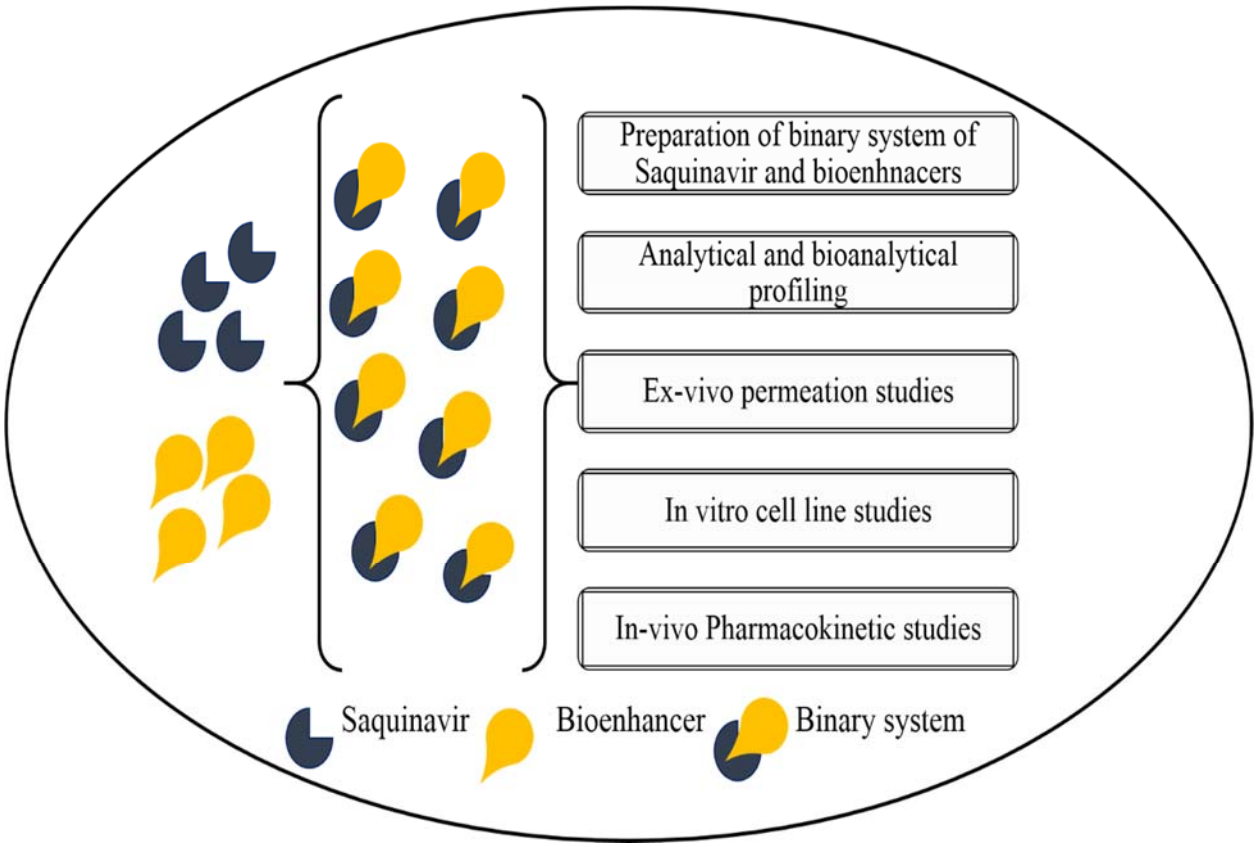
Graphical presentation of Chapter V

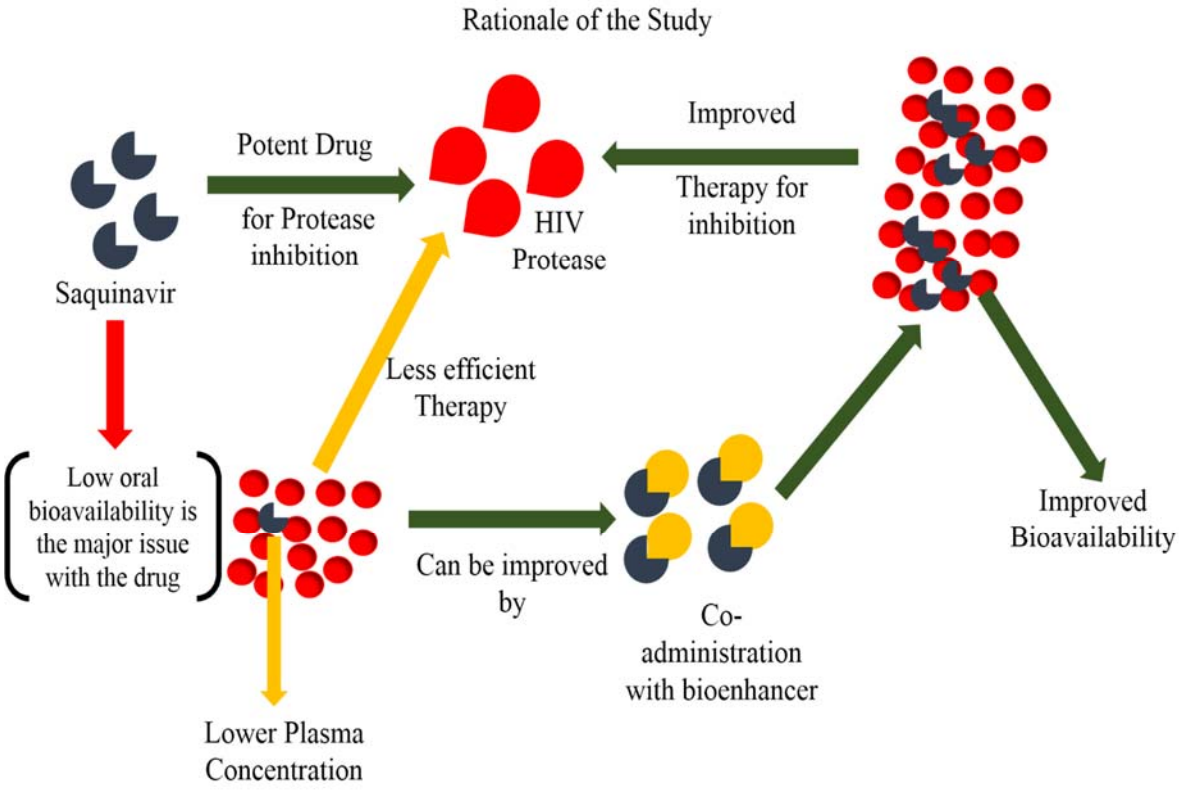


5.0. Introduction

Saquinavir (SQU) was the first drug candidate which gets approved as protease inhibitior for the HIV infection treatment. In the last decade, SQU has become a chief component of highly active antiretroviral therapies (1, 2). SQU having molecular weight 670.8 with partition coefficient (LogP) 4.1. It is class IV drug in the Biopharmaceutical Classification System (BCS) having low permeability and low solubility (3). It is usually administered in the salt form mesylate, the solubility of the SQU is pH-dependent (4). Apart from having the not very much favorable physicochemical properties for permeability, it is also reported that efflux protein (P-gp) also hampers the transport of SQU from the gut wall (5, 6). Inspite of this SQU is also metabolized by both human hepatic and small intestinal enzymes, which also further results in low oral bioavailability (4%) and displays wide inter-individual variability (7, 8).

The main purpose of this study was to study the effect of natural compounds on its bioavailability. In this work, binary systems of SQU with the natural bioenhancers were prepared using physical mixing method. The effect of these compounds were studied using different sophisticated experimental protocols. Firstly the compatability was tested for the three used bioenhancers quercetin (QU), silibinin (Sil), Luteolin (LT). Oral uptake was studied by analyzing the transport of SQU across the human colorectal adenocarcinoma cell line (Caco-2) cell lines. Permeation through the goat intestine tissue was also studied. Pharmacokinetic analysis was also performed in rabbits by administered SQU with different bioenhancers in the form of suspension, and the whole analytical studies for the estimation of SQU in different studies were conducted using LC-MS. In the compatibility

studies, bioenhancers found to be showing no or minimal interaction with the SQU. Permeation in the intestinal tissue of goat was significantly increased as compared to the plain drug. The transport of SQU across the Caco-2 cell lines also found to be improved than the plain drug. Pharmacokinetic study showed there was increase in the Cmax by approx. 3 folds using the different bioenhancers. AUC was also found to be increase by more than 2 folds with the each bioenahncer. The maximum oral uptake enhancement was found with the QU following by the Sil and then LT.



5.1. Materials and Methods

5.1.1. Materials

Saquinavir (SQU) mesylate was obtained as a gift sample from Aurbindo Pvt Ltd, Mumbai. Quercetin, Silibinin and Luteolin was purchased from Sigma-Aldrich. Caco-2 cell lines were obtained from NCCS Pune. All other chemicals and reagents used in the study were of analytical grade and were commercially obtained.

5.1.2. Analytical and Bio-analytical Method development and Validation

5.1.2.1. LC-MS method for estimation of SQU in different studies

The samples were analyzed using ekspertTM ultraLC with ekspertTM ultraLC 100 pump system (eksigent-AB Sciex, USA) coupled with 3200 QTRAP mass spectrometer (AB Sciex, USA). 20 µL of each sample was injected. The autosampler system (ekspertTM ultraLC 100 XL, eksigent-AB Sciex, USA) was tempered to 4°C equipped with column oven (ekspertTM ultraLC 100, eksigentAB Sciex, USA) fixed at 40°C. Chromatographic elution of analyte was achieved using a Phenomenax C18 5µm (250*4.6) mm column at a flow rate of 0.5 mL min^{-1} for having run time 8 mins. The composition of mobile phase was eluent a (20 mM ammonium formate buffer) and eluent b (acetonitrile) was in 55:45 % v/v. The SQU was quantified in the multiple reaction monitoring (MRM) mode at ion transitions m/z 671.32 $\longrightarrow$ 432.80. LC-MS Conditions for analysis were in the positive ion mode with a potential of 5.5 kV applied on the electro spray ionization needle. The ionization source temperature was 600 °C. The curtain gas (CUR) was at 25.0 psi, the nebulizer source gas 1 at 50.0 psi and the turbo ion source gas 2 at 50.0 psi was utilized. The optimized declustering potential and entrance potential were 60.0 V and 5.6 V

respectively. SQU fragmentation was achieved by collision activated dissociation (CAD) with nitrogen gas. The collision gas pressure was fixed at 2.0 psi for MRM quantitation. The collision energy 22.0 V and collision cell exit potential 3.0 V were optimized. Dwell time 200 ms was used. Chromatographic responses were found to be linear over an analytical range of 25–2000 ng/ml and found to be quite satisfactory and reproducible. Extraction efficiency was greater than 88 %. Accuracy data in the present study ranged from 98.42 to 100.07 % indicates that there was no interference from endogenous plasma or other components. Inter-day as well as intra-day replicates of SQU, gave R.S.D. below 5.0 (should be less than 15 according to CDER guidance for Bio-analytical Method Validation), revealed that proposed method is highly precise and accurate.

5.1.2.1.1. Preparation of Stock Solutions, Calibration and Validation Standards

An accurately weighed amount of SQU was transferred into a 10 mL calibrated flask and dissolved in 5 mL of mobile phase. The resulting solution were completed to the mark with mobile phase obtaining stock standard solution containing 1000 µg/mL. Stock solution were then further diluted with mobile phase to obtain the working standard solutions at concentrations over the range of 25–2000 ng/mL. Seven calibration standards were prepared at concentrations of 25, 50, 100, 250, 500, 1000 and 2000 ng/mL. Validation standards were similarly prepared at levels of 100, 250, 500 and 1000 ng/mL.

5.1.2.1.2. Plasma sample preparation

Plasma extraction

SQU was extracted from plasma by protein precipitation, as shown in Figure 5.1. The protein extraction procedure was identical for the all samples (Standard validation samples,

in-vivo samples). In 100 µL of plasma samples 400 µL of acetonitrile was added and then vortexed for 5 mins. Following the vortexing, the samples were centrifuged at 9000 rpm for 12 mins. The supernatant was then transferred in to fresh micro-centrifuged tubes and adequately diluted with the mobile phase prior injecting in to LC-MS.

Extraction efficiency

The extraction efficiency was calculated by adding known amount of SQU in plasma. The known amount of samples were injected in LC-MS and then the peak area of the samples with plasma and without plasma were compared.

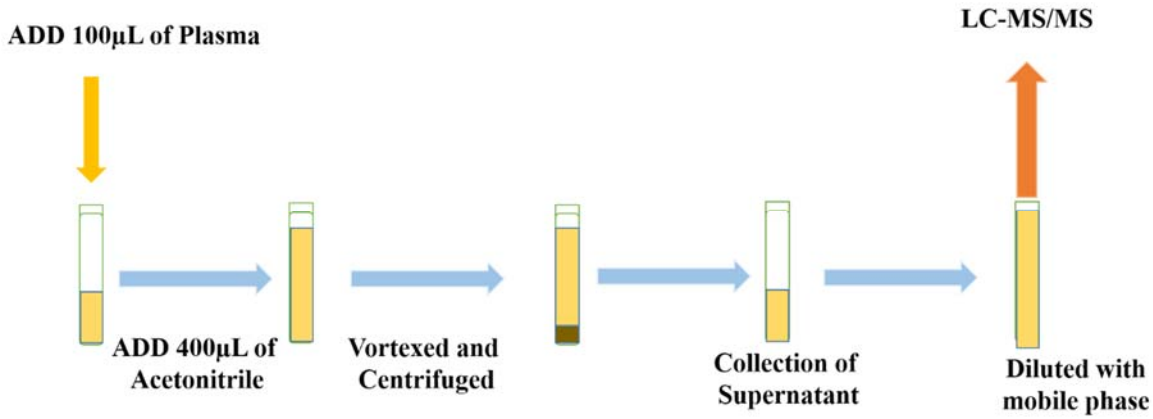


Figure 5.1 Plasma Extraction Procedure for SQU

5.1.2.1.3. Validation

The selectivity of the developed method for plasma samples was investigated by comparing chromatograms of blank plasma, standard and sample were compared as shown in Figure 5.2. Response function in proposed method four sets of calibration curve were plotted between area and different concentrations of SQU and on these four different series regression analysis was performed and series with best coefficient of determination was selected. The recovery study which is the most critical parameter in method validation

requires an extra precautions during study and interpretation of recovery results. Therefore, the results of accuracy studies were interpreted and represented in the β-expectation tolerance limits. In addition to these parameters, risk profile has also been studied to know the future application of the method. Limit of detection and quantification represents the sensitivity of the method.

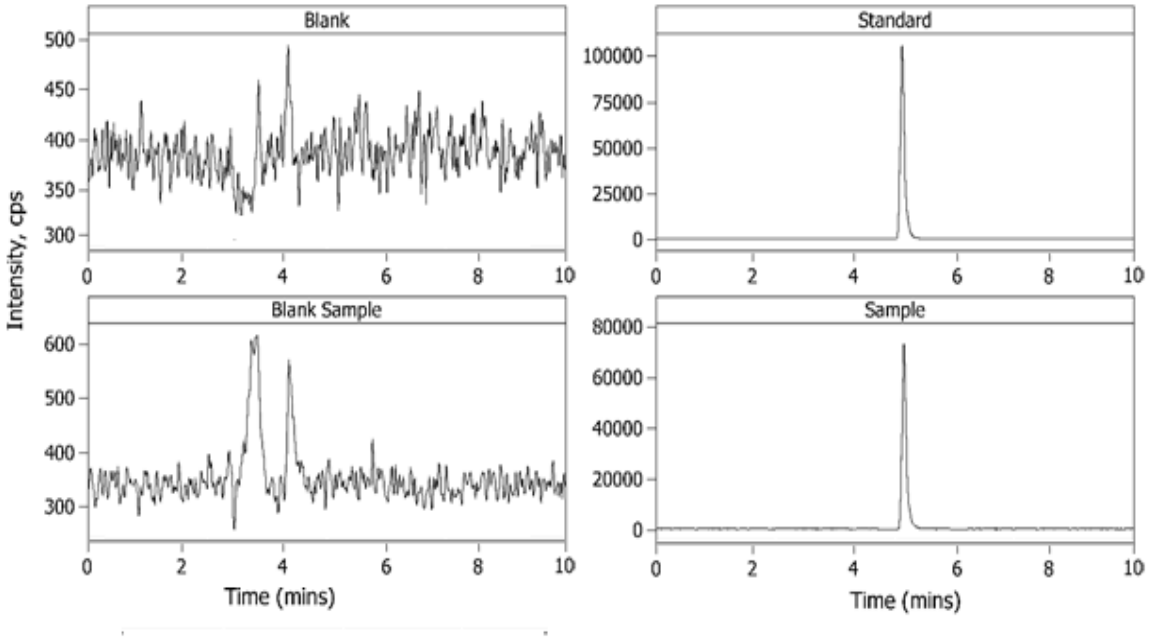


Figure 5.2 Chromatogram of Blank, Blank plasma, Standard and Plasma Sample

5.1.3. Preparation of Physical Mixture of saquinavir and bioenhancers

SQU–QU, SQU–Sil, SQU–LT binary system were prepared at the five different weight ratio levels 5:0.5, 5:1, 5:1.5, 5:2, 5:2.5, 5:3 w/w. Physical mixing method was adapted for the binary system, in this the required amounts were accurately weighed and were sealed in a polythene bag and were blended for 30 minutes. All the prepared mixture were tested for uniformity of content using analytical method.

5.1.3.1. Compatibility studies of binary system

SQU–QU, SQU–Sil, SQU–LT binary system were characterized for their compatibility in different ratios. In this FTIR and DSC were used. The FTIR spectrum of the SQU and binary mixtures were recorded and were interpreted for any physical interaction. Similarly the DSC chromatogram for the SQU and binary mixtures were recorded and were interpreted for physical interaction.

5.1.3.1.1. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR transmission spectra of a pure SQU, QU, Sil, LT, SQU–QU, SQU–Sil and SQU–LT and binary systems of PM method were obtained using Avatar™ 360 E.S.P™ FTIR spectrometer, Thermo Nicolet Corp., Madison, WI, USA. Samples were mixed with dry KBr and converted to a fine powder before compressing into KBr disc. Each sample was scanned for 16 times over a wave number region of 500–4000 cm^{-1}. The characteristic peaks of SQU were observed and compared with the spectrum of binary mixtures to check any physical interaction among the SQU and bioenhancers.

5.1.3.1.2. Differential Scanning Calorimetry (DSC)

DSC thermograms were recorded for SQU, QU, Sil and LT and their respective binary systems using Mettler-Toledo, Schwerzenbach, Switzerland. During the recording of thermograms an empty aluminium pan was used as the reference for the samples. The rate of heating was kept 10 °C min-1.

5.1.4. *Ex-vivo* permeation studies (Franz diffusion cell)

Diffusion cell with an area of 3.80 cm^2 having donor and acceptor compartment were used. The intestinal tissue of goat was collected from local slaughter house and was stored in

normal saline. Tissue was cleaned and intestinal content was removed by a slow infusion of normal saline and air before setting up for the experimentation. The prepared binary systems using the physical mixture method was assessed for the permeation using the intestinal tissue of the goat which is very much morphological similar to human intestine tissue. In this experimentation the tissue was mounted in between the donor and acceptor chamber in the diffusion cell. The donor compartment was filled with the sample (SQU and binary systems) having concentration (5 mg/ml) and acceptor cell was filled up with the simulated intestinal fluid (SIF) with aid to continuous stirring and then samples of the solution (1 mL) were withdrawn at different time intervals (0, 15, 30, 60, 120, 180, 240, 360, 480 min) and filtered using the syringe filters. The SQU amount was quantified using validated LC-MS method for the SQU. The amount of the drug permeated through the tissue was determined. The amount permeated was plotted against the different time points. The permeability coefficients (Peff) and permeation enhancement ratios (Peff values) were calculated as per Eq. 5.1 and 5.2 respectively. The results of experiments performed (n = 3).

$$Peff\,(cm/\sec) = \frac{dQ/dt}{A*Cd} \tag{5.1}$$

Where, A = the surface area, dQ/dt = amount of drug permeated per unit time at steady state, Cd = donor drug concentration.

$$R = \frac{Papp(sample)}{Papp(control)} \tag{5.2}$$

5.1.5. Cellular uptake and Transport of SQU across the Caco-2 cell monolayers

Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with high glucose, fetal bovine serum, penicillin, and streptomycin at 37 °C and 5% CO_2.

The Caco-2 cells were seeded at the density of approximately 1×10^5 cells per well in to a 12-well transwell polycarbonate cell culture inserts having 12-mm diameter and 3-μm pore size purchased from Costar®, (Corning Costar Co, Cambridge, MA, USA). The cells were cultured and used after 25 days so that full maturation and confluence must obtained. It also includes P-glycoprotein (P-gp) expression and the formation of tight junctions in the cell monolayer. For the very first week of the culturing the medium was replaced every other day. After 7 days the medium was changed daily. The basolateral (BL) and apical (AP) compartments contained 1.5 and 0.5 mL of culture medium, respectively.

Cellular uptake

In trailing the cellular uptake of SQU and binary systems the fluorescein labeled samples were prepared using rhodamine B using anti-solvent precipitation method. In this study, cells were treated with test solutions up to 1 hr at 37^{o}C. After 0, 0.5 and 1 hr samples were removed from the well and cell line was washed using HBBS. Cells were then treated with paraformaldehyde solution for 10 min and then stain with 5 μg/ml solution of 4,6-diamidino-2-phenylindole (DAPI) and then images were recorded under the confocal laser scanning microscope (CLSM) (Olympus Japan) (9, 10).

Transport Study

The trans-epithelial electric resistance (TEER) values were measured using Millicell®-ERS (Millipore, Bedford, MA, USA) before and immediately after the transport studies to evaluate the integrity of the Caco-2 cell monolayers. The transport medium (HBSS) resistance was subtracted from the TEER value considering it as the background resistance. The cell monolayers having TEER values below 300 $\Omega \cdot cm^2$ were excluded from the study design. The study design for SQU and binary system has been shown in Table 5.1, 5.2, 5.3 5.4, 5.5. The cell monolayer was equilibrated at 37 °C with warm HBSS (37 °C) for 30 min before starting the experimentation (transport studies). After 30 mins, the HBSS was removed and the sample solutions (SQU, SQU-QU, SQU-Sil, and SQU-LT containing 10 μM/mL SQU in HBSS) were added to the AP compartments. Sample (100 μL) were withdrawn from the receiver chamber at different time points (30, 60, 90, 120, 240, 480 mins) the equal volume (100 μL) of fresh HBSS was added to the chamber so as to maintain a constant volume.

The drug concentration in the samples was determined by validated LC-MS method as described above. The experiments were performed in triplicate. The apparent permeability coefficient (Papp, cm/s) was calculated using the Eq. 5.3

$$Papp(cm/\sec) = \frac{dQ/dt}{A*C0} \tag{5.3}$$

Where, dQ/dt is the transport rate, C0 is the initial drug concentration on the apical side, and A is the surface area of the membrane filter (1.12 cm^2).

Cell Viability estimation

At the end of the experimentation, cell viability for the cell lines were checked using tryphan blue (15) staining technique. The viability of cells treated with SQU and binary systems were greater than 90% and not significantly different from the control cells. Trypan blue chromophore is negatively charged so it does not react with the membrane until it is damaged, so all the cells that exclude the dye are viable

Table 5.1 Study Design for Caco-2 cell lines (Plate No. 1)

A1 **HBSS (Blank)**	**B1** 10 µM SQU	**C1** SQU:QU (5: 0.5)	**D1** SQU:QU (5: 1)
A2 **HBSS (Blank)**	**B2** 10 µM SQU	**C2** SQU:QU (5: 0.5)	**D2** SQU:QU (5: 1)
A3 **HBSS (Blank)**	**B3** 10 µM SQU	**C3** SQU:QU (5: 0.5)	**D3** SQU:QU (5: 1)

Table 5.2 Study Design for Caco-2 cell lines (Plate No. 2)

A1 SQU:QU (5: 1.5)	**B1** SQU:QU (5:2)	**C1** SQU:QU (5: 2.5)	**D1** SQU:QU (5: 3)
A2 SQU:QU (5: 1.5)	**B2** SQU:QU (5:2)	**C2** SQU:QU (5: 2.5)	**D2** SQU:QU (5: 3)
A3 SQU:QU (5: 1.5)	**B3** SQU:QU (5:2)	**C3** SQU:QU (5: 2.5)	**D3** SQU:QU (5: 3)

Table 5.3 Study Design for Caco-2 cell lines (Plate No. 3)

A1 SQU:Sil (5: 0.5)	**B1** SQU:Sil (5:1)	**C1** SQU:Sil (5: 1.5)	**D1** SQU:Sil (5: 2)
A2 SQU:Sil (5: 0.5)	**B2** SQU:Sil (5:1)	**C2** SQU:Sil (5: 1.5)	**D2** SQU:Sil (5: 2)
A3 SQU:Sil (5: 0.5)	**B3** SQU:Sil (5:1)	**C3** SQU:Sil (5: 1.5)	**D3** SQU:Sil (5: 2)

Table 5.4 Study Design for Caco-2 cell lines (Plate No. 4)

A1 SQU:Sil (5: 2.5)	**B1** SQU:Sil (5:3)	**C1** SQU:LT (5: 0.5)	**D1** SQU:LT (5: 1)
A2 SQU:Sil (5: 2.5)	**B2** SQU:Sil (5:3)	**C2** SQU:LT (5: 0.5)	**D2** SQU:LT (5: 1)
A3 SQU:Sil (5: 2.5)	**B3** SQU:Sil (5:3)	**C3** SQU:LT (5: 0.5)	**D3** SQU:LT (5: 1)

Table 5.5 Study Design for Caco-2 cell lines (Plate No. 5)

A1 SQU:LT (5: 1.5)	**B1** SQU:LT (5:2)	**C1** SQU:LT (5: 2.5)	**D1** SQU:LT (5: 3)
A2 SQU:LT (5: 1.5)	**B2** SQU:LT (5:2)	**C2** SQU:LT (5: 2.5)	**D2** SQU:LT (5: 3)
A3 SQU:LT (5: 1.5)	**B3** SQU:LT (5:2)	**C3** SQU:LT (5: 2.5)	**D3** SQU:LT (5: 3)

5.1.6. In vivo pharmacokinetic study in rabbits

5.1.6.1. Animal preparation for *In-vivo* studies

In vivo pharmacokinetic study performed in New Zealand white rabbits (2-3 Kg) provided by the animal house at Pharmacy Department, The Maharaja Sayajirao university of Baroda details has been provided in Table 5.6. All the experiments were performed under guidelines approved by the Institutional Animal Ethics Committee (IAEC Registration number 404/01/a/CPCSEA). The rabbits were given free access to food and water. The rabbits were fasted for 12 h prior to the experiments with free access to water.

Table 5.6 Animals detail used for pharmacokinetic Studies

Species/Common name	New Zealand White Rabbits
Age/Weight/Size	3-4 months/2 – 3 Kg
Gender	Either sex
Number of animals to be used	15
Proposed source of animals	CPCSEA approved breeding facility

5.1.6.2. Dosing and sampling

In pharmacokinetics study rabbits were divided in to five groups (3 per group). The SQU (oral), SQU-QU, SQU-Sil, SQU-LT were orally administered as suspension, at a dose of 50 mg/kg. The rabbits had free access to water during the entire experiment. Blood samples of 600 μL were collected at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12 and 24 hr after dosing via the orbital venous plexus using isoflurane as anesthesia. The whole blood was collected in

heparinized tubes, and the plasma from the sample was separated by centrifugation at 9000 rpm for 12 min and stored at -20 °C prior to analysis by LC-MS.

5.1.6.3. Analysis of plasma samples

The concentration of SQU in the plasma samples were estimated by LC-MS. The extraction for the analysis of sample was carried out using protein precipitation method. The extraction procedure was identical for the all samples (Standard validation samples). In 100 µL of plasma samples 400 µL of acetonitrile was added and then vortexed for 5 mins. Following the vortexing, the samples were centrifuged at 9000 rpm for 12 mins. The supernatant was then transferred in to fresh micro-centrifuged tubes and adequately diluted with the mobile phase prior injecting in to LC-MS. The drug concentrations in the samples were calculated.

5.1.6.4. Pharmacokinetic and Statistical analysis

The drug concentration obtained from the LC-MS analysis was used to derive pharmacokinetic parameters. PK solver was used to determined parameters maximum plasma concentration (Cmax), time for maximum plasma concentration (Tmax), Area under the curve (AUC) etc. All other mathematical calculations were done using the Microsoft Excel 2013.

5.2. Results and Discussion

5.2.1. LC-MS Method for estimation of SQU

5.2.1.1. Method Development and Optimization

Optimization of the chromatographic conditions is the most critical step having a very specific aim to achieve symmetrical peak shapes with short chromatographic analysis time also having high sensitivity and selectivity. Ion transitions at m/z 671.34 for SQU were selected for quantification. The CE, DP, CXP, and EP for SQU were optimized to obtain the greater intensity of the target ion pairs. The CE of 40 and 25, DP of 150 and 100, CXP of 10, and 13 and EP of 11 and 11 for SQU were adopted, respectively.

4.2.2.1.2. Validation parameters

In the proposed method calibration curves from the response of different concentration were prepared using linear regression model. The four different sets were prepared for response function studies with range of SQU from 25-2000 ng/mL, from their regression analysis studies series 2, shows the best results with coefficient of determination (r^2) 0.9996, so this series was selected for further computation for validation and sample analysis. Trueness of method was justified by calculation of %age relative bias which was found to be limited between [-0.0926% -- 1.285%] as illustrated in Table 5.7 from which it has been concluded that trueness of method is adequate. The method precision and reproducibility was authenticated by results obtained from precision studies which were found to be < 2% in terms of RSD for both repeatability and intermediate levels as illustrated Table 5.8. After the conformation of accuracy of all the parameters related to system and developed method, sample matrixes was incorporated in validation process

which includes recovery studies. Recovery studies were carried out using standard addition method in sample matrixes. These recovery studies receipts into account total error of test results and is represented by the β-expectation tolerance limits. The results of accuracy studies has been illustrated in Table 5.9. Further, these recovery studies of the method was justified by plotting risk profile keeping maximum risk level at 5.0% from which it was concluded that risk of outliers are within limits and in future analysis of the samples using this developed and validated method will fall within range. The results of LOD show that this method is sensitive enough to analyze the plasma, LOD was found to be 0.097 ng/mL resp.

4.2.2.1.3. Extraction efficiency Plasma samples

Extraction efficiency of the developed method was calculated for Plasma samples and was found to be in the range of 87-94%. These results justifies the use of developed method for the analysis of Plasma samples. Results of extraction of SQU from plasma samples has been shown in Table 5.10.

Table 5.7 Results of Trueness in terms of relative bias (%)

Nominal concentration (ng/mL)	Back calculated Concentration (ng/mL)	Absolute bias (ng/mL)	Relative bias (%)
25.00	25.04	0.0426	0.01065
50.00	50.01	0.0136	0.0068
100.00	199.91	-0.0926	-0.0926
250.00	250.12	0.1259	0.31475
500.00	500.06	0.0625	0.3125
1000.00	999.90	0.1022	1.022
2000.00	2000.04	0.0425	1.285

Table 5.8 Results of repeatability and intermediate precision and repeatability in terms of (%RSD)

Nominal Conc (ng/mL)	Rep* (%RSD)	Intermediate precision (%RSD)
50.00	0.0026	0.1055
100.00	0.2656	0.1598
200.00	0.2695	0.2689
400.00	0.1595	0.2687
800.00	0.1269	0.2654
1600.0	0.1298	0.2578

Table 5.9 Result of method accuracy in terms of relative beta-expectation tolerance limit and risk assessment obtained by selected regression model in matrix

	Concentration Level (%)	Concentration (ng/mL)	Beta-expectation tolerance limits (ng/mL)	Relative Beta-expectation tolerance limits (%)	Risk (%)
Tablet	80.0	80.00	[79.26 , 80.65]	[-1.661, 1.659]	0.0359
	100.0	100.00	[99.26 , 99.95]	[-1.268 , 1.270]	0.1589
	120.0	120.00	[119.25 , 120.56]	[-1.596 , 1.598]	0.0265

Table 5.10 Extraction efficiency results for plasma samples

	Plasma samples		
Spiked Concentrations (ng/ml)	100	250	500
Mean Extraction (%)	90.66 ± 1.52	93.73 ± 2.08	93.46 ± 2.08
Average Extraction (%)		92.62	

5.2.2. Compatibility and uniformity of content studies of binary systems prepared

The prepared mixture were tested for uniformity of content and results were found to be within the range of 98-100%. As shown in Table 5.11.

Table 5.11 Content of Uniformity of different binary systems

Weight Ratio	% ASSAY					
	5:0.5	**5:1**	**5:1.5**	**5:2**	**5:2.5**	**5:3**
SQU-QU	99.15 ± 0.32	99.58 ± 0.59	98.15 ± 0.45	98.88 ± 0.52	98.65 ± 0.21	99.55 ± 0.44
SQU-Sil	99.02 ± 0.48	98.59 ± 0.45	99.01 ± 0.15	99.62 ± 0.12	99.29 ± 0.15	100.58 ± 0.44
SQU-LT	98.35 ± 0.36	99.56 ± 0.89	100.01 ± 0.54	100.04 ± 0.56	100.21 ± 0.58	99.45 ± 0.26

5.2.2.1. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectrums of SQU, QU, Sil, LT and their binary systems were recorded. The recorded spectrums were interpreted in terms of change in any characteristic peaks of the SQU. The characteristics peaks in the FTIR studies of SQU exhibited C=O stretching at 1623 cm^{-1}, OH stretching at 3528 cm^{-1} and NH_2 stretching at 3102 cm^{-1} which confirms the structure of SQU. All these characteristic peaks remains regular in the prepared different binary systems. Although, there was a slight (not significant) change observed in the intensities of the peaks of SQU. These primary results of the FTIR spectra revealed that there was no

interaction between SQU, QU, Sil and LT in binary system. It also confirms that there is no interaction at the molecular level in the SQU and their binary system. The FTIR spectrum of SQU and different binary system has been shown in Figure 5.3.

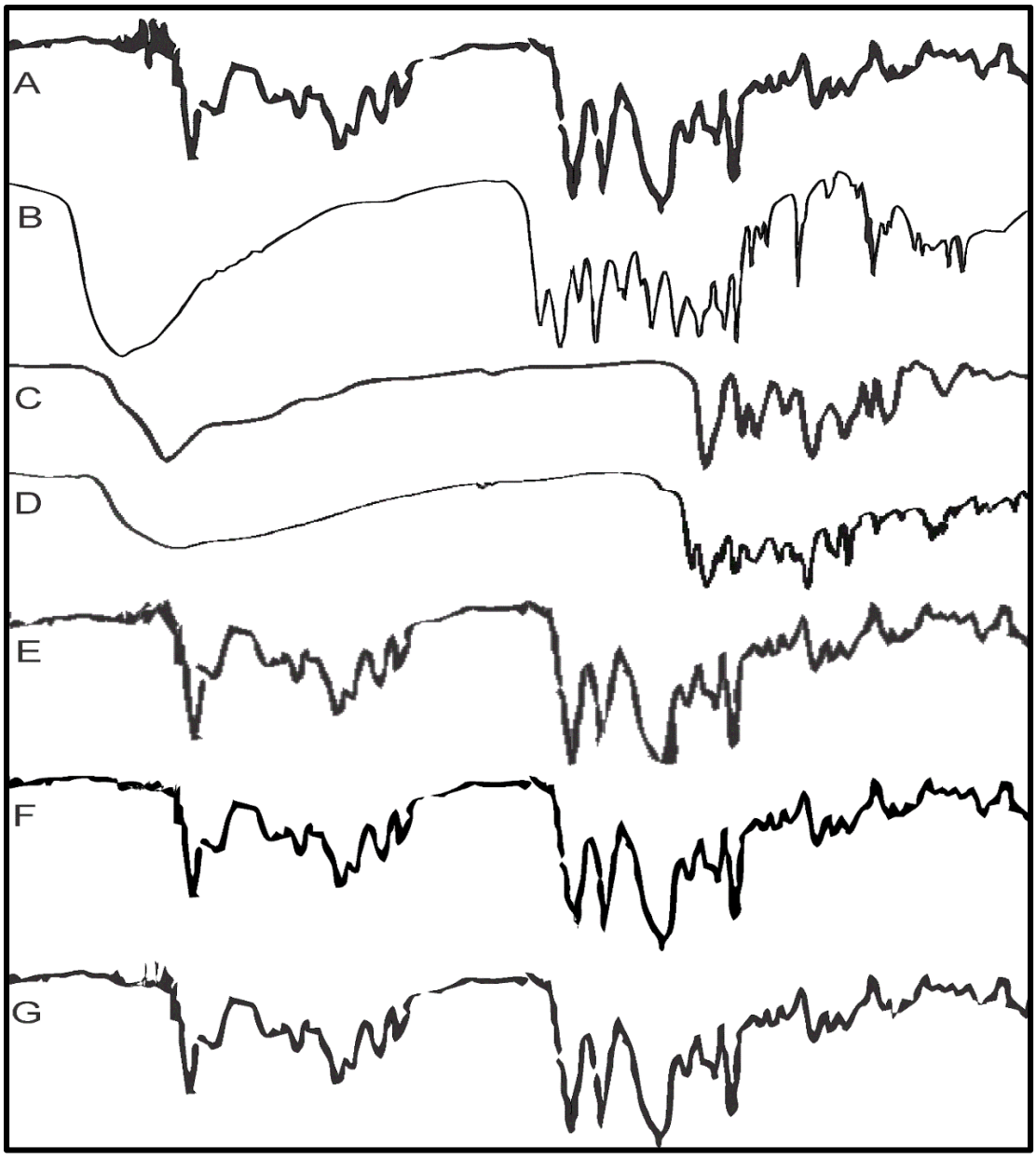


Figure 5.3 FTIR spectrum of (A) SQU (B) QU (C) Sil (D) LT (E) SQU:QU (F) SQU:Sil (G) SQU:LT for compatibility studies.

5.2.2.2. Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry (DSC) thermograms of the SQU, QU, Sil and LT has been recorded. These recorded thermograms were interpretated to know the interactions. Interpretations of the thermograms revealed that there is no physical interaction between the SQU, QU, Sil and LT in their respective binary systems. The DSC thermogram of SQU showed an endothermic peak at 239°C which was corresponding to its melting point. While the thermogram of QU shows an endothermic peak at 322°C. Sil shows a broad endothermic peak in the range of 166-173°C. The luteolin thermogram showed endothermic peak on 328.16°C, which again attributed to its melting point. DSC thermogram of SQU-QU physical mixture shows an endothermic peak at 235°C which is very near to the pure SQU, which reveals that there is no interaction in the QU and SQU. The DSC thermogram of all the pure compounds and binary mixtures has been illustrated in Figure 5.4. DSC thermogram of SQU-Sil physical mixture shows an endothermic peak at 231°C which is slightly lower than the pure SQU, although it is not significant, the endothermic peak of binary mixture suggest minimal or no physical interaction between the QU and Sil. DSC thermogram of SQU-LT physical mixture shows an endothermic peak at 234°C which is at the lower side of the pure SQU, the endothermic peak of SQU-LT binary mixture suggests no physical interaction between the SQU and LT. The DSC data of all the samples encourages and clears the way for the researchers to move further for the permeation studies.

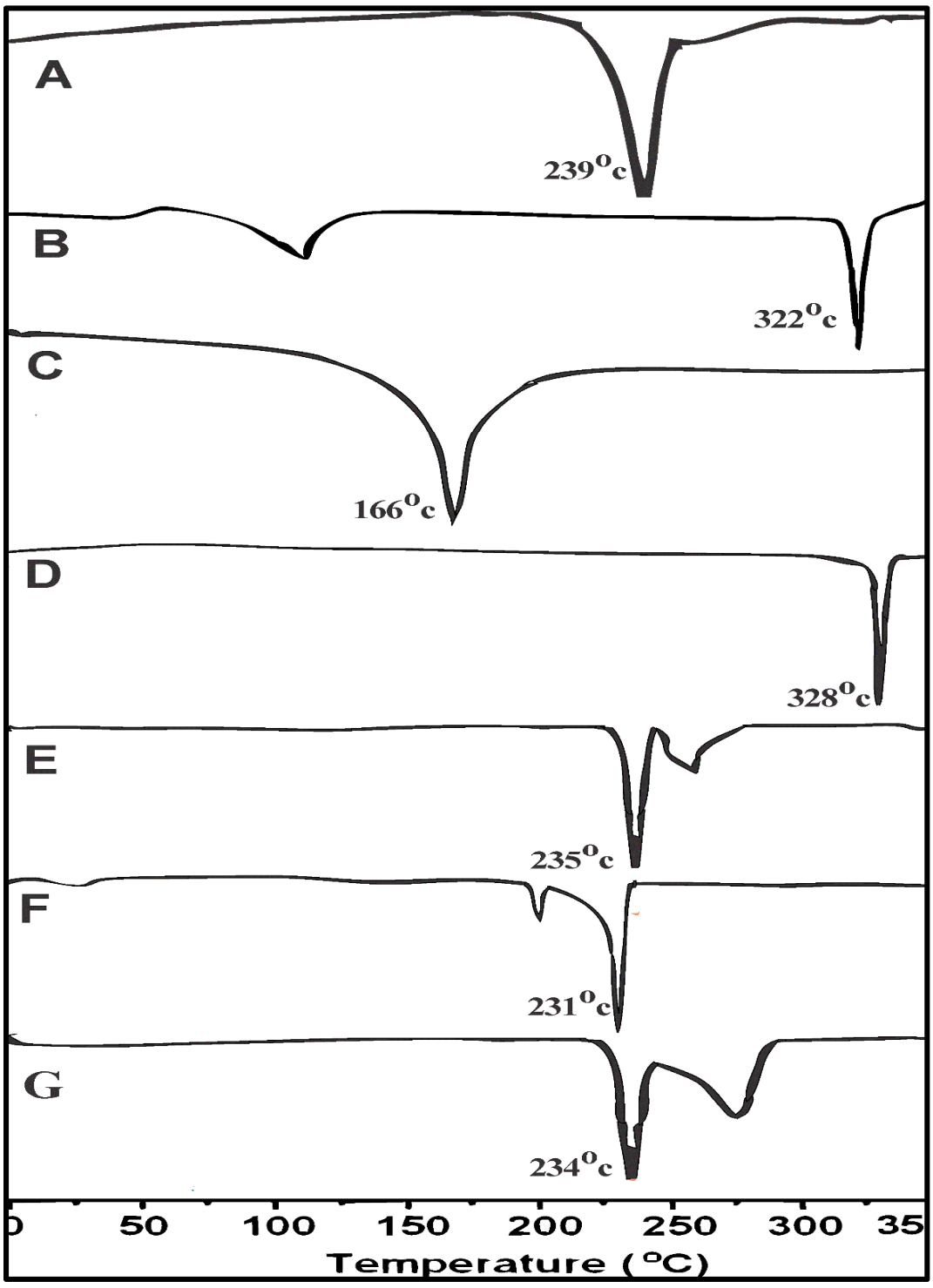


Figure 5.4 DSC thermogram of (A) SQU (B) QU (C) Sil (D) LT (E) SQU:QU (F) SQU:Sil (G) SQU:LT for compatibility studies.

5.2.3. Ex-vivo permeation studies

The permeability of binary systems of SQU-QU, SQU-Sil and SQU-LT in goat intestinal tissue shows a significant rise as compared to the plain SQU. Permeation coefficient (P_{eff}) was calculated for SQU and SQU-QU, SQU-Sil and SQU-LT binary systems. The permeation coefficients calculated for the all weight ratios has been summed up in Table 5.12**.** Release profile of the SQU and binary systems at different time points has been shown in Figure 5.5, 5.6 and 5.7. The permeation coefficient for plain SQU was (2.135±0.387) × 10^{-6} cm/s. The QU shows an increase in the amount permeated having permeation coefficient (4.395±0.15) × 10^{-6} cm/s at weight ratios (5:1), while Sil shows maximum permeation coefficient (4.283±0.18) × 10^{-6} cm/s at weight ratios (5:2) and LT shows maximum enhancement at weight ratio (5:2.5) with permeation coefficient (3.956±0.458) × 10^{-6} cm/s.

Table 5.12 Permeation coefficient for SQU, SQU-QU, SQU-Sil and SQU-LT at different weight ratios

Permeation coefficient and enhancement ratio of SQU and its different binary systems (x 10^{-6} cm/s)						
SQU	2.135 ± 0.38					
Weight Ratio	**5:0.5**	**5:1**	**5:1.5**	**5:2**	**5:2.5**	**5:3**
	Peff	**Peff**	**Peff**	**Peff**	**Peff**	**Peff**
SQU-QU	4.093± 0.18	**4.395± 0.15**	4.217± 0.22	3.898± 0.25	3.764± 0.32	3.561± 0.25
SQU-Sil	2.664± 0.26	2.778± 0.28	2.939± 0.15	**4.283± 0.18**	3.961± 0.34	3.846± 0.28
SQU-LT	2.345± 0.38	2.458± 0.32	3.009± 0.26	3.473± 0.15	**3.956± 0.28**	3.278± 0.32
	ER	**ER**	**ER**	**ER**	**ER**	**ER**
SQU-QU	1.92	**2.06**	1.98	1.83	1.76	1.67
SQU-Sil	1.25	1.30	1.38	**2.01**	1.86	1.80
SQU-LT	1.10	1.15	1.41	1.63	**1.85**	1.54

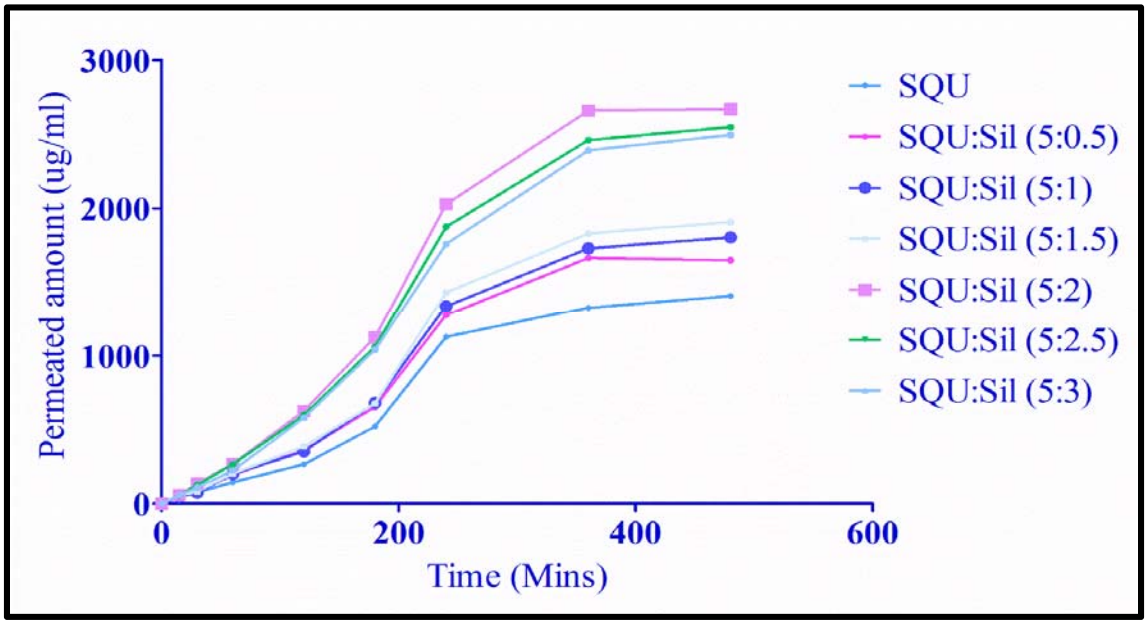


Figure 5.5 Time profile for permeation of the SQU and SQU:QU from the intestinal tissue

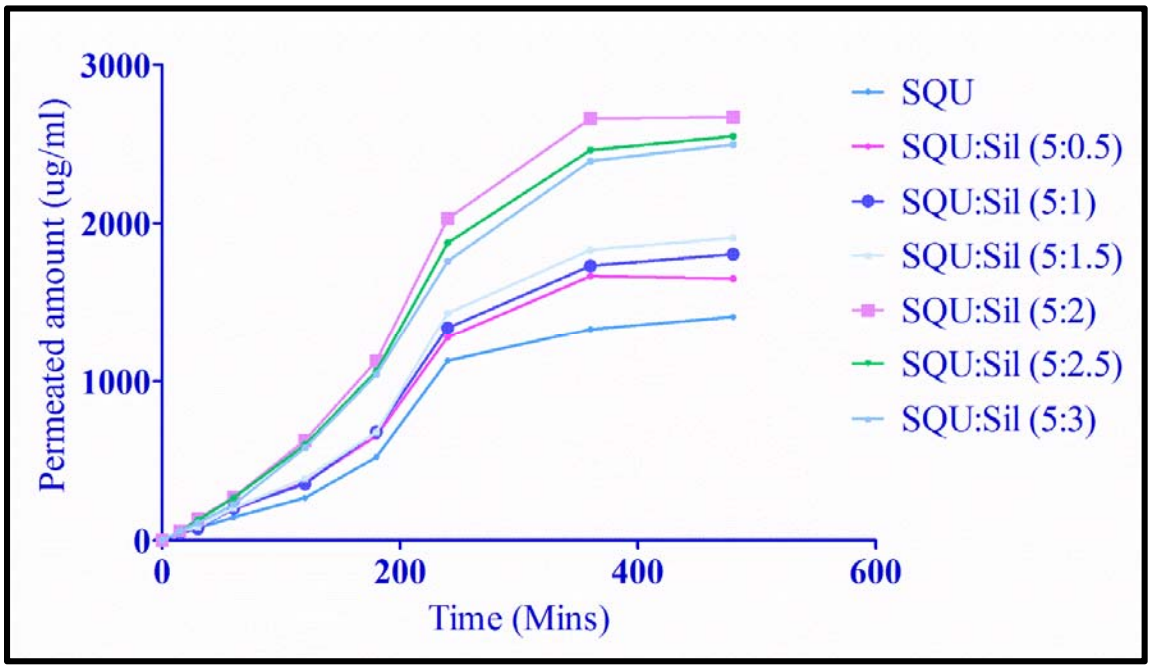


Figure 5.6 Time profile for permeation of the SQU and SQU:Sil from the intestinal tissue.

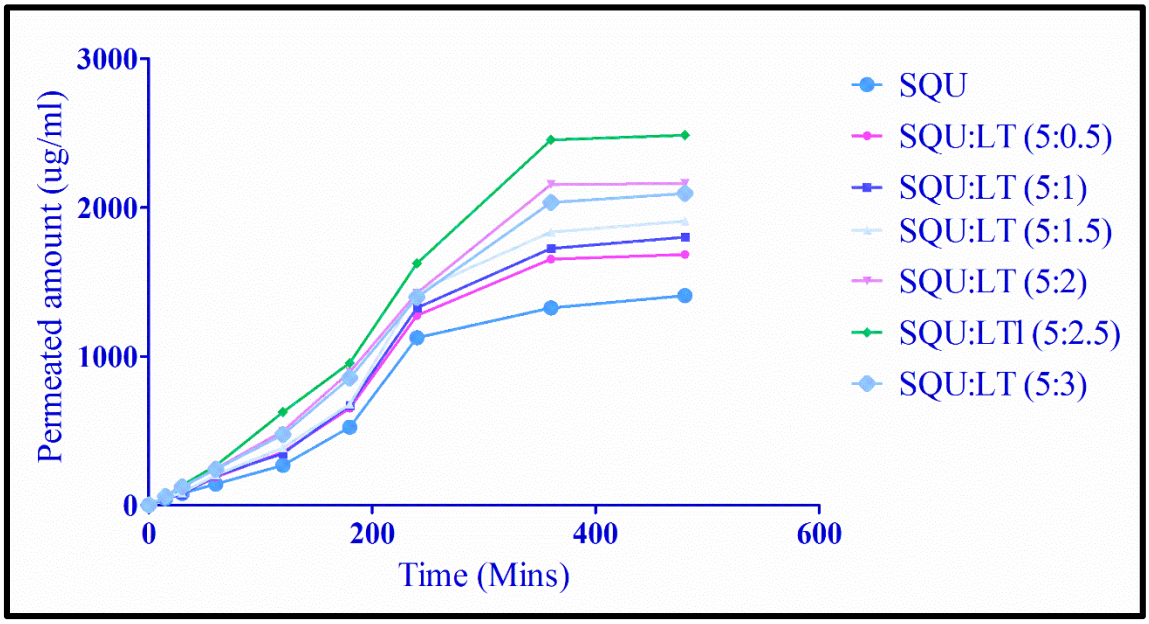


Figure 7 Time profile for permeation of the SQU and SQU:Sil from the intestinal tissue.

5.2.4. Cellular uptake and Transport of SQU across the Caco-2 cell monolayers

Cellular uptake

Cellular uptake of SQU has been shown in Figure 5.8. It can clearly observed from the images that very few individual particles has been seen in the SQU as compare to the binary mixtures. Hence, it shows that there is increase in the uptake of the SQU in presence of the bioenhancers. To know the quantitative effect further transport studies were carried out.

Transport Study

To study the effect of the bioenhancers on SQU transport through cell monolayers, drug transport across Caco-2 cell monolayers from the AP side to the BL side were studied. TEER was determined in all the experiments, which clearly shows there was no cellular damage. Effect of presence of different concentration of QU, Sil and LT on TEER has been

studied. Figure 5.9 shows effect of different concentration of QU, Figure 5.10 and 5.11 shows effect of different concentration of Sil and LT respectively on TEER values post experimental. Figure 5.12, 5.13 and 5.14) shows the time profiles of SQU permeation through the Caco-2 cell monolayers in the presence of QU, Sil and LT respectively. It is clearly seen that the amount of drug at the BL side increased with time for all the samples. Although the concentration in the samples having bioenhancers was higher than the plain SQU sample.

The *P*app (AP to BL side) of SQU and its binary systems were calculated for 24 hrs. As shown in (Figure 5.15, 5.16, 5.17) maximum *P*app was observed with the QU in the ratio of 5:1 while in the Sil and LT maximum was observed in the ration of 5:2 and 5:2.5 respectively. Although it was also observed that the ratio of Sil 5:2 and 5:2.5 was very close, but 5:2 concentration also shows maximum enhancement in the ex-vivo studies so this was chosen as optimum concentration for pharmacokinetic studies.

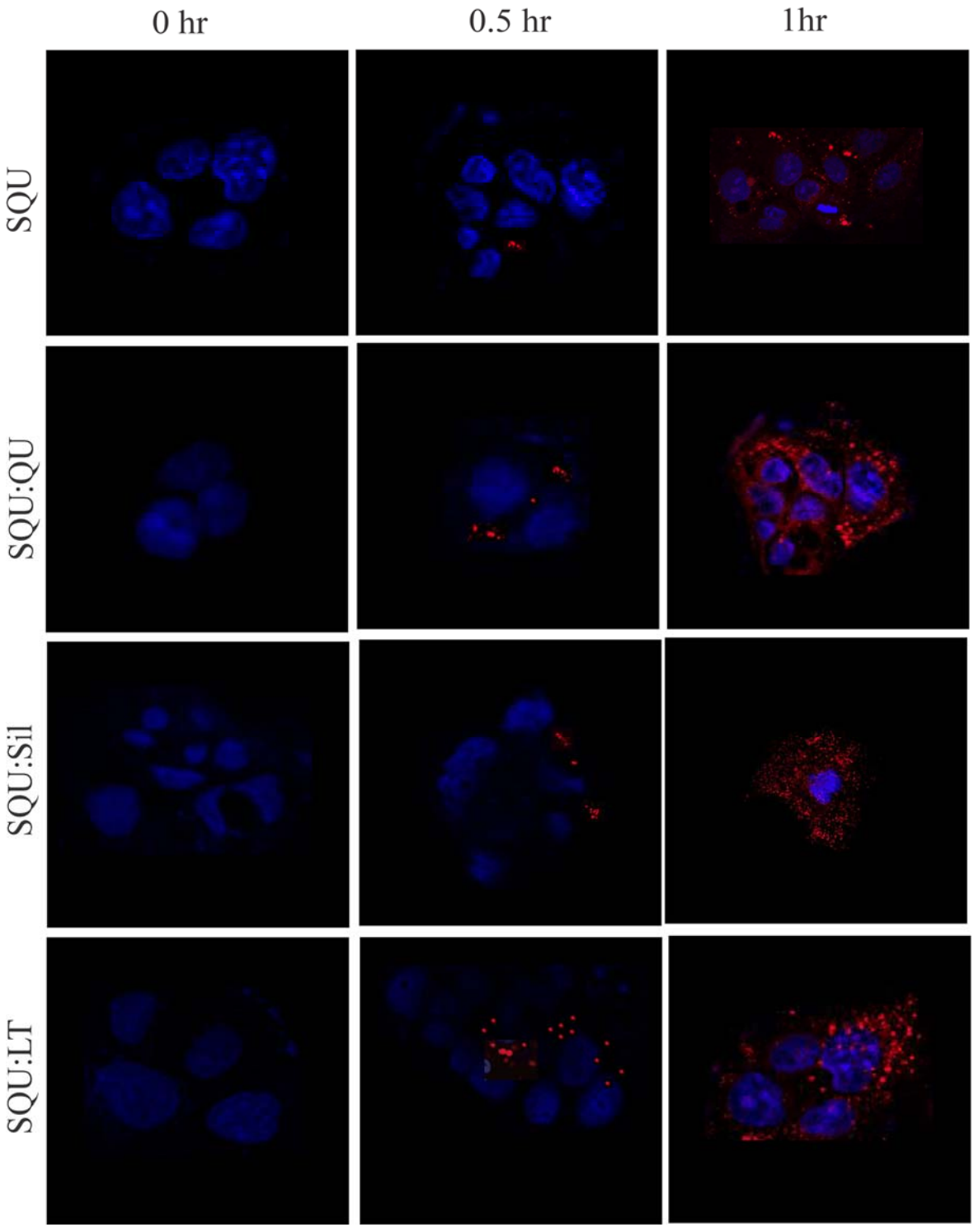


Figure 5.8 Cellular Uptake of SQU and binary system at different time points (0hr, 0.5hr, 1hr)

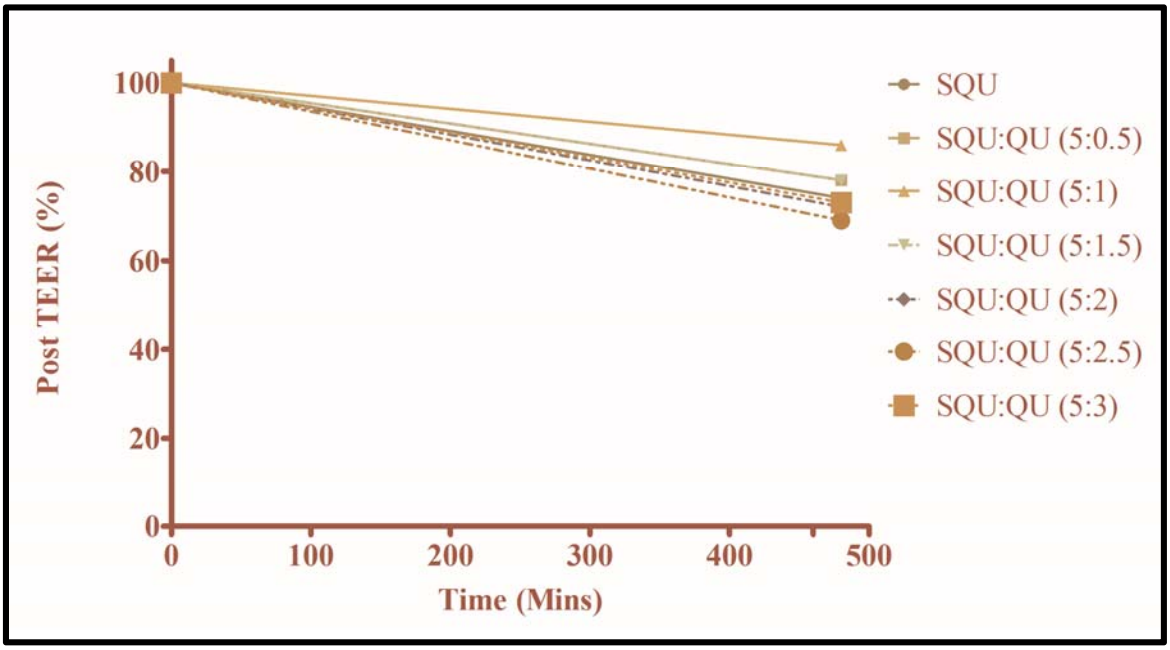


Figure 5.9 Effect of different ratios of Quercetin on TEER Values of Caco-2 cell line

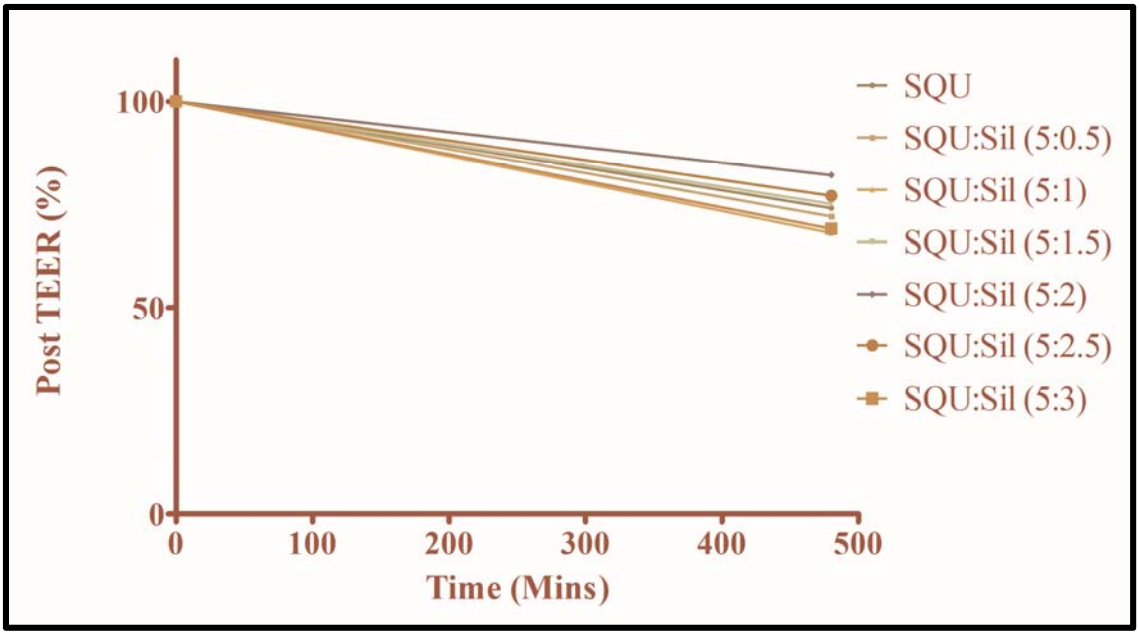


Figure 5.10 Effect of different ratios of Silibinin on TEER Values of Caco-2 cell line

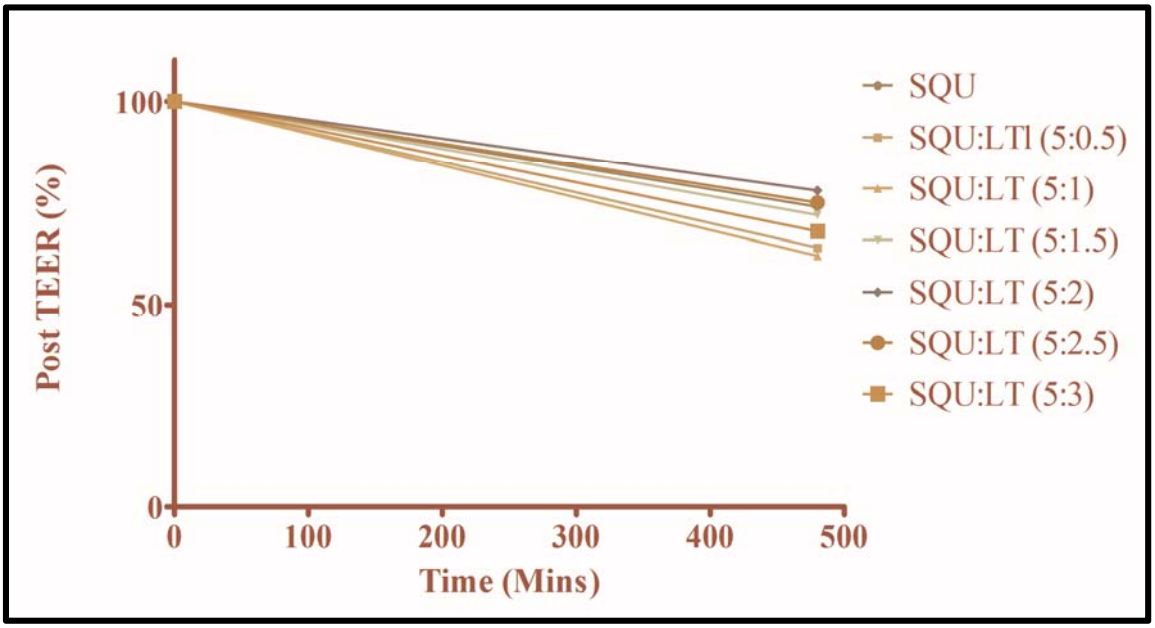


Figure 5.11 Effect of different ratios of Luteolin on TEER Values of Caco-2 cell line

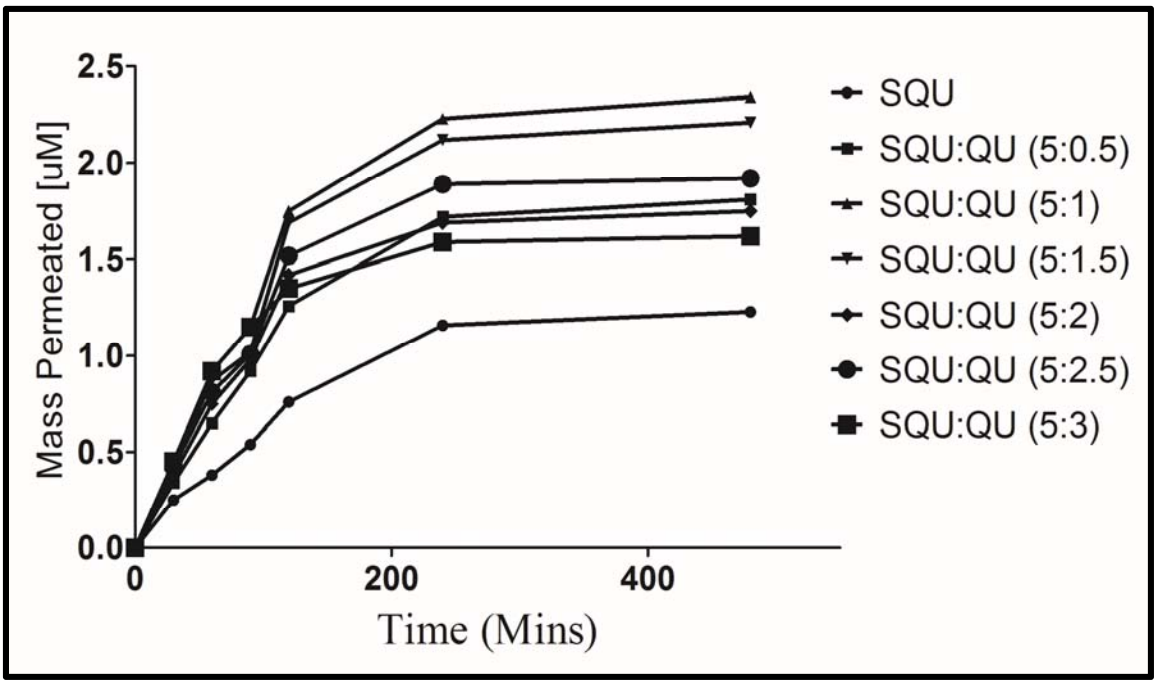


Figure 5.12 Release time profile of SQU and SQU:QU binary system in Caco-2 cell lines

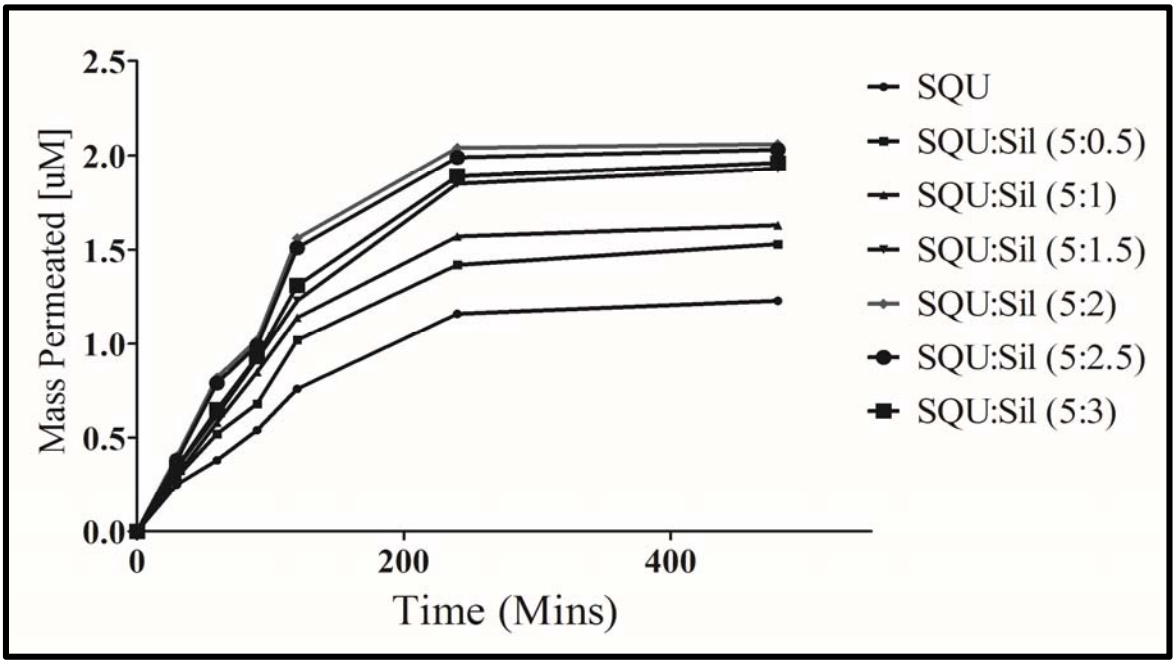


Figure 5.13 Release time profile of SQU and SQU:Sil binary system in Caco-2 cell lines

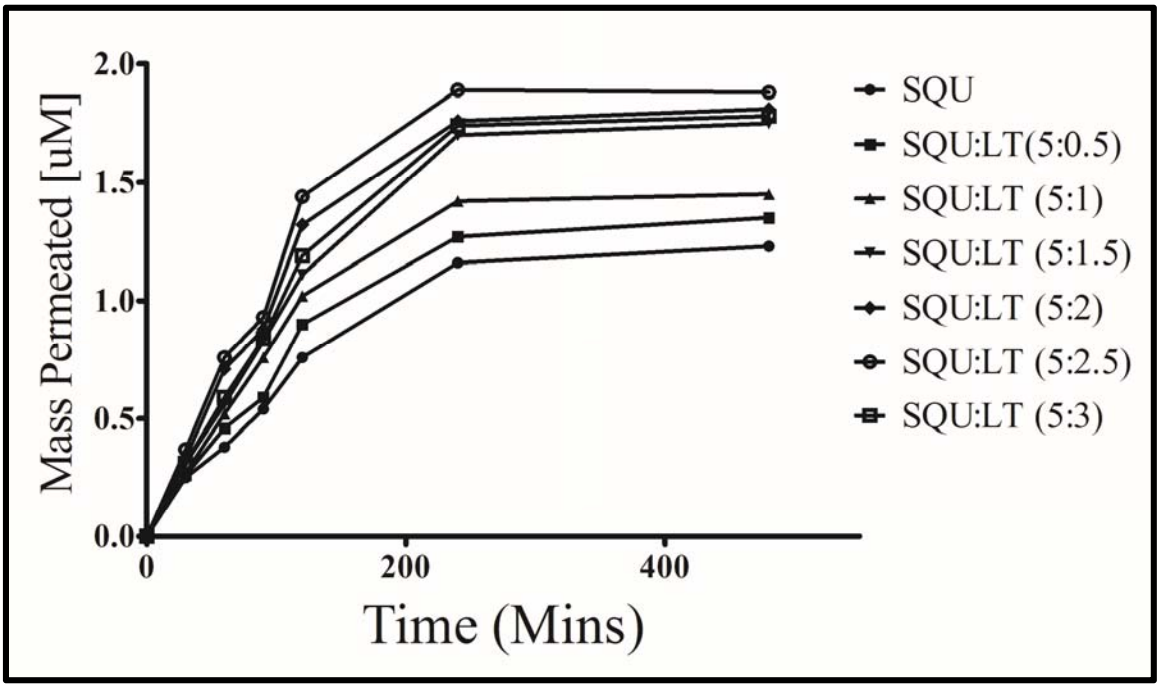


Figure 5.14 Release time profile of SQU and SQU:LT binary system in Caco-2 cell lines

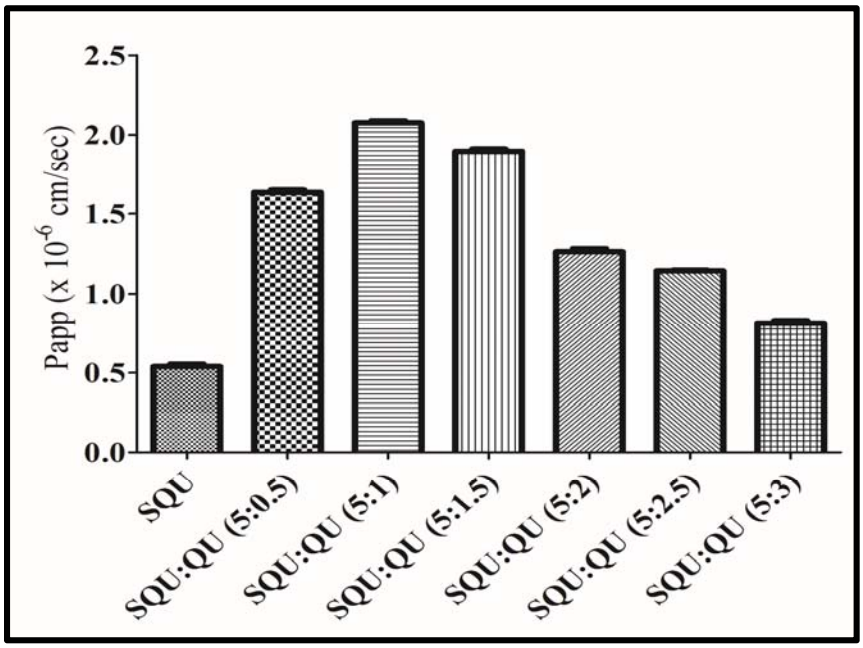


Figure 5.15 Papp comparison of SQU and SQU:QU different weight ratios from AP to BL side

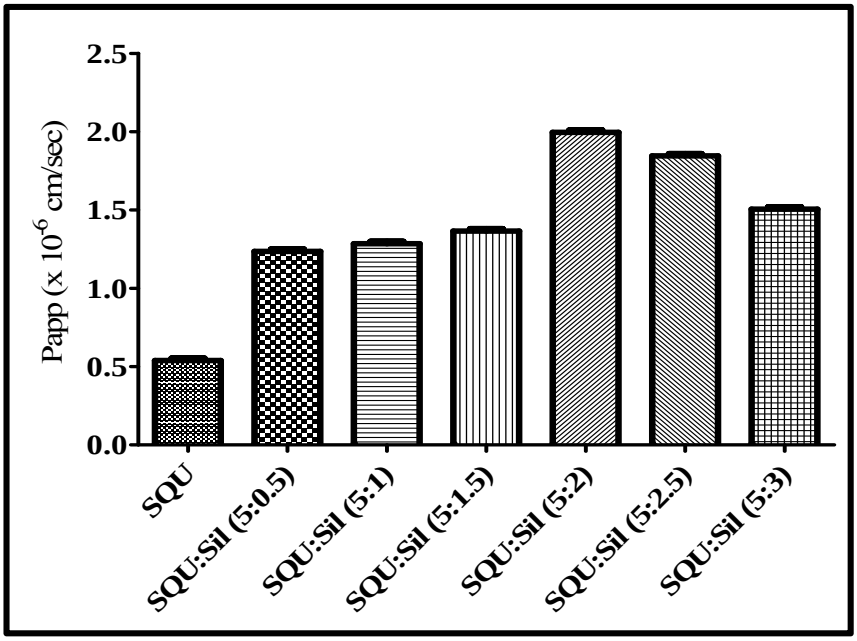


Figure 5.16 Papp comparison of SQU and SQU:Sil different weight ratios from AP to BL side

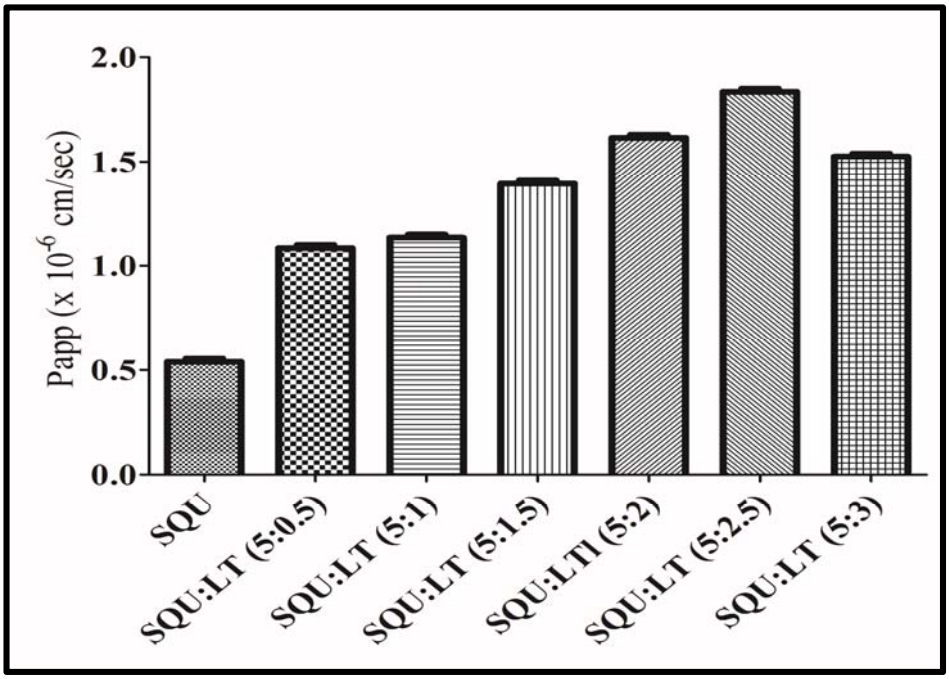


Figure 5.17 Papp comparison of SQU and SQU:LT different weight ratios from AP to BL side

5.2.5. Pharmacokinetic studies of SQU in rabbits

The pharmacokinetics of SQU were studied in rabbits to evaluate the enhancement in the absorption efficiency of the SQU in combination with QU, Sil and LT. Plasma drug concentration versus time profile for SQU and SQU-QU (5:1), SQU-Sil (5:2) and SQU-LT (5:2.5) were plotted as shown in Figure 5.18, 5.19 and 5.20. After the oral administration of the SQU and binary systems there was significant difference was observed in the pharmacokinetic profile. Semi log plot of concentration versus time was plotted which shows significant changes in the concentration of the binary mixture as compare to plain SQU shown in Figure 5.21. AUC increase by 2.51 folds in the case of the QU. While, 2.34 and 2.09 folds increase was observed with the Sil and LT respectively. Enhancement ratio for the different bioenhancers has been compared as shown in Figure

5.22. It is clearly observed that QU shows the maximum enhancement as compare to Sil and LT. The pharmacokinetic parameters for all the three bioenhancers are shown in Table 5.13. Ln concentration time profile including absorption phase and elimination phase has been plotted for SQU, SQU:QU, SQU:Sil, SQU:LT as shown in Figure 5.23, 5.24, 5.25 and 5.26. In these graphs it has been observed that the absorption phase remains almost unchanged although there was a slight change in elimination patter was observed by curve fitting. It can be concluded from the Pharmacokinetic profiling that bioenhancers positively changes the concentration of the SQU in the plasma this may be due to their P-gp inhibition property and also can have some effect due to the CYP inhibition property too. The QU showing the best results in comparison to the Sil and LT, it may be due to its confirmed dual activity of p-gp inhibition and CYP activity which metabolites the SQU. It can be concluded from these studies the above used bioenhancers can be incorporated with the oral dosage forms to have an efficient therapy as compared to the conventional therapy.

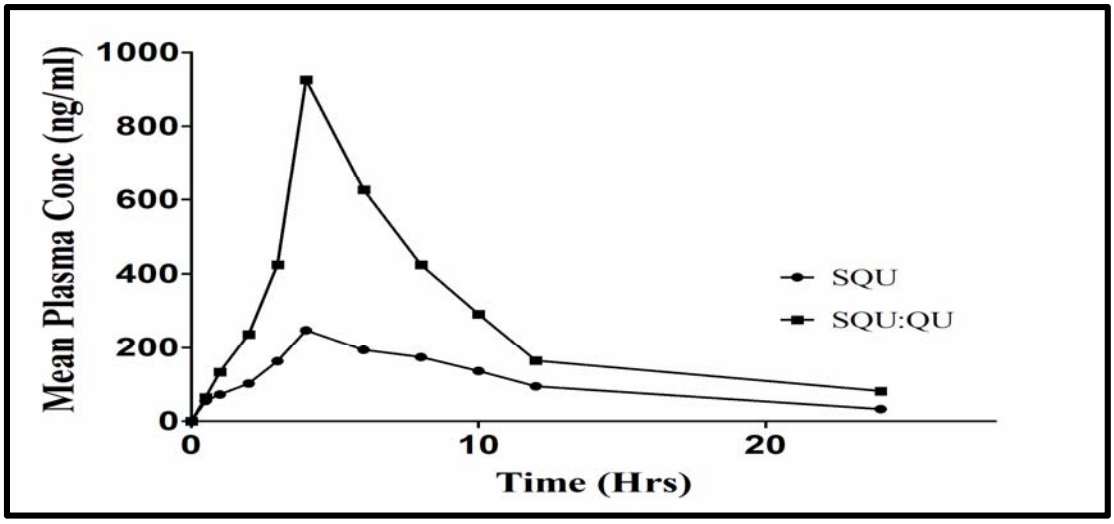


Figure 5.18 Time profile for mean plasma concentration for SQU and SQU:QU

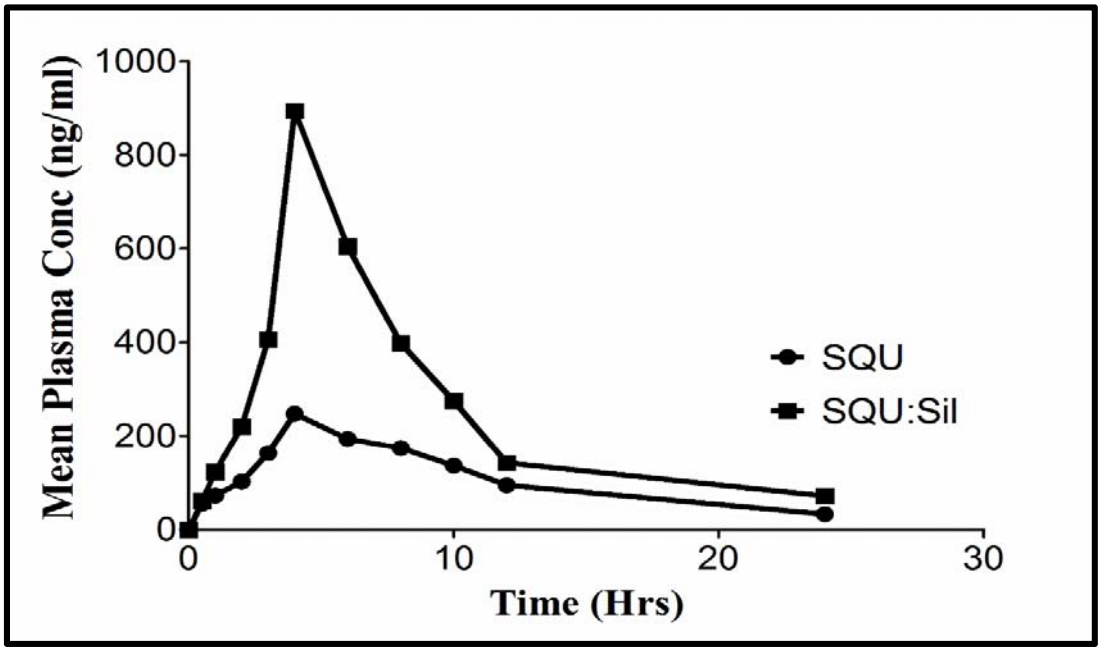


Figure 5.19 Time profile for mean plasma concentration for SQU and SQU:Sil.

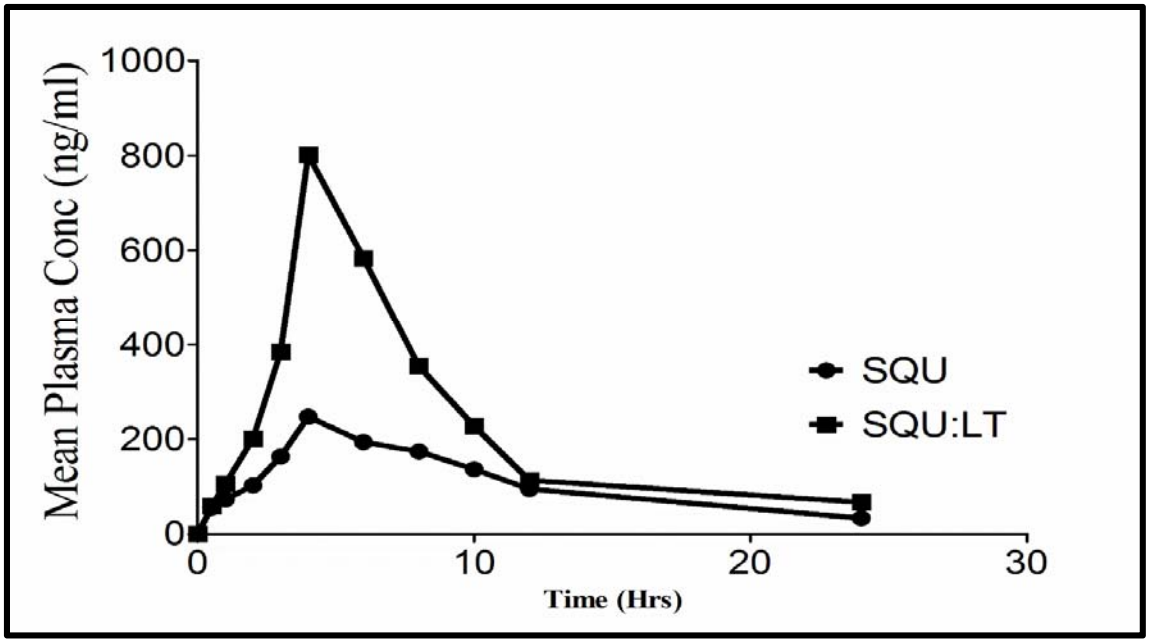


Figure 5.20 Time profile for mean plasma concentration for SQU and SQU:LT

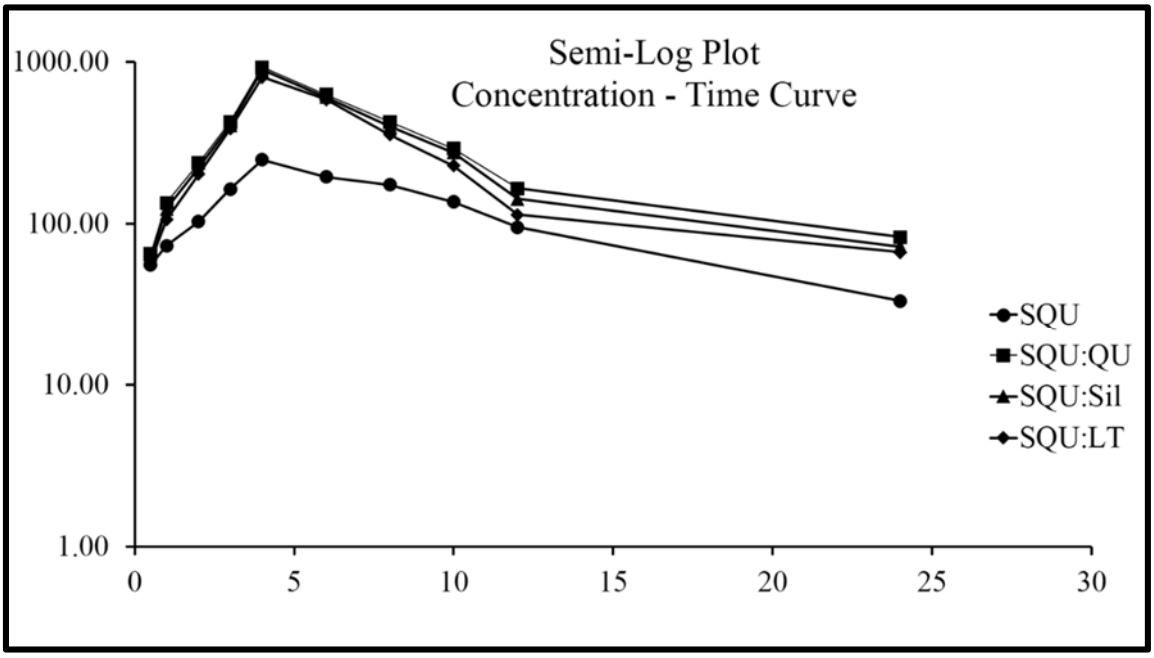


Figure 5.21 Semi Log Plot for Concentration vs Time for SQU, SQU:QU, SQU:Sil, SQU:LT

Table 5.13 Pharmacokinetic Parameters of SQU after a single oral dose of SQU, in absence and presence of each of three different bioenhancers

Parameter	Unit	Value			
		SQU	SQU:QU (5:1)	SQU:Sil (5:2)	SQU:LT (5:2.5)
Half Life (t1/2)	h	6.88	5.87	5.62	5.61
Time for maximum Concentration (Tmax)	h	4	4	4	4
Maximum Plasma Concentration (Cmax)	ng/ml	247.66	925.66	894.33	802
Area under Curve AUC 0-t	ng/ml*h	2593.58	6514.5	6072.33	5424.25
AUC 0-inf_obs	ng/ml*h	2924.23	7212.22	6655.75	5964.19
Relative BA	%	1	2.51	2.34	2.09

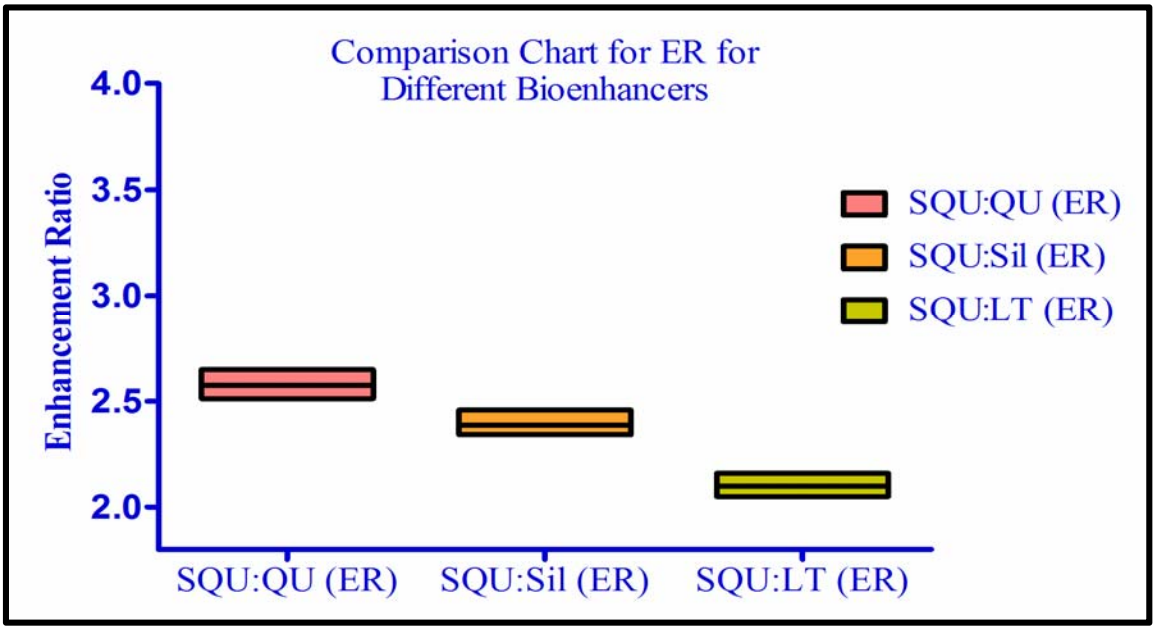


Figure 5.22 Enhancement Ratio (ER) comparison for different bioenhancers with respect to AUC

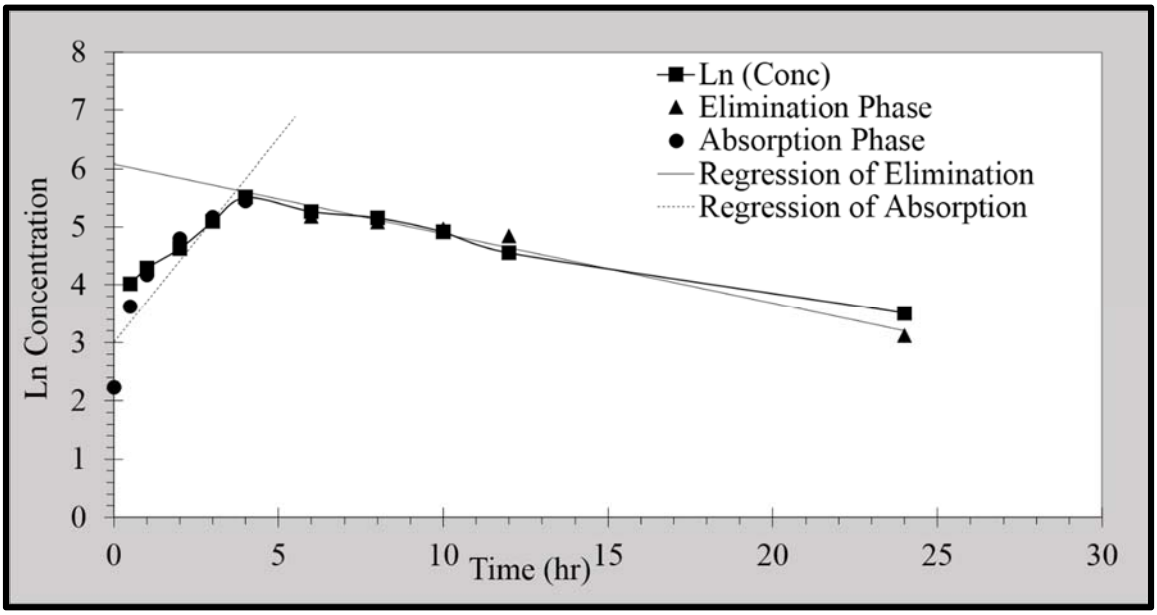


Figure 5.23 Ln concentration time profile including absorption and elimination phase for SQU

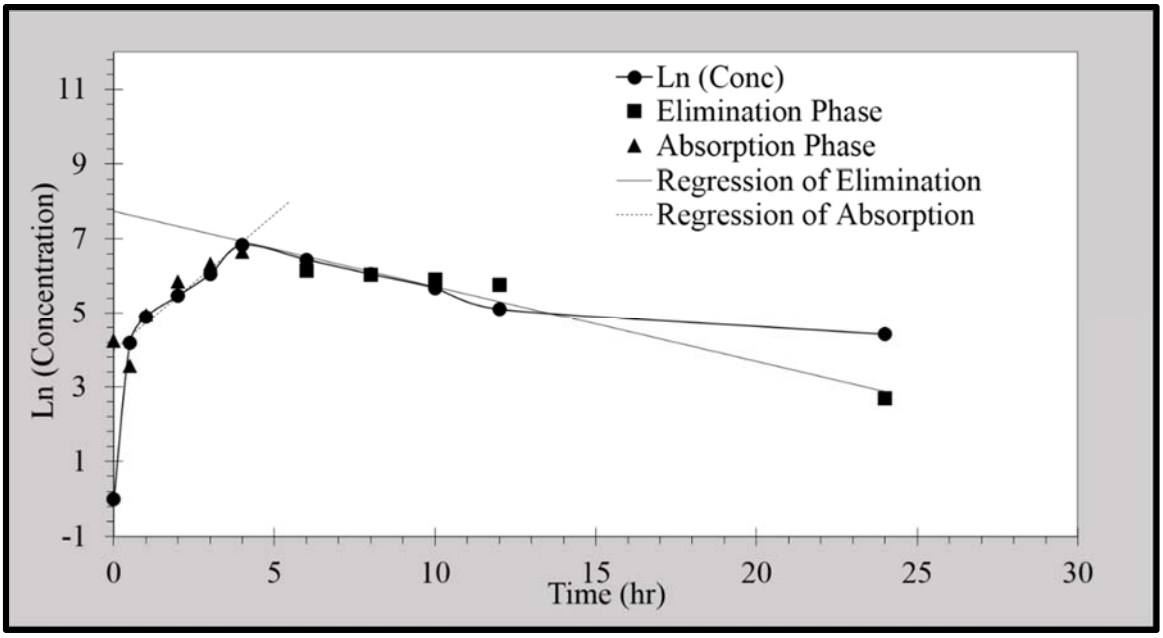


Figure 5.24 Ln concentration time profile including absorption and elimination phase for SQU:QU

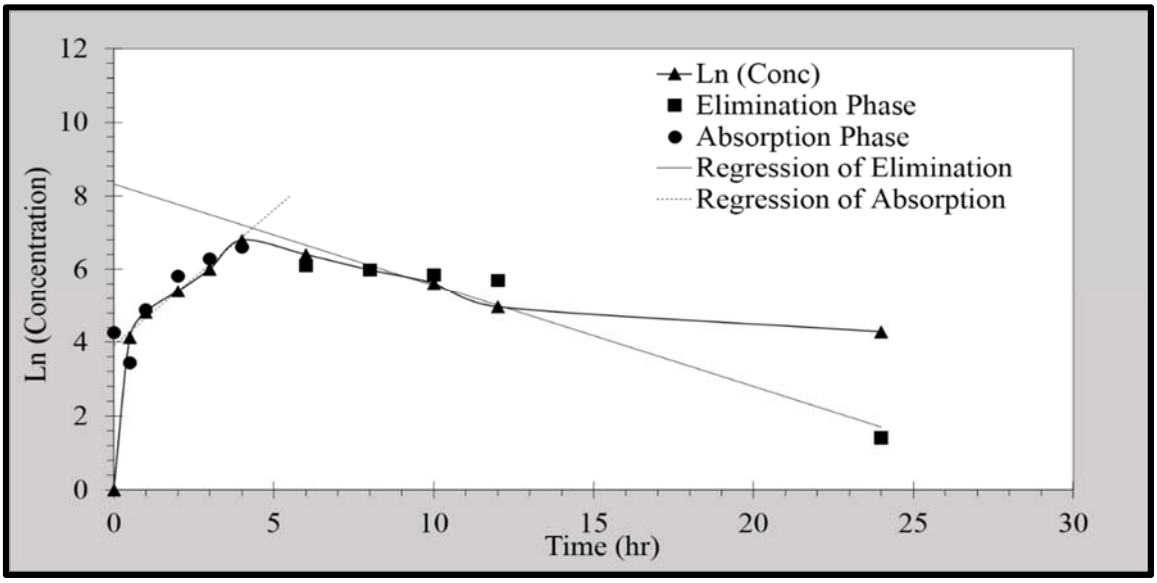


Figure 5.25 Ln concentration time profile including absorption and elimination phase for SQU:Sil

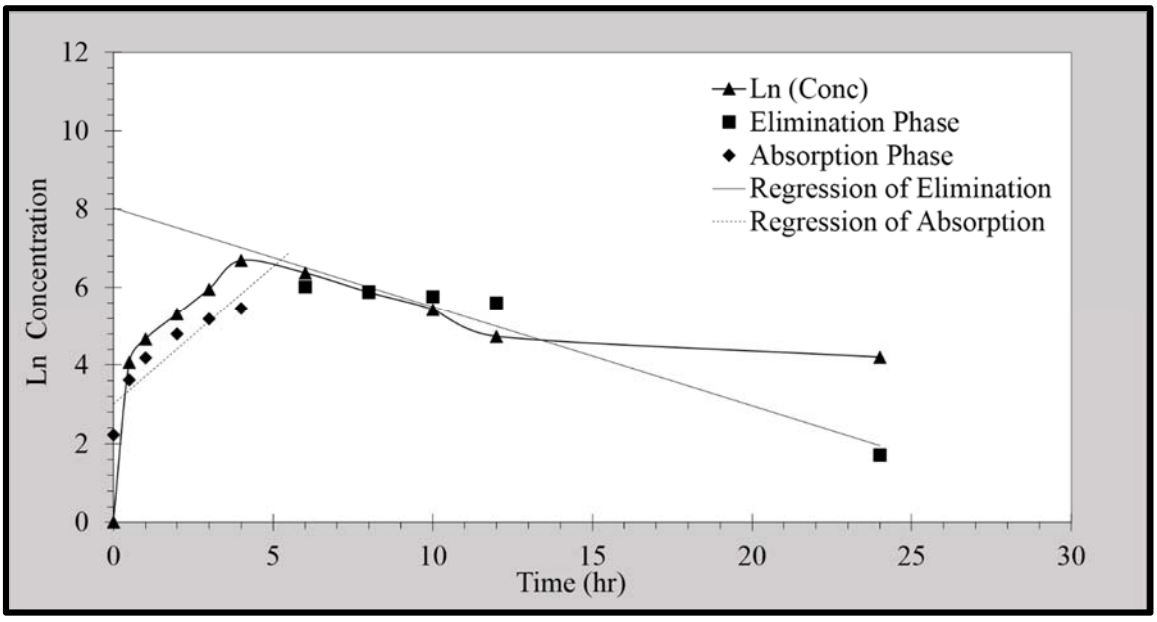


Figure 5.26 Ln concentration time profile including absorption and elimination phase for SQU:Sil

5.3. Discussion

Low bioavailability of SQU presents a considerable challenge for the researcher from the very beginning. As, it is a very important and potent drug in anti-HIV therapy. In our proposed work, binary system of the SQU was prepared with three different bioenhancers using physical mixing method. This method has very crucial step of the blending or mixing as if mixing is not proper it can alters and effects result very much. So, the blending must be proper as to achieve uniformity of content. Analytical method for the estimation of SQU was an important step for the whole research process, so for this purpose the most sophisticated and highly reliable instrument LC-MS was used. The results of LC-MS was very much reproducible and the method was properly validated before using for the samples.

Ex-vivo studies were primary studies and were of important as they confirms the hypothesis behind the research work and encourages the team to go for further sophisticated and reliable technologies such as cell lines and *In-vivo* studies. The results of Ex-vivo studies revealed that the bioenhancers can plays a crucial role in the permeation enhancement of the SQU. The transport studies in caco-2 cell lines suggested that there is increase in the cellular uptake of SQU in the presence of the bioenhancers. The effective transport of the SQU in the presence of the bioenhancers most likely due to their P-gp inhibitory effect. The caco-2 cell lines also helps in optimization of the best ratios that can be used for the pharmacokinetic studies.

Pharmacokinetic studies were conducted in rabbits, these studies shows a significant increase in the plasma concentration of the SQU in the presence of the bioenhancers. It is

well known fact that SQU is the substrate of the P-gp so it limits its oral uptake. As the bioenhancers used in the research work are P-gp inhibitor so the oral absorption has increased in the case of binary systems. In addition, to this it has also been reported that the hepatic and intestinal first pass metabolism also effects on the bioavailability of the SQU. This east meets west technique, in combination with P-gp inhibition also helps in the metabolism as they also shown some effect of the CYP enzymes. So these bioenhancers may further advantage the bioavailability of SQU. Both the cell lines and in-vivo studies shows the same pattern of enhancement for the bioenhancers and is almost similar so with the SQU all the bioenhancers can be used either as food supplement or some other ways for co-administration with the SQU. The study of hepatic metabolism will be our future perspective.

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