Asian Journal of Pharmaceutical and Clinical Research
Vol. 3, Issue 3, 2010 ISSN - 0974-2441

Research Article

SIMULTANEOUS ANALYSIS OF INTESTINAL PERMEABILITY MARKERS, CAFFEINE, PARACETAMOL AND SULFASALAZINE BY REVERSE PHASE LIQUID CHROMATOGRAPHY: A TOOL FOR THE STANDARDIZATION OF RAT EVERTED GUT SAC MODEL

ANAND PATIL, VARUN RAHEJA, ANAGHA DAMRE*

Drug Metabolism and Pharmacokinetics, Piramal Life Sciences Limited, Goregaon (E), Mumbai - 400 063, India. E-mail: anagha.damre@piramal.com

ABSTRACT

A simple, sensitive and specific reverse-phase high performance liquid chromatographic (RP-HPLC) method with UV detection was developed and validated for simultaneous analysis of caffeine, paracetamol and sulfasalazine, markers of high, medium and low intestinal permeability, respectively. The method was used to determine their apparent permeability coefficient (P_{app}) using rat everted gut sac technique. Chromatography was carried out on C-18 column with mobile phase comprising of ammonium acetate buffer (0.01 M, pH 5.0) and acetonitrile gradient pumped at a flow rate of 0.5 ml min^{-1}. The retention times for caffeine, paracetamol and sulfasalazine were 8.85 ± 0.02 min, 7.8 ± 0.02 min and 12.2 ± 0.05 min, respectively. Method validation parameters included specificity, accuracy, precision, sensitivity and stability studies. The applicability and reliability of the analytical method was evaluated by analyzing samples obtained from the rat everted gut sac experiment. P_{app} values obtained for the marker compounds, studied separately or in a cassette, correlated well with human fraction absorption, enabling their classification as high, medium and low permeable compounds. In conclusion, the developed RP-HPLC method for permeability markers caffeine, paracetamol and sulfasalazine can be used for high throughput cassette validation of rat everted gut sac model for intestinal permeability assessment.

Key words: Caffeine, Paracetamol, Sulfasalazine, RP-HPLC-UV, Everted gut sac, Intestinal permeability.

INTRODUCTION

During the development of drugs and formulations intended for oral administration, it is of considerable value to have reliable and predictive *in vitro* methods to quantify drug transport across the intestinal epithelium. In the discovery stage, drug absorption studies can be performed only in laboratory animals and/or in *in vitro* systems where the absorption process can be characterized both qualitatively and quantitatively. Several *in vitro* models [1] are available to determine permeability, such as human Colon Carcinoma (Caco-2) cells, Madin Darby Canine Kidney (MDCK) cells, Immobilized Artificial Membrane (IAM) columns, Parallel Artificial Membrane Permeation Assay (PAMPA), excised animal tissues in Ussing chambers and everted gut sacs. These models show good correlation to the *in vivo* intestinal permeability and fraction of dose absorbed in humans [2].

The everted gut sac model, especially the improved technique using oxygenated TC 199 medium [3], offers advantages *viz.* it is easy and inexpensive to perform and offers the possibility of conducting regional and mechanistic studies [4]. The everted gut sac of the rat small intestine can be used to determine various aspects of drug absorption with high reliability and reproducibility [5, 6] in early stages of drug discovery.

To demonstrate suitability of any permeability model, a rank-order relationship between permeability values and the extent of drug absorption in human subjects needs to be established using a sufficient number of model drugs. US-FDA guidelines[7] suggest the use of low (e.g., < 50 % F_a), medium (e.g., 50 – 89 % F_a) and high (≥ 90 % F_a) permeability markers to evaluate permeability of new chemical entities (NCEs) for classifying them according to the BCS (Biopharmaceutical Classification System). These markers are used in permeability studies to monitor the integrity and functional status of the intestinal membrane [2].

For the standardization of the rat everted gut sac method, we have selected caffeine, paracetamol and sulfasalazine as the markers of high, medium and low permeability, respectively. Chemical structures of these drugs are presented in Figure 1. The basis for selection of these drugs was their reported percent human absorption and BCS classification. Caffeine is a Class I (high solubility, high permeability) drug with 100 % F_a in humans [8,9]. According to the present regulations, paracetamol is a Class III (high solubility, low permeability) drug [8,10], however, due to 80 % F_a in humans, it can be considered as a medium permeable drug. Sulfasalazine is a Class IV (low solubility, low permeability) drug with reported percent human absorption of 13 % [8,11]. Although sulfasalazine has been reported to have efflux potential through drug transporters [12], caffeine and paracetamol are not reported to undergo any transporter mediated efflux. Also the drug-drug interaction, which would affect absorption profile of a compound in the presence of the other, has not been reported among these compounds.

We have reported here a simple and sensitive RP-HPLC-UV method for simultaneous analysis of caffeine, paracetamol and sulfasalazine. The method was validated for routine use and its usefulness and reliability for standardization of rat everted gut sac model is demonstrated using real time rat everted gut sac experiment samples. Also, the method can be utilized for standardization of other *in vitro* (Caco-2, MDCK) or *in situ* (luminal perfusate samples) models where caffeine, paracetamol and sulfasalazine can be used as permeability markers.

EXPERIMENTAL

Reagents and chemicals

Media 199 (D5796) and sodium bicarbonate (S5761) were purchased from Sigma-Aldrich (Ayrshire, UK). TC 199 medium was prepared by adjusting the pH 7.4 of Media 199 solution (9.6 g/L water) using sodium bicarbonate. The following compounds were purchased commercially from Sigma–Aldrich Chemie (Steinheim, Germany): caffeine, paracetamol, sulfasalazine and lucifer yellow. Ammonium acetate (HPLC grade), acetonitrile (HPLC grade), ortho-phosphoric acid (88%) and dimethylsulfoxide were purchased from Merck (Mumbai, India).

Instruments

The liquid chromatographic system consisted of a Waters Alliance Separations Module 2695, Photodiode Array detector 2996 (Waters, USA). All the parameters of HPLC were controlled by Empower software version 1 (Waters Inc., Milford, USA).

Liquid chromatographic conditions

Chromatographic separations were obtained using a stainless steel column, Thermo BDS Hypersil C_{18}, 5 µ, 250 mm×4.6 mm (Thermo Electron Corporation, Germany), which was maintained at 30 ^{0}C. The injection volume of samples was 50 µL. The mobile phase consisted of 100 % acetonitrile (mobile phase A) and 0.01 M ammonium

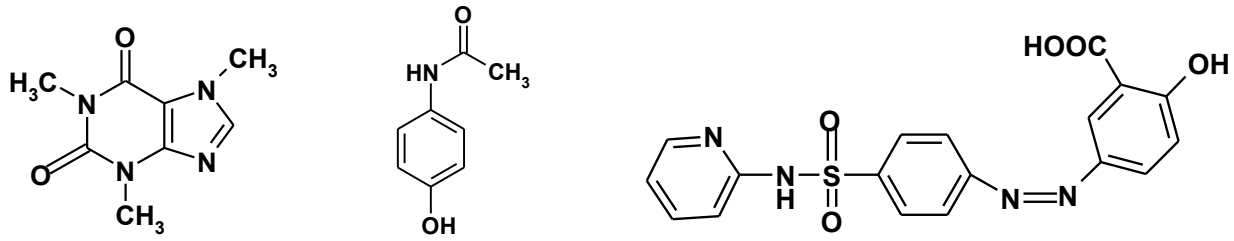

Figure 1: Chemical structures of caffeine (A), paracetamol (B) and sulfasalazine (C)

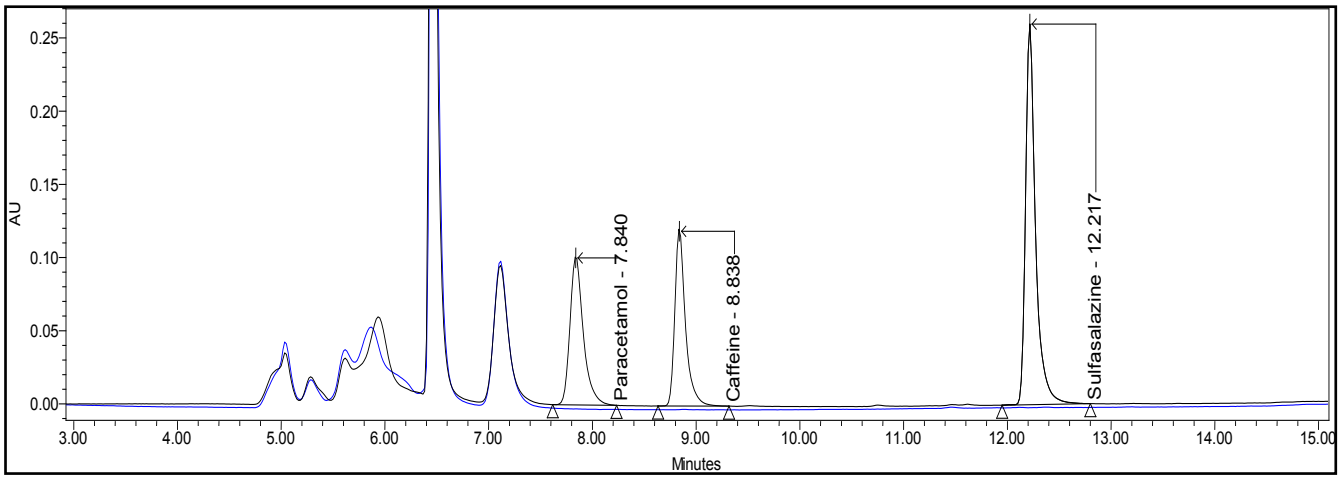

Figure 2: Representative overlayed chromatograms of blank TC 199 media (blue line) and TC 199 Media sample containing paracetamol, caffeine and sulfasalazine (black line). Peaks are annotated with its name and retention times

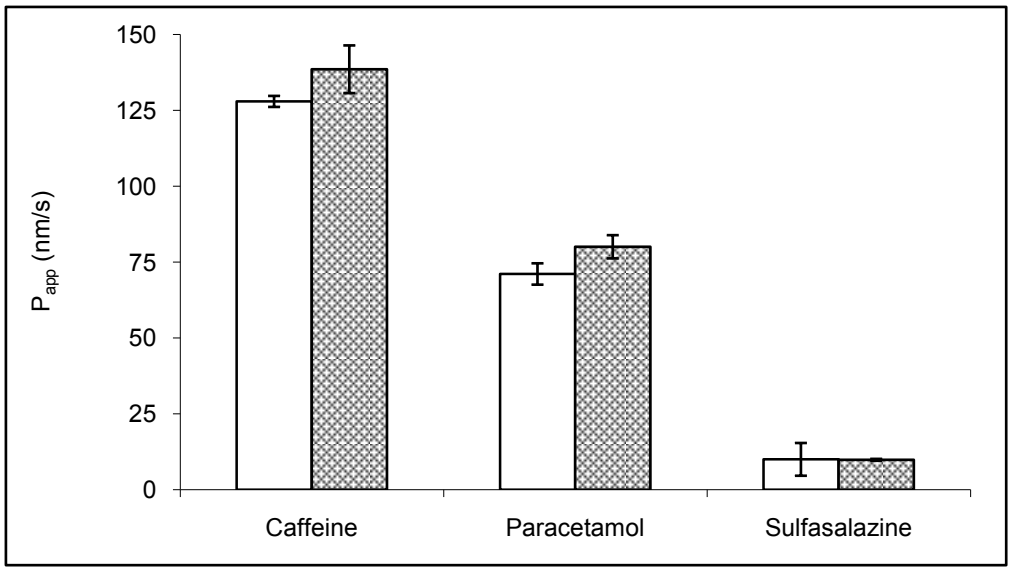

Figure 3: P_{app} of caffeine (100 µM), paracetamol (100 µM) and sulfasalazine (100 µM), conducted separately (empty columns) or in a combination (filled columns), using rat everted gut sac method

Table 1: Regression analysis[a] of calibration curves for caffeine, paracetamol and sulfasalazine in the specified range of 1 µM-100 µM

Analyte	RT[b] (min)	Slope[b] (*m*)	Intercept[b] (*c*)	(R^2) [c]
Caffeine	8.85 (± 0.03)	6984 (± 936)	5390 (± 2146)	0.9992 (± 0.0001)
Paracetamol	7.84 (± 0.02)	6948 (± 948)	3772 (± 1955)	0.9982 (± 0.0005)
Sulfasalazine	12.20 (± 0.05)	71233 (± 2223)	19348 (± 2946)	0.9992 (± 0.0001)

[a] Linear regression analysis with a regression equation of $y = mx \pm c$, in which x is the concentration in µM and y is the peak area,
[b] Retention Time values are mean (± S.D.) of three calibrations curves,
[c] R^2 is the correlation coefficient obtained from eight point calibration curve. The concentrations across the range were evenly distributed.

Table 2: Accuracy and precision data (inter-day and intra-day) of chromatographic method for analysis of caffeine, paracetamol and sulfasalazine in TC 199 medium (pH 7.4)

Analyte	Theoretical Concentration[a] (µM)	Intra-day			Inter-day		
		Measured Concentration (µM)	% RE	% CV	Measured Concentration (µM)	% RE	% CV
Caffeine	75	75.07	100.10	0.83	73.95	98.60	0.91
	25	24.99	99.94	0.85	24.52	98.10	0.75
	0.75	0.78	104.25	1.60	0.79	105.85	2.07
Paracetamol	75	78.08	104.11	2.04	78.77	105.03	1.17
	25	26.51	106.02	1.45	25.93	103.71	1.78
	1	1.04	104.60	2.06	0.99	99.10	1.64
Sulfasalazine	75	78.56	104.75	1.48	76.39	101.85	2.09
	25	25.62	102.47	2.17	24.33	97.31	2.16
	1	1.05	105.85	3.15	1.06	106.04	2.58

Intraday and interday accuracy and precision was determined with n=5 for each concentration. % RE (% relative error) = (Measurec concentration - Theoretical concentration)/Theoretical concentration * 100.
%CV (% coefficient of variation) = (S.D./mean)×100.
a Selected concentrations represent the low, medium and high (HQC, MQC and LQC, respectively) concentrations lying within standard curve range.

acetate containing 0.5 % v/v triethylamine, adjusted to pH 5.0 with o-phosphoric acid (mobile phase B) at a flow rate of 0.5 ml min^{-1}. The column was initially equilibrated at 85 % mobile phase B. After injection, the concentration of mobile phase B was reduced to 30 % over 13 min. After 13 min, the system was returned to original conditions (mobile phase B at 85 %) in next 2 min and further equilibrated for 5 min before the next injection.

Preparation of stock and working standard solutions

Primary standard stock solutions (10 mM) of caffeine and paracetamol were prepared in TC 199 and that of sulfasalazine was prepared in DMSO separately. These solutions were further diluted with TC 199 to obtain mixed working standard solutions of concentrations ranging from 0.25 µM to 100 µM. Final DMSO content in salfasalazine solutions was kept below 0.5 %.

Method Validation

The chromatographic method was validated for specificity, linearity, sensitivity, selectivity, precision and accuracy. All validation runs were performed in five replicates on three consecutive days to assess inter-day and intra-day variation. Calibration curves were constructed in the range 0.25 µM to 100 µM (0.25, 0.5, 0.75, 1.00, 2.50, 5.00, 10.00, 25.00, 50.00, 75.00 and 100.00 µM). Precision and accuracy was assessed at three concentrations, i.e. low (LQC), medium (MQC) and high (HQC), from weighing independent of those used for preparing calibration curves, with five determinations (n = 5) per concentration. Precision and accuracy was further subdivided into intra-day and inter-day precision. Limit of quantification (LOQ) was determined by serial dilution of working standards.

Everted Gut Sac Experiment using Rat Intestine

Intestinal permeability studies using everted gut sac were performed using established methods adopted from literature [13,14] (Ruan *et al.,* 2006, Mariappan and Singh, 2006). Male Wistar rats (body wt. 250-300 g, n = 4) were used for the study. Prior to the surgical procedure, the rats were fasted overnight (16–20 h) with water *ad libitum*. The rats were anesthetized with pentobarbital sodium (60 mg/kg, i.p.). The intestine of the rats was exposed by a midline abdominal incision and a 20-25 cm segment of the proximal rat jejunum was excised and placed in oxygenated TC 199 medium. The intestine was gently everted over a glass rod, divided into segments of length of approximately 4 cm each, filled with oxygenated TC 199 medium and tied using surgical suture (Braided silk wax, Pearsalls Ltd, USA) to prepare sacs. The sacs were placed in flasks containing 20 ml of caffeine, paracetamol and sulfasalazine (prepared in TC 199 at a concentration of 100 µM each) either separately or in a combination of all three drugs. Lucifer yellow (10 µg/ml) was added to all the solutions as an internal standard. The flasks containing sacs were incubated for the period of 60 min, at 37 °C in an oscillating water bath (80 cycles per min). After the incubation period, the sacs were cut open and the contents obtained were centrifuged at 3000 g for 5 min at 4 °C. The supernatants were analyzed for marker compounds using the validated method described earlier. Lucifer yellow was quantified by spectrofluorimetry at excitation and emission wavelengths of 485 nm and 530 nm, respectively, using POLARstar OPTIMA (BMG LABTECH, Germany), controlled by FLUOstar OPTIMA (version 1.30 R3). The apparent permeability coefficient (P_{app}) of the marker drugs was calculated by using the following equation:

$$P_{app} = [V/ (A*T)]* (C_{60}/C_0)$$

Where V is volume of serosal content, A is the area of the intestinal segment, T is the time of incubation, C_0 is the initial concentration on mucosal side, while C_{60} is the concentration of the compound on serosal side after 60 minutes.

RESULTS AND DISCUSSION

Method development

In order to develop and validate a simple and sensitive RP-HPLC method that is suitable for studing markers in intestinal permeability study using everted gut sac model, the following parameters were optimized. The optimum wavelength for detecting all the analytes with adequate sensitivity was ascertained and found to be 260 nm. Buffers with different pH (0.01 M ammonium acetate buffer pH at 2.5, 3.5, 5.0 or 7.0) and acetonitrile were selected for the initial separation studies. At pH 2.5 and 3.5 interference from blank samples was observed for paracetamol but the other analytes were well separated with good peak shapes. At pH 5.0 and 7.0 good resolution off all the peaks with no interference from blank sample was observed (Figure 2). Optimization of composition of the mobile phase for better resolution of all analytes was done with 0.01 M ammonium acetate (pH 5.0) and acetonitrile. Experiments were carried out by changing the flow rate (1.0, 0.8 or 0.5 ml/min) of mobile phase. The best peak shape and maximum separation was achieved with the gradient (Time in min/ % 0.01 M ammonium acetate buffer, pH 5.0) of 0 /85, 13/ 30, 15/ 85, 20/85, at the flow rate of 0.5 ml/min.

The retention times were about 7.8 ± 0.02 min, 8.85 ± 0.02 min and 12.2 ± 0.05 min for paracetamol, caffeine and sulfasalazine, respectively. System suitability was checked by injecting 6 replicates of paracetamol, caffeine and sulfasalazine (25.00 µM each). The relative standard deviation determined from these 6 replicates was not more than 5.0 %. The tailing factor of all the peaks was evaluated using Empower software version 1 (Waters Inc., Milford, USA) and was found to be not more than 2.0.

Method Validation

Specificity

Specificity experiment was carried out using five different blank samples. Chromatogram obtained from the blank sample is presented in Figure 2. There were no interfering peaks of

endogenous compounds observed at the retention time of the analytes.

Range and linearity

Calibration curves were constructed on three consecutive days in the range 0.25 µM-100 µM. The concentration range was selected based on the anticipated drug concentrations in the permeability studies. Linearity was determined by calculation of regression parameters using the method of weighed (1/X) least square analysis. Samples were quantified from the resulting concentration–peak area relationships from regression equation of the calibration curve. The regression parameters are listed in Table 1.

Sensitivity and selectivity

The limit of quantification (LOQ) was defined as the lowest drug concentration that could be determined with acceptable precision (i.e. % CV ≤ 15 %) and accuracy (i.e.RE 100 ± 5 %) [13]. In the present study, the LOQ was found to be 0.25 µM for caffeine and 0.5 µM for paracetamol and sulfasalazine.

Accuracy and precision

The percent relative error (% RE) of the method was found to be 100 ± 10% and the percent coefficient of variation (% CV) for precision determined at each concentration level did not exceed 5 %, indicating that the method is accurate and precise. The data is shown in Table 2.

Utility of method for permeability assay using everted gut sac model

The P_{app} values of caffeine, paracetamol and sulfasalzine obtained, when studied individually or in a cassette of three marker compounds, are shown in Figure 3. Compared to P_{app} obtained in individual study, caffeine and paracetamol, showed slight, but not significant, increase in $P_{app,}$ when studied in combination, while sulfasalazine showed similar P_{app} in both in individual as well as in cassette study. This indicates that permeability of these compounds is not affected in the presence of each other. The P_{app} of Lucifer yellow, an internal standard with intermediate permeability [15], was similar (27 ± 2.1 nm/s) in all the studies. The respective P_{app} values for caffeine and paracetamol were found to be 139 nm/s and 70 nm/s, which are similar to earlier reported [13] values, classifying these compounds as high and medium permeable, respectively. Although P_{app} value for sulfasalazine is not reported in everted gut sac, the value of 10 nm/s classifies this compound as low permeable and it correlates well with its low human $F_{a.}$(13 %), as well as with P_{app} value from Caco-2 (4 ± 1 nm/s) and from PAMPA (2 ± 1 nm/s) [16].

CONCLUSIONS

A reverse phase HPLC method using UV detection was developed and validated for simultaneous analysis of caffeine, paracetamol and sulfasalazine. The method has acceptable accuracy, precision and linearity. It was used successfully to determine the intestinal permeability of high, medium and low permeability markers using rat everted gut sac technique. The method has good resolution and allows analysis of all three analytes in a short run time of 20 min. The present assay is simple in terms of utilizing UV detection, mobile phase preparation and direct sample injection. The application of the method includes its use in routine validation of everted gut sac model for assessment of intestinal permeability of compounds. The intestinal permeability markers can be studied either individually or in a cassette for high throughput purpose.

REFERENCES

1. Balimane PV, Chong S, Morrison RA. Current methodologies used for evaluation of intestinal permeability and absorption. Journal of Pharmacological and Toxicological Methods 2000; 44: 301–312.
2. Venkatesh G, Ramanathan S, Mansor SM, Nair NK, Sattar MA, Croft SL, Navaratnam V. Development and validation of RP-HPLC-UV method for simultaneous determination of buparvaquone, atenolol, propranolol, quinidine and verapamil: A tool for the standardization of rat in situ intestinal permeability studies. Journal of Pharmaceutical and Biomedical Analysis 2007; 43: 1546–1551.
3. Barthe L, Woodley JF, Kenworthy S, and Houin G. An improved everted gut sac as a simple and accurate technique to measure paracellular transport across the small intestine. European Journal of Drug Metabolism and Pharmacokinetics 1998; 23: 313-323.
4. Deferme S, Annaert P, Augustijns P. Drug Absorption Studies *In Situ, In Vitro and In Silico* Models, Springer, 2008
5. Barthe L, Woodley J Houin G. Gastrointestinal absorption of drugs: methods and studies. Fundamental Clinical Pharmacology 1999; 13: 154-168.
6. Acra SA, Ghishan FK. Methods of investigating intestinal transport. Journal of Parenteral and Enteral Nutrition 1991; 15: 93S-98S.
7. Guidance for Industry, Waiver of *in vivo* bioavailability and bioequivalence studies for immediate-release solid oral dosage forms based on a biopharmaceutics classification system, August 2000, CDER/FDA.
8. Oprea TI, Gottfries J, Toward minimalistic modeling of oral drug absorption. Journal of Molecular Graphics and Modeling 1999; 17: 261–274.
9. Sugawara M, Kadomura S, He X, Takekuma Y, Kohri N, Miyazaki K. The use of an in vitro dissolution and absorption system to evaluate oral absorption of two weak bases in pH-independent controlled-release formulations. European Journal of Pharmaceutical Sciences 2005; 26: 1–8.
10. Kalantzi L, Reppas C, Dressman JB, Amidon GL, Junginger HE, Midha KK, Shah VP, Stavchansky SA, Barends DM. Biowaiver monographs for immediate release solid oral dosage forms: Acetaminophen (paracetamol). Journal of Pharmaceutical Sciences 2006; 95: 4-14.
11. Lindenberg M, Kopp S, Dressman JB. Classification of orally administered drugs on the World Health Organization Model list of Essential Medicines according to the biopharmaceutics classification system. European Journal of Pharmaceutics and Biopharmaceutics 2004; 58: 265–278.
12. Dahan A, Amidon GL. Small intestinal efflux mediated by MRP2 and BCRP shifts sulfasalazine intestinal permeability from high to low, enabling its colonic targeting. American Journal of Physiology - Gastrointestinal and Liver Physiology 2009; 297: G371-G377
13. Ruan LP, Chen S, Yu BY, Zhu DN, Cordell GA, Qiu SX. Prediction of human absorption of natural compounds by the non-everted rat intestinal sac model. European Journal of Medicinal Chemistry 2006; 41: 605–610.
14. MariappanTT, Singh S, Positioning of Rifampicin in the Biopharmaceutics Classification System (BCS). Clinical Research and Regulatory Affairs 2006; 23: 1-10.
15. Castella M, Reist M, Mayer JM, Turban J, Testa B, Boursier-Neyret C, Walther B, Delbos J, Carrupt P. Development of an *in vitro* rat intestine segmental perfusion model to investigate permeability and predict oral fraction absorbed. Pharmaceutical Research 2006; 23: 1543-1553
16. Balimane PV, Han Y, Chong S. Current industrial practices of assessing permeability and P-glycoprotein interaction. The AAPS Journal 2006; 8: E1–E13.