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Drug-drug interactions: Influence of verapamil on the pharmacokinetics of sitagliptin in rats and *Ex vivo* models

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ABSTRACT

P-glycoprotein (P-gp) and cytochrome P450 3A4 (CYP3A4) play a significant role in the disposition and elimination of drugs. The objective of this study was to investigate the mechanism underlying the interaction between sitagliptin (substrate of P-gp and CYP3A4) and verapamil (known modulator of P-gp and CYP3A4) using in vivo, ex vivo and in situ models. Rats were treated with sitagliptin (10 mg/kg, oral and/or 5 mg/kg, intravenous) alone and in combination with verapamil (40 mg/kg, oral) for 15 consecutive days. Blood samples were collected from the tail vein on 1st day in single dose pharmacokinetic study (SDS) and on 15th day in multiple dose pharmacokinetic study (MDS). The plasma concentrations of sitagliptin were significantly higher in the verapamil pretreated group when compared to sitagliptin control group. Verapamil pretreatment significantly increased the mean area under the plasma concentration-time curve from 0 to 24h (AUC0-24h), peak plasma concentration (Cmax), percent absolute bioavailability (AB%), elimination half-life (t1/2) and decreased the volume of distribution (Vz/F), clearance (CL/F) and apparent volume of distribution at steady state (Vss/F) of sitagliptin in both SDS and MDS (oral and intravenous). Ex vivo study results showed that the apparent permeability coefficient (Papp), net efflux and efflux ratio values were significantly increased by the known P-gp and CYP3A4 inhibitors (itraconazole and ketoconazole) and verapamil. In single pass intestinal perfusion (In situ) study, the effective permeability coefficient (Peff) and intestinal absorption rate constant (Ka) were increased in the presence of verapamil ($p<0.05$). The present study results revealed that verapamil enhanced the bioavailability of sitagliptin probably by inhibiting its absorption via P-gp and/or the CYP3A4-mediated biotransformation in rats. Verapamil can be co-administered with sitagliptin without dose adjustment due to high safety margin of sitagliptin.

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Keywords
P-glycoprotein,
CYP3A4,
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Everted gut sacs,
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INTRODUCTION

Drug efflux transporters (especially P-glycoprotein, P-gp) and drug-metabolizing enzymes, particularly cytochrome

P450 3A4 (CYP3A4) play an important role in the absorption, distribution and elimination of a wide variety of drugs [1, 2]. In the marketed drugs, upto 50% have been identified to be P-gp substrates and/or inhibitors and 60% identified to be CYP3A4 substrates and/or inhibitors [3, 4]. The broad and overlapping substrate specificity of P-gp and CYP3A4 renders dual substrates prone to numerous potential drug-drug interactions [5, 6]. P-gp and CYP3A4 may act synergistically in limiting the oral bioavailability of drugs thereby the pharmacokinetics of a drugs may be altered when co-administered with compounds which inhibit or induce P-gp and CYP3A4 [7].

Diabetes and hypertension are intertwined clinical conditions that share a significant overlap in underlying risk factors and complications [8]. These complications can be divided into macrovascular (coronary artery disease, myocardial infarction, congestive heart failure, stroke, and peripheral vascular disease) and microvascular (retinopathy, nephropathy, neuropathy and cardiomyopathy) are conventionally linked to hyperglycemia, studies have shown that hypertension constitutes an important risk factor [9]. Sitagliptin is a first selective dipeptidyl peptidase-4 (DPP-4) inhibitor approved for the treatment of type 2 diabetes [10]. In addition to antihyperglycemic activity [11], sitagliptin also has cardioprotective [12], antioxidant [13], renoprotective [14] and neuroprotective [15] activities.

Verapamil, the prototype of the Calcium channel blockers (CCBs) has been extensively used in various cardiovascular conditions including hypertension [16], angina, supraventricular arrhythmias [17], heart failure [18], and congestive heart failure [19]. It has been reported to have cardioprotective [20], nephroprotective [21], neuroprotective [22], renoprotective [23], hepatoprotective [24], antioxidant and anti-inflammatory [25] activities. It is used as second line add-on drug and has beneficial effects in managing hypertension in patients with diabetes [26]. It is a substrate and inhibitor of P-glycoprotein [27] and also inhibits CYP3A4 isoenzymes [28].

Many clinically significant interactions have been reported with verapamil. Previous studies reported that verapamil inhibited the P-gp and CYP3A4-mediated metabolism of levobupivacaine, lidocaine [29], imipramine [30], atorvastatin [31], simvastatin, nilotinib [32] cerivastatin [33], darifenacin, ethosuximide [34], fentanyl, pimozide, sildenafil, terfenadine and zolpidem [35]. Pharmacokinetically, sitagliptin is a substrate of P-gp and CYP3A4 thereby it is considered likely to cause interactions with P-gp and CYP3A4 substrates and/or inhibitors [36]. Cyclosporine A significantly inhibited P-gp mediated transport of sitagliptin [37]. However, data (either clinical or preclinical) are lacking on the pharmacokinetic interactions between sitagliptin and verapamil. Therefore, the study was planned to evaluate the effect of verapamil on the pharmacokinetics of sitagliptin in rats and to explore the roles of P-gp and CYP3A4 in the absorption of sitagliptin using rat intestinal sacs *ex vivo* and *in situ* models.

MATERIALS AND METHODS

Drugs and chemicals

Sitagliptin and verapamil were obtained as gift samples from Actis Pharma, Hyderabad, India and Torrent Pharmaceuticals, Secunderabad, India, respectively. Ketoconazole was obtained from Mylan Pharmaceuticals, Hyderabad. Methanol, acetonitrile and water of high-performance liquid chromatography (HPLC) grade were purchased from Finar Chemicals Ltd, Ahmadabad, India. All other chemicals and reagents used were of analytical grade.

Experimental animals

Animal experiments were performed according to the institutional guidelines for the care and use of laboratory animals, and approved by the animal ethics committee of KVSR Siddhartha College of Pharmaceutical Sciences (SCOPS), Vijayawada, Andhra Pradesh, India (Protocol No: KVSRSCOPS/11-03-04-007). The college was recognized by the Govt. of India (993/PO/E/S/06/CPCSEA). Male wistar rats (180-220 g) were procured from Mahaveer Enterprises, Hyderabad, and Andhra Pradesh, India. Animals were housed six per cage and given free access to food (Hindustan Lever, Mumbai, India) and water *ad libitum* in animal house at the KVSR SCOPS. Before starting the experiments, animals were kept under standard laboratory conditions (12/12 h light/dark cycle, 22±2ºC and 50-60% humidity) for at least a week.

Experimental design

The entire study consists of four experiments as it has been described previously by Pingili *et al*. in our laboratory [38]. First two experiments are single dose pharmacokinetic study (SDS) and multiple dose pharmacokinetic study (MDS) *in vivo*. In these studies, animals were treated with sitagliptin alone and in combination with verapamil once daily for 15 consecutive days [39]. The doses of sitagliptin and verapamil were calculated and selected based upon the observations from the earlier experiments. Third and fourth experiments are *ex vivo* absorption studies using rat gut sacs [40] and *in situ* drug permeability (Single pass intestinal perfusion) studies to determine the role of P-gp on the absorption of sitagliptin using rat small intestine [41, 42]. Finally, the concentrations of sitagliptin were estimated in the plasma and other (*in vitro* & *in situ*) samples by using reversed phase-high pressure liquid chromatography (RP-HPLC).

In vivo oral pharmacokinetic (SDS and MDS) studies

Sitagliptin and verapamil were suspended in sodium carboxymethylcellulose (0.5%) for oral administration. Male Wistar rats were randomly divided into two groups of six animals in each group. Group I (sitagliptin control group), treated orally with sitagliptin (10 mg/kg) alone and Group II (treatment group): pre-treated (30 min) with verapamil (40 mg/kg, oral) followed by sitagliptin (10 mg/kg, oral). The treatment was given once daily for 15 consecutive days. After treatment, 200 µL blood samples were collected from

tail vein at intervals of 0.5, 1, 2, 3, 4, 6, 8, 12 and 24 h on the 1st day in SDS and on the 15th day in MDS. After collection of each blood sample, an equal volume of heparinized 0.9% NaCl (20 IU/mL) was administered to the animals. Blood samples were collected in heparinized eppendorf tubes and kept on ice during the experiment. The plasma was separated by centrifugation (Remi, R4C Compact model, Mumbai, India) at 5000 rpm for 6 min and stored at -20ºC until analysis.

In vivo intravenous pharmacokinetic (SDS and MDS) studies

For intravenous administration, sitagliptin phosphate was directly dissolved in phosphate buffer saline (pH 7.2) and verapamil was suspended in sodium carboxymethylcellulose (0.5%) for oral administration. Wistar rats were randomly divided into two groups of six animals in each group and their lateral tail veins were cannulated for blood withdrawal and sitagliptin administration. Group I (sitagliptin control group), treated intravenously with sitagliptin (5 mg/kg) alone and Group II (treatment group), pre-treated (30 min) with verapamil (40 mg/kg, oral) followed by sitagliptin (5 mg/kg) intravenously. The treatment was given once daily for 15 consecutive days.

After treatment, 200 µL blood samples were collected from tail vein at intervals of 5, 15, 60, 120, 180, 240 and 720 min on the 1st day in SDS and on the 15th day in MDS. After collection of each blood sample, an equal volume of heparinized 0.9% NaCl (20 IU/mL) was administered to the animals. Blood samples were collected in heparinized eppendorf tubes and kept on ice during the experiment. The plasma was separated by centrifugation (Remi, R4C Compact model, Mumbai, India) at 5000 rpm for 6 min and stored at -20ºC until analysis.

Extraction of sitagliptin from plasma

Liquid–liquid extraction method was used to extract the sitagliptin from the rat plasma. To an aliquot of 50 µL plasma, 50 µL of 1M sodium hydroxide and 1.5 mL ethyl acetate were added. Vortex mixed for 5 min on a Remi vortex mixer (Remi CM-101 Cyclomixer, Mumbai, India) and centrifuged (Remi, R4C Compact model, Mumbai, India) at 3000 rpm for 5 min. The supernatant (1.4 mL) was evaporated to dryness at 40ºC under a stream of nitrogen. The dry residue was reconstituted in 200 µL of the mobile phase and a 20 µL aliquot was injected into HPLC system for analysis.

Determination of sitagliptin by HPLC

Sitagliptin concentrations in rat plasma were estimated by RP-HPLC as it has been described by Xiang et al. with minor modifications [43]. A Shimazdu HPLC system consisted of a pump (LC-20AT VP), C18 column (ODS Thermo Hypersil, 150 mm × 4.6 mm, 5.0 µm, Thermo Electron Corp, Beverly, MA) and a dual wavelength UV–visible detector (SPD-10A VP). LC solution software was used to collect and process the data. The mobile phase was composed of methanol: water (60:40, v/v) containing 10 mM tris and 10 mM triethylamine and titrated to pH 9.0 with 1M hydrochloric acid. The mobile phase was vacuum-filtered through 0.45 µm nylon Millipore membranes (Millipore, Billerica, MA), and degassed by ultrasonication (Remi, Mumbai, India) for 20 min before use. The flow of mobile phase was set at 1.0 mL/min and the injection volume was 20 µL. After equilibration with the solvent to obtain a stable baseline, aliquots of samples were injected. The absorbance of the eluent was monitored at 267nm. All the analyses were performed at 25.0 ± 0.5ºC. The retention times of blank plasma and sitagliptin were obtained at 2.883 and 6.181 min, respectively (Fig. 1).

Preparation of standard and stock solutions

Sitagliptin stock solution was prepared by dissolving 1 mg in 10 mL of methanol (100 µg/mL) and stored in amber

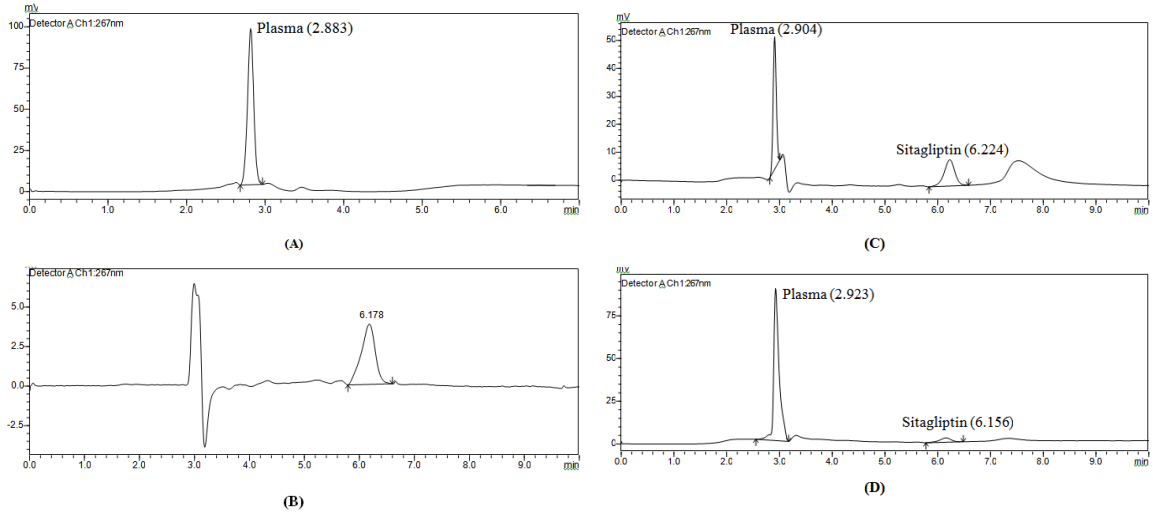

Figure 1. Representative chromatograms of (A) blank plasma (2.883); (B) sitagliptin 2 µg/mL (6.181); (C) plasma (2.904) + sitagliptin 6 µg/mL (6.224); (D) plasma sample obtained from rats treated with 10 mg/kg of sitagliptin monitored at 267 nm.

glass containers at -20ºC. Calibration standards were prepared by spiking appropriate amounts of sitagliptin in 100 µL of rat plasma. The final concentrations of calibration standards (0.1, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8 and 10 µg/mL) were stored at 2-8ºC. Calibration curves were constructed between the concentration and peak area. The lower limit of quantification was 0.75 µg/mL.

Calculation of pharmacokinetic parameters

The plasma concentrations versus time data obtained from each individual rat were submitted to a non-compartmental pharmacokinetic analysis using Thermo Kinetica, Version 5.1 (Thermo Electron Corporation, Beverly, MA). The maximum plasma concentrations (C_{max}) and times to achieve maximum plasma concentrations (t_{max}) were obtained directly from the individual plasma concentration-time curves. Area under the plasma concentration-time curve from time zero to 24 h (AUC_{0-24}) was calculated by the linear trapezoidal rule and $AUC_{0-\infty}$ was determined by the following formula:

$$AUC_{0-\infty} = C_{last}/K_{el} + AUC_{0-24}$$

Where C_{last} is the last quantifiable concentration. The apparent total body clearance or oral clearance (CL/F) was calculated as follows:

$$CL/F = Dose/AUC_{0-24}$$

The elimination half-life ($t_{1/2}$) was calculated as $0.693/K_{el}$ and the mean residence time (MRT) was calculated as follows:

$$MRT = AUMC_{0-\infty}/AUC_{0-\infty}$$

The area under the first moment curve ($AUMC_{0-\infty}$) was calculated from the plasma concentration–time curve. The volume of distribution (Vz/F) was estimated by means of Vz/F = dose/ $AUC_{0-\infty}/K_{el}$. The apparent volume of distribution at steady state (Vss/F) was estimated by means of Vss/F = CL × MRT. The absolute oral bioavailability was determined by using following equation:

$$\text{Absolute bioavailability (AB\%)} = \frac{AUC_{0-24,\ oral} \times Dose_{i.v.}}{AUC_{0-24,\ i.v.} \times Dose_{oral}} \times 100$$

Ex vivo drug absorption studies using rat gut sacs

Rat everted and non-everted gut sacs (NEGS) are simple and useful *ex vivo* models to investigate the role of P-gp and CYP3A4 in drug disposition [44]. Both sacs were prepared using a method described previously by Pingili et al. in our laboratory with some modifications [45]. Healthy male wistar rats (weighing about 180-220 g, n = 3) were deprived of food for 1 day and provided with only double-distilled water before the experiments. The rats were anesthetized with pentobarbital sodium (40 mg/kg, i. v.) and the small intestine was dissected out. The intestine was immediately rinsed with ice-cold Krebs-Ringer-Hensleit bicarbonate (KRH) buffer (7.8 g NaCl, 0.35 g KCl, 0.37 g $CaCl_2$, 1.37 g $NaHCO_3$, 0.32 g NaH_2PO_4, 0.02 g $MgCl_2$, 1.4 g glucose, pH 6.8) in order to discard the intestinal digesta and the distal ileums (approximately 10 cm each) were taken for the study.

Effect of verapamil on the intestinal transport of sitagliptin

NEGS were used to evaluate the transport of sitagliptin from mucosal to serosal (M-to-S) side of the intestine. NEGS were loaded with 1mL of modified Krebs-Ringer (128.5 mM NaCl, 4.7 mM KCl, 3.3 mM $CaCl_2$, 2.3 mM $MgCl_2$, 16.3 mM $NaHCO_3$, 1.87 mM NaH_2PO_4, 7.8 mM glucose, pH 7.4) bicarbonate (KRB) buffer containing sitagliptin (50µg/mL) in the presence or absence of known P-gp inhibitors (itraconazole and ketoconazole) and verapamil (50µg/mL). Each sac was placed in individual 50 mL Erlenmeyer flasks contain 30 mL of oxygenated (O_2/CO_2; 95:5) KRB and incubated at 37ºC for 60 min in a shaker bath. Aliquots (150 µL) of serosal fluid were collected at 10, 20, 30, 40, 50 and 60 min and then replaced by the same volume of buffer. At the end of incubation, all samples were centrifuged (Remi, R4C Compact model, Mumbai, India) at 3500 rpm for 10 min and supernatants were analyzed for sitagliptin by RP-HPLC. Each experiment was triplicate. The same experiment was repeated with everted gut sacs (EGS) to study the transport of sitagliptin from serosal to mucosal (S-to-M) side of the intestine. The intestinal segments were everted using a stain less steel rod. The apparent permeability coefficient (P_{app}), efflux ratio and net efflux were determined by the following formula:

$$\text{Apparent permeability coefficient (cm/s)} = \frac{dQ}{dt} \times \frac{1}{A \times C_0}$$

$$\text{Efflux ratio} = \frac{P_{app}\,(\text{serosal to mucosal})}{P_{app}\,(\text{mucosal to serosal})}$$

$$\text{Net efflux} = P_{app}\,(\text{serosal to mucosal}) - P_{app}\,(\text{mucosal to serosal})$$

Where dQ/dt (µg/min) is the permeation rate of drug; A (cm^2) is the surface area of the intestinal sac and C_0 (µg/mL) is the initial loading concentration.

In situ drug permeability (Single pass intestinal perfusion) studies

To further confirm the involvement of P-gp and CYP3A4 in the intestinal permeability of sitagliptin, single pass intestinal perfusion *(in situ)* experiment was performed as described previously by Yang et al. [46] and Neerati et al. [47]. In brief, healthy male wistar rats (weighing about 180-220 g, n = 6) were maintained on a 12h light-dark cycle and fasted 16-20 h before each experiment. Animals were anaesthetized with an intravenous injection of pentobarbital sodium (40 mg/kg) and placed on a heated surface to maintain a normal body temperature. A midline incision was made on the abdomen and an ileal segment of approximately 13-15 cm was isolated using the ileo-caecal junction as a distal marker. The ileal segment was cannulated at both ends with flexible plastic tubing and rinsed with normal saline (37°C). The segment was infused with KRB buffer for 10 min at a flow rate of 1.0 mL/min using a syringe micro infusion pump (Aspire, Plenum tech Pvt. Ltd, Nagpur,

India). Subsequently, KRB buffer containing sitagliptin (50 µM) with or without 100 µM verapamil was perfused through the intestinal segment at a constant flow rate of 0.25 mL/min for 90 min. Perfusate samples obtained from the outlet of the ileum were collected every 10 min. During the entire course of the experiment, care was taken to avoid disturbing the circulatory system, and the exposed segment was kept moist after cannulation with isotonic saline-moistened gauze (37°C). At the end of the experiment (i.e., following the last sample collection), the radius and length of the ileal segment were measured. The samples were stored at -20°C until HPLC analysis. The effective permeability coefficient (P_{eff}) is a quantitative estimate of the rate of drug permeability across a membrane and is calculated based on the steady state concentration of drug in the collected perfusate as follows:

$$\text{Effective permeability coefficient} = \frac{-Q \times \ln\ (C_{out}/C_{in})}{2\pi rL}$$

Where Q is the perfusion flow rate through the ileal segment; C_{out} and C_{in} are the outlet and inlet concentrations of the drug, respectively; r is the radius of the perfused rat small intestine segment (0.19 cm); and L is the length of the perfused segment (15 cm). Intestinal absorption rate constant (Ka) was calculated by using the following relationship:

$$\text{Intestinal absorption rate constant } (K_a) = \frac{Q_{in}\ (1 - C_{out}/C_{in})}{V_{ps}}$$

Where V_{ps} is the volume of perfused ileal segment ($=r^2\pi L$).

RESULTS

Effect of verapamil on the oral pharmacokinetics of sitagliptin in vivo

The mean plasma concentration-time profiles of sitagliptin after oral administration of sitagliptin (10 mg/kg) alone and with verapamil (40 mg/kg) pretreatment are

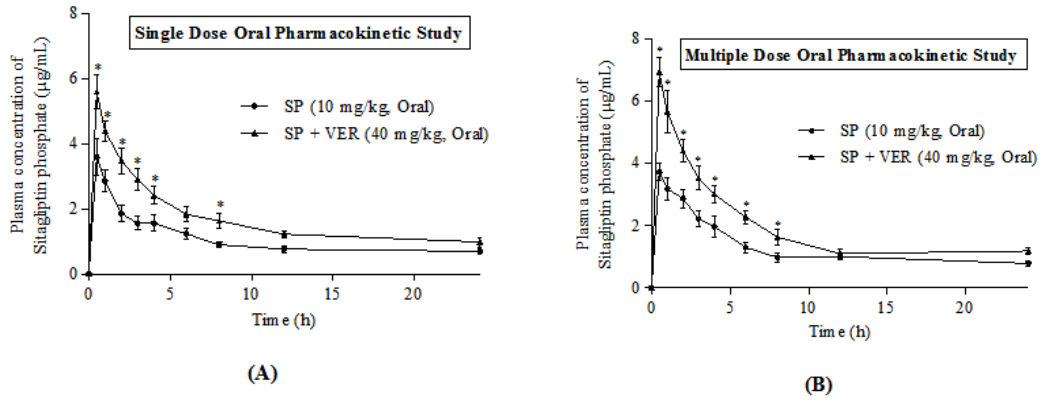

Figure 2. Mean plasma concentration–time profiles of sitagliptin following an oral administration of sitagliptin (10 mg/kg) to rats with or without verapamil (40 mg/kg, oral) pretreatment (A) on 1st day; (B) on 15th day (n=6). (●) SP (10 mg/kg); (▲) SP with verapamil (40 mg/kg) pretreatment. All values are Mean ± SD. Bars represent the standard deviation. *p < 0.01 when compared to the SP control group. SP, Sitagliptin Phosphate; VER, Verapamil. (Two-way ANOVA followed by Bonferroni post-tests to compare to each column to column).

Table 1. Pharmacokinetic parameters of sitagliptin phosphate (10 mg/kg) following an oral administration of sitagliptin to rats with or without verapamil (40 mg/kg) in SDS and MDS

Parameter	*SDS (n=6)*		*MDS (n=6)*	
	SP (10 mg/kg)	*SP + VER (40 mg/kg)*	*SP (10 mg/kg)*	*SP + VER (40 mg/kg)*
C_{max} (µg/mL)	3.651 ± 0.855	6.120 ± 1.524***	3.953 ± 1.210	7.525 ± 1.550***
AUC_{0-24} (µg h/mL)	25.092 ± 5.320	40.175 ± 6.625***	30.404 ± 6.235	45.266 ± 4.632***
$AUC_{0-\infty}$ (µg h/mL)	41.121 ± 7.740	69.830 ± 10.023***	61.146 ± 8.255	82.068 ± 9.624 ***
t_{max} (h)	0.500 ± 0.000	0.500 ± 0.000^{NS}	0.500 ± 0.000	0.500 ± 0.000^{NS}
$t_{1/2}$ (h)	15.863 ± 4.261	20.935 ± 3.145**	25.569 ± 5.241	39.280 ± 2.547***
MRT (h)	23.954 ± 5.620	28.182 ± 3.251*	54.918 ± 7.158	66.055 ± 4.325***
CL/F (mL/h/kg)	0.103 ± 0.052	0.048 ± 0.016*	0.124 ± 0.055	0.024 ± 0.008*
Vz/F (mL/kg)	3.114 ± 0.563	1.113 ± 0.245**	1.668 ± 0.854	1.602 ± 0.472^{NS}
Vss/F (mL/kg)	2.906 ± 0.400	1.165 ± 0.366***	2.000 ± 0.700	1.460 ± 0.551^{NS}
AB (%)	1.161 ± 0.285	1.530 ± 0.186*	1.654 ± 0.166	2.136 ± 0.269*

SP, sitagliptin phosphate; VER, verapamil; SDS, single dose pharmacokinetic study; MDS, multiple dose pharmacokinetic study; C_{max}, peak plasma concentration; AUC_{0-24}, area under the plasma concentration–time curve from 0 h to 24 h; $AUC_{0-\infty}$, area under the plasma concentration–time curve from 0 h to infinity;; t_{max}, time to reach peak plasma concentration; $t_{1/2}$, terminal half-life; MRT, mean residence time; CL/F, apparent total body clearance or oral clearance; Vz/F, apparent volume of distribution; V_{ss}, apparent volume of distribution at steady state; AB, absolute bioavailability. All values are mean ± SD. ***p < 0.001, **p <0.01, *p < 0.05, ^{NS}P > 0.05 when compared to sitagliptin alone group (two-way ANOVA followed by Bonferroni post-tests to compare to each column to column).

depicted in Figure 2 (A, SDS and B, MDS). The pharmacokinetic parameters of sitagliptin are summarized in Table 1. Verapamil pretreatment significantly affected the pharmacokinetics of sitagliptin in both SDS and MDS. The sitagliptin plasma concentrations were significantly higher in the verapamil pretreated rats than rats treated with sitagliptin alone. The $AUC_{0\text{-}24h}$, C_{max}, AB (%), MRT and $t_{1/2}$ of sitagliptin were significantly increased by the verapamil pretreatment. The mean ± SD $AUC_{0\text{-}24h}$ of sitagliptin was significantly increased from 25.092 ± 5.320 to 40.175 ± 6.625 µg h/mL (in SDS, $p<0.001$) and 30.404 ± 6.235 to 45.266 ± 4.632 µg h/mL (in MDS, $p<0.001$), C_{max} increased from 3.651 ± 0.855 to 6.120 ± 1.524 µg/mL (in SDS, $p<0.001$) and 3.953 ± 1.210 to 7.525 ± 1.550 µg/mL (in MDS, $p<0.001$), AB (%) increased from 1.161 ± 0.285 to 1.530 ± 0.186 (in SDS, $p<0.05$) and 1.654 ± 0.166 to 2.136 ± 0.269 (in MDS, $p<0.05$), MRT increased from 23.954 ± 5.620 to 28.182 ± 3.251 h (in SDS, $p<0.05$) and 54.918 ± 7.158 to 66.055 ± 4.325 h (in MDS, $p<0.001$), $t_{1/2}$ increased from 15.863 ± 4.261 to 20.935 ± 3.145 h (in SDS, $p<0.01$) and 25.569 ± 5.241 to 39.280 ± 2.547 h (in MDS, $p<0.001$). The verapamil effect was not statistically significant on the t_{max} of sitagliptin. The Vz/F, CL/F and Vss/F of sitagliptin were significantly decreased in verapamil pretreated rats when compared to sitagliptin control group. The mean ± SD Vz/F of sitagliptin was decreased from 3.114 ± 0.563 to 1.113 ± 0.245 mL/kg (in SDS, $p<0.01$) and 1.668 ± 0.854 to 1.602 ± 0.472 mL/kg (in MDS, $p>0.05$), CL/F decreased from 0.103 ± 0.052 to 0.048 ± 0.016 mL/kg (in SDS, $p<0.05$) and 0.124 ± 0.055 to 0.024 ± 0.008 mL/kg (in MDS, $p<0.05$), Vss decreased from 2.906 ± 0.400 to 1.165 ± 0.366 mL/kg (in SDS, $p<0.05$) and 2.000 ± 0.700 to 1.460 ± 0.551 mL/kg (in MDS, $p>0.05$).

Effect of verapamil on the intravenous pharmacokinetics of sitagliptin in vivo

The mean plasma concentration versus time curves of sitagliptin after intravenous administration of sitagliptin (5

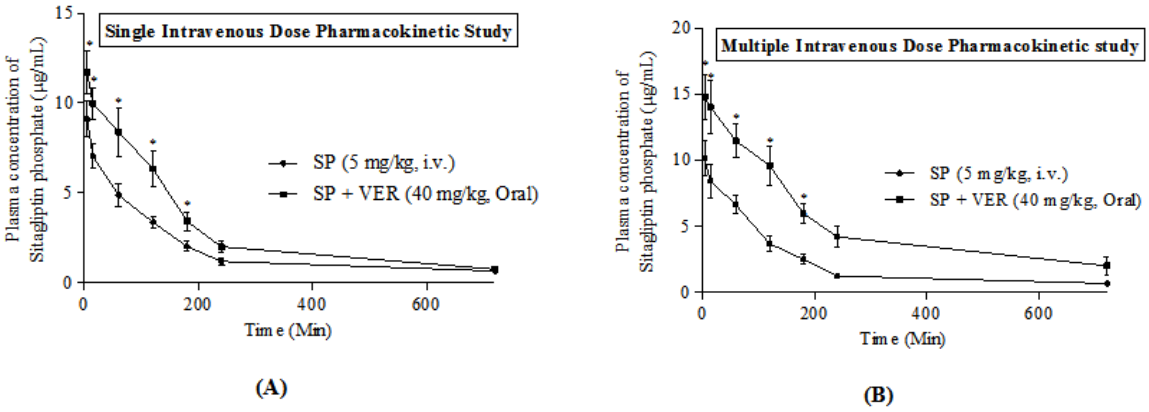

Figure 3. Mean plasma concentration–time profiles of sitagliptin following an intravenous administration of sitagliptin (10 mg/kg) to rats with or without verapamil (40 mg/kg, oral) pretreatment (A) on 1st day; (B) on 15th day (n=6). (●) SP (10 mg/kg); (■) SP with verapamil (40 mg/kg) pretreatment. All values are Mean ± SD. Bars represent the standard deviation. *$p < 0.01$ when compared to the SP control group. SP, Sitagliptin Phosphate; VER, Verapamil. (Two-way ANOVA followed by Bonferroni post-tests to compare to each column to column).

Table 2. Pharmacokinetic parameters of sitagliptin (5 mg/kg) following an intravenous administration of sitagliptin to rats with or without verapamil (40 mg/kg) in SDS and MDS

Parameter	*SDS*		*MDS*	
	SP (5 mg/kg)	*SP + VER (40 mg/kg)*	*SP (10 mg/kg)*	*SP + VER (40 mg/kg)*
C_{max} (µg/mL)	8.657 ± 2.632	11.524 ± 3.415*	10.771 ± 2.400	15.141 ± 3.482**
AUC_{0-24} (µg h/mL)	1318.990 ± 120.451	2079.980 ± 251.362***	1521.115 ± 100.356	3613.370 ± 256.400***
$AUC_{0-\infty}$ (µg h/mL)	1509.766 ± 146.844	2281.145 ± 235.478***	1706.612 ± 154.262	2178.000 ± 325.162***
t_{max} (h)	5.000 ± 0.000	5.000 ± 0.000^{NS}	5.000 ± 0.000	5.000 ± 0.000^{NS}
$t_{1/2}$ (min)	183.225 ± 20.415	201.473 ± 30.487*	189.379 ± 17.255	377.183 ± 40.236**
MRT (h)	263.294 ± 40.855	307.790 ± 50.325*	279.672 ± 30.480	475.332 ± 62.354***
CL/F (mL/h/kg)	0.003 ± 0.0004	0.001 ± 0.002^{NS}	0.0015 ± 0.0001	0.0011 ± 0.0001^{NS}
Vz/F (mL/kg)	0.712 ± 0.250	0.364 ± 0.052***	0.952 ± 0.240	0.436 ± 0.040***
Vss/F (mL/kg)	0.651 ± 0.324	0.276 ± 0.063**	0.865 ± 0.300	0.315 ± 0.050**

SP, sitagliptin phosphate; VER, verapamil; SDS, single dose pharmacokinetic study; MDS, multiple dose pharmacokinetic study; C_{max}, peak plasma concentration; AUC_{0-24}, area under the plasma concentration–time curve from 0 h to 24 h; $AUC_{0-\infty}$, area under the plasma concentration–time curve from 0 h to infinity;; t_{max}, time to reach peak plasma concentration; $t_{1/2}$, terminal half-life; MRT, mean residence time; CL/F, apparent total body clearance or oral clearance; Vz/F, apparent volume of distribution; V_{ss}, apparent volume of distribution at steady state. All values are mean ± SD. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, $^{NS}P > 0.05$ when compared to sitagliptin alone group (two-way ANOVA followed by Bonferroni post-tests to compare to each column to column).

mg/kg) alone and with verapamil (40 mg/kg) pretreatment are shown in Figure 3 (A, SDS and B, MDS). The pharmacokinetic parameters of sitagliptin are presented in Table 2. Verapamil pretreatment was significantly affected the pharmacokinetics of sitagliptin (intravenous) in both SDS and MDS. When sitagliptin was administered with verapamil pretreatment, the plasma concentrations of sitagliptin were significantly higher compared to the sitagliptin control (Fig. 3). The mean ± SD $AUC_{0\text{-}24h}$ of sitagliptin was significantly increased from 1318.990 ± 120.451 to 2079.980 ± 251.362 µg h/mL (in SDS, $p<0.001$) and 1521.115 ± 100.356 to 3613.370 ± 256.400 µg h/mL (in MDS, $p<0.001$), C_{max} increased from 8.657 ± 2.632 to 11.524 ± 3.415 µg/mL (in SDS, $p<0.05$) and 10.771 ± 2.400 to 15.141 ± 3.482 µg/mL (in MDS, $p<0.01$), MRT increased from 263.294 ± 40.855 to 307.790 ± 50.325 h (in SDS, $p<0.05$) and 279.672 ± 30.480 to 475.332 ± 62.354 h (in MDS, $p<0.001$), $t_{1/2}$ increased from 183.225 ± 20.415 to 201.473 ± 30.487 h (in SDS, $p<0.05$) and 189.379 ± 17.255 to 377.183 ± 40.236 h (in MDS, $p<0.01$). Verapamil had no significant effect on the t_{max} of sitagliptin in both SDS and MDS. The Vz/F, CL/F and Vss/F of sitagliptin were significantly decreased in verapamil pretreated rats when compared to sitagliptin control group. The mean ± SD CL/F of sitagliptin was decreased from 0.003 ± 0.0004 to 0.001 ± 0.002 mL/kg (in SDS, $p>0.05$) and 0.0015 ± 0.0001 to 0.0011 ± 0.0001 mL/kg (in MDS, $p>0.05$), Vz/F decreased from 0.712 ± 0.250 to 0.364 ± 0.052 mL/kg (in SDS, $p<0.001$) and 0.952 ± 0.240 to 0.436 ± 0.040 mL/kg (in MDS, $p<0.001$), Vss/F decreased from 0.651 ± 0.324 to 0.276 ± 0.063 mL/kg (in SDS, $p<0.01$) and 0.865 ± 0.300 to 0.315 ± 0.050 mL/kg (in MDS, $p<0.01$).

Effect of verapamil on the intestinal transport of sitagliptin ex vivo

The apparent permeability coefficient (P_{app}), net efflux

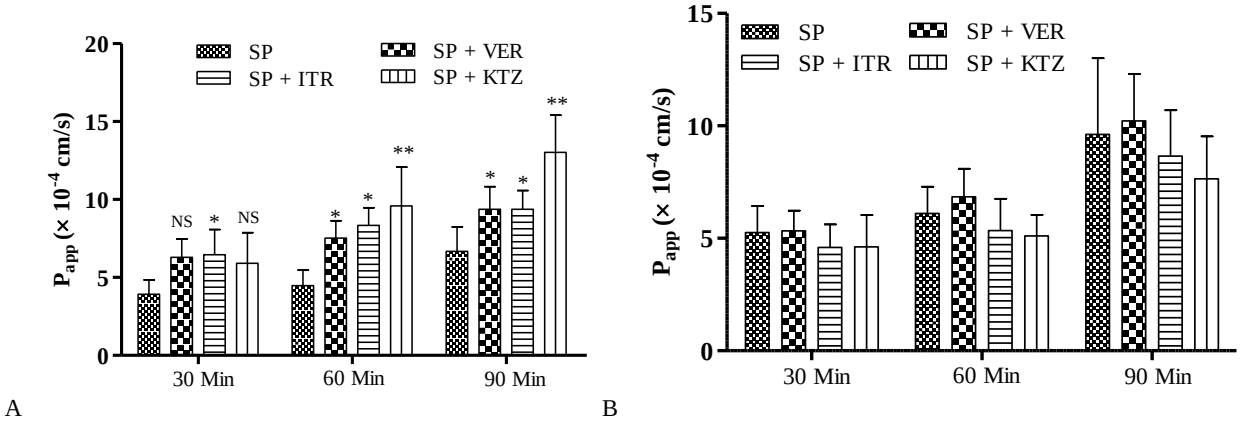

Figure 4. The effect of verapamil on the apparent permeability coefficient (P_{app}) of sitagliptin phosphate from (A) mucosal to serosal side of non-everted gut sacs; (B) serosal to mucosal side of everted gut sacs *ex vivo* (n=3). All values are mean ± SD. $^{**}p<0.01$, $^{*}p<0.05$, $^{NS}P>0.05$ when compared to sitagliptin alone group (two-way ANOVA followed by Bonferroni post-tests to compare to each column to column). SP, Sitagliptin Phosphate; VER, Verapamil; ITR, Itraconazole; KTZ, Ketoconazole.

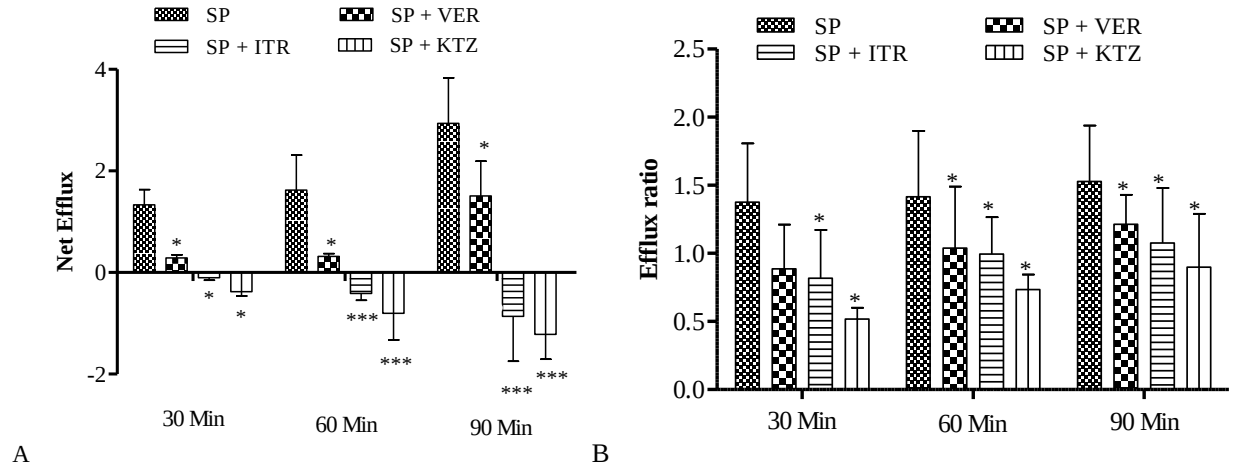

Figure 5. The effect of verapamil on the (A) Net efflux and (B) efflux ratios of sitagliptin using rat gut sacs *ex vivo* (n=3). All values are mean ± SD. $^{***}p<0.001$, $^{*}p<0.05$, $^{NS}P>0.05$ when compared to sitagliptin alone group (two-way ANOVA followed by Bonferroni post-tests to compare to each column to column). SP, Sitagliptin Phosphate; VER, Verapamil; ITR, Itraconazole; KTZ, Ketoconazole.

and efflux ratio values were calculated to evaluate the effect of verapamil on the P-gp mediated intestinal absorption of sitagliptin using *non-everted rat gut sac* absorption models are shown in Figures 4 & 5. The results indicated that known P-gp inhibitors (itraconazole and ketoconazole) and verapamil significantly affected the P_{app} of sitagliptin from mucosal to serosal (M-to-S), net efflux and efflux ratio. But the effect was statistically not significant on the P_{app} of sitagliptin from serosal to mucosal (S-to-M) when compared to sitagliptin alone. The sitagliptin mean ± SD P_{app} from M-to-S increased significantly from 4.479 ± 0.989 ($\times 10^{-4}$ cm/s) to 7.519 ± 1.122 ($\times 10^{-4}$ cm/s) and 6.675 ± 1.567 ($\times 10^{-4}$ cm/s) to 9.381 ± 1.445 ($\times 10^{-4}$ cm/s) with verapamil at 60 and 90 min, respectively. The mean ± SD net efflux of sitagliptin was decreased from 1.623 ± 0.690 to 0.318 ± 0.056 (at 60 min, $P<0.05$) and 2.938 ± 0.893 to 1.509 ± 0.682 (at 90 min, $p<0.05$) in the presence of verapamil. Verapamil was also significantly decreased the mean ± SD efflux ratio of sitagliptin from 1.415 ± 0.483 to 1.038 ± 0.452 ($p<0.05$) and 1.529 ± 0.409 to 1.213 ± 0.218 ($p<0.05$) at 60 and 90 min, respectively.

Effect of verapamil on the intestinal permeability of sitagliptin in situ

The effective permeability coefficient (P_{eff}) and intestinal absorption rate constant (K_a) values were determined to further evaluate the influence of verapamil on the P-gp mediated intestinal permeation of sitagliptin using *single pass intestinal perfusion* models are depicted in Figure 6. The results showed that the P-gp inhibitors (itraconazole and ketoconazole) and verapamil significantly increased the P_{eff} and K_a of sitagliptin when compared to sitagliptin alone. The mean ± SD P_{eff} of sitagliptin was significantly increased from 0.810 ± 0.050 ($\times 10^{-3}$ cm/s) to 1.986 ± 0.514 ($\times 10^{-3}$ cm/s) and 1.163 ± 0.150 ($\times 10^{-3}$ cm/s) to 2.739 ± 0.452 ($\times 10^{-3}$ cm/s) in the presence of verapamil at 60 and 90 min, respectively. The sitagliptin mean ± SD K_a was increased from 0.102 ± 0.025 to 0.187 ± 0.051 (at 60 min, $p<0.05$) and 0.113 ± 0.032 to 0.196 ± 0.045 (at 90 min, $p<0.05$) with verapamil.

DISCUSSION

Oral administration is the most common route for drug administration. Orally-administered drugs have to cross the gastrointestinal epithelial cell membrane and are then transported into the port vein to the liver and eventually into the systemic circulation to exhibit their pharmacological effects [48]. P-gp and CYP3A4 are constitutively expressed in intestinal epithelial cells and form a significant barrier to the absorption of some orally administered drugs like sitagliptin and verapamil. Moreover, P-gp may act with CYP3A4 to increase the metabolism of sitagliptin in the intestine [49, 50]. Previous studies reported that verapamil inhibited the P-gp and CYP3A4-mediated metabolism of levobupivacaine, lidocaine [29], imipramine [30], doxorubicin [51], atorvastatin [31], carbamazepine [52], simvastatin, nilotinib [32], cerivastatin [33], darifenacin, ethosuximide [34], cyclosporine A [53], prazosin [54], digoxin [55], quinine, quinidine and zolpidem [35]; thereby increased their plasma concentrations. In the present study also, the plasma concentration of sitagliptin was significantly increased in SDS and MDS may be due to inhibition of P-gp and CYP3A4.

Risperidone is a substrate of P-gp. The C_{max} of risperidone was significantly increased by 1.69-fold with verapamil, a strong P-gp inhibitor in male healthy volunteers [56]. Another study revealed that the C_{max} of aliskiren (P-gp substrate) was increased by 1.92-fold when co-administered with verapamil (P-gp inhibitor) in healthy participants [57]. In the present study, verapamil pretreatment significantly increased the C_{max} of sitagliptin by 1.67 and 1.90-folds in SDS and MDS, respectively. The C_{max} of sitagliptin was increased by 1.33-fold (SDS) and 1.40-folds (MDS) after intravenous administration of sitagliptin with or without oral

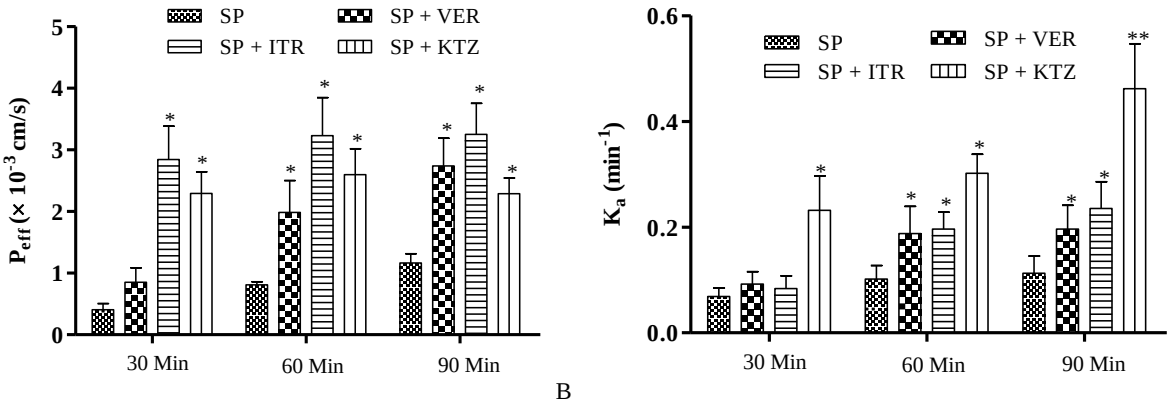

Figure 6. The effect of verapamil on the (A) effective permeability coefficient (P_{eff}) and (B) intestinal absorption rate constant (K_a) of sitagliptin in the presence or absence of verapamil using rat gut sacs *in situ* (n=3). All values are mean ± SD. $^{**}p < 0.01$, $^{*}p < 0.05$, $^{NS}P > 0.05$ when compared to sitagliptin alone group (two-way ANOVA followed by Bonferroni post-tests to compare to each column to column). SP, Sitagliptin Phosphate; VER, Verapamil; ITR, Itraconazole; KTZ, Ketoconazole.

pretreatment of verapamil. Verapamil is a P-gp inhibitor may increase the exposure of afatinib (P-gp substrate) when co-administered with afatinib. Aprepitant is a CYP3A4 substrate. Coadministration of daily oral aprepitant (230 mg, or 1.8 times the recommended single dose) with verapamil (moderate CYP3A4 inhibitor) increased the aprepitant AUC 2-fold [58]. The total AUC of risperidone was increased 1.59-fold by verapamil (strong P-gp inhibitor) in male healthy volunteers [56]. In the present study also the AUC_{0-24} of sitagliptin was significantly increased by 1.60-fold (SDS) and 1.48-fold (MDS) with verapamil pretreatment. The AUC_{0-24} of sitagliptin was also increased by 1.57-fold (SDS) and 2.37-fold (MDS) after intravenous administration of sitagliptin with verapamil pretreatment.

Aliskiren is a substrate for P-gp transporter: The t_{max} of aliskren was not affected significantly when co-administer with verapamil (P-gp inhibitor) in healthy participants [57]. Erik et al. (2014) reported that the t_{max} of fexofenadine (P-gp substrate) was not significantly affected by the concomitant use of verapamil in pigs [59]. Verapamil pretreatment had no significant effect on the t_{max} of sitagliptin in the present study. Colchicine is a P-gp and CYP3A4 substrate and verapamil is a P-gp and CYP3A4 inhibitor. Half-life of colchicine in the serum was found to be 8-fold higher than the normal when used concomitantly with verapamil [60]. In the present study, the sitagliptin half life was 1.31-fold (SDS) and 1.53-fold (MDS) higher in verapamil pretreatment group compared to sitagliptin control group. The half life of sitagliptin was increased significantly after intravenous administration in both SDS and MDS. Verapamil increased the MRT of moxidectin from 17.7 ± 2.56 to 19.7 ± 4.24 days in sheep [61]. In another study, the MRT of irinotecan was increased 1.42-fold by verapamil in rats [62]. The MRT of sitagliptin was significantly increased by 1.17-fold (SDS) and 1.20-fold (MDS) in the present study. The same results were observed in the intravenous pharmacokinetic study also.

Verapamil inhibits the CYP3A4 metabolism and decreases clearance of imipramine [30], Doxorubicin [51], Theophylline [63] and Caffeine [64]. Paclitaxel is a substrate of P-gp. The clearance of paclitaxel was decreased 0.5-fold by verapamil (P-gp inhibitor) in women with breast cancer [65]. The clearance of Polyphyllin I was significantly decreased 0.17-fold with verapamil treatment [66]. Daunomycin clearance was decreased 0.10-fold when co-administered with verapamil in rats [67]. In the present study also, verapamil significantly decreased the clearance of sitagliptin by 0.33-fold (SDS) and 0.19-fold (MDS). The clearance of sitagliptin was significantly decreased by 0.33-fold (SDS) and 0.73-fold (MDS) after intravenous administration of sitagliptin in verapamil pretreated rats. The Vz/F of ivermectin (P-gp substrate) was significantly decreased by 0.58-fold with verapamil in sheep [68]. The Vz/F of sitagliptin was significantly decreased in the present both (oral and intravenous) studies.

The Vss/F of paclitaxel (P-gp substrate) was significantly decreased by 0.49-fold when co-administered with verapamil (P-gp inhibitor) in women with breast cancer [65]. Irinotecan Vss/F was also decreased by 0.23-fold when concomitantly administered with verapamil in rats [62]. In the present study also, the Vss/F of sitagliptin was significantly decreased by 0.40-fold (SDS) after oral administration of sitagliptin to verapamil pretreated rats. In intravenous study also, sitagliptin Vss/F was significantly decreased. Polyphyllin I (PPI), one of the steroidal saponins in Paris polyphylla, is a promising natural anticancer candidate but its oral bioavailability is poor due to P-gp. Verapamil was greatly enhances the bioavailability of PP1 through the inhibition of P-gp [66]. The absolute bioavailability of paclitaxel was increased 1.77-fold with verapamil (P-gp inhibitor) in rats [69]. Another study reported that the irinotecan bioavailability was increased by 4.33-fold with verapamil co-administration in rats [62]. The oral bioavailability of sitagliptin was significantly increased by 1.31-fold (SDS) and 1.29-fold (MDS) in rats pretreated with verapamil.

FB2 is a promising Abl/Src dual tyrosine kinase inhibitor. It bioavailability is poor due to the P-gp mediated active efflux and first-pass metabolism in the rat intestine. The P_{app} (A-B) of FB2 was increased 28.39-fold and the P_{app} (B-A) of FB2 was decreased 0.86-fold with 100µM verapamil in MDCK-MDR1 cell model. The efflux ratio was decreased from 111.71 to 3.37 and the net efflux was decreased from 46.50 to 2.64 with 100µM verapamil in the same model [70]. Verapamil also decreased the efflux ratio of etoposide from 4.01 to 1.33 in everted rat gut sac model [71]. In the presence of 20 µM cyclosporin A, the efflux ratio value of aconitine was reduced from 8.17 to 0.99 by increasing the AP-BL and reducing the BL-AP AC fluxes. Verapamil at 100µM exhibited an effect similar to that of cyclosporin A; however, the decrease in the BL-AP direction was greater, and the increase in the reverse direction was lower. The efflux ratio value of AC at 2 h decreased to 1.31 upon co-incubation with verapamil [72]. In the present study, the P_{app} (M-to-S) of sitagliptin was significantly increased and the P_{app} (S-to-M) of sitagliptin was slightly increased (statistically insignificant) with 50µg/mL verapamil in rat gut sac model. The efflux ratio was decreased from 1.529 to 1.213 and the net efflux was decreased from 2.938 to 1.509 with 50µg/mL verapamil in the same model.

The coperfusion of verapamil (100 µM) and aconitine (5µM) resulted in a significant increase in intestinal permeability (12.9-fold) of aconitine in situ single-pass perfusion experiment in the rat ileum [72]. The effective intestinal permeability (P_{eff}) of fexofenadine (P-gp substrate) was increased 5-fold with verapamil, a P-gp inhibitor in porcine model [73]. In the present study, the P_{eff} of sitagliptin was significantly increased by 2.35-fold with verapamil in situ single-pass perfusion experiment in the rat ileum. The intestinal absorption rate constant (Ka) of fexofenadine (P-gp substrate) was increased by 1.76-fold with verapamil, a P-gp inhibitor in porcine model [73]. The Ka of paclitaxel was increased 1.17-fold with verapamil (P-gp inhibitor) pretreatment in rats [69]. In the present study also, the K_a of

sitagliptin was increased by 1.73-fold with verapamil in the same model.

CONCLUSION

Verapamil pretreatment significantly increased the bioavailability and affected the pharmacokinetics of sitagliptin in rats might be through the inhibition of P-gp and CYP3A4. Further studies are needed to confirm this interaction at cellular level using P-gp and CYP3A4 over expressed cell lines and in human subjects.

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CONFLICT OF INTEREST

The authors declare that this research does not have any conflict of interest with anyone or any Institute.

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