Chapter 7

Parallel Artificial Membrane Permeation Assay (PAMPA) study

7.1 INTRODUCTION

For permeability screening, the Caco-2[1-3] cell monolayer permeation method has been widely and successfully used in drug discovery and early development. With the demand for higher throughput, reduced cost, and increased predictability in drug discovery, some limitations of the Caco-2 method are being discussed. These limitations include the expense of cell culture, 21 days for full cell differentiation, relatively low throughput, variable expression of transport and metabolizing proteins, the necessity of liquid chromatography/mass spectrometry (LC/MS) or high-performance liquid chromatography (HPLC) for quantitation, the complication of interpreting multiple permeation mechanisms in Caco-2, and observations that the drug concentration at which Caco-2 is typically performed does not accurately model in vivo dosing. In light of recent conclusions that 80–95% of commercial drugs are absorbed primarily by passive diffusion, there is strong interest in a rapid and inexpensive permeability assay that provides direct data on the passive diffusion mechanism.

Kansy et al[4,5] introduced a new permeability assay called ''parallel artificial membrane permeability assay'' (PAMPA). This technique involves no cell culture. PAMPA uses two aqueous buffer solution wells separated by an artificial membrane. The artificial membrane consists of a lipid in organic diluent that is supported by a porous commercial filter plate matrix. At the beginning of the experiment, the test compound is diluted in buffer (e.g., 25 mg/mL) and placed in a ''donor'' well. The compound moves from the donor well, by passive diffusion, into the artificial membrane and then into the ''acceptor'' well. The rate of permeation is determined by the compound's effective permeability (Pe). The time for experiment setup is greatly reduced compared with cell-monolayer methods. Only passive diffusion is tested and there is no metabolism. PAMPA is performed in a 96-well format and it can be rapidly quantitated using a UV plate reader. There are no transporter proteins, so saturation is not an issue. It is designed to predict passive, transcellular permeability of drugs in early drug discovery. A comparison of Caco-2 and PAMPA characteristics is shown in table 7.1[6].

Table 7.1. Comparision of PAMPA and Caco-2 permeabiilty assay characteristics.

Characteristic	PAMPA	Caco-2
Membrane composition	Phospholipid in alkane	Caco-2 cell monolayer
Permeability mechanisms	Passive diffusion	Passive diffusion Active transport Active efflux Paracellular
Metabolism	No	Yes
Maximum throughput per instrument	650 compounds per week (three plates/day in duplicate)	50 compounds per week (two plates/day in duplicate in both A>B and B>A directions)
Resources	Robot, plate washer, UV plate reader, 1 scientist	Cell culture lab, robot, HPLC or LC/MS, 1.5 scientists
Supplies	++	+++
Estimated cost/sample	1X	15–20X

Avdeef et al[7] from pION Inc. described an extension of the Roche approach, including a way to assess membrane retention. This method uses 1, 2-dioleoyl-sn-glycer-3-phosphocholine as the lipid component at 2% in dodecane. The experimental data obtained (figure 7.1) was compared with literature values for human jejunum permeability and the fraction absorbed in humans.

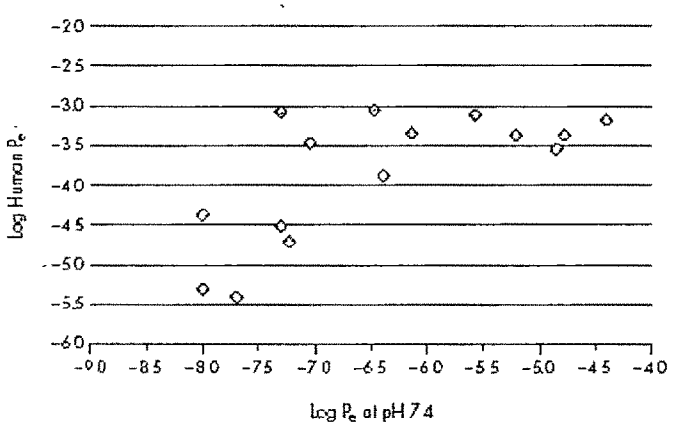

Figure 7.1. PAMPA Log $P_e^{7.4}$ vs. Human Absorption Values 1[5].

Sugano et al[8] endeavored to improve the composition of the lipid solution used in PAMPA for a more precise prediction of oral absorption. In their study, a mixture of several different phospholipids was used to more accurately resemble the mixture found in reconstituted brush-border lipids.

Di et al[9] from Wyeth described a variation of PAMPA designed to predict blood–brain barrier permeation. The artificial membrane for the PAMPA-BBB (blood–brain barrier) assay is formed from a mixture of brain lipids selected to model the composition of the blood–brain barrier

Schmidt et al from Millipore investigated both the Permeability[10] and PAMPA[11] assays for data reproducibility and the affect of different assay conditions. Both assays were tested over the course of several days using different lots of filter plates.

Whohnsland et al[12], and Zhu et al[13] modified the assay and used it to screen compound permeability. Hwang K-K et al[14] used PAMPA for the permeation prediction of M100240, which was unable to be determined by cell-based assays due to compound instability.

7.2 EXPERIMENTALS

7.2.1 Reagents

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, USA). Propranolol, carbamazepine and timolol were gift sample of Torrent Pharmaceutical Limited, Ahmedabad.

7.2.2 Equipments

- MultiScreen filter plate for PAMPA assay with underdrain removed (cat. MAPBMN310; Millipore Corporation, Billerica, MA).
- PTFE Acceptor plate ((cat. MSSACCEPTOR; Millipore).
- Spectramax® Plus microtiter plate reader (Molecular Devices, Sonnyvale, CA).
- SoftMax® Pro software (Molecular Devices).
- UV compatible quartz plate (for example: # R8024; Molecular Devices)

- Polypropylene reagent reservoirs (cat. 175-RBAS-000; ELKay laboratory consumables Shrewsbury, MA).
- Multichannel pipette such as: Biohit™ 8 channel electronic pipettor with polypropylene tips (cat. W67-710-800 and W16- 160045, Vanguard 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 (SarasotaFL).

7.2.3 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 kW.). Data from wells with electrical resistance measurements below 5 kW were excluded.

7.2.4 Validation of the Method

The method was validated using standard compound like propanolol (high permeability), carbamezipine (medium permeability) and timolol (medium permeability).

7.2.5 Method

Prepare a 20% solution (v/v) of hexadecane in hexane (~3 ml/plate). Pipette 20 µl of the hexadecane/hexane mixture carefully on a 3.0µm polycarbonate membrane support of each well, avoiding pipette tip contact with the membrane. Note: use polypropylene reservoir 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 Immediately after the application of the artificial membrane (within 10 minutes maximum), add 125 µl of drug containing donor solutions (salbutamol sulphate dissolved in acetate buffet pH 4.0 (2000 µM), phosphate buffer pH 7.0 (2000 µM) and borate buffer pH 9.0 (2000 µM), ondansetron hydrochloride in pH acetate buffer 4.0 (750 µM), propanolol (500 µM),

carbamazepine (500 μM) and timolol (500 μM) in 5 % dimethyl sulphoxide) to each well of the Donor plate. The drug containing donor solutions were added after 16 hours of incubation as shown in table 7.2 to study the permeation of drug for 6 hours. The drug solutions and blank solutions were added in triplicate in 96 well plate as shown in table 7.3 Add 250 μl of aqueous respective buffer or 5 % DMSO to each well of the PTFE Acceptor plate (MSACCEPTOR).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. Replace the plate lid and incubate at room temperature for 24 hours. After incubation, measure UV/Vis absorption from 250 to 500 nm for 100 μl/well of the Donor solution. 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 190 to 400 nm for 100 μl/well of each.

Table 7.2. The drug solutions and blank solutions in triplicate in 96 well plate.

	Incubation for 24 hour		Incubation for 6 hour	
	Triplicate in column no. 1, 2 and 3.	**Triplicate in column no. 4, 5 and 6.**	**Triplicate in column no. 7, 8 and 9.**	**Triplicate in column no. 10, 11 and 12.**
A	5 % DMSO	Phosphate buffer pH 7.0	5 % DMSO	Phosphate buffer pH 7.0
B	Propanolol in 5 % DMSO	Sabutamol sulphate in buffer pH 7.0	Propanolol in 5 % DMSO	Sabutamol sulphate in buffer pH 7.0
C	Timolol in 5 % DMSO	Acetate buffer pH 4.0	Acetate buffer pH 4.0	Timolol in 5 % DMSO
D	Carbamezepine in 5 % DMSO	Ondansetron in buffer pH 4.0	Ondansetron hydrochloride in buffer pH 4.0	Carbamezepine in 5 % DMSO
E	Acetate buffer pH 4.0		Acetate buffer pH 4.0	
F	Sabutamol sulphate in buffer pH 4.0		Sabutamol sulphate in buffer pH 4.0	
G	Borate buffer pH 9.0		Borate buffer pH 9.0	
H	Sabutamol sulphate in buffer pH 9.0		Sabutamol sulphate in buffer pH 9.0	

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

$$\text{lop}P_e = \log\left\{C\times -\ln\left(1-\frac{[\text{drug}]_{\text{acceptor}}}{[\text{drug}]_{\text{equilibrium}}}\right)\right\} \qquad \text{where}\, C = \left(\frac{V_D \times V_A}{(V_D + V_A)\,\text{Area}\times\text{time}}\right)$$

Variables for the equation are defined in table 7.3.

Table 7.3. Description of variables used to calculate Log P_e.

Term	Definition	Notes
V_D	Volume of donor compartment	Expressed in cm^3, 150 L = 0.15 cm^3
V_A	Volume of acceptor compartment	Expressed in cm^3, 300 L = 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

7.3 RESULTS AND DISCUSSION:

7.3.1 Results of Equilibrium Samples

Figure 7.2 shows the results of UV spectra of the equilibrium samples in 96 well plate. The spectra were measured from 190 nm to 400 nm SPECTRAmax UV plate reader.

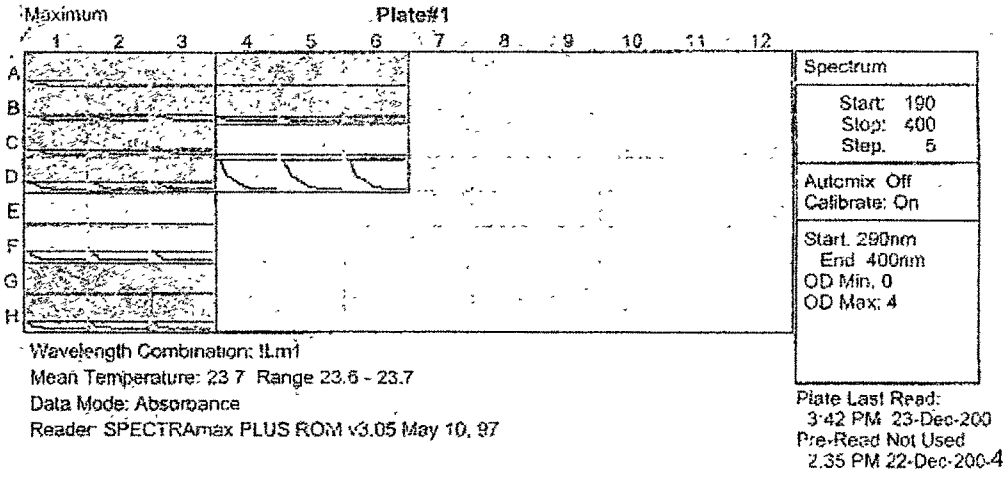

Figure 7.2 UV spectra of the equilibrium samples in the 96-well plate.

Table 7.4 shows the absorbance values of blank solution of 5% DMSO and the equilibrium samples of carbamizepine in 5% DMSO (conc. 166μM), propanolol in 5% DMSO (conc. 166μM) and timolol in 5% DMSO (conc. 166μM) measured at 290 nm using UV plate reader in 96 wells plate.

Table 7.4. The absorbance values of blank 5% DMSO and the equilibrium samples of standard compounds.

Samples	Conc. (μM)	Wells	Absorbance values	Mean absorbance value	Std. Dev.	C.V.%	Lambda max (λ_{max})
5% DMSO blank solution			0.154	0.154			290nm
Carbamizapine in 5% DMSO	166.00	D1 D2 D3	0.843 0.840 0.837	0.840	0.010	3.7	290nm
Propanolol in 5% DMSO	166.00	B1 B2 B3	0.365 0.358 0.351	0.356	0.008	5.8	290nm
Timolol in 5% DMSO	166.00	C1 C2 C3	0.198 0.197 0.187	0.194	0.006	3.3	290nm

Table 7.5 shows the absorbance values of blank solution of borate buffer pH 9.0 and the equilibrium samples of salbutamol sulphate in borate buffer pH 9.0 (conc. 666.6μM) measured at 290 nm using UV plate reader in 96 wells plate.

Table 7.5. The absorbance values of blank borate buffer pH 9.0 and the equilibrium samples of salbutamol sulphate.

Sample	Conc. (μM)	Wells	Absorbance values	Mean absorbance value	Std. Dev.	C.V. %	Lambda max
Borate buffer pH 9.0 blank solution			0.154	0.154			290 nm
Salbutamol sulphate in borate buffer pH 9.0	666.6	H1 H2 H3	0.364 0.369 0.359	0.364	0.005	1.3	290nm

Table 7.6 shows the absorbance values of blank solution of phosphate buffer pH 7.0 and the equilibrium samples of salbutamol sulphate in phosphate buffer pH 7.0 (conc. 666.6μM) measured at 290 nm using UV plate reader in 96 wells plate.

Table 7.6. The absorbance values of blank phosphate buffer pH 7.0 and the equilibrium samples of salbutamol sulphate.

Sample	Conc. (μM)	Wells	Absorbance values	Mean absorbance value	Std. Dev.	C.V. %	Lambda max
Phosphate buffer pH 7.0 blank solution			0.041				290 nm
Salbutamol sulphate in phosphate buffer pH 7.0	666.600	B1 B2 B3	0.159 0.156 0.153	0.156	0.003	1.8	290nm

Table 7.7 shows the absorbance values of blank solution of acetate buffer pH 4.0 and the equilibrium samples of salbutamol sulphate in acetate buffer pH 7.0 (conc. 666.6μM) measured at 290 nm using UV plate reader in 96 wells plate.

Table 7.7. The absorbance values of blank acetate buffer pH 4.0 and the equilibrium samples of salbutamol sulphate.

Sample	Conc. (µM)	Wells	Absorbance values	Mean absorbanceValue	Std. Dev.	C.V. %	Lambda max
Acetate buffer pH 4.0 blank solution			0.039				290 nm
Salbutamol sulphate in acetate buffer pH 4.0	666.60	F1 F2 F3	0.424 0.403 0.405	0.411	0.012	2.8	290nm

Table 7.8 shows the absorbance values of blank solution of acetate buffer pH 4.0 and the equilibrium samples of ondansetron hydrochloride in acetate buffer pH 7.0 (conc. 250.0µM) measured at 290 nm using UV plate reader in 96 wells plate.

Table 7.8. The absorbance values of blank acetate buffer pH 4.0 and the equilibrium samples of ondansetron hydrochloride.

Sample	Conc. (μM)	Wells	Absorbance values	Mean absorbance Value	Std. Dev.	C.V. %	Lambda max
Acetate buffer pH 4.0 blank solution			0.038				290 nm
Ondansetron hydrochloride in acetate buffer pH 4.0	250.00	D1 D2 D3	1.690 1.681 1.696	1.689	0.008	0.4	290nm

7.3.2 Results of Samples Incubated for 6 hours and 24 hours.

UV spectra of the samples incubated for 6 hrs and 24 hrs in the 96-well plate is shown in figure 7.3. The spectra were measured from 190 nm to 400 nm using SPECTRAmax UV plate reader.

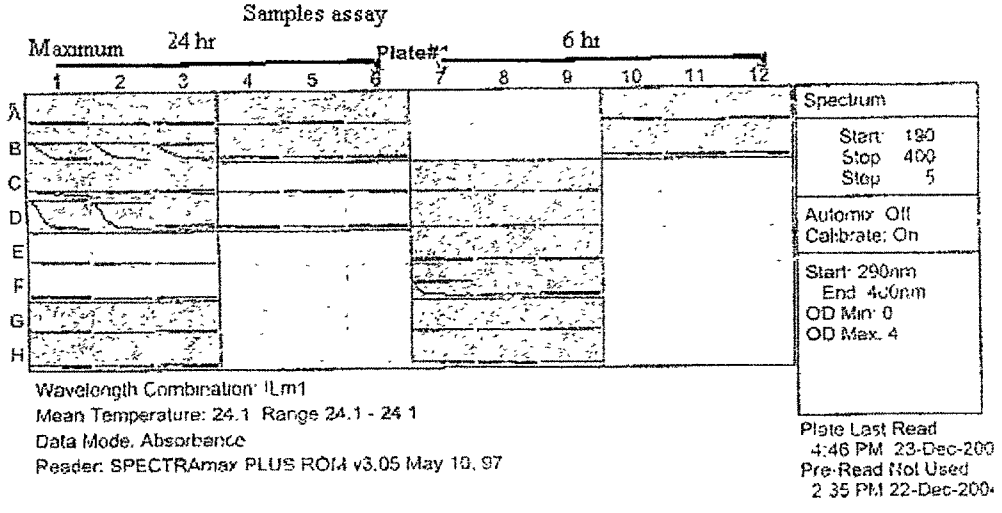

Figure 7.3 UV spectra of the samples incubated for 6 hrs and 24 hrs in the 96-well plate.

7.3.2.1 *Results of the samples incubated for 6 hrs*

Table 7.9 shows the absorbance values samples incubated for 6 hours of salbutamol sulphate in borate buffer pH 9.0 (conc. 2000 µM) measured at 290 nm using UV plate reader in 96 wells plate.

Table 7.9. The absorbance values of salbutamol sulphate in borate buffer pH 9.0 incubated for 6 hours.

Sample	Conc. (µM)	Wells	Absorbance values	Mean absorbance value	Std. Dev.	C.V. %	Lambda max
Goup blank			0.040				290 nm
Salbutamol sulphate in borate buffer pH 9.0	2000	H7 H8 H9	0.048 0.018 0.0.28	0.0.31	0.01 5	49.3	290nm

Table 7.10 shows the absorbance values samples incubated for 6 hours of salbutamol sulphate in phosphate buffer pH 7.0 (conc. 2000 µM) measured at 290 nm using UV plate reader in 96 wells plate.

Table 7.10. The absorbance values of salbutamol sulphate in phosphate buffer pH 7.0 incubated for 6 hours.

Sample	Conc. (µM)	Wells	Absorbance values	Mean absorbance value	Std. Dev.	C.V. %	Lambda max
Group blank			0.041				290 nm
Salbutamol sulphate in phosphate buffer pH 7.0	2000	B10 B11 B12	0.000 -0.003 0.009	0.002	0.006	337.3	290nm

Table 7.11 shows the absorbance values samples incubated for 6 hours of salbutamol sulphate in acetate buffer pH 4.0 (conc. 2000 µM) measured at 290 nm using UV plate reader in 96 wells plate.

Table 7.11. The absorbance values of salbutamol sulphate in acetate buffer pH 4.0 incubated for 6 hours.

Sample	Conc. (µM)	Wells	Absorbance values	Mean absorbance value	Std. Dev.	C.V. %	Lambda max
Group blank			0.039				290 nm
Salbutamol sulphate in acetate buffer pH 4.0	2000	F7 F8 F9	0.193 -0.002 -0.002	0.063	0.113	178.6	290nm

Table 7.12 shows the absorbance values samples incubated for 6 hours of ondansetron hydrochloride in acetate buffer pH 4.0 (conc. 2000 µM) measured at 290 nm using UV plate reader in 96 wells plate.

Table 7.12. The absorbance values of ondansetron hydrochloride in acetate buffer pH 4.0 incubated for 6 hours.

Sample	Conc. (µM)	Wells	Absorbance values	Mean absorbance value	Std. Dev.	C.V. %	Lambda max
Group blank			0.039				290 nm
Ondansetron hydrochloride in acetate buffer pH 4.0	750.00	D7 D8 D9	-0.003 -0.003 -0.002	-0.003	0.000	17.7	290nm

7.3.2.2 Results of the samples incubated for 24 hours

Table 7.13 shows the absorbance values samples incubated for 24 hours of 5% DMSO blank solution and of carbamizepine in 5% DMSO (conc. 500μM), propanolol in 5% DMSO (conc. 500μM) and timolol in 5% DMSO (conc. 500μM) measured at 290 nm using UV plate reader in 96 wells plate.

Table 7.13. The absorbance values samples incubated for 24 hours for 5% DMSO blank solution and of standard compounds.

Samples	**Conc. (μM)**	**Wells**	**Absorbance values**	**Mean absorbance value**	**Std. Dev.**	**C.V.%**	**Lambda max**
5% DMSO blank solution			0.154				290 nm
Carbamizapine in 5% DMSO	500	D1 D2 D3	0.425 0.365 0.030	0.273	0.213	78.0	290nm
Propanolol in 5% DMSO	500	B1 B2 B3	0.243 0.231 0.200	0.224	0.022	9.8	290nm
Timolol in 5% DMSO	500	C1 C2 C3	0.014 0.024 0.014	0.017	0.006	34.5	290nm

Table 7.14 shows the absorbance values samples incubated for 24 hours of salbutamol sulphate in borate buffer pH 9.0 (conc. 2000 μM) measured at 290 nm using UV plate reader in 96 wells plate.

Table 7.14. The absorbance values of salbutamol sulphate in borate buffer pH 9.0 incubated for 24 hours.

Sample	Conc. (μM)	Wells	Absorbance values	Mean absorbance value	Std. Dev.	C.V. %	Lambda max
Group blank			0.040				290 nm
Salbutamol sulphate in borate buffer pH 9.0	2000	H1 H2 H3	0.015 0.011 0.010	0.012	0.003	22.2	290nm

Table 7.15 shows the absorbance values samples incubated for 24 hours of salbutamol sulphate in phosphate buffer pH 7.0 (conc. 2000 μM) measured at 290 nm using UV plate reader in 96 wells plate.

Table 7.15. The absorbance values of salbutamol sulphate in phosphate buffer pH 7.0 incubated for 24 hours.

Sample	Conc. (μM)	Wells	Absorbance values	Mean absorbance value	Std. Dev.	C.V. %	Lambda max
Group blank			0.041				290 nm
Salbutamol sulphate in phosphate buffer pH 7.0	2000	B4 B5 B6	0.009 0.009 0.010	0.009	0.001	6.7	290nm

Table 7.16 shows the absorbance values samples incubated for 24 hours of salbutamol sulphate in acetate buffer pH 4.0 (conc. 2000 µM) measured at 290 nm using UV plate reader in 96 wells plate.

Table 7.16. The absorbance values of salbutamol sulphate in acetate buffer pH 4.0 incubated for 24 hours.

Sample	Conc. (µM)	Wells	Absorbance values	Mean absorbance value	Std. Dev.	C.V. %	Lambda max
Group blank			0.039				290 nm
Salbutamol sulphate in acetate buffer pH 4.0	2000	F1 F2 F3	-0.002 -0.005 -0.009	-0.005	0.004	67.2	290nm

Table 7.17 shows the absorbance values samples incubated for 24 hours of ondansetron hydrochloride in acetate buffer pH 4.0 (conc. 2000 µM) measured at 290 nm using UV plate reader in 96 wells plate.

Table 7.17. The absorbance values of ondansetron hydrochloride in acetate buffer pH 4.0 incubated for 24 hours.

Sample	Conc. (µM)	Wells	Absorbance values	Mean absorbance value	Std. Dev.	C.V. %	Lambda max
Group balnk			0.039				290 nm
Ondansetron hydrochloride in acetate buffer pH 4.0	750.00	D4 D5 D6	0.004 0.006 0.011	0.007	0.004	52.8	290nm

7.3.2.3 *Calculation of the permeability coefficients*

The permeability coefficients (Pe) and log Pe values of salbutamol sulphate in buffer pH 4.0, 7.0, 9.0 and ondansetron hydrochloride in buffer pH 4.0 were calculated using the equation reported by Faller et al[11]. The results of the calculations of the permeability coefficients are given in table 7.18 and table 7.19.

Table 7.18. Calculation for permeability coefficient for 24 hrs incubation.

Compound	Buffer	pH	Absorbance after incubation			Average	Absorance equilium
			1	2	3		
Propanolol	5% DMSO		0.243	0.231	0.2	0.224	0.35(
Carbamezapine	5% DMSO		0.425	0.365	0.03	0.273	0.84
Timolol	5% DMSO		0.014	0.024	0.014	0.0173	0.19
Salbutamol sulphate	acetate buffer	4.0	0.000	0.000	0.000	0.000	0.41
Salbutamol sulphate	phosphate buffer	7.0	0.009	0.009	0.01	0.009	0.15(
Salbutamol sulphate	borate buffer	9.0	0.048	0.018	0.028	0.031	0.36
Ondansetron hydrochloride	acetate buffer	4.0	0.004	0.006	0.011	0.007	1.68

Table 7.19. Calculation of permeability coefficients.

Compound	r	1-r	ln(1-r)	Pe (cm/sec)	logPe	Reported value of log Pe
Propanolol	0.6310	0.3689	-0.99719	0.0000249	-4.60	-4.6
Carbamezapine	0.3253	0.6746	-0.39363	0.00000984	-5.00	-9.3
Timolol	0.0893	0.9106	-0.09359	0.00000234	-5.63	-5.0
Salbutamol sulphate(pH4.0)	0.0421	0.9578	-0.04309	0.00000108	-5.96	
Salbutamol sulphatepH 7.0)	0.0598	0.9401	-0.06169	0.00000154	-5.81	
Salbutamol sulphatepH 9.0)	0.0860	0.9139	-0.09001	0.00000225	-5.64	
Ondansetron hydrochloride (pH 4.0)	0.0041	0.9958	-0.00415	0.000000104	-6.98	

The value of the permeability coefficients of salbutamol sulphate at pH 4.0, 7.0 and 9.0 were 1.08×10^{-6}, 1.54×10^{-6} and 2.25×10^{-6} respectively. The values of the permeability coefficients of salbutamol sulphate calculated using the PAMA assay increased with increasing the pH from 4.0 to 9.0. The results of increase in the permeability values of salbutamol sulphate with increase in pH by the PAMPA assay was further confirmed by the in vitro buccal permeation study using guinea pig buccal mucosa (chapter 6).

The permeability coefficient (Pe) of ondansetron hydrochloride in at pH 4.0 was 1.04×10^{-4}. The permeability of ondansetron hydrochloride was less than that of salbutamol sulphate at pH 4.0. This may be due to less solubility of the ondansetron hydrochloride at pH 4.0 than that of salbutamol sulphate.

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