In-silico assisted development of supersaturable preconcentrated isotropic mixture of atazanavir for augmenting biopharmaceutical performance in presence of H₂-receptor antagonist

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Abstract

The therapeutic potential of atazanavir (BCS Class II drug), a highly selective inhibitor of human immunodeficiency virus (HIV-1) has been largely limited due to its low intrinsic solubility at elevated pH resulting in low oral bioavailability. Thus, the current work describes the systematic development, optimization and evaluation of hydroxypropyl methylcellulose acetate succinate (HPMC-AS) based supersaturable preconcentrate isotropic mixture (SP-IM) containing long chain triglyceride to improve intestinal lymphatic transport and augment oral bioavailability of atazanavir (ATZ). A D-optimal mixture design was employed for optimization of plain IM containing Corn Oil, Oleic acid, Tween 80 and Propylene Glycol, evaluating various critical quality attributes (CQAs) like particle size, PDI, self-emulsification time, % transmittance and drug content. *In-silico* analysis and *in-vitro* supersaturation test facilitated the selection of HPMC-AS as a best suited polymeric precipitation inhibitor (PPI) for formulating ATZ loaded SP-IM (ATZ-SP-IM). *In-vitro* dissolution data indicated that ATZ-SP-IM exhibits superior performance in 0.025N HCl and pH 6.8 over pure drug. *Ex-vivo* permeation and *in-vivo* pharmacokinetic study of ATZ-SP-IM corroborated enhanced permeation (2.03 fold) and improved drug absorption via lymphatic transport in wistar rats. Further, the pharmacokinetic performance of ATZ-SP-IM was not affected in presence of H₂ receptor antagonist. Therefore, the results showed that ATZ-SP-IM can significantly improve the biopharmaceutical attributes of ATZ so as to lay a foundation of further research on the new dosage form of ATZ.

1. Introduction

Acquired immunodeficiency syndrome (AIDS) commonly referred as Human immunodeficiency virus (HIV), constitutes one of the serious infectious disease challenging the public health globally, as around 37.7 million people have been infected with HIV till 2020. As per the World Health Organization (WHO) report submitted in the year 2020, around 1.5 million new cases appeared and 0.68 million people lost their lives due to HIV [1]. The entry of HIV into the human body weakens the immune system by destroying the vital cells and further resides in the body by making its reservoir in various anatomical (central nervous system, lymphatic system, spleen, liver, lungs and genital tract) and cellular (macrophages, CD4 + lymphocytes and dendritic cells) sites [2, 3]. Interventions such as AIDS counselling, educational tools and antiretroviral drug therapy have contributed to transform HIV infection from a fatal to a manageable chronic infectious disease [4]. It has been reported that around 73% of the total infected people are receiving highly active antiretroviral therapy (HAART). The administration of HAART to the infected patients has significantly reduced the mortality and morbidity rate [5, 6]. However, the insufficient amount of the drug concentration at the reservoir site may enhance the chances of viral mutations and drug resistance [7]. Due to these possible reasons, the current therapy lacks in offering the complete eradication of the virus from the host body.

Atazanavir (ATZ) is an azapeptide derivative and the seventh addition to the protease inhibitors used for the treatment and management of HIV [8]. ATZ selectively inhibits the virus-specific processing of viral Gag and Gag-Pol polyproteins in HIV-1 infected cells by binding onto the active site of HIV-1 protease, thus prevents the formation of mature virions. Atazanavir possess advantage over that of other protease inhibitors because of distinct resistance profile and 2 to 20 fold higher antiretroviral activity [9, 10]. Similar to the other drugs in this class, atazanavir (BCS Class II drug) is a weak base that exhibits pH dependent solubility over the physiologic pH range [11]. The ATZ exhibits low oral bioavailability (60%) potentially owing to its low solubility which might get decreased to less than 1 µg/mL when acid reducing agents are co-administered [12]. In a routine practice, administration of antiretroviral drugs generally cause secondary effects like heart burn and stomach pain, so to overcome this problem, acid reducing agents are co-administered [13–15]. The *in-vitro* solubility profile of ATZ and available clinical data suggests that elevation in the gastric pH by acid reducing agents (ARAs) can alter the drug absorption resulting in lower systemic exposure [13, 16, 17]. Besides, ATZ undergoes extensive hepatic metabolism, efflux by P-gp transporter and high metabolism by cytochrome P450 (CYP) 3A. To augment the oral bioavailability, atazanavir is generally administered with low-dose ritonavir (known to be P-gp efflux inhibitor) [8]. Despite of improvement in the oral bioavailability, atazanavir possesses poor access into the lymphatics, which acts as a viral

sanctuary for persistent HIV infection. To circumvent the aforementioned limitations, various formulations of atazanavir have been reported *viz* solid lipid nanoparticles (SLNs) [18], nanostructured lipid carriers (NLCs) [1], solid dispersion [8], mesoporous silica particles [15], SEDDS [19], SNEDDS [19] and nanoparticles [20]. All these formulations were reported to improve biopharmaceutical attributes by increasing the solubility and thereby dissolution in absence of ARAs. Thus, it can be interpreted that none of these strategies were able to overcome the issue of reduction in the biopharmaceutical performance of ATZ when co-administered with acid reducing agents and the lymphatic delivery simultaneously. Hence, a need was felt to develop a promising ATZ formulation that has capability to resolve the problem of low drug absorption due to precipitation of drug at an elevated pH and the targeted delivery of the atazanavir to the lymphatics.

To promote the intestinal lymphatic transport of atazanavir, lipid-based drug delivery could be exploited. Amongst various lipid-based delivery systems, an isotropic mixture (IM) of suitable oil containing long chain triglycerides, surfactant and co-surfactant in this regard have proved as promising drug delivery system for promoting the lymphatic transport of drug [21]. However, after oral administration of atazanavir loaded isotropic mixture, the drug could be precipitated when exposed to the gastrointestinal fluids [22]. To prevent the precipitation of the lyphophilic drugs, polymeric precipitation inhibitors (PPIs) are incorporated into the IM, which are generally said to be supersaturable preconcentrate IM (SP-IM) [23]. The SP-IM represents to be a newer technology in which the PPIs intended to generate and maintain a meta-stable supersaturated state *in-vivo* by preventing or minimizing the drug precipitation [24]. However, to the best of our knowledge, hydroxypropyl methylcellulose acetate succinate (HPMC-AS) based SP-IM have not been investigated yet for the lymphatic uptake of atazanavir.

The aim of the present investigation, therefore, focused on QbD steered systematic development of the supersaturated preconcentrate IM (SP-IM) of ATZ containing long chain triglycerides that have a capability to augment intestinal permeation and oral bioavailability at an elevated pH and assist in promoting the lymphatic uptake for the eradication of virus from the viral sanctuaries. For this, we developed SP-IM of ATZ by using different types of polymeric precipitation inhibitors (PPIs) published in the literature reports. The developed supersaturable formulations were tested for its efficiency to inhibit precipitation with regard to dissolution efficiency and dissolution-retaining time. The best suited PPI based supersaturable formulation was extensively evaluated by *in-vitro*, *ex-vivo* and *in-vivo* study.

2. Materials And Methods

2.1 Materials

Atazanavir Sulfate was provided *ex-gratis* by Sun Pharmaceutical Industries Ltd., Gurugram, India. Corn Oil, Oleic Acid, Castor Oil, Sesame Oil, Soyaben Oil And Olive Oil were received as a kind gift samples from Croda Inc, Mumbai, India. Labrafac Lipophilic WL 1349, Capryol PGMC, Labrafil M 2125CS, Labrasol, Labrasol ALF, Maisine 35 – 1, Lauroglycol, Labrafac PG, Plurol Oleique CC497, Labrafil M 1944 CS and Transcutol P were received as the gift samples from Gattefosse, Saint-Priest, France. Kolliphor EL, RH 40, HS 15, Vitamin E TPGS, PVP K17, Poloxamer 407 and Poloxamer 188 were received as the gift sample from BASF, Mumbai, India. Capmul MCM C10, Capmul PG8, Captex 355 and Captex 200p were received as the gift samples from Abitec Corp., Wisconsin, USA. PEG 400, Tween 80 and Propylene glycol were procured from Loba Chemie, Mumbai, India. Sefsol 218 was obtained as a gift sample from Nikko Chemical Co., Ltd., Tokyo, Japan. Grapeseed Oil, Muskmelon Oil, Watermelon Oil and Poppyseed Oil were purchased from Satpal & Sons, Patiala, India. HPMC E15 was obtained as a gift sample from Colorcon Asia Pvt. Ltd., India. HPMC-AS was received as a gift sample from Shin-Etsu Chemical Co. Ltd., Japan. The solvents and chemicals employed for liquid chromatographic studies were all of HPLC grade. All other materials employed during the studies were of analytical grade and were used as such as obtained.

2.2 Methods

2.2.1 Validation of analytical method

The analytical method of ATZ employing high-performance liquid chromatography (HPLC) system was validated according to US FDA and European Medical Agency guidelines of bioanalytical method validation [25]. The analytical method was validated using 50 mM Potassium dihydrogen phosphate Buffer pH 4.5: Acetonitrile: Methanol::40:45:15 and 0.07% TFA (trifluoroacetic acid) as mobile phase at 249 nm λ_{max} ; 0.7 mL/min flow rate; Xbridge 3.5 μm , 4.6 x 150 mm analytical column. Further, this estimation of ATZ was validated for linearity (200 ng/mL- 100000 ng/mL), limit of detection (13.62 ng/mL), limit of quantification (45.40 ng/mL), Inter-assay and intra-assay Precision and Accuracy. The linearity equation ($y = 72.26x + 3542$; $R^2 = 0.99$, where "y" is the area under curve and "x" is the concentration in ng/mL) was then employed to obtain unknown ATZ concentration in various samples.

2.2.2 Equilibrium solubility of ATZ in various oils, surfactants and co-surfactants

The equilibrium solubility of ATZ was quantified in various natural oils, synthetic/semi synthetic oils, surfactants, and co-surfactants. For this purpose, an excess amount of ATZ was added to each capped glass vial formerly containing 1 g of surfactant and/or co-surfactant and/or oil and vortexed for 5 min after every 2 h for 24 h at a constant temperature ($40 \pm 0.5^\circ\text{C}$) in shaking incubator at 50 rpm until the attainment of equilibrium phase [26]. An aliquot of 0.5 mL sample was withdrawn at 72 h and centrifuged at 2,000 rpm ($\sim 180 \times g$) for 10 minutes. The resultant supernatant was appropriately filtered through 0.45 μm membrane filter, diluted with methanol and was examined by utilizing validated HPLC method.

2.2.3 Ternary phase diagram

In order to determine the levels of the material attributes (MA) for D-optimal mixture design, ternary phase diagram with selected surfactant and co-surfactant along with combination of oils was constructed; each of them positioned at an apex of the triangle using PCP-Disso software (Poona College of Pharmacy, Pune, India). After equilibrium, the time of self-micro emulsification efficiency, dispersibility, appearance, percentage transmittance (at 560 nm) and flowability was observed [27, 28]. The clear isotropic mixture regions in the diagrams were plotted, and the wider region indicated the better self-emulsification efficiency was determined.

2.2.4 Systematic optimization of ATZ loaded isotropic mixture as per mixture design

D-Optimal mixture design (Design-Expert® software version 11; Stat-Ease, Inc., Minneapolis, MN) was used to optimize the composition of the critical material attributes (CMAs) of the isotropic mixture. The range of Corn oil: Oleic acid (CO: OA) (1:1) (oil; X_1), Tween 80 (T80) (surfactant; X_2), and Propylene glycol (PG) (co-surfactant; X_3) were set to 0.1–0.2 g, 0.5–0.7 g and 0.2–0.4 g respectively (Table 1). For each formulation, the total of the composition of X_1 , X_2 and X_3 was summed to 100% i.e. 1 g. Briefly, the ATZ loaded IM were prepared by simple admixture method. An amount of ATZ (50 mg) was dissolved in propylene glycol at 80°C by stirring on a magnetic stirrer. Subsequently, the Tween 80 and corn oil: oleic acid (1:1) was further added as per the design matrix to form IM. Particle size (Y_1 ; nm), polydispersity index (PDI) (Y_2), Self emulsification time (SET) (Y_3 ; sec), percent transmittance (%T) (Y_4 ; %) and Drug content (DC) (Y_5 ; %) were evaluated as the critical response variables (CRVs) to optimize the ATZ loaded IM by utilizing the desirability function. The design consisted of 16 experimental runs to find an appropriate polynomial model and to evaluate the effects of CMAs on the CRVs.

2.2.5 Determination of critical response variables (CRVs) as per D-optimal mixture design

The developed formulations were determined for various critical response variables (CRVs) namely particle size (Y_1), Poly dispersity index (Y_2), Percent transmittance (Y_3), Drug Content (Y_4) and Self emulsification time (Y_5). The method for

estimating Y_1 , Y_2 , Y_3 and Y_4 has been discussed in Supplementary data.

Self Emulsification Time (SET) (Y_5)

The self-emulsification time of IM pre-concentrates were determined through aqueous dilution method using a standard US Pharmacopeia dissolution apparatus type II (Electrolab, Mumbai, India) [29]. The time required by IM pre-concentrates to completely emulsify in the dilution media was noted.

2.2.6 Selection of polymer precipitation inhibitor (PPI)

(a) In-silico analysis

The 3D-structure of ligand (ATZ) and different PPIs like Poloxamer 188, Poloxamer 407, HPMC E15, HPMC-AS and PVP K17 were downloaded from freely available chemical structure database i.e. chemspider. Further, the 3D structure of ligand and polymers were clean up in Chem Draw Ultra 12.0 software. Each polymer containing one repeating unit was docked with ligand (ATZ) respectively by using the MGL tool (Autodock software version 1.5.6) (M/s GNU General Public Licence, CA, USA). For all docking calculations, the size of grids was set as $40 \text{ \AA} \times 40 \text{ \AA} \times 40 \text{ \AA}$ with grid spaces of $0.375 \text{ \AA}$. Lamarckian genetic algorithms (LGA) was applied to probe the most favorable drug-exciipient complex geometry. The other docking parameters were set to the default values [30–32].

After molecular docking study, the stability of the different ligand-PPIs complex was accessed by molecular dynamics simulations (MDS) for a period of 30 ns, using a freely available academic version of “Desmond” program. The solvent system was built around the ligand-PPIs complex using the TIP3P water model and the shape of the box was kept as orthorhombic with dimensions $10 \times 10 \times 10 \text{ \AA}$. The neutralization of physiological pH of the system was done by adding the Na^+ counter ions and the salt concentration was set as 0.15 M. After the energy minimization, the constant temperature was maintained with Nose-Hoover chain thermostat to attain NPT equilibration at 310K, while, constant pressure was acquired using the Martyna-Tobias-Klein barostat. Thereafter, RMSD was calculated using the desmond protocol [33–35].

(b) In-vitro supersaturation test

To prevent the ATZ loaded IM from precipitation in the intestinal condition, supersaturable IM (SP-IM) were prepared by adding different types of polymeric precipitation inhibitors (PPIs) (i.e., Poloxamer 188, Poloxamer 407, HPMC E15, HPMC-AS and PVP K17) to the optimized IM. The SP-IM was prepared by adding different type of PPIs along with ATZ in propylene glycol. Subsequently, the Tween 80 and Corn oil: Oleic acid (1:1) was further added to form ATZ loaded SP-IM. PPIs are incorporated in an amount equivalent to 2.5% of the total weight of optimized IM. Further, the prepared formulations were subjected to *in-vitro* supersaturation test in simulated intestinal fluid (pH 6.8) under non-sink conditions [36, 37]. A 500 mL of dissolution media was added to a 1000 mL dissolution basket equipped with a rotating paddle maintained at $37 \pm 0.5^\circ \text{C}$ and 50 rpm (Electrolab, Mumbai, India). An aliquot of 3 mL was withdrawn from different formulations at specific time intervals without volume replacement and the amount of drug at each time interval was estimated by utilizing the validated in house HPLC method. The apparent drug concentration-time profile and the duration of the supersaturated state were subsequently determined [23, 24, 38, 39]. Further, the PPI with a higher degree of supersaturation was selected for further studies.

2.2.7 Evaluation of atazanavir loaded supersaturable isotropic mixture (ATZ-SP-IM)

2.2.7.1 Determination of particle size (PS), poly dispersity index (PDI) and Zeta potential

The particle size, PDI and zeta potential of the ATZ-SP-IM were determined by dynamic light scattering technique using particle size analyzer (Nano ZS 90, M/s Malvern, Worcestershire, UK).

2.2.7.2 Transmission electron microscopy

The morphological and structural behavior of the microemulsion droplet of the ATZ-SP-IM was examined by using high-resolution transmission electron microscopy (JEM 2100 Plus, Jeol, Tokyo, Japan) [27].

2.2.7.3 Stability testing of ATZ-SP-IM

The storage stability of ATZ-SP-IM was assessed as per ICH Q1A (R2) guidelines under accelerated study conditions ($40 \pm 2^\circ\text{C}/75 \pm 5\%$). The 10 g of ATZ-SP-IM sample was packed in an air tight culture tube and maintained in a stability chamber (Remi, SC-10 Plus, India). At specific time points, particle size, PDI, drug content, cloud point, signs of drug precipitation, self-emulsification time and % Transmittance were estimated to confer stability [35]. In addition, the thermodynamic stability testing of ATZ-SP-IM was also assured by freeze thaw cycle and centrifugation test as per method reported by Kamboj et al. [40].

2.2.8 *In-vitro* drug dissolution study

Dissolution studies of ATZ-SP-IM and ATZ powder were conducted using USP apparatus II (Electrolab, Mumbai, India) employing 900 ml of office of generics media (OGD media) 0.025N HCl and phosphate buffer (pH 6.8) as the dissolution medium maintained at $37 \pm 0.5^\circ\text{C}$ and 50 rpm [15, 37]. ATZ-SP-IM containing ATZ equivalent to 50 mg or ATZ powder (50 mg) loaded into hard gelatin capsule were subjected to dissolution testing. At pre-determined time intervals, an aliquot of 3 mL sample (each) was withdrawn with subsequent replacement of equivalent volumes of the medium. The percentage ATZ released at specific time intervals were determined by in house validated HPLC method.

2.2.9 Drug permeation study across rat's everted intestinal sac

An everted rat gut sac experiment was performed as described in the literature reports [27, 41]. Cognizance was taken that the research work, involving drug permeation and pharmacokinetic study adheres to the guidelines for care and use of the laboratory animals. Thus, all the animal investigations were performed as per the requisite protocol approved by the Institutional Animal Ethics Committee (IAEC), Punjabi University, Patiala, India (107/99/ CPCSEA/2018-05).

Male wistar rats weighing 200–250 g were used for this study. Prior to surgical procedure, the rats were fasted overnight with free access to water. Overnight-fasted rats were sacrificed by cervical dislocation. The excised medial jejunum segment (length ~ 6 cm; internal diameter ~ 0.3cm; area ~ 5.8 cm^2) was immediately flushed with ice cold Krebs's Ringer phosphate buffer (KRPB). After washing, one end of the tissue segment was then ligated with thread to one end of the thin glass rod and carefully everted on a glass rod. The prepared formulations equivalent to 50 mg of ATZ was transferred to donor compartment. The everted gut sac filled with 2 mL of KRPB solution was submerged inside the aerated (10–15 bubbles/min) bath (50 mL; $37 \pm 0.5^\circ\text{C}$) containing ATZ-SP-IM or ATZ powder. An aliquot of drug solution was withdrawn from the serosal compartment at a predetermined time intervals up to 2 h and replaced with fresh KRPB solution. The amount of ATZ permeated was determined by analyzing samples using in-house validated HPLC method. The values of flux, apparent and relative permeability of different formulations were calculated.

2.2.10 *In-vivo* pharmacokinetic and lymphatic tissue distribution study

For the *in-vivo* pharmacokinetic study, male wistar rats (weighing 250–280 g) were randomly divided into five groups (four rats in each group) according to the two way cross over design ($n = 3$, 8 time points). For accessing the ATZ concentration into the lymph nodes, rats were randomly divided into two groups ($n = 3$, 3 time points). All animals were fasted overnight before dosing, water was provided ad libitum throughout the study and received the following treatment:

Group A and Group B rats received ATZ-SP-IM (equivalent to 7 mg/kg of ATZ) and ATZ suspension (7 mg/kg) [8]. While, the rats in Group C were pretreated with cycloheximide (3 mg/kg; i.p; 0.6 mg/mL solution in normal saline) one hour prior to the administration of ATZ-SP-IM [42]. Further, rats in Group D and E were pretreated with famotidine (25 mg/kg; per oral; 5 mL/kg in 100 mM phosphate buffer, pH 6.5) one hour prior to the administration of ATZ-SP-IM and ATZ suspension [12]. For estimating the drug concentration into the lymphatic tissue, Group F and G received ATZ-SP-IM and ATZ suspension formulation. All the animals received a dose equivalent to 7 mg/kg of atazanavir.

Aliquots of blood samples (0.5 mL) were periodically withdrawn from the retro-orbital plexus of the animals at predetermined time intervals. Plasma was then harvested by centrifugation at 6000 rpm for 20 min and stored at -20°C till further analysis. In case of tissue distribution study, lymph nodes from rats at different time intervals were collected after cervical dislocation and lymph nodes were blotted with a tissue paper. The excised tissue was homogenized in an ice-cold phosphate buffer saline solution. The extraction of ATZ from plasma and tissue homogenate was carried out by liquid-liquid extraction method [43]. The drug content was analyzed by HPLC and standard non-compartmental pharmacokinetic parameters were calculated using in house pharmacokinetic program.

3. Results And Discussion

3.1 *Equilibrium solubility of ATZ*

Formation of stable and robust ATZ-IM without any precipitation depends upon the equilibrium solubility of ATZ in various oils, surfactants and co-surfactants. Solubility of ATZ in different oils, surfactants and co-surfactants has been illustrated in Fig. 1A & B. ATZ exhibited maximum solubility in oleic acid and corn oil. Oleic acid and corn oil chosen from the list of oils will assist in the lymphatic uptake of the atazanavir [44]. In case of surfactants and co-surfactants, ATZ possesses higher solubility in Tween 80 and propylene glycol. Being a non-ionic surfactant, Tween 80 is considered to be safe, effective, and an inhibitor of the P-gp efflux of atazanavir after oral ingestion [45]. In addition, propylene glycol being a co-surfactant is non volatile and compatible with gelatin capsules compared to other alcoholic co-surfactants. Based upon the equilibrium solubility studies of atazanavir, oleic acid, corn oil, Tween 80 and propylene glycol were chosen for delineating a stable and clear IM region during the ternary phase diagram studies.

3.2 Ternary Phase Diagram

Based on solubility study, ternary mixtures were firstly prepared by taking individual oil i.e. oleic acid or corn oil in combination with tween 80 and propylene glycol. It was observed that oleic acid and corn oil alone doesn't able to form a clear and stable ternary mixture. Further, oleic acid: corn oil was used in a ratio of 1:1, 1:2 and 1:3 along with surfactant and co-surfactant to prepare a ternary mixture. In a ratio of 1:1, ternary mixture formed was clear and stable with a transmittance of more than 97%. Beyond that concentration, the ternary mixtures so prepared were turbid and undergoes phase separation after 24 h. So, the ternary phase diagram comprising of Oleic acid: Corn oil (1:1), Tween 80 and propylene glycol was constructed (Fig. 1C). It was observed that when oil phase was taken from 0.1–0.2 g, the IM formed after reconstitution was clear and stable with a particle size < 100 nm and transmittance approaches towards 100%. But, when the concentration of oil phase increased to 0.25 g, the particle size of IM was increased significantly. Beyond that, the IM was translucent having particle size more 200 nm and got turbid when left aside for some time. Hence, the ternary phase diagram showed that 0.1–0.2 g Oleic acid: Corn Oil (1:1), 0.5–0.8 g Tween 80 and 0.1–0.4 g Propylene glycol could be further used to optimize the ATZ-IM

3.3 Systematic optimization of ATZ-IM as per D-optimal mixture design

On the basis of phase diagram and screening studies (supplementary data), 16 IM were developed as per D-optimal mixture design each containing 50 mg of ATZ/g of IM. The developed formulations were further evaluated for various

critical response variables (CRVs) namely particle size (Y_1), PDI (Y_2), Self Emulsification Time (Y_3), % Transmittance (Y_4) and Drug Content (Y_5). Various CMAs and CRVs of D-optimal mixture design are summarized in Table 1. The CRVs obtained were given into Design Expert® 11 software for statistical analysis that generates polynomial equation for each respective CRVs. The magnitude of each estimated regression coefficient in the polynomial equation indicated the relative contribution of the corresponding independent variable to the response variable. Larger magnitude of the coefficient signifies that the CMAs have greater influence on the CRVs. A positive sign of the coefficient indicates a synergistic effect, while a negative sign indicates an antagonistic effect of the coefficient on the CRVs. The mathematical models used for all the CRVs were found to be appropriate and acceptable (Model p value > F is less than 0.01). The value of R^2 for all mathematical models indicates the magnificent fit of the polynomials produced by statistical analysis to the response variables ($p < 0.0001$). The values of “lack of fit” were found to be insignificant in all the mathematical models, suggesting that the models are appropriate. Further, the closeness in the magnitude of predicted (Pred) and adjusted (Adj) R^2 to the actual model R^2 also affirms the magnificent fit to the data. The equation obtained after critical material attributes (CMAs) with critical response variables (CRVs) has been illustrated below:

$$\text{P.S} = + 60.23X_1 + 15.51X_2 + 17.14X_3 - 27.44X_1X_2 - 76.69X_1X_3 - 5.51X_2X_3 \text{ (Eq. 3) } R^2 = 0.791; \text{ (model = Quadratic) (Eq. 1)}$$

$$\text{PDI} = + 1.52X_1 + 0.3196X_2 + 0.1824X_3 - 2.22X_1X_2 - 2.82X_1X_3 - 0.5241X_2X_3 \text{ (Eq. 4) } R^2 = 0.8680; \text{ (model = Quadratic) (Eq. 2)}$$

$$\text{\% Transmittance} = + 69.64X_1 + 100.21X_2 + 98.91X_3 + 38.99X_1X_2 + 51.04X_1X_3 + 1.82X_2X_3 - 199.14X_1X_2X_3 \text{ (Eq. 5) } R^2 = 0.8523; \text{ (model = Special Cubic) (Eq. 3)}$$

$$\text{Drug Content (\%)} = 388.33X_1 + 32.53X_2 + 444.18X_3 - 289.10X_1X_2 - 1310.18X_1X_3 - 377.44X_2X_3 \text{ } R^2 = 0.9860; \text{ (model = Quadratic) (Eq. 4)}$$

$$\text{Self-emulsification time} = + 8.61X_1 + 20.76X_2 + 23.79X_3 + 52.15X_1X_2 + 19.91X_1X_3 + 3.45X_2X_3 - 24.91X_1X_2X_3 \text{ (Eq. 5) } R^2 = 0.9130; \text{ (model = Special Cubic) (Eq. 5)}$$

The results obtained from the polynomial equations were in concordance to the 3D response surface plot (Fig. S2).

Numerical Optimization

Numerical optimization of ATZ loaded IM utilizing desirability approach with an aid of Design Expert 11 software was carried out to determine the optimized concentration of CMAs to generate a high-quality robust product. In the formulation design space, five critical response variables namely particle size (minimum), PDI (minimum), self-emulsification time (minimum), % transmittance (maximize), and drug content (maximize) with optimum goals were selected. The outcome of numerical optimization suggested formulation composition comprised of oleic acid: corn oil (0.1 g), Tween 80 (0.5 g), and propylene glycol (0.4 g) fulfilled all the requirements of an optimized formulation because of better regulation of critical response variables (Table 2). The desirability plot of the optimized ATZ loaded IM has been illustrated in Fig. S2; supplementary data. For the above optimized formulation composition, 0.763 desirability was obtained. Table 3 manifested the predicted and the observed value of CRVs after developing the optimized formulation (F_1). The current findings revealed that not more than 5% biasness was observed when observed response values were correlated with predicted one. The optimized formulation was then subjected to further study.

3.4 Selection of PPI (polymer precipitation inhibitor)

(a) In-silico analysis

ATZ being a weakly basic drug undergoes drug precipitation in the intestinal pH. To prevent drug precipitation in the intestinal media, polymeric precipitation inhibitors (PPIs) were used to maintain the drug in the supersaturated state. ATZ being a ligand was docked with different types of PPIs via molecular docking study [46]. In the present investigations, the lowest binding energy and the intermolecular H-bond formation were considered as important criteria for inhibiting the crystallization of ATZ [47]. The H-bond surfaces between PPIs and ATZ were evident in Fig. 2. The results revealed that the intermolecular H-bonds were formed between the O-atom present in the glycosidic linkage of the PPI and H-atom of the ATZ. During molecular docking, the free energy (ΔG) is used to investigate the strength of interactions between ATZ and PPIs [48]. The binding energies of all the ligand-PPIs complexes have been shown in Table 3. From the above results, it was observed that, ATZ-HPMC-AS complex possesses lowest binding energy (-2.98 Kcal/mol) and had a strong interaction with ATZ than any other PPIs in inhibiting the crystallization of ATZ and prolonging the stabilized supersaturation state in intestinal pH. However, the docking study was usually established for ligand-PPIs interaction in the absence of H₂O molecules that would be a demerit for the theoretical verification of our results. This flaw of docking study was rectified by carrying out further dynamics simulations for the period of 30 ns in the build system.

While running the MD simulations, the root-mean-square deviation (RMSD) is usually used to study the stability of the ligand-PPIs complex [31]. From Fig. S3; supplementary data, it was concluded that all the ATZ-PPIs complexes could arrive at the equilibrium state but ATZ-HPMC-AS complex was able to attain the stability at the earliest with a RMSD fluctuations less than 1.5Å. The solvent-accessible surface area (SASA) of solute was also calculated to compare the hydrophilic property of the ATZ-PPI complex [46]. After carrying out the MD simulation, it was observed that all polymers except HPMC-AS were able to interact with the system constituting the H₂O molecules. The existence of carbonyl and the ester moiety in the HPMC-AS exhibited a large hydrophobic area than other PPIs, which could help HPMC-AS to interact greatly with the hydrophobic moiety of the ATZ molecule [47]. Thus, the above observations provide an insight into the potential of the HPMC-AS to inhibit the crystallization of ATZ in the intestinal media.

(b) In-vitro supersaturation test

To validate the results obtained from *in-silico* analysis, *in-vitro* supersaturation test was conducted. The *in-vitro* supersaturation test was conducted for optimized ATZ-IM containing different polymeric precipitation inhibitors (PPIs) in 500 mL of simulated intestinal fluid (pH 6.8) under non-sink conditions. The performance of PPIs was evaluated by comparing the apparent concentration of the drug v/s time, as illustrated in Fig. 3A. The initial drug concentration of ATZ was found out to be 0.1 mg/mL (based on the dilution factor of 500, i.e. 1 g of ATZ-IM containing 50 mg of ATZ dissolved in 500 mL of simulated intestinal fluid) before the commencement of supersaturation test. After initiating the experiment, at time $t = 5$ min, the apparent drug concentration was found out to be 0.034 mg/mL for HPMC E15, 0.086 mg/mL for HPMC-AS, 0.024 mg/mL for PVP K17, 0.028 mg/mL for Poloxamer 188 and 0.024 mg/mL for poloxamer 407, respectively. A rapid decline in the apparent drug concentration was observed in case of HPMC E15, PVP K17, Poloxamer 188, and Poloxamer 507 (0.004 mg/mL for all the PPIs), while the HPMC-AS maintains the supersaturated state (0.041 mg/mL) to a great extent. From the outcome of *in-vitro* supersaturation test, it was concluded that HPMC-AS was found successful in inhibiting the crystallization of ATZ in intestinal media. Hence, HPMC-AS was selected as PPIs in formulating ATZ-SP-IM for further investigation.

3.5 Evaluation of optimized ATZ-SP-IM

3.5.1 Determination of particle size (PS), poly dispersity index (PDI) and Zeta potential

The developed formulation was evaluated for particle size, PDI and zeta potential. During the particle size and PDI analysis, it was observed that ATZ-SP-IM exhibited a mean particle size of 14.42 ± 1.9 nm and a PDI of 0.186 ± 0.02 (Fig. 3B). No significant difference was observed in particle size and PDI when HPMC-AS was incorporated into ATZ-IM.

The above results clearly vouch the presence of nano-sized globules in the IM with a monodisperse nature of their distribution, thus fulfilling the requisite of SP-IM as per the published literature reports. The zeta potential of the SP-IM was estimated to be -25.7 ± 2.4 mV (Fig. 3C). The negative zeta potential was ascribed to the presence of free fatty acids in the formulation [49]. The above results suggested that ATZ-SP-IM generated a more stable system.

3.5.2 Transmission electron microscopy

The TEM image of reconstituted ATZ-SP-IM illustrated in Fig. 3D exhibited the presence of spherically shaped oil globules. In addition, no sign of drug precipitation was observed suggesting that ATZ is present in stable and solubilized form. Although, the TEM analysis also revealed that oil droplets retain their integrity in terms of morphology, uniformity in size and shape after reconstituting ATZ-SP-IM.

3.5.3 Stability testing of ATZ-SP-IM

The ATZ-SP-IM was subjected to accelerated stability studies for a time period of 3 months. The results of particle size analysis and PDI at different time intervals shows that no significant change in the values of particle size and PDI was observed when ATZ-SP-IM was kept in a stability chamber. No significant change in the values of drug content, cloud point, self-emulsification time and % Transmittance was observed during the study (Table 3). Hence, it can be concluded that ATZ-SP-IM exhibited good shelf life. During thermodynamic stability study, no signs of phase separation or drug precipitation were observed when ATZ-SP-IM was subjected to centrifugation test (Table 3). The freeze thaw cycles did not able to change the clarity and stability of ATZ-SP-IM. The above results suggested that the developed formulation is an isotropic mixture with high stability attributes.

3.6 *In-vitro* drug release study

To demonstrate the superiority of the SP-IM, dissolution profiles of ATZ from ATZ-SP-IM and ATZ powder loaded in hard gelatin capsule were determined in both 0.025N HCl (OGD media) and intestinal pH 6.8 (Fig. 4). Being a weakly basic drug, ATZ powder exhibited higher drug release in 0.025M HCl (Fig. 4A). It was observed that more than 90% of ATZ was release in 45 min. In contrast, ATZ-SP-IM exhibited similar behavior in 0.025N HCl but persistently at a faster release rate. The ATZ-SP-IM releases more than 90% drug and achieved a plateau state within 10 min (Fig. 4A). This predominantly occurs due to the presence to non-ionic surfactant (Tween 80) in ATZ-SP-IM. It has been reported that surfactants with higher HLB values shows higher emulsification ability and allows rapid dispersion of oil in the aqueous phase, producing nanosized oil-in-water emulsion. In case of intestinal pH, ATZ powder undergoes only 36% of drug release in 3 h of time course (Fig. 4B). The above results has been evident by the literature reports that ATZ solubility gets reduced to less than $1\mu\text{g/mL}$ when pH goes beyond 3 [12]. On the other hand, the ATZ-SP-IM showed more than 80% of drug release in 15 min with a maximum of 93% within 90 min (Fig. 4B). The presence of HPMC-AS in the IM decelerates the crystallization of ATZ and maintaining the drug in the supersaturated state leading to the higher drug release in the intestinal pH. Overall, the above results clearly demonstrate the improved dissolution characteristics of ATZ-SP-IM.

3.7 Drug permeation study across rat's everted intestinal sac

The *ex-vivo* performance of ATZ-SP-IM and ATZ powder was carried out by using rat's everted intestinal sac. To compare the results, flux ($\mu\text{g}/\text{cm}^2/\text{min}$), apparent permeability (cm/min) and relative permeability were calculated (Table 4). The rate of permeation (flux) of pure ATZ across intestinal wall was calculated to be $0.032\mu\text{g}/\text{cm}^2/\text{min}$, suggested that ATZ belongs to BCS-Class II drug. While, the ATZ release from SP-IM was found to be $0.065\mu\text{g}/\text{cm}^2/\text{min}$, which was calculated to be 2.03 fold enhancement in flux. The results also suggested a 3.63-fold enhancement in relative permeability of ATZ in case of ATZ-SP-IM formulation. The enhanced permeability of the can be attributed to the larger concentration gradient created by the increased solubility of the ATZ into the SP-IM. From the above results, it can be postulated that larger surface area of nanoparticulates provided by ATZ-SP-IM increases the adherence to the intestinal membrane which in turn enhances the intestinal transport of the drug [27].

3.8 *In-vivo* pharmacokinetic and lymphatic tissue distribution study

The *in-vivo* animal pharmacokinetic study employing two way crossover design was carried out to compare the pharmacokinetic profile of ATZ-SP-IM with pure ATZ. The observed plasma concentration-time profile after oral administration of ATZ in the form of pure drug and ATZ-SP-IM is depicted in Fig. 4C and the non-compartmental pharmacokinetic parameters have been summarized in Table 4. The peak plasma concentration ($C_{\max}$) value of atazanavir after a single dose of suspension as a reference product and SP-IM as a test formulation were found to be 14.46 ± 3.72 $\mu\text{g/mL}$ and 28.76 ± 5.43 $\mu\text{g/mL}$, respectively. A significant enhancement in $C_{\max}$ (1.98 fold) was evident, when ATZ-SP-IM was administered. The area under curve (AUC) was also found to be 1.8 fold enhanced for ATZ-SP-IM (95.47 ± 5.77 $\mu\text{g/mL}$) than for ATZ suspension (60.25 ± 4.01 $\mu\text{g/mL}$). The half-life ($t_{1/2}$) of ATZ-SP-IM was found to be 1.47 fold increased as compared to pure ATZ. In line with the AUC values, the mean residence time (MRT) in case of ATZ-SP-IM was found to be increased when compared with pure ATZ. The improvement in the pharmacokinetic performance of ATZ could be attributed to the high solubility of ATZ into the SP-IM components and a large surface area of microemulsion formed after reconstitution with GI fluids may be responsible for promoting gastrointestinal absorption and improving the ATZ oral bioavailability.

The effect of histamine H_2 receptor antagonist (famotidine) on the pharmacokinetic performance of ATZ-SP-IM and pure ATZ was also investigated. A sharp decline in the value of $C_{\max}$ and AUC was observed when pure ATZ was administered to the rats pretreated with famotidine (Table 4). This sudden decrease in the pharmacokinetic parameters could be due to the steep decline in the solubility of ATZ with respect to the change in the gastric pH. At elevated pH, the solubility of ATZ decreases that may lead to insufficient dissolution in the stomach due to which the absorption of ATZ from the GIT decreased [12]. However, the absorption of ATZ doesn't get affected when drug was administered into SP-IM (Table 4 and Fig. 4D). Such type of behavior was ascribed to the presence of HPMC-AS in the SP-IM that induced and maintained the drug in the supersaturated state over a period of time till absorption. This augmentation in the rate and extent of drug bioavailability from SP-IM would eventually results in significant escalation in the intensity of therapeutic effect of atazanavir too.

The intestinal lymphatic transport of any formulation is elucidated by comparing the pharmacokinetic profiles in animals pretreated with cycloheximide, a chylomicron flow blocker. As compared to pure ATZ, the ATZ-SP-IM exhibited significant increase in the extent of oral bioavailability in rats (i.e., nearly 1.58 fold), evident from the corresponding values of the AUC (Table 4). However, a remarkable change in the value of AUC was observed when ATZ-SP-IM formulation was administered to rats group pretreated with cycloheximide over ATZ-SP-IM control group (Fig. 4E). A considerable 1.31 fold decrease in the value of AUC could be ascribed solely to the inhibition of the lymphatic pathways caused by blockage of chylomicron flow. Hence, it can be said that absorption of ATZ was highly affected by cycloheximide and intestinal lymphatic system was involved in the absorption of ATZ-SP-IM. The intestinal lymphatic uptake of ATZ-SP-IM occurs due to the presence of high concentration of surfactant and the long chain triglycerides in the formulation. In our case, presence of corn oil and oleic acid as long chain triglycerides assists in the lymphatic transport of ATZ [44]. Besides, Tween 80 being a surfactant has been reported to inhibit P-glycoprotein (P-gp) activity which might be another benefit for enhanced permeability and intestinal lymphatic transport, resulting in enhanced oral bioavailability [50]. To validate the results of chylomicron flow blocking approach in analyzing the drug transport to the lymphatics, concentration of ATZ in the lymph nodes was also estimated. For this, rats were administered orally with ATZ-SP-IM and ATZ suspension at a dose of 7 mg/kg. The concentration of ATZ in the lymph nodes at specific time intervals was estimated by excising the lymph nodes after cervical dislocation. It was observed that the rats administered with supersaturable preconcentrated isotropic mixture (ATZ-SP-IM) exhibited higher concentration of ATZ in the lymph nodes over that of putative form. The enhanced drug concentration in the lymph nodes could be ascribed to the presence of long chain fatty acids in the supersaturable formulation which assists in the transport of ATZ to the lymphatics. The improved distribution of ATZ to the lymphatics

would potentially results in the site-specific targeted drug delivery, inhibition of the HIV and further prevents the suppression of biodistribution of the virus to the other vital organs.

4. Conclusion

The current investigation successfully vouch the QbD based systematic development of optimized ATZ loaded SP-IM with enhanced biopharmaceutical attributes coupled with lymphatic targeting of BCS Class-II drug, atazanavir, exhibiting pH dependent solubility. The optimized ATZ loaded IM exhibited nanometric particle size, uniform particle distribution (0.186 ± 0.02) and faster emulsification efficiency. A combination of *in-silico* and *in-vitro* analysis indicated that presence of HPMC-AS in SP-IM had inhibitory effect on drug precipitation and manifested immediate drug release as compared to putative atazanavir. Further, extensive evaluation through *ex-vivo* and *in vivo* studies with ATZ-SP-IM revealed remarkable augmentations in the permeability, lymphatic uptake and systemic availability. In addition, the presence or absence of ARAs did not alter the biopharmaceutical performance of ATZ when loaded into SP-IM. Overall, the present investigation suggested the overwhelming influence of HPMC-AS and IM in improving the biopharmaceutical attributes of ATZ. Further, the strategic formulation technology employed in this present investigation can be extrapolated to other similar antiretroviral drugs for embarking upon their targeting potential to the lymphatic system for AIDS management.

Declarations

Ethical Approval

The research work involving *ex-vivo* permeation and *in-vivo* pharmacokinetics adheres to the guidelines for care and use of the laboratory animals. Thus, all the animal investigations were performed as per the requisite protocol approved by the Institutional Animal Ethics Committee (IAEC), Punjabi University, Patiala, India (Approval No. 107/99/ CPCSEA/2018-05).

Consent for Publication

No human volunteers or data related to human volunteers were involved in this research work, as the proposed study was performed on rodents. Therefore, no consent was taken from human volunteers to publish manuscript.

Availability of data & Materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Declaration

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Author's Contribution

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by all authors. All authors read and approved the final manuscript.

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Tables

Table 1

D-optimal mixture design for the formulation and optimization of ATZ loaded isotropic mixture

F.No.	(X ₁) Oil (CO:OA) (1:1) (g)	(X ₂) Surfactant (T80) (g)	(X ₃) Co-Surfactant (PG) (g)	(Y ₁) PS (nm)	(Y ₂) PDI	(Y ₃) % T (%)	(Y ₄) DC (%)	(Y ₅) SET (s)
1.	0.1	0.5	0.4	14.6±0.7	0.191±0.009	98.2±0.7	91.3±2.7	23±1
2.	0.1	0.5	0.4	16.7±0.8	0.125±0.006	99.5±0.3	89.3±2.4	25±2
3.	0.175	0.5875	0.2375	18.8±0.6	0.098±0.004	91.33±0.9	58.2±1.7	26±1
4.	0.1	0.6	0.3	13.3±0.5	0.105±0.005	99±0.6	69.5±1.9	24±2
5.	0.15	0.6125	0.2375	20.6±1.1	0.095±0.004	94.1±0.8	55.0±1.6	26±3
6.	0.1	0.7	0.2	15.9±0.7	0.375±0.018	100±0.2	52.7±1.3	20±1
7.	0.1	0.6	0.3	15.5±0.6	0.121±0.006	99.7±0.2	65.2±1.9	22±2
8.	0.125	0.5375	0.3375	19.5±0.9	0.085±0.004	99.5±0.3	71.9±2.1	24±2
9.	0.2	0.6	0.2	30.9±1.5	0.385±0.019	95.8±0.8	54.9±1.5	28±3
10.	0.2	0.6	0.2	35.7±1.7	0.425±0.021	93.8±0.9	52.0±1.3	27±2
11.	0.15	0.5	0.35	16.2±0.8	0.051±0.002	100±0.3	74.6±2.2	23±1
12.	0.125	0.6375	0.2375	14.1±0.7	0.101±0.005	100±0.2	53.8±1.6	26±3
13.	0.2	0.5	0.3	17.2±0.8	0.099±0.004	96.8±0.9	63.8±1.9	21±1
14.	0.15	0.65	0.2	17.5±0.7	0.132±0.006	100±0.2	51.1±1.2	28±2
15.	0.2	0.5	0.3	18.8±0.9	0.136±0.005	97.8±0.8	61.2±1.8	22±1
16.	0.1	0.7	0.2	17.5±0.6	0.302±0.015	100±0.3	50.2±1.3	21±2

Model fitting and statistical analysis

Responses	Model	C.V. (%)	p-value	PRESS	R-squared	Adj R-squared	Pred R-squared	Adeq Precision
PS	Quadratic	17.44	0.0035	265.22	0.7911	0.6866	0.5101	8.4877
PDI	Quadratic	30.76	0.0004	0.0783	0.8680	0.8021	0.6498	11.3143
%T	Special Cubic	1.38	0.0025	43.85	0.8523	0.7538	0.6076	9.6573
DC	Quadratic	2.96	<0.0001	93.13	0.9860	0.9789	0.9630	34.0784
SET	Special Cubic	4.03	0.0003	28.28	0.9130	0.8550	0.7107	10.8259

*CO: Corn Oil; OA: Oleic Acid; T80: Tween 80; PG: Propylene Glycol; PS: Particle Size; PDI: Polydispersity Index; % T: Percent Transmittance; DC: Drug Content; SET: Self-emulsification Time

Table 2

Results of numerical optimization for the preparation and evaluation of ATZ loaded isotropic mixture

Constraints

Material Attributes/Response Variables	Goal	Lower Limit	Upper Limit	Predicted Value	Observed Value	% Bias
Corn oil: Oleic acid (X_1) (g)	is in range	0.1	0.2	0.1	0.1	
Tween 80 (X_2) (g)	is in range	0.5	0.7	0.5	0.5	
Propylene Glycol (X_3) (g)	is in range	0.2	0.4	0.4	0.4	
Particle Size (Y_1) (nm)	minimize	13.37	35.73	17.13	15.73	<5%
PDI (Y_2)	minimize	0.051	0.425	0.201	0.222	
Self-emulsification time (Y_3) (s)	minimize	20	28	23.78	25	
% Transmittance (Y_4) (%)	maximize	91.33	100	98.91	99.6	
Drug Content (Y_5) (%)	maximize	50.29	91.3	90.42	94.7	

Table 3

Results of ATZ-polymer precipitation inhibitor's surface docking

S.No.	Drug-PPIs Complex	Lowest binding energy (kcal/mol)			
1.	ATZ-Poloxamer 188	+1.51			
2.	ATZ-Poloxamer 407	+0.44			
3.	ATZ-PVP K17	-1.49			
4.	ATZ-HPMC AS	-2.98			
5.	ATZ-HPMC E15	-1.53			
Stability study of ATZ-SP-IM as per ICH Q1A (R2) guidelines					
Stability Study					
Time (months)		0	1	2	3
Particle Size		14.42±1.9	14.5±1.4	15.65±1.2	16.55±1.5
PDI		0.186±0.02	0.262±0.01	0.274±0.03	0.302±0.02
Drug Content (%)		94.6 ± 3.2	94.5 ± 3.1	94.1 ± 2.8	93.8 ± 3.3
Cloud point (°C)		73 ± 4	74 ± 2	73 ± 3	72 ± 3
Drug Precipitation		X	X	X	X
Self-emulsification time (s)		28 ± 3	27 ± 2	25 ± 3	27 ± 2
% Transmittance (%)		99.7 ± 1.1	99.7 ± 1.2	99.3 ± 1.3	99.3 ± 1.1
Thermodynamic stability					
Centrifugation	Precipitation	X	X	X	X
	Phase Separation	X	X	X	X
Freeze Thaw Cycle	4°C	X	X	X	X
	40°C	X	X	X	X

Table 4

Ex-vivo performance of ATZ pure drug and ATZ-SP-IM across everted rat gut sac method

Parameters		ATZ pure drug		ATZ-SP-IM	
Flux (µg/cm ² /min)		0.032 ^a		0.065 ^b	
Relative permeability (µg*mL/cm ²)		3.00 ^a		10.92 ^b	
Apparent permeability (cm/min) (x10 ⁻⁶)		0.64 ^a		1.3 ^b	
Pharmacokinetic parameters of ATZ suspension and ATZ-SP-IM after 7 mg/kg single dose to various wistar rats groups					
Parameters	Group-A	Group-B	Group-C	Group-D	Group E
	ATZ Suspension	ATZ-SP-IM	ATZ+Famotidine	ATZ-SP-IM+Famotidine	ATZ-SP-IM+Cycloheximide
C _{max} (µg/mL)	14.46±3.72 ^a	28.76±5.43 ^b	7.87±2.39 ^c	26.25±4.31 ^d	20.39±3.01 ^e
T _{max} (h)	0.25±0.01 ^a	0.5±0.02 ^b	0.25±0.01 ^c	0.5±0.02 ^d	0.5±0.02 ^b
AUC (µg*h/mL)	60.25±4.01 ^a	95.47±5.77 ^b	28.18±3.40 ^c	96.51±4.82 ^d	72.49±3.92 ^e
AUMC (µg*h ² /mL)	374.18±21.7 ^a	685.35±34.2 ^b	152.82±15.6 ^c	676.37±39.6 ^d	474.69±23.7 ^e
T _{1/2} (h)	4.28±0.21 ^a	6.30±0.31 ^b	4.14±0.20 ^c	6.27±0.29 ^d	6.22±0.30 ^b
MRT (h)	6.07±0.30 ^a	6.75±0.33 ^b	5.28±0.26 ^c	6.71±0.27 ^d	6.26±0.28 ^e
K _e (h ⁻¹)	0.161±0.008 ^a	0.11±0.005 ^b	0.167±0.008 ^c	0.110±0.005 ^d	0.111±0.004 ^b
rF	1	1.58	1	3.42	-
* ^{a, b} Means within the same row, labeled with the same letter in ATZ pure drug and ATZ-SP-IM group, do not statistically differ from each other (p > 0.05); C _{max} , peak plasma concentration; T _{max} , time to peak plasma concentration; AUC, area under the concentration-time curve; AUMC, Area under the moment curve; MRT, mean residence time; K _e , elimination rate constant; T _{1/2} , Half-life; rF, relative oral bioavailability; ^{a, b} Means within the same row, labeled with the same letter in Group A and B, do not statistically differ from each other (p > 0.05); ^{c, d} Means within the same row, labeled with the same letter in Group C and D, do not statistically differ from each other (p > 0.05); ^{b, e} Means within the same row, labeled with the same letter in Group B and E, do not statistically differ from each other (p > 0.05)					

Figures

Figure 1

Solubility analysis of ATZ in various oils (A), surfactants and co-surfactants (B) and ternary phase diagram constituting corn oil, oleic acid, tween 80 and propylene glycol (C).

Figure 2

The surface mode of ATZ docked with (A) Poloxamer 188, (B) Poloxamer 407, (C) PVP K17, (D) HPMC AS and (E) HPMC E15. The color is based on the atom type: in gray are the carbon atoms, in red are oxygen atoms, in blue are nitrogen atoms, in white are hydrogen atoms and green dotted line representing H-bond.

Figure 3

(A) Apparent drug concentration-time profile for various PPIs; (B) Particle size, (C) Zeta potential and (D) TEM image of ATZ-SP-IM.

Figure 4

Dissolution profile of ATZ from ATZ powder and SP-IM in (A) 0.025N HCl, (B) pH 6.8; Pharmacokinetic performance of ATZ-SP-IM and ATZ suspension after 7 mg/kg single oral dose administration in (C) Normal rats group (D) Famotidine rats group (E) Cycloheximide rats group and (F) Drug concentration of ATZ at different time intervals in lymph nodes (n = 3).

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