

## EFFECT OF HERBAL BIOENHANCERS ON SAQUINAVIR IN HUMAN Caco-2 CELL MONOLAYERS AND PHARMACOKINETICS IN RATS

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### ABSTRACT

**Purpose:** Membrane-bound efflux transporters, such as P-glycoprotein (P-gp), may limit the entry and distribution of HIV-1 protease inhibitors saquinavir (SQN) and may be the reason for low and variable oral bioavailability. The purpose is to investigate *in vitro* mechanisms of gastrointestinal absorption of saquinavir mesylate, in presence and absence of herbal bioenhancers using human Caco-2 cells. To investigate the correlation of Caco-2 out come with animal pharmacokinetic studies for bioavailability enhancement.

**Method:** Confluent epithelial layers of human Caco-2 cells mimicking the intestinal barrier. Pharmacokinetic studies using Male Sprague-Dawley (SD) rats.

**Results:** Saquinavir showed polarized transport through Caco-2 cell monolayers in the basolateral to apical direction (secretory pathway). Saquinavir has shown an efflux ratio (B-A / A-B) of > 25 and reduced to ~ 2 when saquinavir co-administered with herbal bioenhancers. Active efflux was temperature dependent, saturable and inhibited by verapamil. In presence of herbal bioenhancers the permeability of saquinavir was increased. Oral bioavailability was also increased in rat by ~ 10 folds.

**Conclusions:** Saquinavir is a substrate for an efflux mechanism in the gut, most likely P-glycoprotein, which acts as a counter-transporter for the drug. This may partially account for the low and variable oral bioavailability of saquinavir. After co-administration with herbal enhancers the permeability of saquinavir was increased. These herbal bioenhancers may be acting as an inhibitor of P-gp and may help by improving the oral bioavailability of saquinavir.

**KEY WORDS:** Saquinavir; Piperine, Gallic acid; Cinnamic acid; Herbal bioenhancers; Caco-2 cells; Pharmacokinetics

## INTRODUCTION

Bioavailability is the rate and extent to which a therapeutically active substance enters systemic circulation and becomes available at the required site of action. Intravenous drugs attain maximum bioavailability, while oral administration yields a reduced percentage due to incomplete drug absorption and first-pass metabolism. Methods of increasing bioavailability of a drug correspondingly increase levels in the bloodstream, and thus the efficacy, which in turn reduces the drug dosage required to achieve a given therapeutic effect. Until now, methods of increasing drug bioavailability have operated within a narrow manipulative framework, mainly based on physical processes including micronization, deaggregation of micronized molecules, timed/site release preparations, solubilization of active drug and polymorphic/crystal form selection and nanotechnology (nanotechnology is at the experimental stage so it is a promising future method).

The oral bioavailability of the human immunodeficiency virus (HIV) protease inhibitor saquinavir mesylate (SQN) is low and variable in patients [1]. The reasons for low oral bioavailability are unclear. One explanation is that absorption is decreased by an active efflux pump in the liver and intestine such as P-glycoprotein (P-gp). P-gp is a 170 kDa transmembrane protein [2]. It is localized at the apical secretory surface of various tissues where it mediates the active transmembrane transport of a variety of lipophilic substrates. It appears to act as a general detoxification system protecting tissues from a broad spectrum of lipophilic endogenous or exogenous toxic compounds, most of which tend to be large, aromatic and amphiphilic [3,4]. Several studies have demonstrated the possible use of P-gp inhibitors that reverse P-gp-mediated efflux in an attempt to improve the efficiency of drug transport across the epithelia, thus resulting in enhanced oral bioavailability. P-gp inhibitors may also influence absorption, distribution, metabolism and elimination of P-gp substrates in the process of modulating pharmacokinetics [5].

Bioenhancers are drug facilitators they are the molecules which by themselves do not show typical drug activity, but when used in combination enhance the activity of drug molecules in several ways. Moreover, efficacy is enhanced by increased bioavailability. Many studies have clearly shown that diets rich in plant foods protect humans against various diseases such as cancer, neurodegenerative disorder and cardiovascular diseases [6]. Plant foods contain a variety of medicinally active constituents such as alkaloids (piperine) and polyphenols (gallic acid and cinnamic acid), which are increasingly regarded as herbal medicines [7]. Piperine (1-piperoyl piperidine) is a major component of the Piper species. This species has been used widely as spices and in various systems of Indian herbal medicine [8]. Piperine (PIP) has various pharmacological activities namely anti-asthmatic along with inhibition of hepatic enzymes such as CYPs, monooxygenase and UDP-glucouronyl transferase and intestinal glucouronidation [9, 10], and inhibition of P-glycoprotein (P-gp) [11]. In addition, piperine has also been shown to enhance the bioavailability of drugs like vasicine, sparteine, curcumin, barbiturate and oxyphenylbutazone, zoxazolamine, propranolol and theophylline in animal experiments [12-15]. Polyphenols are abundant micronutrients in our diet, and evidence for their role in the prevention of

many diseases [16]. Bioavailability differs greatly from one polyphenol to another [17]. Gallic acid (GA) and cinnamic acid (CA), isoflavones are the well-absorbed polyphenols, followed by catechins, flavanones, and quercetin glucosides, but with different kinetics [18, 19]. Due to the many apparent health benefits, piperine, various vegetable/fruit juices (GA and CA present in juices), and green tea beverages and extract supplements are widely used, that creating an increased risk of adverse/beneficial herb drug interactions [20, 21].

The exact mechanism of action for increasing bioavailability by the bioenhancers is unknown. The objective of the study was to evaluate bidirectional permeability of saquinavir alone and in presence of bioenhancers (piperine, gallic acid and cinnamic acid). Another objective was to investigate whether these bioenhancers inhibit the P-gp and can enhance the oral bioavailability of saquinavir in rats. For this the permeability assay of saquinavir was performed in presence and in absence of P-glycoprotein inhibitor verapamil and along with other herbal bioenhancers. Loperamide was run as a positive control to assess the functionality of P-gp [22-24]. The pharmacokinetics study was performed using Male Sprague-Dawley (SD) rats.

## **MATERIALS AND METHODS**

### **Materials**

The reference standard, saquinavir mesylate, loperamide, verapamil and internal standard imipramine were purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA). Piperine (lot no. # U13423-458), gallic acid (lot no.# 53540), cinnamic acid (lot no. # W228826), ammonium acetate buffer (lot no. # BCBB1360V), propylene glycol (lot no. #1359720) and formic acid (lot no. # BCBC4518V) were procured from Sigma-Aldrich (Aldrich, St. Louis, MO). Acetonitrile and methanol were of HPLC grade procured from JT Baker (Phillipsburg, NJ). Caco-2 cell line was procured from National Center for Cell Sciences (NCCS, Pune, India). Dulbecco's Modified Eagles medium (Cat # D5671), Trypsin-EDTA solution (Cat # T4049), and HBSS Buffer (Cat # H6648) were purchased from Sigma, Germany. Fetal Bovine Serum (Cat # 14-502F) was purchased from Lonza (Walkersville, MD USA). T-75 flasks & serological pipettes were purchased from Grenier-Bio-one, Germany. Mill cell -24 well PET membrane 1  $\mu$ m plates (Cat # PSRP010 R5) were purchased from Millipore Corporation (Billerica MA). All the other reagents or solvents used were either analytical or high-performance liquid chromatography (HPLC) grade. Absolute ethanol (99.9%) was purchased from Tedia Company. Inc (Fairfield, OH, USA). Reverse osmosis water from Millipore (Bedford, MA) was used for all the preparations.

### **Seeding and Maintenance of Caco-2 Cells**

Caco-2 cells were grown in DMEM (20% FBS) until 85-90% confluence. After attaining required confluence, cells were trypsinized and seeded into 24 well mill cell plates at a density of  $0.6 \times 10^5$  cells/insert. The plates were maintained for 21 days at 37 °C in CO<sub>2</sub> incubator with change of medium every alternative day. Monolayer integrity was monitored by measuring TEER from day 15.

### Bidirectional Transport Studies

Caco-2 cell monolayer was washed twice with HBSS buffer and incubated for 30 min in CO<sub>2</sub> incubator. TEER values were measured and wells showing TEER values above 230 ohms.cm<sup>2</sup> were taken for experiments. Bi-directional permeability study was initiated by adding appropriate volume of HBSS buffer containing the test compounds or probe P-gp substrate loperamide to either the apical (A-B transport) or basal side (B-A transport) of the monolayer. At selected time points (0, 30 and 60 minutes) an aliquot of 50 µl was collected from the receiver compartment for determination of test compound concentrations. The volume withdrawn was replaced immediately with plain HBSS buffer (pH 7.4). TEER was measured at the end of the experiment to test the post experiment monolayer integrity. The samples were stored at -70°C until LC-MS/MS analysis. The apparent permeability coefficients (P<sub>app</sub>) of all compounds in both apical to basolateral (P<sub>app</sub>, A-B) and basolateral to apical (P<sub>app</sub>, B-A) directions were measured in triplicate. The apparent permeability coefficient was calculated as follows:  $P_{app} = (dQ/dt) / [1/(A * C_0)]$ , where A is the membrane surface area, C<sub>0</sub> is the donor drug concentration at t = 0, and dQ/dt is the amount of drug transported within a given time period. Efflux ratio = P<sub>app</sub> B-A / P<sub>app</sub> A-B.

### Animals

Male Sprague-Dawley (SD) rats (body weight 250 - 280 gm) were procured from Reliance life Science, Mumbai, India. Animals were housed in the room, which was environmentally-controlled at a temperature of at 22 ± 2 °C, 50–60% relative humidity, under a 12 h light: 12 h dark cycle. All the animals were provided laboratory rodent diet (Nutrilab Rodent diet, Vetcare, Netherlands) except for 10 to 12 hours before treatment and 2 hours post dosing. Reverse osmosis water treated with ultraviolet light was provided *ad libitum* during experimental period. Temperature and humidity were recorded by auto-controlled data logger system. All the animals were acclimatized to the experimental conditions for at least a week prior to dosing.

### Formulation Preparation

Solution formulation of Saquinavir mesylate (SQN) was prepared in 20% ethanol / 20% aqueous hydroxyl β cyclodextrene (β CD) + 20µL of ortho phosphoric acid (OPA)(v/v/v) for intravenous (i.v.) and for per oral (p.o.), suspension formulation prepared using 0.1% tween 80 and 0.5% sodium CMC in water (v/v). The strength of the dose formulations were 0.2 and 5 mg/ml of saquinavir for i.v and p.o., respectively. For all the experiments the dose of saquinavir was 1 mg/kg and 50 mg/kg for i.v and p.o respectively. Piperine (PIP) was dissolved in 10% ethanol /40% propylene glycol /50% Milli Q water (v/v/v) and formed as solution formulation for per oral (p.o.) administration in the concentration of 1 mg/ml of piperine. Gallic acid (GA) for p.o. administration was prepared by dissolving gallic acid in Milli Q water to obtain a final concentration of 1 mg of gallic acid/ml. Cinnamic acid (CA) was dissolved in 10% ethanol /90% Milli Q water (v/v) and formed as solution formulation for p.o. administration at the concentration of 1 mg/ml of cinnamic acid. All solution formulations were prepared freshly prior to dosing. The dose volume for p.o. routes was 10 ml/kg, for PIP, GA and CA.

### Pharmacokinetic Studies

Three groups of rats were formed and each group having three rats (n=3). Group one of three animals each was given 1 mg/kg saquinavir intravenously (i.v.), alone. Group two of three animals each were given 50 mg/kg saquinavir per oral (p.o.), alone. Group three of three animals each were given 50 mg/kg saquinavir orally, along with coadministered (30 minutes prior) with piperine or gallic acid or cinnamic acid (10 mg/kg) by oral gavage using 16-gauge stainless steel needle. saquinavir doses (1 and 50 mg/kg) were chosen to keep the plasma concentrations above the limit of detection at the time variation from 0 to 72 h in rat's plasma and based on in-house data. The dose of co-administered drugs (piperine, gallic acid and cinnamic acid) was chosen at 10 mg/kg since the maximal bioenhancement effect was observed at this dose (based on in-house study, data not shown).

For i.v. group (group one), a dose of 1 mg/kg saquinavir was given to the rats via tail vein injection. Three rats were used this group. Blood samples (150  $\mu$ l) were collected at 0.08, 0.25, 0.5, 1, 3, 5, 8, 12, 24, 48 and 72 h after saquinavir administration. The blood samples (150  $\mu$ l) were taken from the retro-orbital plexus into labeled tubes, containing 10  $\mu$ l of 20 % w/v K<sub>2</sub>EDTA solution, as anticoagulant. Plasma was harvested from the blood by centrifugation at 4000 rpm for 10 min at 4  $\pm$  2  $^{\circ}$ C and stored below -70 $^{\circ}$ C (Thermo Scientific, USA) deep freezer until bioanalysis. For oral drug interaction studies, rats were fasted overnight and the fasting continued up to 2 h post dosing with free access to drinking water. For p.o. group (group two), a dose of 50 mg/kg saquinavir was given to the rats by oral gavage using 16-gauge stainless steel needle. Three rats were used this group. For herb-drug interaction study (group three), a specified dose (10 mg/kg) of the herbal bioenhancer compounds (piperine, gallic acid and cinnamic acid, solution formation) was administered separately to the respective group of rats by oral gavage using 16-gauge stainless steel needle. Thirty minutes later, the animals were administered 50 mg/kg saquinavir through per oral (p.o.) route. Three rats were used for each group and total nine animals were used for this group. The blood samples (150  $\mu$ l) were taken for group two and three from the retro-orbital plexus into labeled tubes, containing 10  $\mu$ l of K<sub>2</sub>EDTA solution (20%), as anticoagulant. Blood samples were collected at 0.25, 0.33, 0.67, 1, 2, 4, 8, 12, 24, 48 and 72 h after saquinavir administration. Plasma was harvested from the blood by centrifugation at 4000 rpm for 10 min at 4  $\pm$  2  $^{\circ}$ C and stored below -70 $^{\circ}$ C (Thermo Scientific, USA) deep freezer until bioanalysis.

### LC-MS/MS ANALYSIS OF SAQUINAVIR

Analysis was performed using API 4000 Applied Biosystem-Sciex LC/MS/MS (Concord, Ontario, Canada) triple quadrupole mass analyzer system with an turbo ion spray atmospheric pressure ionization interface connected to a Shimadzu LC-20AD LC system (Shimadzu Corp., Japan). A Shimadzu LC20AD system (Kyoto, Japan), equipped with two pumps, a controller module, a vacuum degasser and an auto-sampler (SIL HTc, Kyoto, Japan), was used in the study. The mobile phase was consisted of acetonitrile (A) and water (B), both containing 0.1% formic acid, for gradient elution. Chromatographic separation was performed on Waters SymmetryShield RP 18 column (75mm x 4.6 mm, 3.5  $\mu$ m, Waters, Massachusetts, Ireland) at 40  $^{\circ}$ C temperature. The following gradient was

employed for the separation: 30% A for 1 min, 90% A at 2 min, and hold to 2.5 min, 30% A at 3.5 min and hold to 5 min. The flow rate remained at 0.8 ml/min throughout the run. The auto-sampler was kept at 4 °C and 5µl samples were injected.

The instrument was operated in the multiple reaction monitoring mode (MRM). The best sensitivities and minimum interferences were achieved by monitoring the molecular precursor and daughter ions, for saquinavir  $m/z$  671.7  $\rightarrow$  367.4, 671.7  $\rightarrow$  570.9, 671.7  $\rightarrow$  433.6, 671.7  $\rightarrow$  416.5 and similarly for imipramine as internal standard (IS) 281.4  $\rightarrow$  86.0. The optimum operating parameters of electro spray ionization (ESI) interface in positive ion mode were: Interface temperature 550 °C, Nebulizing gas 60 psi, Drying gas 55 psi and detector ESI probe voltage 5000 volts. The voltage parameters like declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP), Dwell time were optimized for each analyte and fragment to get better sensitivity. The optimized voltage parameters are mentioned in the Table 1. Instrument control, peak integration, regression, data acquisition and calculation of analytes concentration were computed using Analyst Classic, Version 1.5 software (ABI-SCIEX, Toronto, Canada). The calibration was performed by linear curve fit of the peak area ratio (analyte/internal standard) as a function of the concentration in the respective matrix. A weighting of  $1/x^2$  (where x is the concentration) was found to be optimal. The lower limit of quantification (LLOQ) in plasma was 1.02 for saquinavir. Based on some in house study we have selected this calibration curve (1.02 -2070 ng/ml) range and LLOQ.

The extraction procedure for Caco-2 transport assay samples, plasma samples for pharmacokinetic study or the spiked calibration standards/quality control samplers in to transport buffer/plasma were identical. A 25 µl sample of either study sample or spiked calibration standard/quality control samples were added to individual pre-labeled micro-centrifuge tubes. 150 µl of internal standard (imipramine, 200 ng/ml) prepared in acetonitrile was then added to the micro-centrifuge tubes except in blank sample where acetonitrile was added and vortexed for 5 minutes. Samples were centrifuged for 10 minutes at the speed of 15000 rpm (20600 g) at  $4 \pm 2$  °C. Following centrifugation, 100 µl of supernatant was transferred to clean insert vial and analyzed by LC-MS/MS system. Sample preparation was performed at room temperature.

### Pharmacokinetic Analysis

The pharmacokinetic parameters of saquinavir were obtained by noncompartmental analysis using WinNonlin version 2.1 (Pharsight, Mountain View, CA). The area under the plasma concentrationtime curve (AUC) was calculated using the trapezoidal method. The terminal half-life ( $t_{1/2}$ ) was calculated as  $\ln 2/k$ , and k was determined from the slope of the terminal regression line. The systemic clearance (CL) and apparent oral clearance (CL/F) were calculated as the i.v. and p.o. dose divided by AUC, respectively. Peak saquinavir concentrations ( $C_{max}$ ) and the times when they occurred ( $t_{max}$ ) were derived directly from the data. T Absolute bioavailability was taken into consideration when clearance was determined for the oral route (CL/F). The absolute bioavailability (F) was determined by  $(AUC_{p.o.}/Dose_{p.o.})/(AUC_{i.v.}/Dose_{i.v.})$ .

## RESULTS

The integrity of cell layer was maintained well throughout the experiment, which is reflected by consistent TEER values before and after experiment. P-gp substrate loperamide used in the study showed an efflux ratio of 5.6. This indicates that the batch of Caco-2 cells used for the assay have functional P-gp. The efflux ratio dropped down to 1.1 in presence of 100  $\mu$ M verapamil.

### Influence of Piperine, Gallic Acid and Cinnamic Acid on Caco-2 Cells

Bi-directional Caco-2 permeability of saquinavir alone and in presence of bioenhancers was determined. saquinavir has shown an efflux ratio (B-A / A-B) of > 25. This efflux ratio reduced to 2.6 when saquinavir co-administered with verapamil, a positive control P-gp inhibitor. This result confirms that saquinavir is a P-gp substrate. Similarly experiments with saquinavir in presence of piperine, gallic acid and cinnamic acid reduced the efflux ratio to ~2 suggesting potential inhibition of P-gp. Caco-2 mediated saquinavir transport were completely blocked by 100  $\mu$ M piperine (PIP), gallic acid (GA) and cinnamic acid (CA), which is reflected by a decrease of efflux ratios down to 2.7, 2.1 and 2.3 respectively. The summary results (Papp, recovery and efflux ratio) of saquinavir and loperamide alone and with verapamil and potential P-gp inhibitors are presented in Table 2 and Figure 1.

### Effects of Piperine, Gallic Acid and Cinnamic Acid on Saquinavir Pharmacokinetics in SD Rats

The plasma concentration–time profiles of saquinavir following i.v., p.o. and p.o. co-administration with oral piperine, gallic acid and cinnamic acid was shown in Figure 2, and the pharmacokinetic parameters were shown in Table 3. After 1 mg/kg intravenous (i.v.) dose of saquinavir, the area under the plasma concentration–time ( $AUC_{last}$ ) observed was  $3390.914 \pm 1091.791$  (ng/ml\*h) and volume of distribution ( $V_{ss}$ ) was observed  $0.291 \pm 0.086$  (L/kg). The  $t_{1/2}$  and plasma clearance (CL) was  $0.723 \pm 0.019$  h and  $5.217 \pm 1.417$  (ml/min/kg) respectively. After 50 mg/kg per oral (p.o.) dose of saquinavir the area under the plasma concentration–time ( $AUC_{last}$ ) observed was  $3789.49 \pm 1089.49$  (ng/ml\*h) and apparent volume of distribution ( $V_{ss}/F$ ) was observed  $63.98 \pm 18.27$ . The  $t_{1/2}$  and apparent oral plasma clearance (CL/F) was  $3.195 \pm 0.138$  h and  $229.80 \pm 57.51$  respectively. The peak plasma concentration ( $C_{max}$ ) after oral administration was observed  $950.03 \pm 254.12$  ng/ml.

Ten milligrams per kilogram (10 mg/kg) piperine, gallic acid and cinnamic acid significantly increased the area under the plasma concentration–time ( $AUC_{last}$ ) of saquinavir by 10.3, 9.49 and 10.61 folds respectively. The oral co-administration of piperine, gallic acid and cinnamic acid (10 mg/kg) also increased significantly the peak plasma concentration ( $C_{max}$ ) of saquinavir by 3.6, 3.8 and 3.5 folds respectively. The apparent oral plasma clearance (CL/F) of saquinavir decreased significantly by 9.2%, 10.0% and 9.2% in presence of piperine, gallic acid and cinnamic acid, respectively. Other oral pharmacokinetic parameters were also altered significantly such as the apparent volume of distribution ( $Vd/F$ ) and the mean residence time (MRT). The oral  $t_{1/2}$  of saquinavir was also changed from saquinavir alone and after co-administration with piperine, gallic acid and cinnamic acid from 3.12 h to 6.8, 7.7 and 6.5 h respectively.

The observed absolute oral bioavailability (F) of saquinavir alone is 2.4 % whereas saquinavir co-administered with per oral dose (10 mg/kg) of piperine, gallic acid and cinnamic acid is 23.12, 21.20 and 23.71%, respectively.

## DISCUSSION

Co-administration of herbals with medicinal drugs is frequent and the likelihood of a clinical relevant interaction is high [25]. There are many sources of phytochemicals, including herbal medicines, vegetables and food materials and it is not feasible to check the intake. Despite of the data shown by most of the literature it is likely that such interactions are common than generally thought, but are under-reported and appearance of adverse effects may be attributed to the disease for which the treatment is taken. Therefore, interaction between herbal drugs and medicinal drugs are not any more just a theoretical possibility. Therefore knowingly or unknowingly these agents act on the physiological targets resulting in herb-drug interaction and any untoward effect is considered as adverse effect of drug. Piperine has been shown in clinical trials to increase plasma concentration of phenytoin, propranolol, and theophylline [12, 13]. Poly phenols (gallic acid and cinnamic acid) present in many fruits and vegetables [26]. These herbal medicines (piperine, gallic acid and cinnamic acid) were not investigated for its ability to increase bioavailability of saquinavir and also checked the active apical secretory efflux in presence and absence of herbal bioenhancers in caco-2 cell monolayers.

Co-administration of drug candidates with herbal medicines, which might be the inhibitors of known drug transporters and metabolizing enzymes, may represent a strategy to improve the bioavailability of the co-administered drug. The mechanisms underlying most of the reported herb-drug interactions have been ascribed to the inhibition of various types of efflux transporters such as P-glycoprotein (P-gp), multi drug resistance proteins (MRPs), organic anionic transporter polypeptides (OATPs), breast cancer resistance protein (BCRP) and/or drug-metabolizing enzymes, cytochrome P450s (CYPs) [27-30]. The piperine, gallic acid and cinnamic acid were selected for this study, since little or no information is available in the literature of their used either as CYP inhibitor and /or efflux transporters.

As shown in Table 2, piperine, gallic acid and cinnamic acid at concentration of 100  $\mu$ M, significantly decreased apically directed transport and increased basolaterally directed transport of saquinavir in Caco-2 cells, resulting in efflux ratios close to unity. At the 100  $\mu$ M concentration, these compounds almost completely inhibited the activity of P-gp. These results suggested that piperine, gallic acid and cinnamic acid are appeared to be an inhibitor of P-gp. Further study is needed to identify they might be an inhibitor for other transports.

To further investigate the interactions of piperine, gallic acid and cinnamic acid with saquinavir *in vivo*, pharmacokinetic studies of saquinavir after i.v. and p.o. administration were performed in rats with and without the co-administration of piperine or gallic acid or cinnamic acid. As shown in Table 3, the presence of piperine, gallic acid or cinnamic acid at oral dose of 10 mg/kg significantly increased the AUC (exposure) and  $C_{max}$  of saquinavir and reduced apparent oral clearance (CL/F) of p.o. administered



saquinavir. The increased AUC and reduced CL/F of saquinavir might be mainly due to the inhibition of P-gp and other transporters (BCRP, OATP1B1, OATP2B1, OATP1B3, and NTCP), which are located in the intestine and liver.

Since piperine, gallic acid and cinnamic acid are well absorbed through intestine, non-toxic and has many health-beneficial activities; these might be good candidates of efflux transporter(s) modulator in improving the bioavailability of low bioavailable compound viz. saquinavir. Previous pharmacokinetic studies in rats using topotecan as a probe BCRP substrate have shown that the contribution of Bcrp1 transporter in rat is significant [27, 31]. Therefore, we cannot exclude the possibility that approximately 10-fold increase in saquinavir AUC in rats. This may represent the P-gp inhibition by piperine, gallic acid and cinnamic acid, possibly attributed to the enhanced intestinal absorption and/or decreased systemic elimination of saquinavir. After p.o. administration of piperine, gallic acid and cinnamic acid separately, the altered p.o. pharmacokinetics of saquinavir is more likely because of the intestine and/or systemic interactions of piperine, gallic acid and cinnamic acid with saquinavir. Further study is needed in the HIV protease inhibitors therapy to demonstrate the potency of piperine or gallic acid or cinnamic acid on the pharmacokinetics of saquinavir in humans.

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#### **Conflict of Interest Statement**

The authors confirm that there are no conflicts of interest.

#### **REFERENCES**

1. Noble S. and Faulds D. (1996). Saquinavir; A review of its pharmacology and clinical potential in management of HIV infection. *Drugs* 52: 93-112.
2. Germann U.A. (1996). P-glycoprotein – a mediator for multidrug resistance in tumor cells. *Eur J. Cancer*. 32A: 927-944.
3. Pajeva I.K., Wiese M., Cordes H.P. and Seydel J.K. (1996). Membrane interactions of some cataphilic drugs and relation to their multi-drug resistance reversing ability. *J. Cancer. Res. Clin. Oncol.* 122: 27-40.
4. Ecker G., Chiba P. (1997). Structure activity relationship studies on modulators of multidrug transporter P-glycoprotein-an overview. *Wien. Klin. Wochenschr.* 107(22): 681-686.
5. Varma, M.V., Ashokraj, Y., Dey, C.S., Panchagnula, R.. (2003). P-glycoprotein inhibitors and their screening: a perspective from bioavailability enhancement. *Pharmacol. Res.* 48: 347–359.

6. Patwardhan B., Mashelkar R.A. (2009). Traditional medicine-inspired approaches to drug discovery and development: can Ayurveda show a way forward?, *Drug Discov. Today* 14: 804-811.
7. Navin P., Atal B., Bedi K.L. (2010). Bioenhancers: Revolutionary concept to market, *J. Ayur. Intergrat. Med.* 1: 96-99.
8. Atal C.K., Zutshi U., Rao P.G. (1981). Scientific evidence on the role of Ayurvedic herbals on bioavailability of drugs. *J. Ethnopharmacol.* 4: 229-232.
9. Khajuria A., Zutshi U., Bedi K.L. (1998). Permeability characteristics of piperine on oral absorption—an active alkaloid from peppers and a bioavailability enhancer. *Indian J. Exp. Biol.* 36: 46–50.
10. Atal C.K., Dubey R.K., Singh J. (1985). Biochemical basis of enhanced drug bioavailability by piperine: evidence that piperine is a potent inhibitor of drug metabolism, *J. Pharmacol. Exp. Ther.* 232: 258–262.
11. Bhardwaj R.K., Glaeser H., Becquemont L., Klotz U., Gupta S.K., Fromm M. F. (2002). Piperine, a major constituent of black pepper, inhibits human P-glycoprotein and CYP3A4, *J. Pharmacol. Exp. Ther.* 302: 645-650.
12. Bano G., Amla V., Raina R.K., Zutshi U., Chopra C.L. (1987). The effect of piperine on pharmacokinetics of phenytoin in healthy volunteers, *Planta Med.* 53: 568-569.
13. Bano G., Raina R.K., Zutshi U., Bedi K.L., Johri R. K., Sharma S. C. (1991). Effect of piperine on bioavailability and pharmacokinetics of propranolol and theophylline in healthy volunteers, *Eur. J. Clin. Pharmacol.* 41: 615-617.
14. Shobha G., Joseph T., Majeed M., Rajendran R., Srinivas P.S.R. (1998). Influence of piperine on the pharmacokinetics of curcumin in animals and human volunteers, *Plant Med.* 64: 353-356.
15. Majumdar A.M., Dhuley J.N., Deshmukh V.K., Naik S.R. (1999). Effect of Piperine Bioavailability of Oxyphenylbutazone in rats, *Indian Drugs.* 36: 123-124.
16. Middleton E. Jr., Kandaswami C., Theoharides T.C. (2000). The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer, *Pharmacol. Rev.* 52: 673–751.
17. Havsteen B.H. (2002). The biochemistry and medical significance of the flavonoids, *Pharmacol. Ther.* 96: 167–202.
18. Zhang S., Morris M.E. (2003). Effects of the flavonoids biochanin A, morin, phloretin, and silymarin on P-glycoprotein-mediated transport. *J. Pharmacol. Exp. Ther.* 304: 1258–1267.
19. Feng H.P., Huang C.L., Zhang L.T., Li R.F. (2004). Pharmacokinetics and bioavailability of cinnamic acid in mice, *J. China Pharm. Univ.* 35: 328-330.

20. Foti S., Wholstrom J.L. (2008). The role of dietary supplements in cytochrome P450 – mediated drug interactions, *Bol Latinoam Caribe Plant Med Aromaticas*. 7: 66-84.
21. Ioannides C. (2002). Pharmacokinetic interactions between herbal remedies and medicinal drugs, *Xenobiotica* 32: 451- 478.
22. Hunter J., Jepson M.A., Tsuruo T., Simmons N.L. and Hirst B.H. (1993). Functional expression of p-glycoprotein in apical membranes of human intestinal Caco-2 cells. *J. Biol. Chem.* 268: 14991-14997.
23. Wils P., Phung Ba.V, Warney A., Lechardeur D., Raeissi S.I., Hidalgo J. and Scherman D. (1994). Polarized transport of docetaxel and vinblastine mediated by p-glycoprotein in human intestinal epithelial cell monolayers. *Biochem. Pharmacol.* 48: 1528-1530.
24. Hosoya K., Kim K. and Lee V.H. (1996). Age-dependent expression of p-glycoprotein gp 170 in caco-2 cell monolayers. *Pharm. Res.* 13: 885-890.
25. Harris R.Z., Jang G.R., Tsunoda S. (2003). Dietary effects on drug metabolism and transport, *Clin. Pharmacokinet.* 42: 1071–1088.
26. Kang M. J., Cho J.Y., Shim B.H., Kim D.K., Lee J. (2009). Bioavailability enhancing activities of natural compounds from medicinal plants. *J. Med. Plants Res.* 13: 1204-1211.
27. Zhang S., Yang X., Morris M.E. (2004). Flavonoids are inhibitors of breast cancer resistance protein (ABCG2)-mediated transport, *Mol. Pharmacol.* 65: 1208–1216.
28. Fuchikami H., Satoh H., Tsujimoto M., Ohdo S., Ohtani H., Sawada Y. (2006). Effects of herbal extracts on the function of human organic anion-transporting polypeptide OATP-B, *Drug Metab. Dispos.* 34: 577-582.
29. Greenblatt D.J. (2009). Analysis of drug interactions involving fruit beverages and organic anion-transporting polypeptides, *J. Clin. Pharmacol.* 49: 1403-1407.
30. Hirofumi T., Hiroki S., Satoko H., Hisakazu O., Yasufumi S. (2010). Inhibitory Effects of Herbal Extracts on Breast Cancer Resistance Protein (BCRP) and Structure-Inhibitory Potency Relationship of Isoflavonoids, *Drug Metab. Pharmacokinet.* 25: 170–179.
31. Jonker J.W., Smit J.W., Brinkhuis R.F., Maliepaard M., Beijnen J.H., Schellens J.H., Schinkel A.H. (2000). Role of breast cancer resistance protein in the bioavailability and fetal penetration of topotecan, *J. Natl. Cancer. Inst.* 92: 1651–1656.

**Table 1: Positive product ion mass parameter of saquinavir and imipramine**

Name of the Analyte	Q1 Mass (Da)	Q3 Mass (Da)	DP	Dwell time (msec)	CE	CXP
Saquinavir	671.7	367.4	10	50	48.24	5.98
	671.7	570.9			45.13	6.67
	671.7	433.6			48.22	9.43
	671.7	416.5			51.65	11.06
Imipramine	281.4	86.0	102	50	25.26	12.04
EP: 10						

Note: DP indicates Declustering Potential, CE is collision energy, CXP is Cell Exit Potential and EP indicates Entrance Potential. All are voltage dependent parameters of instrument.

**Table 2: Summary table representing Papp, recovery and efflux ratios of saquinavir alone and in presence of bioenhancers (PIP, GA and CA) along with positive control loperamide**

Compounds	Permeability direction	Replicates	Spiking	Papp	Recovery	Efflux Ratio
			Concentration	(10 <sup>-6</sup> cm/sec)		(B-A / A-B)
			(μM)			
Loperamide	A-B	3	5	3.9 ± 0.4	100	5.6
	B-A	3		21.7 ± 0.2	93	
Loperamide + Verapamil	A-B	3	5	15.6 ± 0.6	100	1.1
	B-A	3		16.6 ± 1.2	98	
Saquinavir	A-B	3	10	0.47 ± 0.2	100	27.5
	B-A	3		12.92 ± 1.2	100	
Saquinavir + Verapamil	A-B	3	10	5.16 ± 0.7	100	2.6
	B-A	3		13.29 ± 1.0	100	
Saquinavir + Piperine	A-B	3	10	5.07 ± 0.8	100	2.7
	B-A	3		13.55 ± 1.1	98	
Saquinavir + Cinnamic acid	A-B	3	10	6.39 ± 0.4	100	2.3
	B-A	3		14.76 ± 1.2	99	
Saquinavir +	A-B	3	10	5.70 ± 0.3	98	2.1
Gallic acid	B-A	3		12.16 ± 0.9	93	

PIP is Piperine, GA is Gallic acid and CA indicates Cinnamic acid.

**Table 3: Pharmacokinetic parameters of saquinavir (SQN) in male SD rats following single (1 mg/kg intravenous (i.v.) and (50 mg/kg) per oral (P.O.) dose and co-administration with per oral dose (10 mg/kg) of PIP, GA and CA**

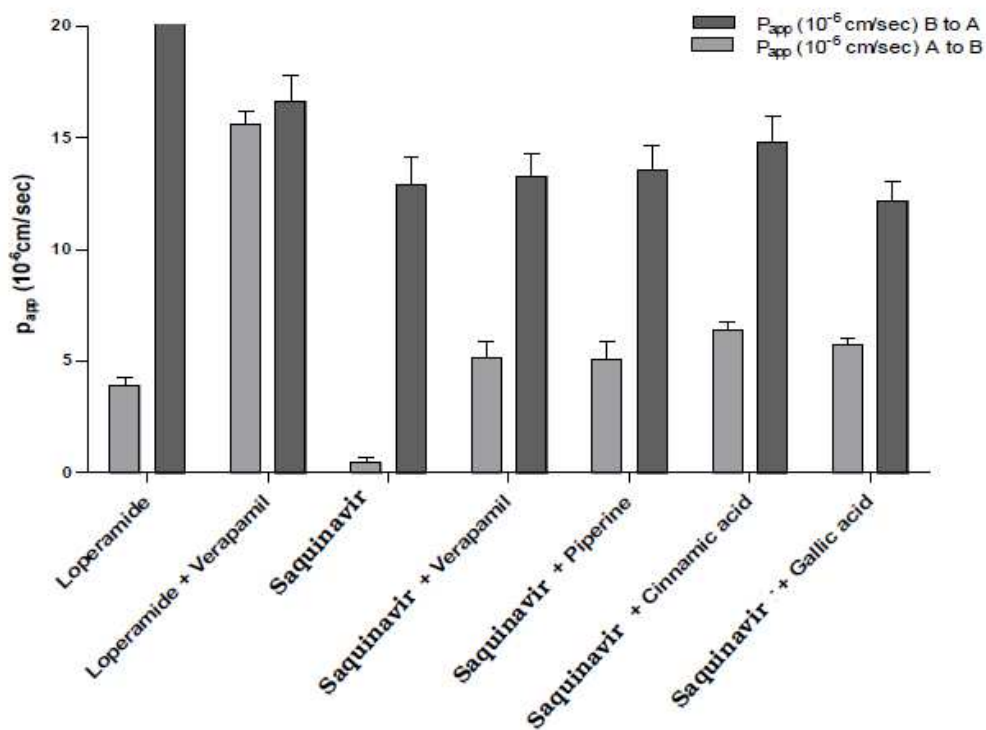
Dose	Pharmacokinetic parameters of saquinavir in male SD rats (n=3)				
	SQN ALONE	SQN ALONE	SQN +PIP	RSV + GA	RSV + CA
<b>PK Parameters</b>	i.v.	p.o.	p.o.	p.o.	p.o.
<b>SQN Dose (mg/kg)</b>	1	50	50	50	50
<b>Bioenhancers Dose (mg/kg)</b>	-	-	10	10	10
<b>Formulation</b>	Solution	Solution	Solution	Solution	Solution
<sup>a</sup> C <sub>max</sub> (ng/ml)	3928.189 ± 1093.858	950.03 ± 254.12	3409.95 ± 481.67	3611.17 ± 264.11	3450.18 ± 540.12
T <sub>max</sub> (ng/ml)	-	0.89 ± 0.19	0.67 ± 0.34	0.56 ± 0.19	0.55 ± 0.39
AUC <sub>last</sub> (0- 72h) (ng/ml*h)	3390.914 ± 1091.791	3789.49 ± 1089.49	39197.19 ± 6354.09	35939.38 ± 8160.09	40207.0 ± 6432.73
AUC <sub>last</sub> (0- inf) (ng/ml*h)	3392.200 ± 1092.077	3808.03 ± 1092.18	39232.93 ± 6334.95	36021.88 ± 8121.61	40236.53 ± 6431.84
T <sub>half</sub> (h)	0.723 ± 0.019	3.195 ± 0.138	6.80 ± 1.42	7.692 ± 1.621	6.500 ± 0.848
MRT <sub>last</sub> (h)	0.924 ± 0.050	3.94 ± 0.51	11.94 ± 2.39	13.66 ± 1.73	12.12 ± 1.41
<sup>b</sup> CL/F	5.217 ± 1.417	229.80 ± 57.51	21.63 ± 3.66	23.86 ± 4.82	21.09 ± 3.55
<sup>c</sup> V <sub>ss</sub> /F	0.291 ± 0.086	63.98 ± 18.27	13.03 ± 4.71	16.25 ± 6.2	11.97 ± 3.06
%F	-	2.24	23.12	21.2	23.71

<sup>a</sup>C<sub>0</sub> back extrapolated concentration of IV profile

<sup>b</sup>CL (ml/min/kg) for IV profile

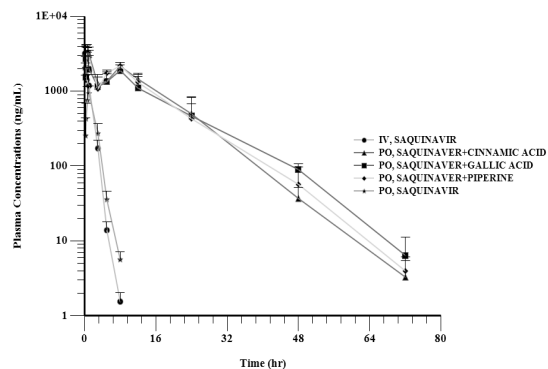
<sup>c</sup>V<sub>ss</sub> (L/kg) for IV profile

SQN indicates saquinavir, PIP is Piperine, GA is Gallic acid and CA indicates Cinnamic acid.



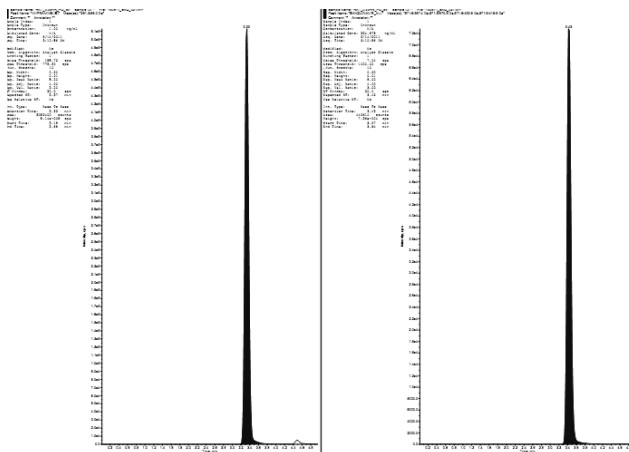
**Figure 1: A-B and B-A permeability (P<sub>app</sub>; 10<sup>-6</sup> cm/sec) of saquinavir alone and in presence of bioenhancers (PIP, GA and CA) along with positive control loperamide**

**PIP is Piperine, GA is Gallic acid and CA indicates Cinnamic acid.**



**Figure 2: Mean plasma concentration–time profiles of saquinavir after i.v. administration of saquinavir (1 mg/kg) to rats with p.o. (50 mg/kg) co-administration in the absence or presence of piperine (10 mg/kg), or gallic acid (10 mg/kg), or cinnamic acid (10 mg/kg).**

Bars represent the standard deviation (n = 3). The data are expressed as mean  $\pm$  S.D. \*



**Figure 3: Representative chromatogram of saquinavir and imipramine (internal standard) from rat plasma**