MRP), lung cancer associated resistance protein
Receptors	Vitamin B12, vitamin D3, epidermal growth factor, sugar transporters (GLUT1, GLUT2, GLUT3, GLUT5, SGLT1)

Cell culture:

The cell culture phase consists of growing of cells in flasks and cultivating the cell monolayer on semi-porous membranes.
The cells are cultivated in high glucose Dulbecco's Modified Eagle's medium (DMEM)

Ingredients	**Quantity**
Glucose	4.5g/litre
L-glutamine	
$NaHCO_3$ supplemented with heat inactivated **F**etal **B**ovine **S**erum (**FBS**)	10%
Streptomycin	0.1 mg/ml
Penicillin	100 units
HEPES buffer	10 mM
Non essential amino acids(NEAA)	1x

Medium should be exchanged every 48-72 hours.

CULTURE CONDITIONS FOR USE OF Caco-2 CELLS

Inactivated serum in the medium on the apical site	20% cells are maintained in Dulbecco's modified eagle medium (DMEM)
Inactivated serum in the medium on the basolateral site	20% cells are maintained in Dulbecco's modified eagle medium (DMEM)
Coating	Type 1 collagen (Nucell; pore size 0.4 µm), or to insert made of mixed cellulose esters (milicell-HA; pore size 0.45 µm).
C02	10% or 5%
temperature	37 ^{o}C
pH	Usually 7.4 at both sides or 7.4 in basolateral side and 6.5 in apical side
Additive	Non-essential amino acids -1% Glucose - 25mM Glutamine - 2mM Antibiotics - Penicillin Streptomycin
Cell density at seeding	2.5 x 105 - 4 x 105 cells/cm2
Number of passages	25-100
To detach from flask	The cell are detached from the flask by trypsin (0.25% in phosphate-buffer saline; PBS) containing 0.2 % EDTA.
Cell expanded	The cells are expanded in tissue culture flasks (225-cm2 growth area).

ADVANTAGE

- To rapidly evaluate the potential permeability and metabolism of a drug.
- To elucidate the mechanism of the drug transport and the pathway of drug degradation.
- To rapidly evaluate strategies for achieving drug targeting, enhancing the drug transport and minimizing the drug metabolism.
- To minimizing time consuming and sometime controversial animal study.

LIMITATIONS OF Caco-2 METHOD

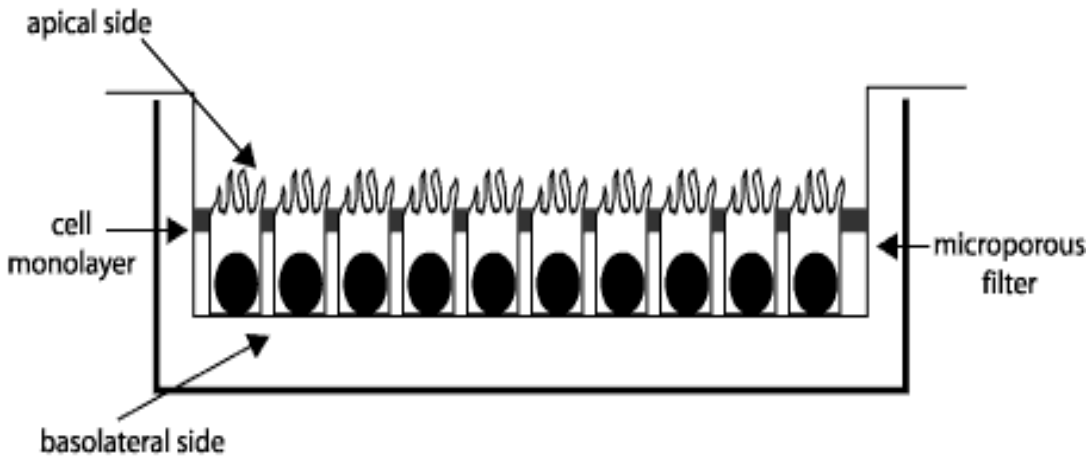
- The expense of cell culture
- 21 days for full cell differentiation
- Relatively low throughput
- The necessity of LC / MS or HPLC for quantitation
- Static model.
- Cells have a tumoral origin.
- Influence of P-gp difficult to estimate
- In Caco-2 cell cultures have large differences reported for actively transported substrates from different laboratories.

INTRINSIC LIMITATION OF THE Caco-2 CELL CULTURE SYSTEM

LIMITATION	ALTERNATIVES
Absence of mucus	Mucus producing cellines, cocultures (Co-cultures of HT29- MTX and Caco-2 cells)
No cellular heterogeneity	Cocultures
No CYP3A4	Transfection or Upregulated by adding 1α,25-dihydroxy vitamin-D3 to the growth medium.
Inability to study regional difference	Using chambers, perfuged intestinal segments
Thickness of unstirred water layer	shaking

- ♣ Variable transporter expression
- ♣ Tightness of epithelium
- ♣ Variable expression of metabolic enzymes

A SCHEMATIC REPRESENTATION OF CULTURE OF Caco-2 CELLS ON A MICROPOROUS FILTER



Caco-2 cells grow as a monolayer and differentiate on a semi-permeable membrane. Thus, separating the apical compartment from the basolateral compartment which corresponds to the intestinal lumen side and the serosal side respectively is possible.

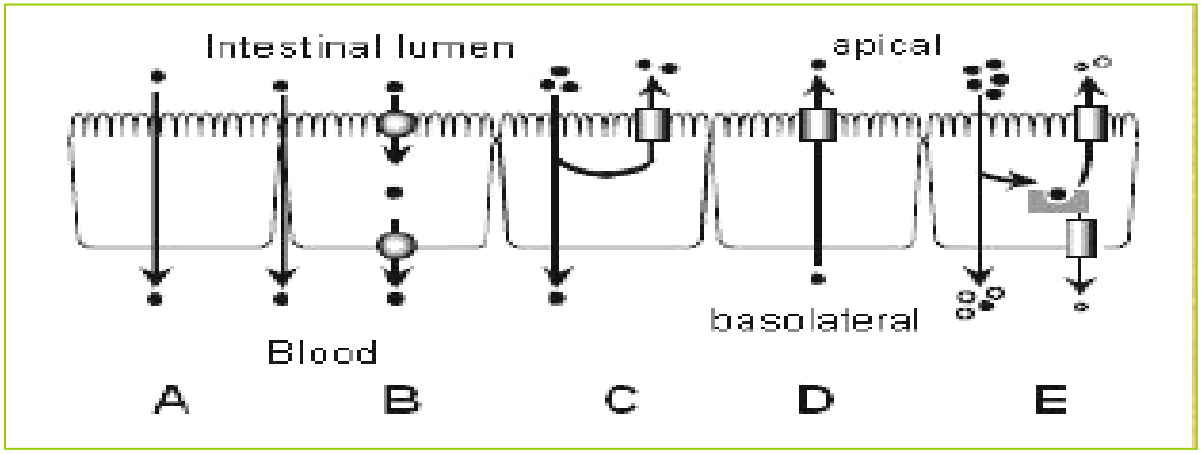
TRANSPORT MECHANISM COMMON TO CELLULAR BARRIER

1. Diffusion flux down an electrical gradient, energy independent.

Passive: - Flux proportional to the concentration gradient.

Transcellular (membrane Permeation) & Paracellular (tight junction passage)

Facilitated: - Carrier mediated flux is saturable with increase concentration of Competitive substrate.



2. Active carrier-mediated:-
Flux can be against an electrochemical gradient. Energy dependent directly or indirectly.

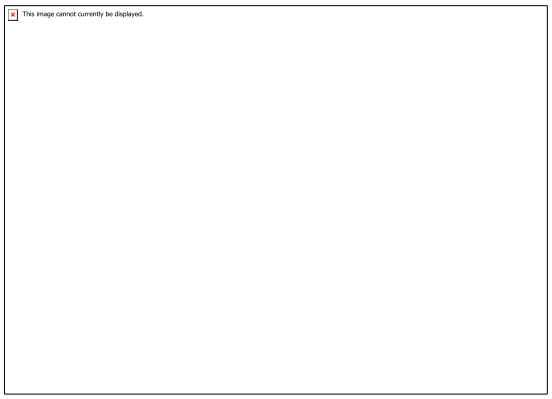
3. Other mechanism include endocytosis
Receptor mediated, Adsorption, Fluid phase

A: Passive trans- and paracellular diffusion;
B: Carrier mediated absorption
C: Active efflux transporter on apical membrane, acting during absorption
D: Active efflux transporter on apical membrane, route for drug clearance from the circulation
E: Intracellular metabolising enzymes localized inside the enterocytes, possibly combined with an active efflux transporter on apical and basolateral membranes

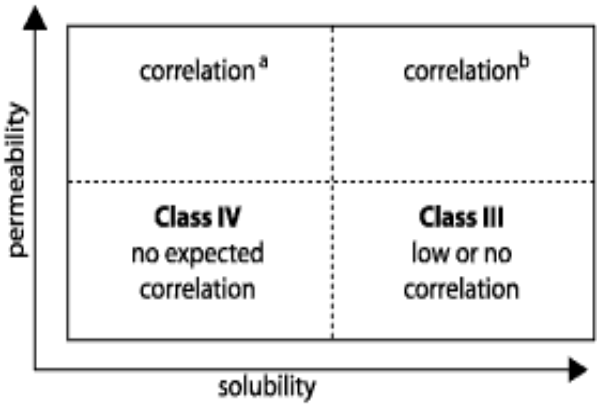
- Paracellular flux occurs strickly by passive diffusion.
- Transcellular flux can occur either Passive ,Facilitated or Active transport process

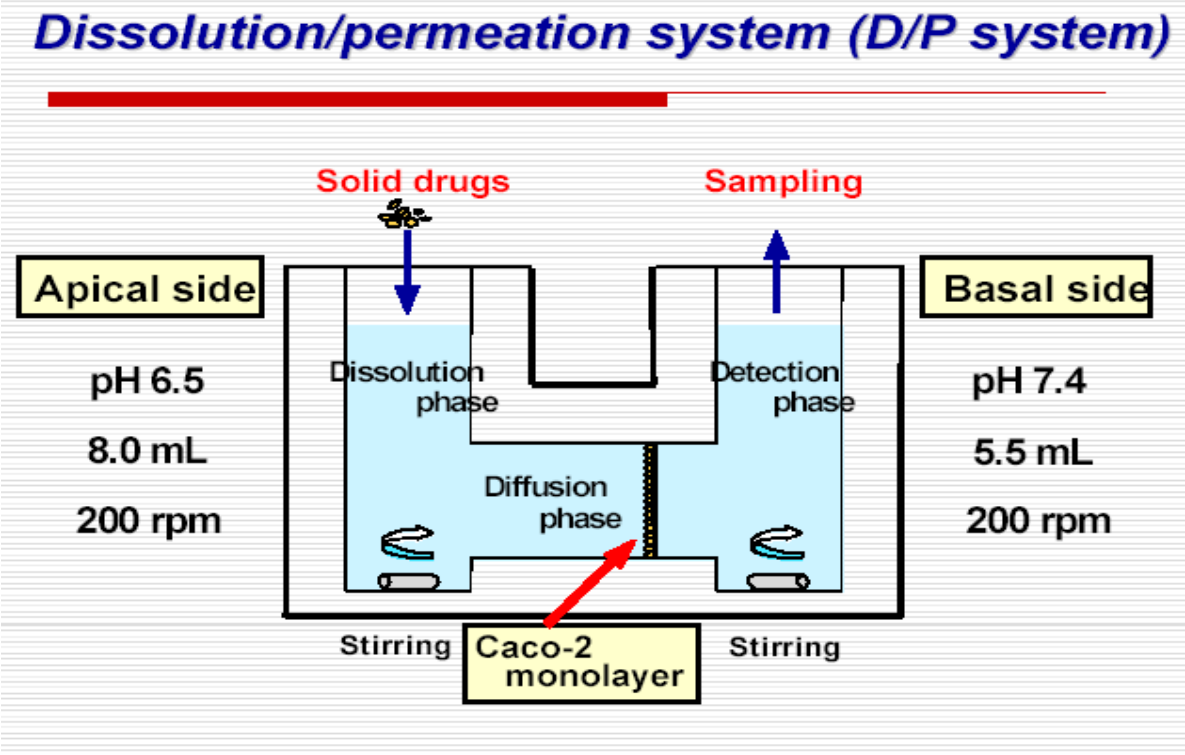
(2) Caco-2: PHARMACEUTICAL CONSIDERATIONS

Biopharmaceutical Classification system



In vitro/in vivo correlation





EFFECT OF COMMON EXCIPIENTS ON Caco-2 TRANSPORT OF LOW-PERMEABILITY DRUGS

Influence of Individual Excipients on Permeability of Drugs:

- Lactose monohydrate, HPMC, EDTA, Propylene glycol, PEG 400, Anhy. Cherry flavor: **No effect on permeability**
- **SLS**: Sodium lauryl sulfate moderately increased the permeability of almost all the drugs.
- **Tween 80**:Increased the apical-to-basolateral direction permeability of furosemide, cimetidine, and hydrochlorothiazide, presumably by inhibiting their active efflux
- **Dioctyl-Sodium-Sulfosuccinate**: increases cimetidine permeability.

ADSORPTION AND NON-SPECIFIC BINDING

- Adsorption to the culture device and/or non-specific binding into the cell mono-layer can lead to under-estimation of the apparent permeability coefficient (Papp) of the compounds as well as to poor recovery of the compound and a low mass balance.
- Minimized absorption include

  - Pretreatment of the device with albumin,
  - Post experiment wash step,

  By acetonitrile, methanol etc.

  - The calculation of the apparent permeability coefficient based on the disappearance of the compound from the donor compartment

SINK CONDITIONS

For low solubility compounds, maintenance of sink conditions avoids the saturation of the acceptor compartment,

For the sink conditions

-We can change the acceptor solvent more frequently at well-defined time points

-To use a medium in the acceptor compartment that is able to decrease the free concentration of the drug.

THE LIMITED SOLUBILITY OF HIGHLY LIPOPHILIC DRUGS

Solubility Enhancing Additives:

Sr. No.	EXCIPIENT	DRUGS
1.	Cyclodextrin	Mefenamic acid, phenytoin
2.	Dimethyl-acetamide	Hydrochlorthiazide
3.	Ethanol	Taxol, quercetin
4.	Propylene glycol	Frusemide, atenolol
5.	Polysorbate 20	Metformin
6.	Sodium docusate	Frusemide, atenolol
7.	SLS	Frusemide, atenolol
8.	Tween 80	Frusemide, atenolol

EVALUATION OF CREATINE TRANSPORT USING Caco-2 MONOLAYERS AS AN IN-VITRO MODEL FOR INTESTINAL ABSORPTION

- Creatine is a nutraceutical. In the present study, creatine transport was examined using Caco-2 monolayer.
- The disadvantages of creatine monohydrate formulations are as follows:
  - Its poor oral bioavailability.
  - Creatine monohydrate has low aq. solubility
- Interestingly, the basolateral to apical permeability of radiolabeled creatine was substantially greater than that observed in the apical to basolateral direction

Transport of levovirin prodrugs in the human intestinal Caco-2 cell line

- The prodrugs were designed to improve the permeability of LVV across the intestinal epithelium by targeting the di/tri-peptide carrier, PepT1.
- Caco-2 cell monolayers were employed to study the transport and hydrolysis properties of the prodrugs.
- Among all mono amino acid ester prodrugs studied, the LVV-5-(L)-valine prodrug (R1518) exhibited the maximum increase (48-fold) in permeability with nearly complete conversion to LVV within 1 h.
- Di-amino acid esters did not offer significant enhancement in permeability comparing with mono amino acid esters and exhibited slower conversion to LVV in Caco2 cell monolayers.

Effect of different surfactants in Biorelevant medium on the secretion of a lipophilic compound in lipoproteins using Caco-2 Cell Culture

- Filter-grown monolayers of Caco-2 cells were incubated for 20 hr with 3H-retinol and 14 C-oleic acid and with increasing concentrations of lyso-phosphotidylcholine (lyso-PC), Cremophor RH40, Tween 80 or Pluronic L81.
- The cell incubated with lyso-PC and Tween 80 increased the incorporation of 3H-retinol and 14C-lipid into chylomicrons and very low density lipoproteins (VLDL) which are concentration dependent manner.
- Lyso-PC and Tween 80 increase lipoprotein secretion in Caco-2 cell
- Cremophor RH40 and Pluronic L81 inhibit lipoprotein secretion in Caco-2 cell.

(3) Caco-2: ANALYTICAL CONSIDERATIONS

- The analytical method should be sensitive, simple and rapid.

- ♣ To minimize the analytical workload including the use of radiolabeled compounds of LC-MS.
- ♣ The use of different additives to the media should not compromise the analytical method.
- ♣ Not require additional manipulations for sample preparation.
- ♣ To increase the relevance of the inter-batch and inter-lab comparisons, the United States Pharmacopoeia Convention (USP) proposes to use reference molecules (methotraxate, propranolol-HCl and testosterone) to validate the system, and gives for each, a standard and an absolute P_{app} apparent permeability value.
- ♣ Each lab should have a reference set of 10 –20 different model drugs including,
    - High and low permeability model drugs
        - (e.g.: propranolol and atenolol)
    - Model drugs for active transport carriers
        - (e.g.: talinolol for Pgp activity).
- ♣ If consider physiological relevance of the media,
  We suggest using bio-relevant apical media and analytically basolateral media, containing additive (e.g.: surfactant) which should not effect on analytical testing.

SAMPLE ANALYSIS

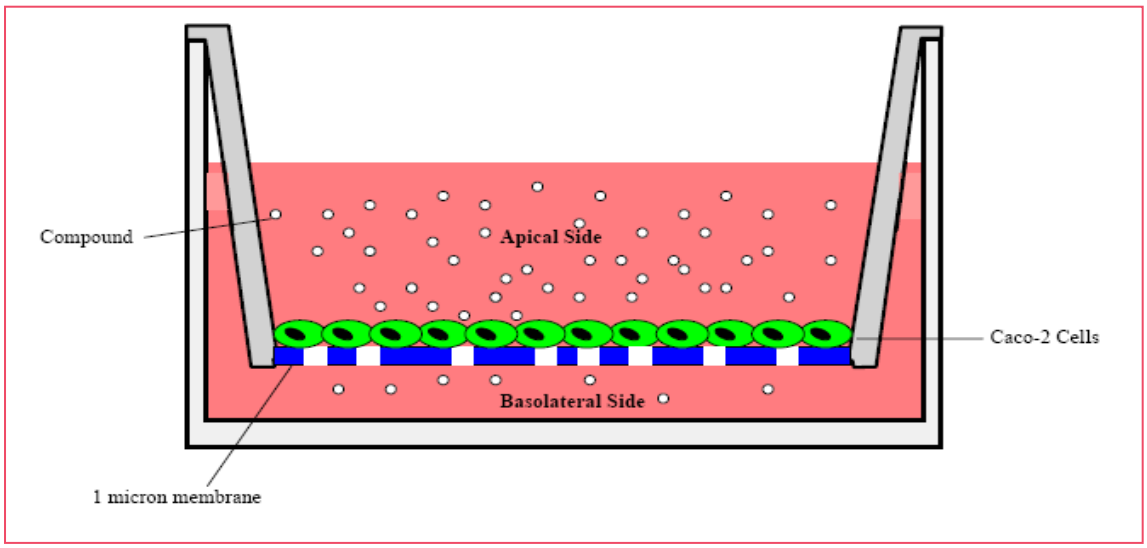
- ♣ The drug concentration can be detected by various analytical methods.
- ♣ Early the radio labeled compounds are used and can be detected by radioactivity measurement by scintillation counters.
- ♣ For the detection of drug concentration,
    - HPLC
    - MS/MS
    - LC-MS/MS Systems are used.

ASSAY DEVELOPMENT

- The preparation time of a fully functional Caco-2 monolayer can be shortened to 3 days using a modified system " **biocoat intestinal epithelium differentiation environment(BIEDE)**

➢ BD BioCoat™ HTS Caco-2 Assay System:

- Reduces caco-2 cell differentiation to 3 days.
- By modifying both the coating material and growth medium Caco-2 monolayers with acceptable barrier properties were obtained.



Calculation Of Apparent Permeability:

- Papp= {V_A/Area ×time} {[Drug]acceptor/[Drug]initial, donor}

Where,
V_A = volume in ml in the acceptor well
Area =the surface area of the membrane
Time= total transport time in seconds

❖ PHARMACOLOGICAL AGENTS FOR USE IN PASSAGE STUDIES

Mechanism	Agents			Effects
P-gp involvement	Inhibitor	Verapamil	≤ 0.5mM in apical (A) and basolateral (B) sides	Increase the transport from the apical side to the basolateral side (if the drug is added on the apical side)
		Quinidine	0.5-1mM in A and B	
		Cyclosporin A	50µ in A and B	
	Substrate	Rhodamine 123	1mM	Efflux of rhodamine at the apical side
MRP involvement	Inhibitor	As for substrates		
	Substrate	Leucotriene C_4, S-2,4-dinitrophenyl glutathione, PAH, doxorubicin, etoposide, vinblastine, methotrexate		
LRP involvement	Inhibitor			
	Substrate	Anthracycline		
Paracellular transport (by action on tight junctions)	EGTA, cytochalasine			Increase the transport, if paracellular

P-gp = permeability glycoprotein, MRP = multidrug resistance associated protein, LRP = lung cancer associated resistance protein.

❖ CORRELATIONS BETWEEN VARIOUS TECHNIQUES

Technique	Number of compounds tested	Comment	Correlation	Currently tested compounds
Caco-2 vs MDCK cells	55	The P_{app} correlation coefficient calculated by these two techniques is 0.79. Moreoever, comparison of the P_{app} values calculated in MDCK and Caco-2 cells in relation to the human org route is 0.58 and 0.54, respectively, indicating that both cells are suitable.	++	D-Glucose, poly(ethylene glycol) (PEG), furosemide, propranolol, atenolol, metoprolol, terbutaline, enalapril, L-dopa, D-mannitol
TC7 vs Caco-2 cells	20	Based on morphological and biochemical parameters and also on transport characteristics, it appears that TC7 cells are a reliable alternative to Caco-2 parental lines for transport studies.	+++	
Caco-2 cells vs human oral route	20	Caco-2 and TC7 cells are used for the prediction of passive human passage	+++	

+ = limited correlation, ++ = median correlation, +++ = high correlation. MDCK = Madin Darby canine kidney. P^{app} = apparent permeability.

(4) OTHER CELL CULTURE MODELS

PARALLEL ARTIFICIAL MEMBRANE PERMEABILITY ASSAY(PAMPA)

- The method has gathered considerable interest in the pharmaceutical industry. PAMPA offers a fast and robust tool for screening permeability of drug in early discovery phase.
- This method was introduced in 1998 and it uses a phospholipid-coated filter separating two aqueous compartments to mimic the passive transport of small molecules.
- Because of its speed, low cost, and versatility, it is a particularly helpful complement to cellular permeability models, such as Caco-2.

PAMPA technique

- The donor solutions were varied in pH, while the acceptor solution had the same pH 7.4 (*p*ION Inc., PN 110139). The buffers are automatically prepared by the robotic system.

- ♣ The *p*ION Gut Box™ (PN 110205) was used to effect stirring and enable environmental control.
- ♣ The plate sandwich was formed and allowed to incubate in the Gut-BoxTM at 23±1 ºC for 30 min in an atmosphere saturated in humidity, and scrubbed free of oxygen and carbon dioxide.
- ♣ Each donor well of the plate had its own stirrer. The speed setting dial on the magnetic stirring device had been calibrated in units of the thickness of the expected unstirred water layer (UWL).
- ♣ After the short incubation time, the sandwich plates were separated, and both the donor and acceptor compartments were assayed for the amount of material present, by comparison with the UV spectrum (225–500 nm) obtained from reference standards. Mass balance was used to determine the amount of material remaining in the membrane barrier.

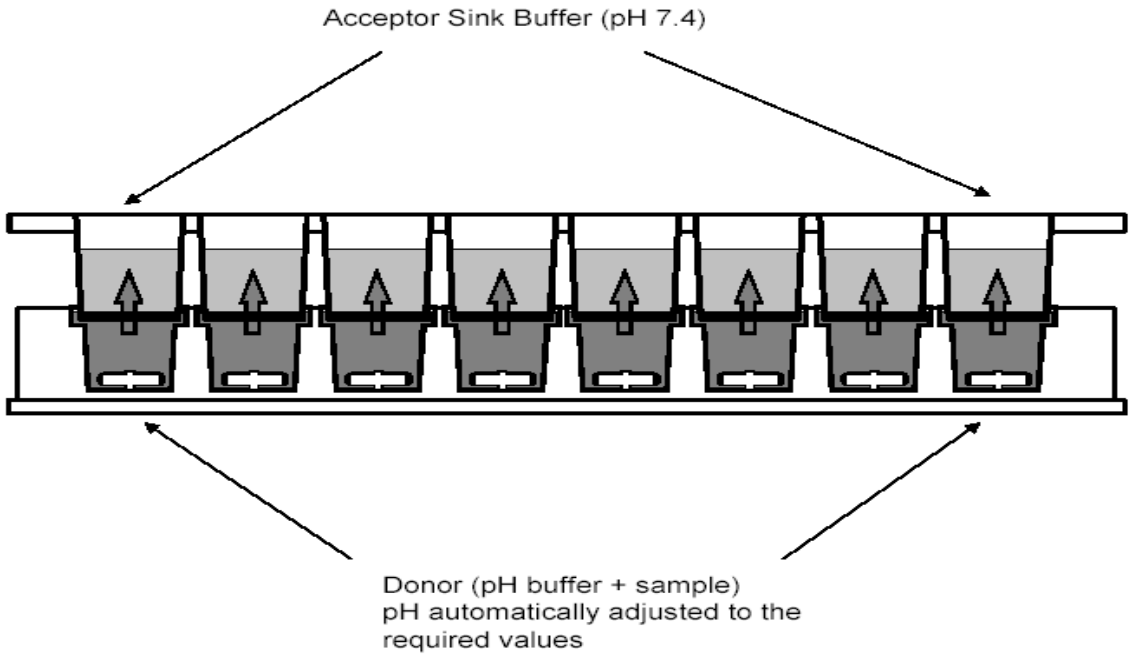
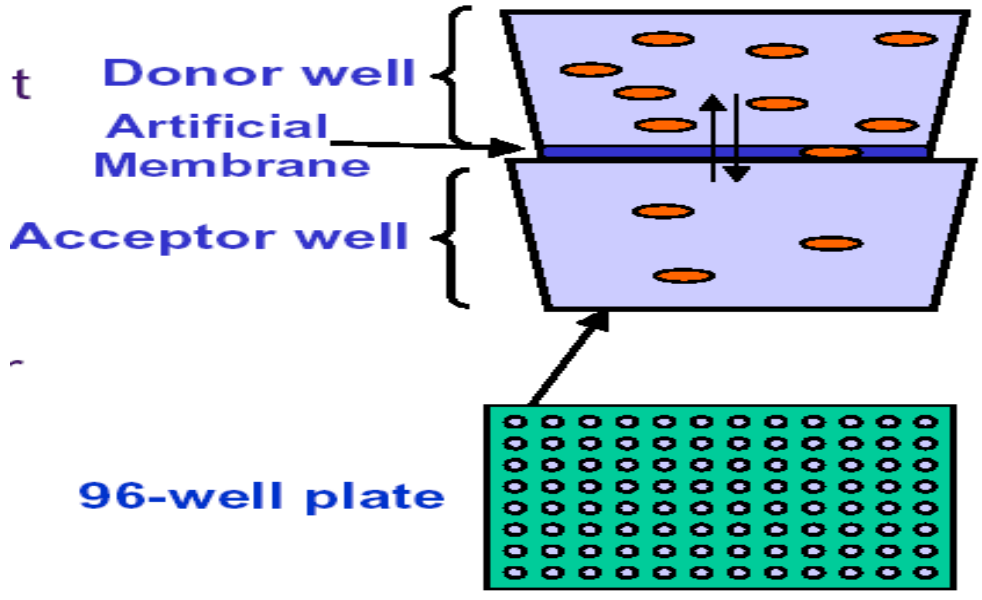
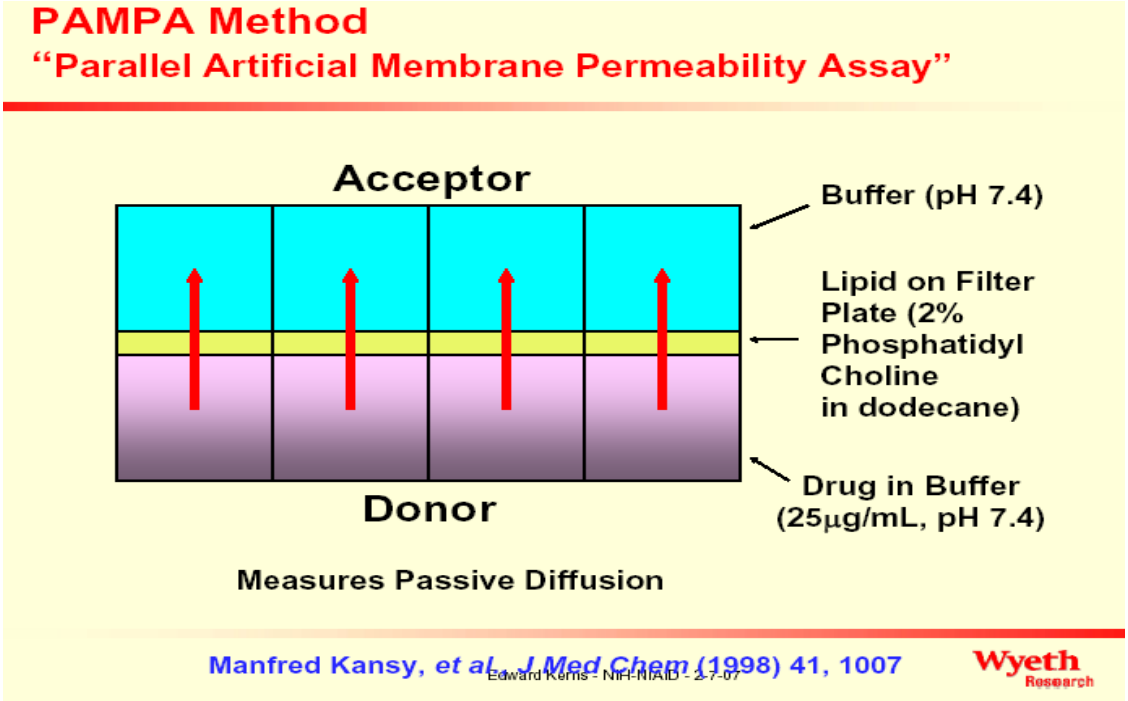


Figure 1. Assembled Donor and Acceptor Plates (PAMPA sandwich)

ENLARGE DIAGRAM OF ONE CHAMBER:





COMPARISON OF PAMPA AND Caco-2 PERMEABILITY ASSAY CHARACTERISTICS

Characteristic	PAMPA	Caco-2
Membrane composition	Phospholipids in alkane	Caco-2 cell mono-layer
Permeability mechanisms	Passive diffusion	Passive diffusion, Active transport, Active efflux, Paracellular & trans-cellular
Metabolism	No	Yes
Maximum throughput	650 compounds/week	50 compounds/week
Resources	Robot, plate washer, UV plate reader, 1 scientist	Cell culture lab., robot, HPLC or LC/MS, 1-2 scientists
Use in	Useful tool in early drug discovery to assess the permeability potential of large no. of compounds.	Method is more suitable during lead optimization or preclinical development stages, where true transepithelial permeability is needed

- PAMPA & Caco-2 should not be considered as **competing permeability methods.**
- **Good correlation** between PAMPA & caco-2 data for a compound indicates a predominance of **passive diffusion in its permeation.**
- **Lack of correlation**
  - **PAMPA <<Caco-2 ;absorptive** (active, paracellular ,gradient effect for acids) transport or
- **PAMPA>>Caco-2** ; **secretary** (efflux, gradient effect for bases) permeation mechanism

MADIN-DARBY CANINE KIDNEY (MDCK) CELL MODEL

- One of the commonly used cell monolayer systems to **assess the human intestine barrier**.

Advantage

- ✓ Can be used for screening
- ✓ Can be used for measurement of passive diffusion.
- ✓ Fast and simple method
- ✓ Do not express P-gp.
- ✓ MDCK cells were seeded at high density and cultured for 3 days

Limitation

- ✓ It is an animal model originate from **dog kidney**
- ✓ **Expression of transporters** is quite different from human intestine.

TC7 cells

Advantage

- ✓ Express CYP3A4.
- ✓ Growth faster than Caco-2 cells.
- ✓ Need less glucose than Caco-2 cells.

CHO cells (Chinese hamster ovary cell cultures)

- ✓ By using this cell line, the membrane transport characteristics of several D- and L-amino acid esters of acyclovir and zidovudine, several di- and tripeptides, amino acids and ß-lactam antibiotics has been characterised.
- ✓ This cell line is used mainly for characterizing substrates and inhibitors of hPepT1.

2/4/A1 cells

- ✓ A conditionally immortalized rat intestinal cell line 2/4/A1 forms polarized monolayer 4-6 days after seeding onto permeable supports.
- ✓ The cells formed continuous multilayer at 33ºC, but at 37ºC monolayer are formed.
- ✓ The paracellular permeability of hydrophilic model drugs is very well comparable to that of small intestine, as well as the diffusion of low permeable model drugs

Limitation

- ✓ The origin of the cells; as they are of rodent origin, the regulation of gene expression during differentiation is probably not similar to that in humans.

Caco-2 / HT 29 – MTX CO-CULTURE CELL LINES

- ✓ Several mucus-producing cell lines have been established from human intestinal HT29 cells.
- ✓ It have been seen that co-culture of HT29 and Caco-2 shows that both cell type forms monolayer with tight junction when grown together on culture.
- ✓ HT29 cells are goblet cell and they secrete the mucus and gives good amount of correlation with in vivo condition.
- ✓ Co-cultures of HT29- MTX and Caco-2 cells offer the opportunity of modifying the permeability barrier of the cell monolayer both w. r. t. Para-cellular resistance and secretary transport via P-gp.

QUESTIONS BANK

- Describe the Biological-, Pharmaceutical- and Analytical-aspects of Caco-2 cells.
- What is Caco-2 Cell? Enlist Advantages, Limitations and intrinsic limitation of the use of Caco-2 cell monolayer.
- Write on general characteristic of Caco-2 cells.
- Describe other cell culture models.
- Comparison of PAMPA and Caco-2 permeability assay characteristic.
- Write note on analytical aspect of Caco-2 cell.

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