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Evaluation of frog as an animal model to study the effective permeability co-efficient: Application to CYP3A4 substrates

Neelima Yerasi[a*] Krishna Devarakonda[b], Himabindu Vurimindi[a]

[a] *Institute of Science and Technology, Jawaharlal Nehru Technological University, Hyderabad, India.*
[b] *Director, Clinical Pharmacology and Pharmacokinetics, Covidien Pharmaceutials, Hazelwood, MO, 63042, USA*

ABSTRACT

To evaluate the reliability of using in situ frog intestinal perfusion technique for permeability assessment of drugs which are substrates for CYP3A4 enzymes. Felodipine was used as the probe drug. Single Pass Intestinal perfusion (SPIP) studies were performed in frogs of the species Rana tigrina using established method for rats with some modifications. Initially the contents of the intestine were flushed out with blank perfusion solution, then with the test solution and then perfused with test solutions at a flow rate of 0.2ml/min using syringe pump for 90 min after 20 min of equilibration. The perfusate was collected at every 10 min. Water flux was quantified using phenol red, a non-absorbable inert marker. Permeability was determined in 6 frogs and the results were presented as mean ± SD. Effective permeability coefficient (P_{eff}) of felodipine was calculated in the presence and absence of inhibitors (ketoconazole and verapamil) using the parallel-tube model. P_{eff} of felodipine when perfused alone was 1.31± 0.04× 10^{-4}cm/s, which was increased to 2.06± 0.37× 10^{-4}cm/s in presence of ketoconazole but was almost unaffected (1.28± 0.14× 10^{-4}cm/s) in presence of verapamil. Mass spectrum of a test sample from perfusion studies showed M+2H peak corresponding to pyridine metabolite of felodipine (mol wt-382) in humans. The results obtained in this study lead us to conclude that it is possible to determine the P_{eff} value for compounds which are substrates of CYP3A4 using in situ frog intestinal perfusion technique.

Key words: Intestinal permeability, CYP3A4enzymes, Frog perfusion model, Effective permeability co-efficient.

INTRODUCTION

Cytochrome P4503A (CYP3A) enzymes metabolize a wide variety of xenobiotics including many drugs. Because CYP3A is localized in both the liver and intestine, it can make a major contribution to the presystemic elimination of substrate drugs after oral administration ('first-pass metabolism'). It has been suggested that the metabolism/ active efflux in the small intestine is involved in the poor absorption of many drugs. A significant amount of CYP3A is expressed in the enterocytes to metabolize drugs during their transit across the intestinal epithelium [1]. CYP3A4, the major isoform of the CYP3A subfamily, is one of the most important drug metabolizing enzymes in humans. CYP3A4 has a very broad substrate specificity and is involved in the phase-I metabolism of about 50% of currently used drugs. The primary site of expression of CYP3A4 is the liver, but intestinal enterocytes also contain high amounts of CYP3A4 [2, 3]. CYP3A4-mediated biotransformation in the gut wall has been shown to substantially contribute to the overall first-pass metabolism of a large number of therapeutic drugs [4,5].

An estimated 40% of new molecular entities fail to be new drugs because of poor biopharmaceutical properties, namely solubility and permeability [6]. Solubility is easily quantifiable in vitro and can be manipulated by formulation strategies, while permeability is more complex to be altered for improved performance in human beings. Moreover Permeability is an important factor, which governs absorption of orally administered drugs. Hence, screening of drug candidates for permeability properties is imperative to select right candidate for development to prevent late surprises [7].

A rapid, time and resource sparing technology to predict human oral absorption has been a goal of biopharmaceutical scientists for generations. Both early discovery and early development teams would benefit from such models. The savings in time and resources clearly justify the continued effort to improve existing models and investigate / validate new models. Different in vitro methods are available to assess the absorption potential of early-stage compounds such as cell lines which over-express CYP enzymes (MDCK, Caco-2) either cDNA transfectants over expressing CYP enzymes or non- transfected cell lines and also the *ex vivo* Using Chamber model using excised rat intestinal Segments [8]. Though *in vitro* techniques offer the most ready means of generating large volumes of data with minimal resources, they are not thoroughly standardized and are associated with several limitations, hence less predictive. The Caco-2 cell model is one of several immortalized cell lines commonly used to study drug transport because of its morphological and biochemical similarity to the intestinal epithelium. However, quiescent Caco- 2 cells do not normally express CYP3A4. And also they do not always express appropriate amounts of transporters or enzymes, which may introduce bias in the determination of their transport via a carrier mediated process or their metabolism via a particular pathway. Additional genetic manipulation of the Caco-2 cells will be needed to further advance the utility of this model in the drug development process.

In situ single pass intestinal perfusion (SPIP) technique using different animal species including rat, rabbit, pig, dog and monkey has been reported in literature to study the intestinal absorption of drugs. Among these animal models, SPIP in rat is a well established technique to study the intestinal passive absorption of drugs with good correlation between human and rat intestinal absorption. Expression profiles of transporters and metabolizing enzymes in both rat and human intestines (duodenum and colon) were measured using Gene Chip analysis. There was no correlation between rat and humans (r2 =0.29) in oral drug bioavailability, which also agreed

with previous results [9]. Hence there is a clear need for the development of a new simple model for the study of intestinal absorption of drugs involving CYP3A4 enzymes.

The objective of this study was to evaluate the reliability of using *in situ* frog intestinal perfusion technique for permeability assessment of drugs, which are substrates for CYP3A4 enzymes. We have chosen felodipine, an antihypertensive agent, as probe drug as it is a specific probe used for assessing the metabolic activity of the CYP3A4 enzymes and is considered gold standard for assessing CYP3A4 activity. In this study we have developed SPIP using frog as the animal model for the first time, for the assessment of intestinal permeability of drugs, which are substrates of CYP3A4 in humans. Moreover frog small intestine has the same composition and villi as vertebrate membranes and same transport mechanisms and luminal enzymes have evolved from lower vertebrates to humans. This is further supported by a study, in which *in vitro* permeability coefficient deduced from isolated frog intestinal sac showed to be a reasonable predictor of *in vivo* oral absorption in humans for compounds that are passively absorbed [10]. Another study also indicated the expression of specific transporter system in frog intestine [11]. When compared with *in vitro* methods, SPIP provides an advantage of experimental control (e.g. permeate concentration, intestinal perfusion rate), intact intestinal blood supply and barrier function of the intestine are not lost or compromised during the entire length of the experiment. Tissue viability is much longer when compared with *in vitro* isolated intestinal segment models. In another study conducted by us using probe drug losartan, which is a CYP3A4 and P-glycoprote (P-gp) substrate, P_{eff} calculated with SPIP method in frog model showed high correlation with P_{eff} values previously reported in man [12]. To further investigate the authenticity of this model to determine the permeability of drugs which are substrates for CYP3A4 enzyme, we selected felodipine as the probe drug to study the CYP3A4 activity, which is a specific probe used for assessing the metabolic activity of the CYP3A4 enzymes and is not a substrate for P-gp [13, 14].

In this model, a known concentration of drug is perfused through a section of the jejunum with intact blood supply. P_{eff} was calculated according to parallel tube model also referred to as an 'open' system, where the perfusion solution, containing a drug, flows down a cylindrical segment and at steady state a constant concentration gradient is established along the length of the segment. Disappearance of drug from the perfusate is attributed to intestinal absorption [15, 16].

MATERIALS AND METHODS

Materials

Felodipine pure drug was obtained as gift sample from Alkem Laboratories, Noida, India. Verapamil and ketoconazole pure substances were obtained as gift samples from Lupin Laboratories, Pune, India. All the solvents were of HPLC grade obtained from Rankem India. Potassium dihydrogen orthophosphate, orthophosphoric acid was purchased from E. Merck Limited, Mumbai, India and all other chemicals were obtained from Hi Media, India.

Composition of Perfusion Solutions

The perfusion buffer composition was as follows: $CaCl_2$ ×$2H_2O$ 0.98 mM, KCl 2.58 mM, Na_2HPO_4 0.66 mM, NaH_2PO_4 5.1 mM, NaCl 84 mM, d-glucose 3.0mM with pH 6.8 (with NaOH). Phenol red (50 mg L^{-1}) was added to the solution as a nonabsorbable marker. The pH was adjusted to 7.4 and the osmolality, measured by the freezing point depression method was 230±10 mOsm kg^{-1} (Osmette A, Precision Systems Inc., Natick, MA), isotonic for amphibian

[11]. Preliminary experiments showed that there was no adsorption of the compounds to the catheters and the tubing. Test drug concentration used in the perfusion studies was determined by dividing the highest prescribed dose by 250 ml, the accepted gastric volume, in order to represent the maximal drug concentration present in the intestinal segment. Solutions of probe drug were prepared with blank perfusion buffer. Probe drug and inhibitors used in the study, were solublised in perfusion buffer using less than 1% methanol.

Analytical methods

Samples were analyzed by reverse-phase HPLC**.** A liquid chromatographic system (HPLC) (Agilent) with a solvent pump (Agilent 1100 binary pump) and UV–VIS multiple wavelength detector (MWD-G1365B, was used. Chemstation® software was used for data acquisition, reporting and analysis. The C-18 reverse phase column used for chromatographic separation was Zorbax (particle size 5 µ, pore size 10 nm, dimension 4.6mm×250 mm) of Agilent technologies. Mass spectral analysis of the perfusates, was done by Agilent LC-MSD ion trap in positive ion mode.

Chromatographic conditions

The mobile phase was a mixture of 15mM Sodium dihydrogen phosphate monohydrate-methanol-acetonitrile 40:30:30, v/v/v, at pH 6.5. The mobile phase was filtered through sintered glass filter P5 (1–1.6 µm) (Winteg, Germany) and degassed in sonicator (Liarre, Italy). The mobile phase was pumped in isocratic mode at a flow rate of 1ml/min at ambient temperature. The UV detection was accomplished at 230 nm. Quantification of the compounds was carried out by measuring the peak areas in relation to those of standards chromatographed under the same conditions.

Frog *in situ* single pass intestinal perfusion Technique

Frog *in situ* perfusion studies were performed using established single pass intestinal perfusion method for rats with few modifications [17]. Animal care and handling throughout the experimental procedure were performed in accordance with the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised in 1985). Frogs of the species *Rana tigrina*, were used for the experiments. Frogs were fasted for 24 hours prior to the start of the experiment. Each frog was anaesthetized and maintained with a combination of intra-peritoneal injection of 230 mg/kg of phenobarbitone sodium and 25 mg/kg of thiopentone sodium. After the onset of deep anesthesia, abdomen was opened by a midline longitudinal incision and approximately 15 to 20 cm length of intestine immediately after stomach was selected, rinsed with frog's ringer and cannulated on both sides. Care was taken in handling the small intestine to minimize the blood loss. Initially the contents of the intestine were flushed out with blank perfusion solution, then with the test solution and then perfused with test solutions at a flow rate of 0.2ml/min using syringe pump for 90 min after 30 min of equilibration. The perfusate samples were collected at every 10 min. Water flux was quantified with the help of concentration change of phenol red (non-absorbable inert marker). The length and radius of the perfused segment was measured at the end of the experiment and the animal was euthanized by quick removal of the heart. Permeability for each drug was determined in 6 frogs and the results were presented as mean ± SD. Samples were stored at -20°C until analysis.

Data analysis

Effective permeability coefficient (P_{eff})

Effective permeability coefficient (P_{eff}) was calculated from the steady-state concentration of compounds in the collected perfusate [18]. Steady state, which was assessed by a constant

concentration of phenol red, was reached 30±10 min after the beginning of the experiment. P_{eff} value was calculated using equation – (i), according to the parallel tube model [19].

$$P_{eff} = - Q \ln [C_{out\ corr} / C_{in}] /2\pi rl \quad \longrightarrow \quad (i)$$

where Q is perfusion flow rate (ml/min). C_{in} is inlet concentration (µg/ml). $C_{out\ (corr)}$ is outlet concentration of compound which is corrected for water flux using phenol red concentration (µg/ml) [$C_{out\ (corr)} = C_{outmeasured}$ x $[\text{phenol red}]_{in} / [\text{phenol red}]_{out}$], r is the radius of the frog intestine (cm), l is the length of the intestinal segment (cm). The concentrations obtained from the perfusate were corrected for changes in the water flux at each time interval using the above equation.

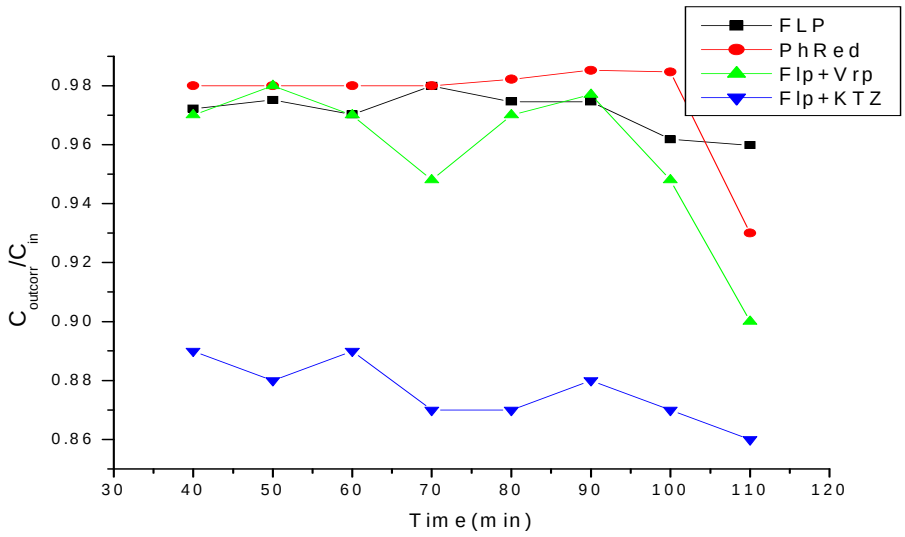
RESULTS AND DISCUSSION

The effective permeability coefficient (P_{eff}) values of felodipine were determined at steady state in the absence of inhibitor and also in the presence of 10µM of ketoconazole, a CYP3A4 inhibitor and also 10µM of verapamil, inhibitor of P-gp. Ketoconazole was selected since it is a potent inhibitor of CYP3A4 enzyme [20]. Verapamil was selected since it inhibits only P-gp but not Cyp3A4 [21]. The data is shown in table-I Equilibrium was attained in about 30 min, after the beginning of perfusion which can be noted from figure-1. Mean intestinal net water flux NWF, (µl/h/cm) calculated according to Fagerholm et al was found to be + 60µl/h/cm, indicating that there is a secretion of fluid into the segment. This was in accordance with that of Fagerholm reported value in rat SPIP [-56 (±59) µl/h/cm] [22].

Table-I Effective permeability coefficient X 10^{-4}(cm/s) of felodipine

Compound	C_{in} (µg/ml)	Frog No.	P_{eff} (× 10^{-4} cm/s)	Mean ±S.D
Felodipine	100	1	1.56	1.31± 0.366
		2	1.24	
		3	1.87	
		4	0.97	
		5	0.89	
		6	1.32	
Felodipine in presence of ketoconazole	100	1	2.87	2.94± 0.355
		2	3.24	
		3	2.47	
		4	3.16	
		5	2.59	
		6	3.32	
Felodipine in presence of Verapamil	100	1	0.97	1.27± 0.208
		2	1.24	
		3	1.37	
		4	1.16	
		5	1.9	
		6	1.32	

Figure-1 Plot of concentration ratio of outlet and inlet concentrations vs. time of felodipine in presence and absence of inhibitors



In figure-2 the representative chromatogram of a sample from intestinal perfusion containing felodipine and phenol red are presented. There was no interference from the blank matrix. The retention times were 3.41 min and 4.38 min for felodipine and phenol red respectively. The inhibitors did not interfere with the analysis. Linearity was found by the six-point calibration curves prepared in the range of 50 to 150 % of assay concentration. Concentration range was selected based on drug concentration used in permeability studies. The correlation coefficient of the calibration curves was found to be 0.999 for both the substances. The HPLC method was validated and was found to be selective, precise, accurate, robust and rugged. The LOD and LOQ were found to be 0.023 µg/mL & 0.037 µg/mL for felodipine, 0.015 µg/mL and 0.024 µg/mL for phenol red respectively. The analytes were found to be stable throughout the study period.

Figure-2 Representative Chromatogram of Felodipine & phenol red

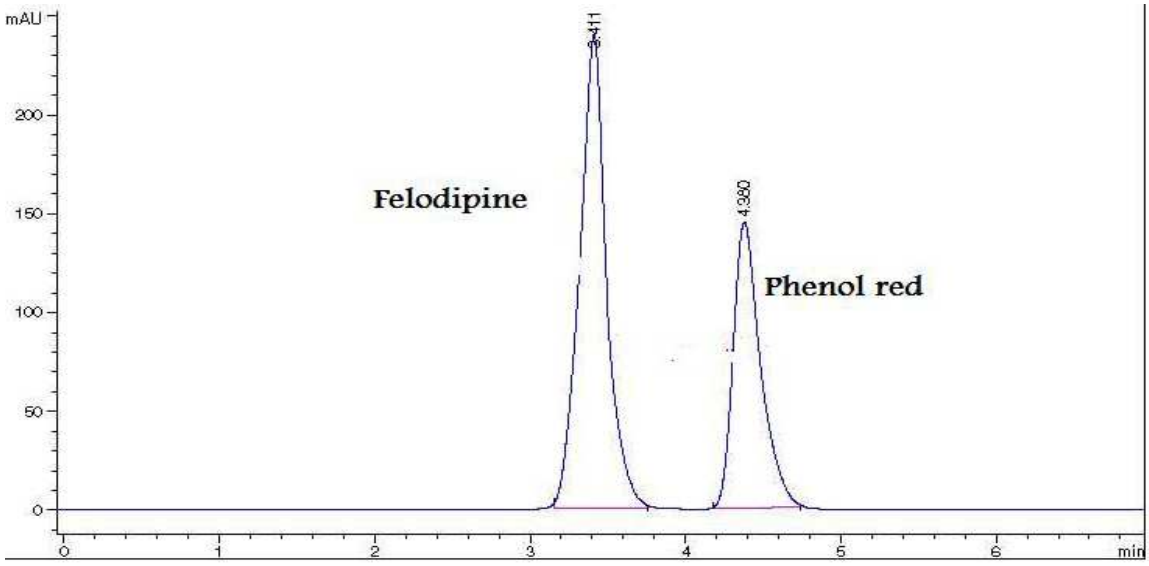


Figure-3a Mass spectrum of a blank sample from Felodipine intestinal perfusion studies

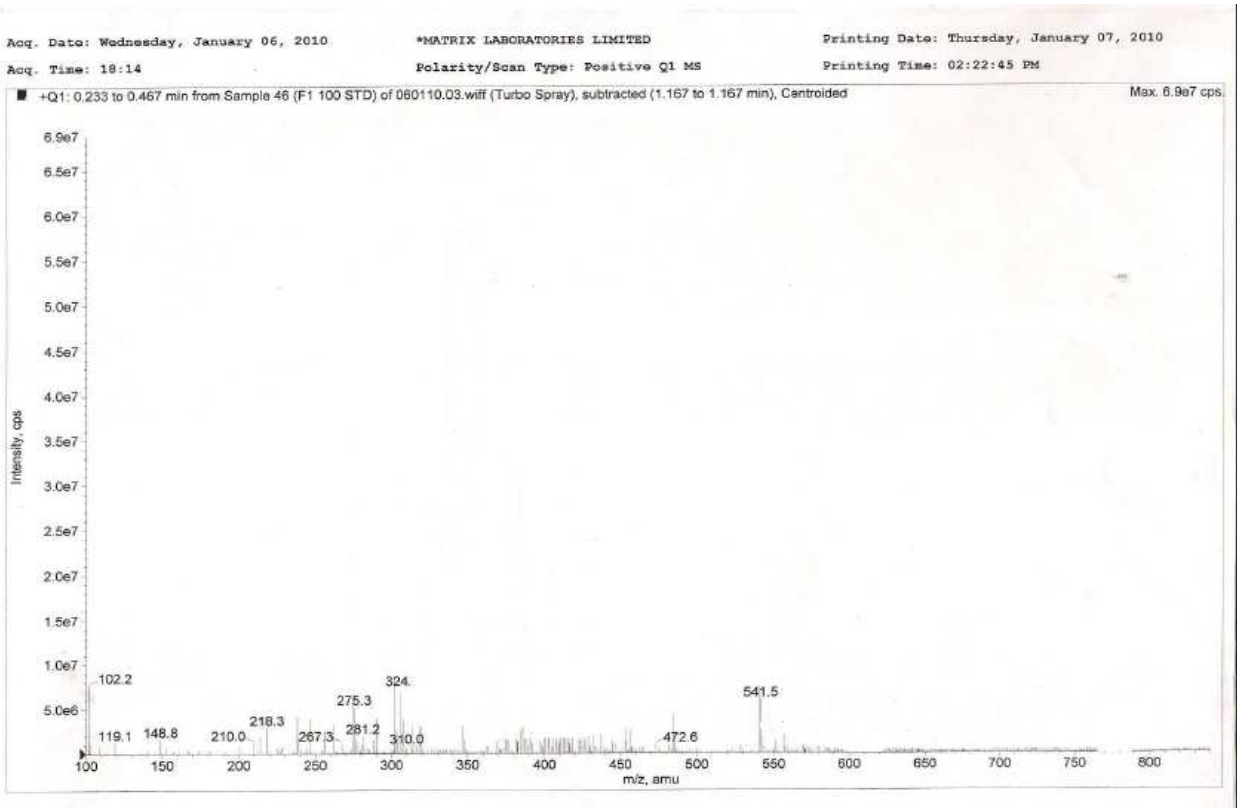


Figure-3b Mass spectrum of a standard sample from Felodipine (M.wt-384.26) intestinal perfusion studies

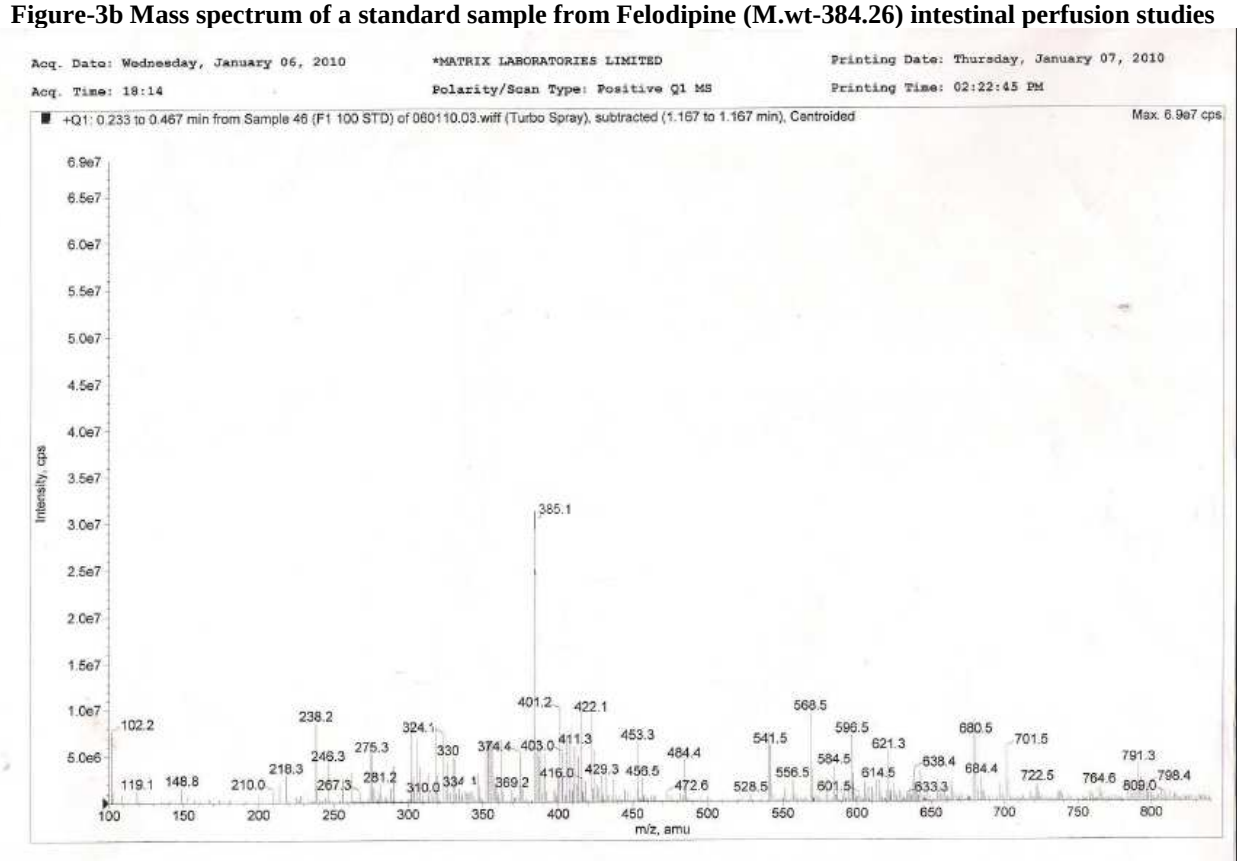


Figure-3c Mass spectrum of a test sample from Felodipine intestinal perfusion studies showing peak corresponding to pyridine metabolite.

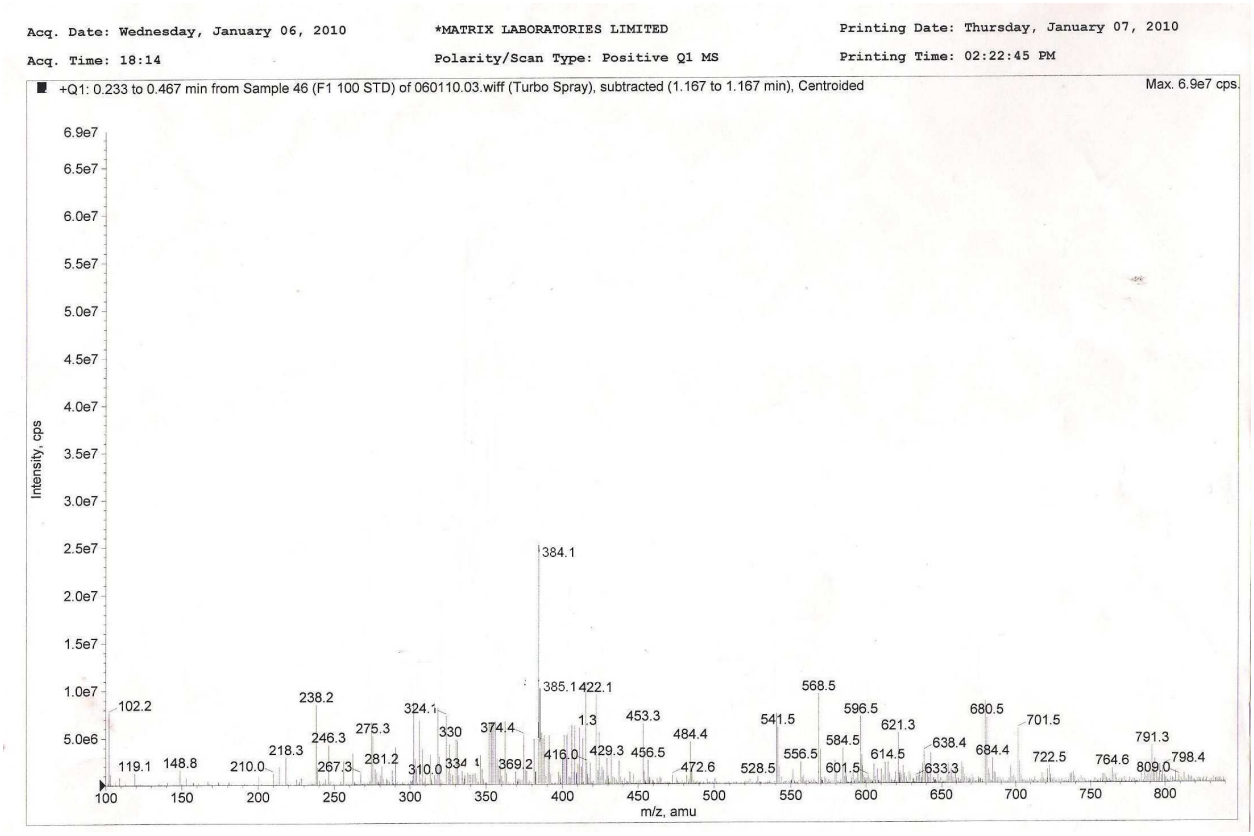
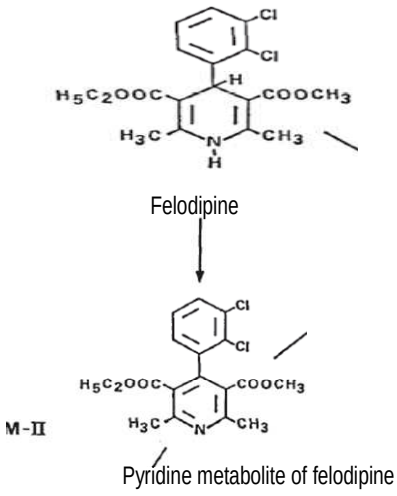


Figure-4: Conversion of felodipine in to its pyridine metabolite



The effective permeability coefficient (P_{eff}) of felodipine when perfused alone was found to be 1.31± 0.04 ×10^{-4}cm/s, in presence of ketoconazole it was found to be 2.06± 0.37 × 10^{-4}cm/s and in presence of verapamil it was found to be 1.28± 0.14×10^{-4}cm/s. Thus a two fold increase in P_{eff} was observed in presence of ketoconazole, a potent CYP3A4 inhibitor and in presence of verapamil it remained unaltered. This suggests that enzymes similar to CYP3A might have been expressed in frog intestine. To support this argument further, the perfusate samples were

analyzed by mass spectroscopy. M+2H peak corresponding to pyridine metabolite of felodipine (mol wt-382) is seen in test sample which is absent in blank and standard samples. The mass spectra are shown in figures.3a, 3b, and 3c. In humans felodipine mainly forms pyridine metabolite after phase-I metabolism by CYP3A4 as depicted in figure-4.

These results authenticate that enzymes similar to CYP3A4 are expressed in frog intestine and hence frog intestinal model can be reliably used as an animal model for studying intestinal permeability of drugs especially drugs which are substrates for CYP3A4 enzymes. This model serves as a valuable alternative for permeability estimation of drugs which are substrates for CYP3A4 since use of rat model and Caco- 2 cell line model for such drugs are associated with limitations, though they are gold standards for permeability estimation of passively absorbed drugs. Limitations of Caco-2 model include model being static, absence of a mucus layer due to lack of mucous secreting cells, with different or no expression of metabolic enzymes (e.g., absence of CYP enzymes), "Tighter" monolayer compared to human small intestine, wide inter- and intra laboratory variability of permeability data, Long differentiation period, Low expression of efflux transporters. Limitations of rat model include different expression profiles of transporters and metabolizing enzymes when compared with humans and the model is not suitable for high throughput screening. Moreover unlike rat model, frog model can be used for high throughput screening since frog being a cold blooded animal doesn't require maintenance of body temperature and perfusion solutions also need not be maintained at 37°C, as a result the experimental burden will be reduced much when we replace rat with frog model.

CONCLUSION

The frog model reported here appears to be promising for permeability assessment of drugs which are substrates for CYP3A4 enzymes. It is possible to determine the P_{eff} value for compounds which are substrates of CYP3A4 using *in situ* frog intestinal perfusion technique. The presence of an apical mucus layer in the frog intestine is an advantage as it may influence the absorption of compounds. Moreover, the small size and the relative ease of maintaining large stocks of animals enable cost-effective screening of numerous compounds. It may represent a valuable alternative to the low speed and high cost of conventional animal models (typically rodents) for the assessment of intestinal permeability.

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