Comparative Single Dose Pharmacokinetics and Bioavailability Studies of Saquinavir, Ritonavir and their Optimized Cyclodextrin Complexes after Oral Administration into Rats using LC-MS/MS.

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ABSTRACT

Purpose: To compare pharmacokinetics and bioavailability of saquinavir (SQV) and ritonavir (RTV) and their optimized cyclodextrin complexes of anti-retro viral drugs after oral administration into rats. **Methods:** Rats were fasted overnight and dose equivalent to 10 mg/kg was administered orally via feeding tubes. Serial blood samples were collected, and plasma concentrations of both drugs and their complexes were determined using liquid chromatography tandem mass spectrometry, LCMS/MS. **Results:** After oral administration, half-life, apparent volume of distribution, total body clearance and bioavailabilities were calculated for both drugs and their optimized cyclodextrin complexes. The cyclodextrin complexes of both saquinavir (3860.93 ± 138.50 ng.h/ml) and ritonavir (2300.19 ± 118.21 ng.h/ml) had shown higher $AUC_{0-\infty}$ values compared to pure drugs, saquinavir (2293.04 ± 82.13 ng.h/ml) and ritonavir (1636.07 ± 162.51 ng.h/ml). **Conclusion:** Orally administered cyclodextrin complexes of SQV and RTV had shown higher bioavailabilities and higher peak plasma concentrations compared to SQV and RTV.

Key words: Oral, LCMS/MS, Plasma, Bioavailability.

INTRODUCTION

Human Immunodeficiency virus (HIV) gradually impairs human immune system and leads to acquired immune deficiency syndrome. There are several combinations of drug treatments available to reduce the replication of virus. Among them, protease inhibitors are effective in reducing the infection by binding to protease enzyme and preventing multiplication of virus. Because of the probability of developing viral resistance, side effects and low patient compliance, different protease inhibitors are prescribed in anti-retro viral therapy.[1] Saquinavir and Ritonavir, extensively prescribed protease inhibitors belong to BCS class II drugs and exhibit low and highly variable oral absorption because of their low aqueous solubility. For increasing their bioavailability, there is a need to enhance solubility and dissolution rate as their oral absorption is dissolution rate limited.

Among various techniques available, complexation with cyclodextrins (CD) gained importance in improving the solubility, dissolution rate, stability, and bioavailability of poorly water-soluble compounds. The solubility is enhanced because of forming water soluble inclusion complexes by encapsulating hydrophobic moiety of the drug which results in changing physicochemical properties and hence enhanced solubility.[2-4]

In the present study, different cyclodextrin complexes were prepared using methods like physical mixture, kneading, solvent evaporation, spray drying and freeze drying.

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All complexes were evaluated for increase in rate of dissolution and statistical analysis was employed for optimization. Both SQV and RTV showed the highest dissolution by freeze drying method. SQV showed better dissolution with sulfo butyl ether β cyclodextrin (SBE7βCD) prepared using freeze drying method and RTV showed highest dissolution with freeze dried randomly methylated β cyclodextrin complex (RMβCD).[1] The purpose of this study was to investigate the bioavailability of SQV and RTV in complex form with optimized CD derivatives in comparison with the free SQV and RTV through the gastrointestinal absorption. We have applied a recently reported specific and sensitive LCMS/MS assay[6] to determine pharmacokinetics of cyclodextrin complexes. In the present investigation, plasma concentrations of saquinavir and ritonavir and their optimized cyclodextrin complexes were determined by using LC-MS/MS.[7-9]

MATERIALS AND METHODS

Materials

Saquinavir and Ritonavir were provided by Hetero Chemicals, Hyderabad. Sulfo butyl ether β cyclodextrin was kindly gifted by Cydex Pharmaceuticals, USA. Randomly methylated β cyclodextrin was provided by Roquette Pharma, Italy. Acetonitrile and other solvents were of HPLC grade and procured from Himedia Labs, Bangalore. All other reagents used were of Analytical grade.

Preparation of inclusion complexes

Freeze drying/lyophilisation[10,11]

The dispersion of drug and optimized cyclodextrin derivatives were prepared with sufficient quantity of water at 1:1 molar ratio and kept for stirring at 200 rpm for 72 h at neutral pH in closed vials for complex formation which results in getting a clear solution. The resulting solution was fast frozen at -20°C using liquid nitrogen and dried at -50°C and 0.0070 mbar pressure in a freeze dryer (Model MODUL YOD 230, Thermo Electron Corporation, India) for 48 h. The product was stored in the desiccator for further pharmacokinetic analysis.

Animals

The pharmacokinetic study protocol was approved by the Institutional Animal Ethics Committee, SreeSiddaganga College of Pharmacy, Tumkur. The animals were obtained from Central Animal House, SreeSiddaganga College of Pharmacy. The study was conducted in male Wistar rats (150-200g) following oral administration of both drugs and their optimized complexes. The animals were housed in cages with proper environmental control with free access to standard diet and water.

Study design

The animals were divided into four groups, each group containing four animals. Group 1 was given SQV, Group 2 was administered with SQV-SBE7βCD complex. Group 3 was given RTV and Group 4 was administered with RTV-RMβCD complex. After keeping animals for overnight fasting, drug equivalent to 10 mg/Kg of rat was calculated and both drugs and their complexes were dispersed in 1 ml of sodium carboxy methyl cellulose (sodium CMC) solution (0.1% w/v) and was given orally via feeding tube.[12]

Blood sampling

Serial blood samples (1 ml) were collected from retro orbital plexus of rats into micro centrifuge tubes containing EDTA coated tubes. Samples were taken at 0, 0.5, 1, 2, 4, 8, 12 h post-dose. The animals were fed with standard diet after 4th h sampling. After each withdrawal of blood, equal volume of intra peritoneal saline was replaced. For separation of Plasma, centrifugation (2000 g) under refrigeration (2–8°C) for 10 min was performed and each plasma sample was immediately collected and stored at -20°C until drug analysis.

Chromatographic Conditions

For detection, Atmospheric pressure ionization (API) 4000 triple quadrupole mass spectrometer detector equipped with turbo ion spray source and operated in the positive mode. Analysis was performed with electrospray ionization using a turbo ion spray ionization source. The turbo ion spray ionization source was operated at 550°C with an ionization voltage of 5500 V with ultrahigh-purity nitrogen as curtain gas 60 PSI, nebulizer gas (40 PSI) and auxiliary gas (40 PSI). Nitrogen was used as collision activated dissociation (CAD) gas and was set at 6 L/h. Quantification was performed using multiple reaction monitoring (MRM) mode based on the precursor m/z and its fragment m/z (MRM transition) for each analyte. Analyst 1.5.2 software was used for system operation and data handling. Samples were chromatographed on a Waters BEH C18 (Ethylene bridged Hybrid particles with 1.7 μm particle size), 50 x 2.1mm (length x dia) column. The temperature of the column was maintained at 35°C.

Formic acid buffer (0.1%) and acetonitrile in the ratio of 50:50 v/v were used as mobile phase. The mobile phase components were filtered before use through a 0.45 μm membrane filter and pumped isocratically at a flow rate of 1.0 ml/min. The volume of the sample analyzed was

10 µl. The drug concentrations (analytes) were detected by monitoring the transactions 675.5 ± 0.5 amu to 570.5 ± 0.5 amu and 721.5 ± 0.5 amu to 296.30 ± 0.5 amu with collision energy of 4 and 27 V for both the drugs and lopinavir which was used as internal standard (IS) respectively. The analytical time for each run was 3 min in total.

Analytical Method

The determination of SQV and RTV plasma samples was performed using a previously described LCMS/MS method.[6] Lopinavir was chosen as internal standard because it showed similar chromatographic behavior to both SQV, RTV with no interference with both saquinavir and ritonavir by admixture in rat plasma. Parent and daughter ion spectra of SQ< RTV and LPV are given in Figure 1. The chromatograms of SQV, RTV were clearly distinguishable and retention times of SQV, RTV and LPV were found to be 0.73, 1.96 and 2.39 min respectively, under specified chromatographic conditions. The assay was validated with a detection limit of 20ng/ml for both drugs and a lower limit of quantification and

upper limit of quantification were found to be 71.7 and 1570 ng/ml (SQV) and 60.9 and 1650 ng/ml (RTV). The correlation coefficients (r) of the calibration curves greater than 0.99 for all analytes as determined by linear regression analysis for saquinavir and for ritonavir, with a $1/x^2$ regression, where x is the concentration, over a concentration range of 50-1500 ng/ml for both the drugs. A representative calibration curve of SQV and RTV for resulted in the linear least squares regression equations, SQV: y=0.000368x+-0.0154, RTV: y=0.000646x+4.14e^{-005}, where x is the concentration of SQV and RTV (ng/mL) and y is the peak area ratio of both SQV and RTV to the IS. Both drugs were found to be stable in rat plasma at least one month at -20°C.

Pharmacokinetic analysis

20 µl of internal standard solution was added to aliquots of 100 µl of plasma from study samples in 1.5 ml microcentrifuge tubes, and mixed gently. Each tube was added with 400 µl of acetonitrile and vortexed for 20 sec at high speed. The tube was centrifuged at 12,000×g for 5 min to pellet the precipitated proteins and give a clear supernatant. These clear supernatants were transferred to vials and were placed in the auto sampler tray for injection onto the LC column and 5 µl plasma volume was injected into LC-MS/MS system.

Data analysis

Pharmacokinetic parameters were analyzed using standard, non-compartmental techniques. From the plasma concentration-time curves, maximum plasma drug concentrations (Cmax) and the time to reach these concentrations (T_{max}) were determined. The pharmacokinetic parameters C_{max}, T_{max}, Area Under the Curve (AUC), Apparent terminal elimination rate constant (K_e), Apparent terminal elimination half-life ($t_{1/2}$), Area under the first moment of plasma concentration-time curve (AUMC), Mean residence time (MRT),Apparent total body clearance, Apparent volume of distribution (V_d/F)of both drugs were estimated form the individual plasma drug concentration-time profile using non-compartmental analysis.[13-16]

Statistical analysis

The pharmacokinetic parameters of the tested formulations were statistically analysed using unpaired t-test for C_{max}, $AUC0_{-12h}$ and $AUC_{0-\infty}$ values.[17] All tests were performed using Graph Pad Prism 5.03 (Graph Pad Software, Inc.CA, USA) software (trial version). The level of significance was set at p<0.05.
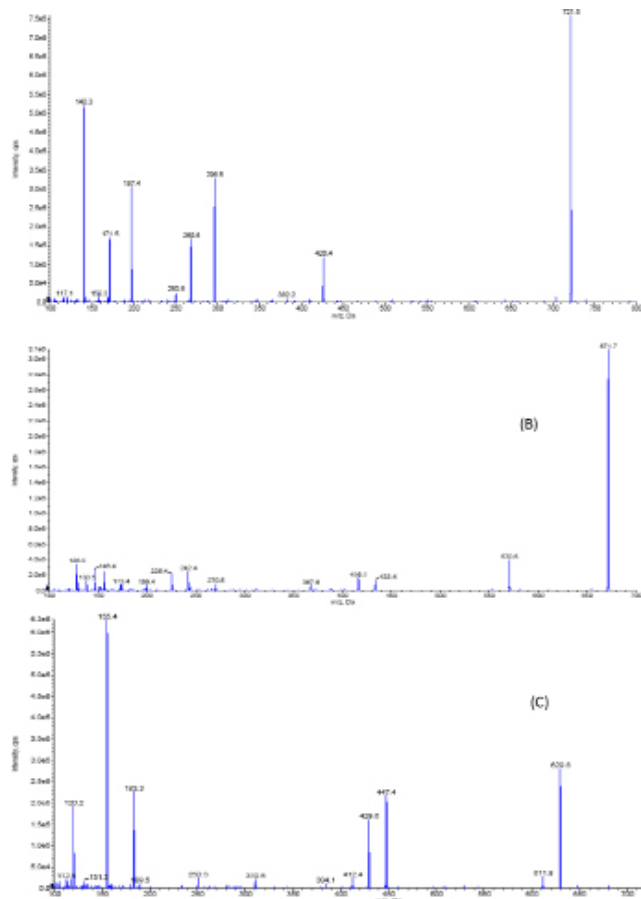
Figure 1: Parent molecule and daughter ion mass spectrum of A) ritonavir B) saquinavir C) lopinavir.

RESULTS AND DISCUSSION

Pharmacokinetic evaluation of SQV-SBE7βCD complex

LCMS chromatograms of SQV, RTV and LPV in blank plasma is given in Figure 2 and 3.The comparative mean plasma concentration-time profiles of SQV for both treatments are shown in Figure 4.

C_{max} and the time to reach C_{max} (T_{max}) were read directly from the observed plasma concentration vs. time data. The plasma elimination half-life was estimated from the log-linear regression of the terminal plasma concentrations as a function of time after dosing. The area under the plasma concentration vs. time curve was calculated by using the linear trapezoidal rule over a single hour dosing interval. All the calculated pharmacokinetic parameters such as C_{max}, T_{max}, AUC_{0-12h}, $AUC_{0-\infty}$, $AUMC_{0-\infty}$, MRT, K_e, $t_{1/2}$, V_d/F and Cl/F for each subject following single oral administration of SQV and its complex and mean, s.d.,% CV values of pharmacokinetic parameters for each subject are given in Table 1. The C_{max} of SQV from SQV and SQV complex with SBE7βCD was found to be 254±18.81 and 515.25±28.65 ng/ml respectively. The T_{max} values were unchanged and found to be 4 h for
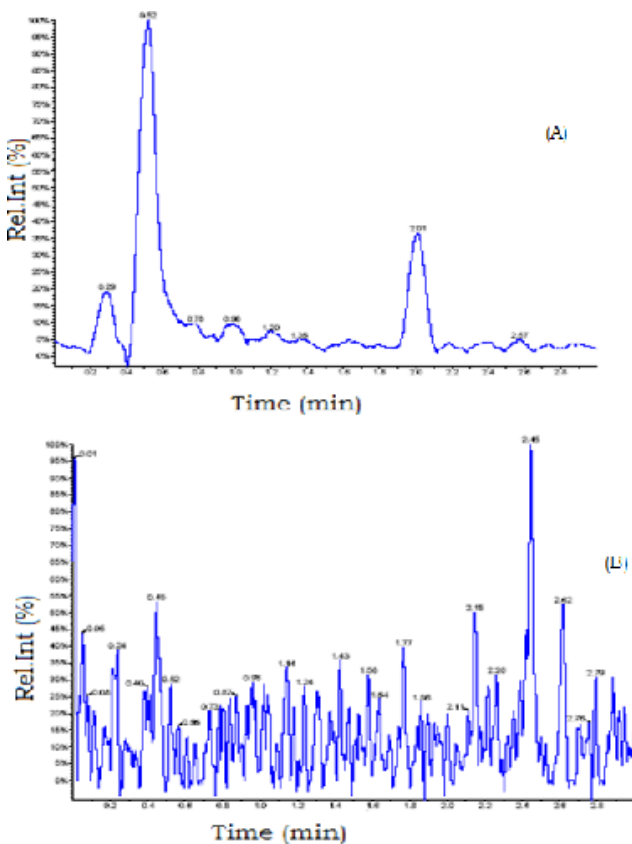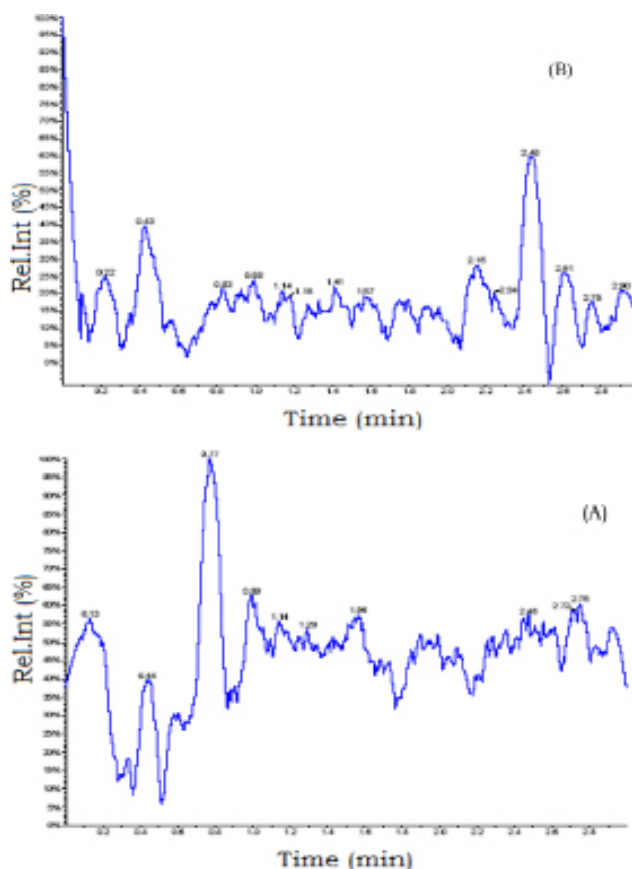

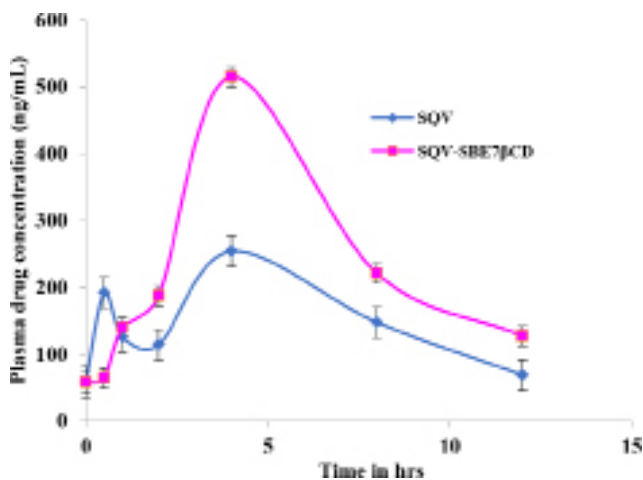**Figure 3: LCMS Spectra of A) RTV and B) LPV (IS) in blank plasma.**

Figure 4: Comparative mean SQV plasma concentration-time profile.

both groups. The elimination rate constant and $t_{1/2}$ were found to be in the range of 0.163 to 0.175 h^{-1} and 3.99 to 4.26 h respectively for both groups. The obtained $t_{1/2}$ of SQV was found to be in the range of earlier reported values (3.5-4.5 h).[18]

AUC_{0-12h} and $AUC_{0-\infty}$ values for SQV were found to be 1867.5±42.07 and 2293.04±82.13 ng.h/ml respectively. For SQV complex, the respective values were found to be 3116.99±116.46 and 3860.93±138.50 ng.h/ml.

**Figure 2: LCMS spectra of A) SQV B) LPV (IS) in blank plasma.**

Table 1: Plasma concentration following oral administration of SQV and SQV-SBE7βCD complex in Wistar rats.

Parameters	Units	SQV Mean± SD (n=4)	%CV	SQV-SBE7βCD complex Mean± SD (n=4)	%CV
K_e	hr^{-1}	0.163±0.01	7.83	0.175±0.02	13.71
$t_{1/2}$	hr	4.26±0.32	7.68	3.99±0.50	12.59
T_{max}	hr	4.00±0.00	--	4.00±0.00	--
C_{max}	ng.hr/mL	254.00±18.81	7.40	515.25±28.65	5.56
AUC_{0-12hr}	ng.hr/mL	1867.5±42.07	2.25	3116.99±116.46	3.73
$AUC_{0-\infty}$	ng.hr/mL	2293.04±82.13	3.58	3860.93±138.50	3.58
$AUMC_{0-\infty}$	ng.hr/mL	21679.30±1486.90	6.85	36970.91±4044.98	10.94
MRT	hr	9.44±0.38	4.04	9.55±0.75	7.91
V_d/F	L	26.83±1.69	6.31	14.92±1.56	10.47
Cl/F	L/hr	4.36±0.158	3.62	2.59±0.09	3.58

Two fold increment in C_{max} value and 1.66 fold increment in AUC_{0-12h} were obtained for SQV complex than SQV alone. The relative percent bioavailability (F_{rel}) of SQV complex was 168% indicating enhanced oral bioavailability of SQV complex. Plasma concentration-time profile of SQV showed low and double peak phenomenon whereas SQV-SBE77βCD showed smooth elevation of plasma levels with one C_{max} value i.e. single peak.

The V_d/F and Cl/F were found to be 26.83±1.69 L and 4.36±0.15 L/h respectively for SQV, 14.92±1.56 L and 2.59±0.09 L/h respectively for SQV complex.

The variability in oral pharmacokinetics of SQV could also be explained by its limited solubility. Because the water solubility of SQV is negligible, its dissolution in GIT is dependent on the pH and the inherent solubilization capacity of the intestine. In rats receiving SQV suspension, a small variation of these factors could lead to a significant difference in dissolution properties and subsequently in the plasma drug concentration. However, in rats receiving SQV-SBE7βCD, as SBE7βCD could solubilize SQV, the impact of such factors became non-determinative and thus, the oral kinetics in rats receiving SQV-SBE7βCD showed less variability than that in rats receiving SQV suspension. It was noticed that there was a primary peak at 0.5 h and a secondary peak at about 4 h (T_{max}) after dosing in the concentration vs. time graph for rats given the SQV suspension where as such a phenomenon was not observed for the rats receiving SQV-SBE7βCD. Several possible mechanisms have been proposed to explain double peak phenomenon of orally administered protease inhibitors such as entero-hepatic recycling, regional distribution differences of active absorption/efflux proteins in the gut, and variable gastric emptying. Earlier literature on SQV also reported similar type of double peak absorption phenomenon as observed in the present investigation.[12,19,20]
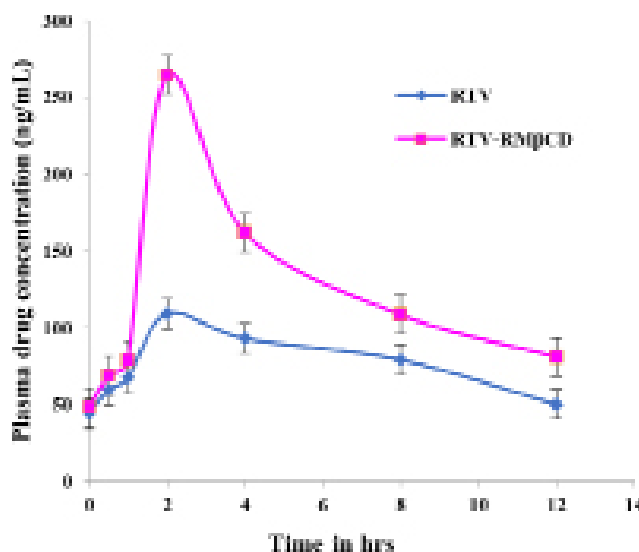
Figure 5: Comparative mean RTV plasma concentration-time profile.

Pharmacokinetic evaluation of RTV-RMβCD complex

The comparative mean plasma concentration-time profiles of RTV for both treatments are shown in Figure 5. All the calculated pharmacokinetic parameters such as C_{max}, T_{max}, AUC_{0-12h}, $AUC_{0-\infty}$, $AUMC_{0-\infty}$, MRT, K_e, $t_{1/2}$, V_d/F and Cl/F for each subject following single oral administration of RTV and its CD complex and mean, s.d., % CV values of pharmacokinetic parameters for each subject are given in Table 2. The peak plasma concentration (C_{max}) RTV in the pure drug and its complex were 109.17±9.6 and 264.74±16.52 ng/ml, while AUC_{0-12h} and $AUC_{0-\infty}$ were found to be 951.20±28.54, 1636.07±162.51 ng.h/ml, 1585.16±56.22 and 2300.19±118.21 ng.h/ml respectively. These values indicated maximum plasma concentration and area under the curve were achieved by RTV complex formulation. C_{max} value was 2.4 times and AUC_{0-12h} was 1.66

Table 2: Pharmacokinetic parameters of RTV and RTV-RMβCD complex in Wistar rats.

Parameters	Units	RTV Mean± s.d. (n=4)	RTV %CV	RTV-RMβCD complex Mean± s.d. (n=4)	RTV-RMβCD complex %CV
K_e	hr^{-1}	0.075±0.011	15.86	0.115±0.0144	12.5
$t_{1/2}$	hr	9.37±1.59	17.00	6.07±0.69	11.43
T_{max}	hr	2.00±0.0	--	2.00±0.00	--
C_{max}	ng.hr/mL	109.17±9.60	8.80	264.75±16.52	6.23
AUC_{0-12hr}	ng.hr/mL	951.20±28.54	3.00	1585.16±56.22	3.54
$AUC_{0-\infty}$	ng.hr/mL	1636.07±162.51	9.93	2300.19±118.20	5.13
$AUMC_{0-\infty}$	ng.hr/mL	25466.92±6451.36	25.33	26866.09±3505.39	13.04
MRT	hr	15.40±2.25	14.61	11.64±0.97	8.38
V_d/F	L	82.35±6.45	7.83	38.06±3.10	8.15
Cl/F	L/hr	6.15±0.56	9.16	4.35±0.22	5.09

Table 3: Statistical summary of pharmacokinetic parameters (p<0.05).

Drug	Parameter	Units	Calculated t value	P value (two tailed)
SQV	C_{max}	ng/mL	15.24*	<0.0001
	AUC_{0-12hr}	ng.hr/mL	34.37*	<0.0001
	$AUC_{0-\infty}$	ng.hr/mL	19.47*	<0.0001
RTV	C_{max}	ng/mL	16.28*	<0.0001
	AUC_{0-12hr}	ng.hr/mL	20.11*	<0.0001
	$AUC_{0-\infty}$	ng.hr/mL	6.609*	<0.0006

*significant difference.

times higher for RTV complex than RTV. The relative percent bioavailability (F_{rel}) of RTV complex observed was 140.58% indicated enhanced oral bioavailability of RTV complex. Pure RTV also exhibited slightly variable bioavailability, which is commonly observed with protease inhibitors.[21] However, with RTV-RMβCD complex, such phenomenon was not observed.

The T_{max} values were unchanged and found to be 2 h for both groups. The elimination rate constant was found to be in the range of 0.011 to 0.144 h^{-1} and $t_{1/2}$ for RTV was found to be 9.37±1.59 h and for RTV complex it was 6.07±0.69 h respectively for RTV complex and is nearer to the reported values (6.07-7.35 h).[21]

For pure drug, the V_d/F and Cl/F were found to be 82.35±6.45 L and 6.15±0.56 L/h respectively and for its complex, 38.06±3.1 L and 4.35±0.22 L/h respectively.

Statistical analysis

To test the significant difference if any between complexes and pure drug, pharmacokinetic parameters C_{max}, AUC_{0-12h} and $AUC_{0-\infty}$ were subjected to statistical analysis (Table 3). The results of statistical analysis showed significant difference as the p value is <0.05. The C_{max} and AUC values of the experimental formulation were significantly higher compared to the pure drug indicating the higher bioavailability of the experimental formulation of SQV prepared with SBE7βCD and RTV prepared with RMβCD. Thus, the results of the present study clearly indicated the applicability of cyclodextrin complexation in the improvement of oral bioavailability of SQV and RTV compared to pure drugs alone.

CONCLUSION

Pharmacokinetic studies were conducted in healthy male Wistar rats showed marked increase in the AUC of saquinavir and ritonavir when both the drugs were administered orally in combination with cyclodextrins. Significant enhancement in bioavailability of both drugs was observed with optimized CD complexes compared to pure drugs. The pharmacokinetic parameters like $t_{1/2}$, T_{max} were in the range of the reported values. The absorption profile of SQV from oral SQV suspension was highly irregular and variable due to its poor solubility. This variation was not seen with SQV-SBE7βCD complex due to improvement in solubility.

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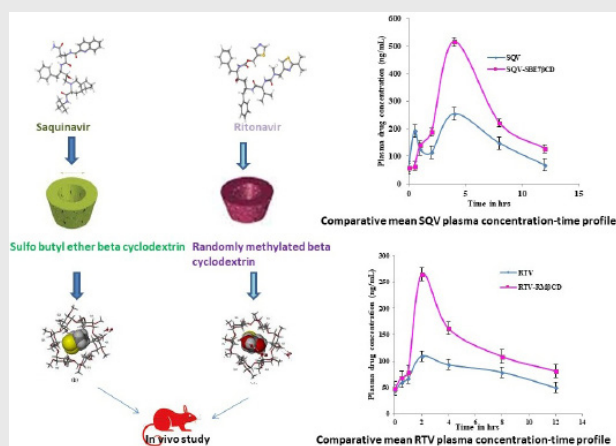
CONFLICT OF INTEREST

Authors have none to declare.

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PICTORIAL ABSTRACT

Comparative mean SQV plasma concentration-time profile

Comparative mean RTV plasma concentration-time profile

SUMMARY

- A comparative study was performed on pharmacokinetics and bioavailability of saquinavir (SQV) and ritonavir (RTV) and their optimized cyclodextrin complexes of anti-retro viral drugs after oral administration into rats. Plasma concentrations of both drugs and their complexes were determined using liquid chromatography tandem mass spectrometry, LCMS/MS. The cyclodextrin complexes of both saquinavir (3860.93±138.50 ng.hr/ml) and ritonavir (2300.19±118.21 ng.hr/ml) had shown higher $AUC_{0-\infty}$ values compared to pure drugs, saquinavir (2293.04±82.13 ng.hr/ml) and ritonavir (1636.07±162.51 ng.hr/ml). Higher bioavailability and higher peak plasma concentrations were obtained with cyclodextrin complexes of both SQV and RTV compared to SQV and RTV alone.

About Authors

Dr. Bharani S Sogali, is presently working as Professor in Krupanidhi College of Pharmacy, Bengaluru and She is having 13 years of teaching and research experience and having publications in national and international journals.