Ritonavir loaded surface modified stealth solid lipid nanoparticles: Full factorial design and pharmacokinetic studies

Pavan Rudhrabatla VSA¹, Beeravelli Sudhakar² and Suresh Reddy KVN*¹

¹Department of Chemistry, GITAM Institute of Technology, GITAM (Deemed to be University), Visakhapatnam - 530045, Andhra Pradesh, India
²Department of Pharmaceutical Technology, A.U. College of Pharmaceutical Sciences, Andhra University, Visakhapatnam - 530003, Andhra Pradesh, India

ABSTRACT

This work aims at developing and optimizing the ritonavir loaded stealth solid lipid nanoparticles for parenteral delivery using full factorial design to improve the systemic circulation. The ritonavir loaded stealth solid lipid nanoparticles (SLN) were developed by hot melt homogenization method using tristearin, HSPC and poloxamer 188. The SLNs were evaluated for various physicochemical evaluation parameters such as percent drug content, particle size, zeta potential, % entrapment efficacy and in-vitro drug release studies. The composition of the formulation was finalized by applying constraints (goals) on independent variables (factors) and dependent (response) and. The Optimization was done based on the criteria of minimum particle size (nm), maximum %EE and maximum zeta potential. The optimized SLN converted into a stealth form using different concentrations of 1,2-distearoyl phosphatidyl ethanolamine-methyl-polyethylene glycol conjugate 2000(sodium salt). The drug encapsulation range of stealth solid lipid nanoparticles (SSLN) is between 94.12% and 98.12%. The mean size of particles was found between 126.6 to 146.2 nm. This indicates particles are in uniform distribution. The zeta potential was found in the range of -38.9 to -54.6 mV for stealth solid nanoparticles. The in vivo studies revealed that the drug-loaded SSLN has prolonged circulation time compared to pure ritonavir (PR) when administered to rats through intravenous route. The optimized SSLNs were further evaluated for drug-excipients compatibility, stability and pharmacokinetic studies by Wistar rats. The PEGylation technique improved pharmacokinetic parameters of ritonavir from stealth solid lipid nanoparticles when compared to pure ritonavir. The Paired t-test was used for comparison of the pharmacokinetic parameters. All the statistical assessments have done at a significant level p < 0.05.

INTRODUCTION

Antiretroviral drugs (ARV) play a major role in controlling of human immunodeficiency virus [HIV] and prevent its progression toward AIDS. Aqueous solubility, poor bioavailability and short half-life are major disadvantages of ARV drugs. They require frequent dosing of larger dose leading to improve patient compliance. Human immunodeficiency virus (HIV) is located in various unreachable parts of the body, i.e. central nervous system, lymphatic system and spleen. Majority of the drugs cannot reach these compartments at
required concentration and cannot be retained at the resident site of HIV for the necessary duration. Owing to all these limitations of ARV drugs which require large doses for achieving a therapeutic effect with increased side effects. Ritonavir is one of the frequently prescribed drugs among antiretroviral drugs (ARV) drugs to treat AIDS (Cohen et al., 2008). Ritonavir is a (BCS-II) drug which has low solubility and high permeability. Solubility is a major obstacle to improving its bioavailability and pharmacological activity. Many attempts are made to increase the solubility, bioavailability and targeting the lymphatic system for effective treatment of AIDS (Muller et al., 2000; Shidhaye et al., 2008).

To overcome all the obstacles of HIV drugs, different nanoparticulate delivery systems were introduced, which include solid lipid nanoparticles (SLNs), polymeric nanoparticles, nanostructured lipid carriers (NLC), nanoemulsions and liposomes for efficient drug delivery. Among all, solid lipid nanoparticles (SLNs) became promising materials for the efficient delivery of various drugs (Muller et al., 2000; Shidhaye et al., 2008). SLNs enhance the solubility of lipophilic drugs and increase the entrapment efficiency and protect the drugs from various destructive conditions such as gastric fluids, light, pH, and rapid clearance by opsonization (Cerpnjak et al., 2013; Fahr et al., 2007; Bunies, 2010).

Javed Ali et al., developed ritonavir loaded solid dispersion using PVP K-30, PEG 4000 and PEG 6000 by solvent evaporation and melting method. They reported that pharmacokinetic parameters are altered from the solid dispersions based formulations compared to the pure drug (Sinha et al., 2010). Alireza Vatanara et al., developed the ritonavir loaded SLNs using soya lecithin and glycercyl monostearate (GMS) as lipids, poloxamer 188 and tween 80 as surfactants by solvent emulsification evaporation (SE) and multiple emulsion methods (DE). They reported that SLNs overcome the disadvantages of ritonavir and sustain its antiviral activity (Javan et al., 2017). Swapnil Kumar et al., developed ritonavir loaded solid lipid nanoparticles using Compritol 888 (lipid carrier) for oral delivery. They reported that SLNs increased the rate of absorption of ritonavir and pharmacokinetic parameters (Kumar et al., 2018). Sudhakar et al., have developed the ritonavir loaded stealth liposomes for parenteral delivery using DSPE as lipid and PEG-10000 for stearic stability (to avoid opsonization). The circulation half-life and MRT of ritonavir were increased 6.65 and 3.39 folds in comparison with conventional liposomes and pure ritonavir solution. As per the literature, no work was reported on ritonavir loaded stealth solid lipid nanoparticles for the parenteral delivery (Sudhakar et al., 2016).

Here in this study, ritonavir loaded solid lipid nanoparticles were prepared by hot melt homogenization method using full factorial design using design expert trial version 11 and their surface was modified by PEGylation technique using DSPE-mPEG-2000 to get the stealth solid lipid nanoparticles (SSLN). The objective of the current research is to assess the in-vitro and in-vivo pharmacokinetic studies of both the conventional solid lipid nanoparticles and stealth solid lipid nanoparticles and comparison of their efficiencies.

**MATERIALS & METHODS**

**Materials**

Tristerine purchased from Sigma-Aldrich. HSPC (Hydrogenated soybean phosphatidylcholine) and DSPE-mPEG-2000 were supplied by Lipoid Pvt Ltd, Germany. Poloxamer 188 received from Sigma Aldrich India, Ritonavir gift sample was received from Hetero Drugs Pvt Ltd, Hyderabad, India.

**Experimental design**

A 3^2 full factorial design was adopted in this study and two factors were studied each at three levels. Experimental trials were carried out in all possible combinations (Nine combinations). The amount of HSPC \((X_1)\) and the amount of poloxamer 188 \((X_2)\) was selected as independent variables for SLN. These variables were varied at three levels, medium level \((0)\), high level \((+1)\) and low level \((-1)\). Amount of drug ritonavir \((20\, \text{mg})\), triglyceride (tristearin) \((100\, \text{mg})\) concentrations and dispersion medium water \(10\, \text{mL}\) were kept constant. All the calculations were done at milligrams level (mg).

Particle size (nm) \((Y_1)\), % EE (\%) \((Y_2)\) and zeta potential \((Y_3)\) were selected as dependent variables. Values of variables and batch codes are shown in the Table 1. Design Expert® DX 11 trial version software was used for the production and assessment of statistical experimental design (Kumar et al., 2018; Sudhakar et al., 2016).

**Solid lipid nanoparticles preparation**

Ritonavir loaded SLN were prepared by hot homogenization technique (Raju et al., 2014) and corresponding schematic sketch presented in Figure 1. The tristearin and HSPC were heated above their melting temperature and added ritonavir to this lipid mixture and mixed rapidly with a glass rod in a hot molten condition. The required quantity of poloxamer 188 was dissolved in water, heated to an equal temperature of lipid solution, mixed with lipid phase and high-pressure homogenization was carried out. The high-pressure homogenization was run for 10 cycles at 800 to 1000 bar at temperature 5°C using a chiller (Cole-Parmer) to control the temperature.
Table 1: Full factorial design of ritonavir loaded Solid lipid nanoparticles

<table>
<thead>
<tr>
<th>Formulations</th>
<th>HSPC (mg)</th>
<th>Poloxamer 188 (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1 (10:5)</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>F2 (10:10)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>F3 (10:15)</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>F4 (20:5)</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>F5 (20:10)</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>F6 (20:15)</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td>F7 (30:5)</td>
<td>30</td>
<td>5</td>
</tr>
<tr>
<td>F8 (30:10)</td>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>F9 (30:15)</td>
<td>30</td>
<td>15</td>
</tr>
</tbody>
</table>

Pure ritonavir solution

Pure ritonavir solution was prepared by dissolving 30 mg of ritonavir in 10 mL of 5% v/v concentration of ethanolic phosphate buffer (pH 7.4) and subjected to filtration with 0.22 µm PVDF sterile filters (Millipore PVT LTD) under aseptic conditions into a 10 CC vial (Sudhakar et al., 2016).

HPLC method for the analysis of drug sample

The samples of in vitro and in vivo were analyzed by high-performance liquid chromatography using reversed phase C-18 column (Gemini TM 5 µm, 250x4.6 mm). The drug was extracted from plasma by precipitation method and injected into the HPLC system. An aliquot of 100 µL of drug-free rat plasma spiked with different concentrations of ritonavir. The spiked samples were vortexed for 30 sec and internal standard (didanosine) (200 ng/mL) was added and vortexed for 5 min. Methanol was added (double the volume of plasma) as a precipitating agent and vortexed for 5 min and then centrifuged for 15 min at 5000 rpm. The supernatant solution was separated and filtered through 0.45 µm and 20 µL of the solution was injected into the HPLC. HPLC (Shimadzu, Class VP series) with two LC-10ATvp pumps, variable wavelength programmable Photo Diode Array (PDA) detector, SPD-M10Avp was used. The HPLC system was equipped with the Shimadzu LC Solution software (Version 1.23). The linearity was in the range of 50-5000 ng/mL. The linear regression equation was found to be $Y = 0.001x$ at 235 nm wavelength and the correlation coefficient value was found to be 0.9999.

Determination of percentage of drug content

1 mL of SLN suspension was pipetted out and dissolved in the methanol. The final volume was made up with methanol and ritonavir content was determined by HPLC at 210 nm (Sudhakar et al., 2016; Raju et al., 2014).

Determination of zeta potential, particle size distribution and polydispersity index (PDI) and of SLN

The ritonavir loaded SLNs after dilution (1:100) with 0.1M sodium chloride solution was taken in a cuvette and size was measured using Malvern Nano ZS90, Malvern, UK. The observations of vesicle size were recorded at 90° light scattering angle and 25°C. The ζ was measured based on the mobility of particles (Sudhakar et al., 2016; Raju et al., 2014).

Determination of % EE

The percentage of drug entrapped in the lipid is determined by ultrafiltration method using sartorius centrist devices which are equipped with a membrane filter at the base of the sample recovery chamber. The unit is centrifuged at 20000 rpm for 15-20 min. The solid lipid nanoparticles along with the encapsulated drug remain in the outer chamber and the aqueous phase is moved into the sample recovery chamber through the membrane. The amount of drug in the aqueous phase is estimated by HPLC at 210 nm using the below equation (Raju et al., 2014; Patravale et al., 2003 ; Arjun et al., 2013).

\[
E(\%) = \left(\frac{C_d - C}{C_d}\right) \times 100
\]  

(1)

Where $C_d$ and $C$ are total drug concentration and unentrapped drug concentrations respectively.

In vitro studies by dialysis and release kinetics

Cellulose membrane (DM 60 from HiMedia, Mumbai, India, with 12000D) was soaked in pH 7.4 phosphate buffer overnight and 2 mL of SLN suspension (equivalent to 4 mg of ritonavir) has taken into dialysis membrane and hanged into a beaker containing pH 7.4 phosphate buffer (500 mL at 37±0.5°C) on magnetic stirrer with 100 rpm using Teflon coated bead. 5 mL of sample was collected at different time intervals from the beaker and the samples were analyzed through HPLC (Sudhakar et al., 2016; Raju et al., 2014). The results were fitted to different release kinetic models, i.e. zero order and first order. The drug release mechanism was confirmed with the help of Higuchi’s model and Korsmeyer-Peppas models (Costa et al., 2001; Peppas, 1985; Lledo et al., 2007).
Preparation of stealth solid lipid nanoparticles

The optimized SLNs formulation subjected for PEGylation to modify into stealth SLN using 10, 20 and 30 mg of DSPE-mPEG-2000 (Sudhakar et al., 2016; Fundaro et al., 2000). The physicochemical evaluation of SLNs was followed same as above mentioned characterization from section 2.3 to 2.9.

Visualization by field emission scanning electron microscopy (FESEM)

The size of the stealth SLNs was studied by FESEM. Before going to study, the formulations were sputtered with gold for 2 min to make them conducting (Rosa et al., 2008).

Drug and excipients compatibility studies

FTIR, DSC and XRD studies

The FTIR spectra of compounds were recorded on a Bruker FTIR spectrophotometer using Opus software. Thermal analysis was carried out for ritonavir, triglycerides (tristearin) and SLNs formulations using Pyrus DSC Perkin Elmer with 10°C/min heating rate between 20–240°C. The Powder X-ray diffractometer (Siemens) was used for diffraction studies. The powder XRD studies were performed for the samples by exposing them to CuKα radiation (50kv, 34 mA) and scanned from 3 to 45°, 2θ at a scan step of 0.02° and step time of 3°/min (Kumar et al., 2018; Sudhakar et al., 2016; Raju et al., 2014).

Sterilization by two-step technique: lyophilization followed 0.22µm filtration

Two-step sterilization technique was applied for sterilization of tested products before the in-vivo study (Raju et al., 2014). Lyophilization was carried out for selected conventional SLN, stealth SLNs and pure ritonavir solution using Christ Alpha 1-2 LD Freeze Dryer. 10% w/v sucrose was used as a cryoprotecting agent for selected products. The samples were filtered through 0.22µm EDF filter into a 10 CC vials (PALL life sciences Pvt LTD) and closed with half sealed stoppers under aseptic conditions. This process involves three steps. The first sample was cooled to -50°C by adjusting condenser temperature for 3 hrs. This primary drying process was taken for 15 hrs whereas the secondary drying process was taken for 6 hrs at 20 to 30°C temperature. While running lyophilization cycle, the pressure was maintained around in the range of 200 to 300Torr. After the lyophilization process, the tested lyophilized products were analysed again reconstitution time, percent drug content, zeta potential, particle size and pH etc.

Pharmacokinetic study by rats

The pharmacokinetic study was conducted by taking Wistar rats (200–250g) as an animal model through parenteral administration (Kumar et al., 2018; Sudhakar et al., 2016; Fundaro et al., 2000). The selected SLN and stealth SSLN were tested against the pure ritonavir solution. These tested products were sterilized by two-step sterilization technique, i.e. lyophilization followed 0.22µm filtration. EDF filters (Pall Life sciences) were used for SLNs formulation whereas the PVDF filters (Millipore Pvt Ltd) are used for pure ritonavir solution. The rats were divided into three groups, each group containing four rats. Group 1 was treated with pure ritonavir solution, Group 2 administered with conventional SLNs and Group 3 was administered with SSLN. The animal dose was calculated as 10 mg/Kg for rat (Sinha et al., 2010). The required dose was administered through the tail vein of the male Wistar rat with an administration rate of 0.3 to 0.4 ml/min. The 300 µL aliquots of blood samples were collected from the rat through the retro-orbital sinus and stored in centrifuge tubes containing K2-EDTA (dipotassium ethylenediaminetetraacetic acid). The samples were collected at 0, 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, 24 and 28 hrs post dose and plasma was separated by ultracentrifugation at 5°C with 5000 rpm for 15 min, and separated sample was stored at -20°C until drug analysis. Initially, plasma samples were thawed and 20 µL of didanosine was added as internal standard to 100 µL of plasma (200 ng/mL) in 2 mL Eppendorf microcentrifuge tubes and mixed gently. Methanol (200 µL) was added to each tube, vortexed for 5 min and then centrifuged for 15 min at 5000 rpm. The supernatant solution was filtered through 0.45µm and 20 µL of this sample was injected the HPLC loop injector.

RESULTS

The present work reports the development of ritonavir loaded SSLN for parenteral delivery to improve the patient compliance (improve the systemic availability of the drug) by reducing frequent administration. In this work, we have selected the tristearin (triglyceride) which has carbon long chain and high phase transition temperature (53°C). Hydrogenated soya phosphatidylcholine (HSPC) is a saturated bilayered lipid considered as an emulsifier and it has a high phase transition temperature (55°C). Poloxamer 188 selected as co-emulsifier. The selected excipients are available as solid form at room temperature. Ritonavir is a more potent antiretroviral agent in ARV therapy, on oral delivery; its bioavailability is uneven due to its poor solubility in water. The key factor in the formulation of poorly water-soluble drugs for parenteral administration is to provide an efficient
loading technique leads to the required high dose. The parenteral dosing of ritonavir is essential in HIV patients who are unable to accept oral therapy.

From the experimental study, the assay was estimated by HPLC. The assay was found in the range of 93 to 103% for SLNs and pure ritonavir solution (P R) respectively. The pH of tested products (SLNs and PR) was found around pH 7.4 which are suitable to administer by intravenous administration (blood pH range of 7.35 to 7.45). At the lower concentrations of HSPC and poloxamer 188 of the (F1, F2 and F3) SLNs shows particle size from 191 to 221 nm, at the middle levels of HSPC and poloxamer 188, the particle size of SLNs (F4, F5 to F6) was found 112 nm to 136 nm and at the higher level concentration (HSPC and poloxamer 188) of the SLNs (F7, F8, F9) size of particle was found between 156 to 171 nm. The mean size of SLNs was found in the range of 112 to 171 nm and PDI was in the range of 0.102-0.350. The particles with nanometer range and low polydispersity (PDI) indicate that the particles are in uniform size distribution. It was strongly affected by the selected independent variables. The results indicated a profound effect of poloxamer 188 and HSPC on the SLN particle size.

The values of the zeta potential of SLNs were in the range of -11 mV to -34 mV (Raju et al., 2014). Higher values of zeta potential (-34 mV) may be caused by the repulsive forces with the large negatively charge domains of the particle surface. Zeta potential values are showed that SLNs had enough charge to stop aggregation of particles by electric repulsion. As a purified solid form of ritonavir in distilled water (1:100 dilution) shows particles size around 10 µm and zeta potential was found around ~ 6 mV. The solubilized ritonavir solution particles size and zeta potential were found 68 nm and -13 mV respectively. It indicates that the drug was solubilized in the 5% v/v concentration of ethanolic phosphate buffer (pH 7.4).

The percent entrapment efficiency of SLNs was determined after separating entrapped and unentrapped drug by centrifists (ultrafiltration). The percent entrapment efficiency varied from 41% to 86% for all the formulations. The drug was uniformly intercalated in the tristearin lipid matrix. The percent encapsulation efficiency of SLNs varied from each other due to the influence of HSPC and poloxamer 188.

An inversely proportional relation was observed between the particle size and %EE of SLNs. The lower particles size distributed SLNs shows higher % of EE which near to 86 % because the lower particles SLNs increase the surface area of particulate systems helps to intercalate of the maximum amount of ritonavir in the particle matrix. The higher the particle size of the SLNs shows lower encapsulation due to low surface area.

**In vitro release studies**

Drug release studies of prepared SLNs were conducted by cellulose membrane as a semi-permeable membrane. The study revealed that drug release from SLNs depends on the relative amounts of tristearin, surfactant (HSPC) and co-surfactant (Poloxamer 188) present. The selected variables showed the effect on in-vitro drug release of ritonavir from SLNs. The formulations from F1 to F3 show the drug release in the range of 42 % to 59%, formulations from F4 to F6 show in the range of 68.62% to 98.6%. Formulations from F7 to F9, drug release was found in the range of 44.3 to 72.3%. Among 9 formulations of SLNs, SLN5 shows 98.63% up to 30hrs. The drug release profile of ritonavir loaded solid lipid nanoparticles indicates that drug release influenced by physico-chemical properties of the SLNs and thermal properties of melted lipid, HSPC and Poloxamer 188. The in vitro cumulative drug release profiles of SLNs are shown in Figure 1.

![Cumulative percentage of drug release](image)

**Figure 1: In vitro drug release profiles of SLNs**

The drug release followed First order as the ‘r’ value (0.9819) higher than zero order (0.9064). Higher ‘r’ value (0.9925) of Higuchi plot indicates that the drug is released by diffusion mechanism and the type of diffusion was non-Fickian as ‘n’ value of Peppas plot is 0.407.

**Statistical data analysis**

The objective of full factorial design is to study the effect of lipid (HSPC (X1)) and poloxamer 188 (X2) and their interactions using a Design Expert® DX11 software tool by applying two way ANOVA at 0.05 levels. First order polynomial equation was obtained using below-given equation depending on the significant influences of two factors X1 and X2 of the factorial design model as mentioned in Eq. 2:

\[ Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 \]  

All the 3 responses observed for 9 runs of SLNs were simultaneously fitted to the quadratic model.
This was estimated since the p-values of the quadratic model was smallest of the three viz., linear, interactive and quadratic. Further, the R-squared value of the quadratic model was 0.9982, 0.9972 and 0.9352 to the size, %EE and zeta potential. The quadratic equations for three dependent responses obtained are:

\[ Y_{\text{Particle Size}} = 112.54 - 20.00X_1 + 7.17X_2 - 3.75X_1X_2 + 59.88X_1^2 - 16.38X_2^2 \] (1)

\[ Y_{\text{EE}} = 85.38 + 8.83X_1 + 0.67X_1^2 - 1.75X_1X_2 + 26.12X_2 - 9.62X_2^2 \] (2)

\[ Y_{\text{Zeta Potential}} = -33.46 - 5.83X_1 + 2.00X_2 - 1.25X_1X_2 + 12.37X_1^2 + 7.87X_2^2 \] (3)

From table 3, particle size \((Y_1)\), Model F-value of 654.12 indicate that the model is significant. In this case, \(X_1, X_2, X_1X_2, X_1X_1, X_2X_2\) are significant model terms. The "Pred R-Squared" of 0.9826 is in reasonable agreement with the "Adj R-Squared" of 0.9966. For encapsulation efficiency \((Y_2)\), The Model F-value of 105.85 implies the model is significant. In this case, \(X_1, X_1X_1\) and \(X_2X_2\) are significant model terms. The "Pred R-Squared" of 0.8940 is in reasonable agreement with the "Adj R-Squared" of 0.9794. For zeta potential \((Y_3)\), the Model F-value of 72.15 implies the model is significant. In this case, \(X_1, X_2, X_1X_1\) and \(X_2X_2\) are significant model terms. The "Pred R-Squared" of 0.8538 is in reasonable agreement with the "Adj R-Squared" of 0.9700. The above three dependent response's Predicted R-Squared values are reasonable agreement with observed R-Squared which indicates that the design model is validated.

Figure 2: Contour plots for the mount of lipid (HSPC) \((X_1)\) and amount of poloxamer-188 \((X_2)\) in SLNs a) on particle size \((Y_1)\), b) on %EE \((Y_2)\) c) on zeta potential \((Y_3)\)

Figure 3: Response surface plots for the amount of lipid (DSPE) \((X_1)\) and amount of Poloxamer188 \((X_2)\) SLNs a) on particle size \((Y_1)\), b) on %EE \((Y_2)\) c) on zeta potential \((Y_3)\)

The coefficients of the terms HSPC\((X_1)\) Poloxamer 188\((X_2)\) and Poloxamer 188 \(X_1X_2\) indicated a positive effect on vesicle size with HSPC showing more effect than poloxamer 188. The % EE of the SLN factorial batches was found in the range of 41-86%, a good correlation coefficient of 0.9972 was observed between the % EE and independent variables. The rise in the poloxamer 188 content increased the entrapment efficiency of the drug beyond certain limit encapsulations was decreased. An opposite effect was exhibited by the interaction terms HSPC\((X_1X_1)\) and Poloxamer 188 \(X_1X_2\) wherein the interaction between HSPC molecules affected predominantly than poloxamer 188 interactions.

Both the HSPC and poloxamer 188 synergistically influence the zeta potential of SLNs. Zeta potential of SLNs was found to increase with an increase in

---

Suresh Reddy KVN et al., Int. J. Res. Pharm. Sci., 10(1), 77-89

© Pharmascope Publications | International Journal of Research in Pharmaceutical Sciences

82
interaction effects HSPC(X1) and Poloxamer 188 (X2). There was a very good relationship between the zeta potential and independent variables (HSPC and poloxamer 188), i.e., R-squared 0.9352. The response surface plot and contour plots for % EE, particle size and zeta potential are shown in Figure 2 & Figure 3.

**Table 2: Optimized formula of solid lipid nanoparticle**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSPC(X1)</td>
<td>23.04 mg</td>
</tr>
<tr>
<td>Poloxamer 188(X2)</td>
<td>10.00 mg</td>
</tr>
<tr>
<td>Tristearin</td>
<td>100.00 mg</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>20 mg</td>
</tr>
</tbody>
</table>

*Amount of Tristearin and ritonavir is constant in all formulations*

From the Table 2, predicted formulations composition is matching with F5 formulation. The percent relative error was calculated between the predicted values and the experimental values. The prepared optimized formulations were found to be good quality formulation by fulfilling all the requirements of SLNs. The percent relative error values for the three response parameters were within 5%. The experimental results were in agreement with the predicted values, which confirmed the predictability and validity of the model.

The best values of the chosen dependent variables, such as, size, % encapsulation efficiency and zeta potential. The SLNs of these compositions were prepared and were shown to have the desired characteristics. The utility of the technique was proved by the point that the percent relative error was extremely low (below 5%) and desirability was found 0.992 shown in Figure 4. The formulations identified by this technique as those with the best properties were the same as F5 formulation of SLNs. Hence these were considered as optimized and were taken for modification into stealth formulation and for proceeding for further work.

The objective of the PEGylation use to overcome opsonization process of in vivo tested products (to maintain long blood circulation time of tested formulations). The DSPE-mPEG-2000 selected for the development of stealth solid lipid nanoparticles (SSLNs). Stealth solid lipid nanoparticles (SSLN1, SSLN2 and SSLN3) were developed by using DSPE-mPEG-2000 was taken as 10, 20 and 30 mg respectively.

The percent drug content of three SSLNs (SSLN1, SSLN2 and SSLN3) was found in the range of 94.12 to 98.12 % as given in Table 5. This indicates that the drug is uniformly distributed in all the formulations. The pH of SSLNs was found to be around pH 7.4. The DSPE-mPEG-2000 doesn’t alter the stealth vesicular dispersions pH and suitable for parenteral drug delivery.

The mean particle size of SSLNs was found in the range of 126.6 nm to 146.2 nm. The stealth particle size was increased compared to conventional SLNs due to the formation of the thick surface layer by DSPE-mPEG-2000 on the surface of the solid lipid nanoparticles. The zeta potential of SSLNs was found in the range of -38.9 to -54.6 mV for stealth solid nanoparticles. These results show that SSLNs have sufficient charge to inhibit aggregation of particles through repulsion. The SSLNs show more zeta potential values, due to the effect of DSPE-mPEG-2000 was partly intercalated on to the lipid particles by a covalent bond. The DSPE-mPEG-2000 on the surface of SLNs may lead to shielding by ions present on its slipping plane. The %EE of SSLNs was slightly enhanced compared to the conventional SLNs. The DSPE-m-PEG-2000 promotes the solubility of ritonavir leads to enhance the %EE of SSLNs.

**Visualization by FE-SEM**

The surface morphology of selected SSLN1 was characterized by Field emission scanning electron microscopy and the corresponding image is presented in Figure 5. The SSLNs are spherical at 30000 X magnification with about 100 nm size.
in vitro drug release studies

The in vitro drug release pattern of SLN (F5) and SSLN1 formulations were studied using dialysis membrane. It showed burst release (i.e. 17-20%) first one hr and then release was released in diffusion manner. The in vitro release profile of tested formulations is shown in Figure 6. The DSPE-mPEG-2000 was extending the in vitro drug release time from 30 hr to 34 hr. The conventional SLN and SSLN1 are followed as first order release kinetics as indicated by higher first order correlation coefficient (r) values than zero order r-value and the release mechanism was followed non-Fickian diffusion mechanism indicated by the 'n' value.

Figure 6: Comparative in vitro drug release profiles of ritonavir SLN and SSLN

Powder X-Ray Diffraction

The X-ray powder diffraction studies were carried out ritonavir API, tristearin and SLN formulation and are presented in Figure 7. The X-ray diffractogram of ritonavir shows sharp peaks at 17°, 20° and 23° and 38° which confirm that the drug is present as a crystalline material. In the X-Ray diffractogram of tristearin, sharp peaks are observed at a diffraction angle (2θ) of 4.5°, 65° and 85°. In the X-Ray diffractogram of ritonavir, peaks at a diffraction angle (2θ) of 17°, 20° and 23° and 38° were present but the peak intensity was reduced inSSLN1 formulation. This X-ray diffraction study confirms that pure ritonavir was uniformly intercalated into tristearin matrix, it leads to increase the solubility of lipophilic ritonavir in the lipophilic lipid material.

Differential scanning calorimetry

The DSC thermogram of ritonavir in Figure 7 shows a sharp endothermic peak at 124.2°C corresponding to its melting point, indicating that it is in the form of a crystalline nature. A sharp peak obtained at 61.2°C corresponds to pure tristearin. In ritonavir loaded SLNs, sharp peak obtained at 59.8°C represents triglyceride (tristearin). The ritonavir was uniformly intercalated in tristearin matrix which is indicated by disappearance of ritonavir peak (124.2°C). It was observed that the decline in melting peak of ritonavir in SLNs formulation might be the physical interaction between the excipient and drug which helps to increase the solubility by converting ritonavir into an amorphous form.

FT-IR Studies

The FTIR studies were performed to detect the possible interactions between the drug and the excipients and the corresponding spectra are presented in Figure 8. The band at 3480 cm⁻¹ is the N-H stretching of an amide group, 1716 cm⁻¹ corresponding to ester linkage, The peaks observed at 1522, 1645 and 1622 cm⁻¹ are corresponding to stretching vibrations of stretching aromatic carbons. 1. N-H stretching vibration of secondary amine observed at 3357 cm⁻¹ C=O stretching vibration of amide observed at 1644 cm⁻¹, C=O ester stretching vibration at 1716 cm⁻¹. The above major functional group stretching vibrations have confirmed the structure of ritonavir. The FTIR spectrum of DSPE-mPEG 2000, C-H stretching at 2955.03 cm⁻¹, 2917.38 cm⁻¹, 2850.29 cm⁻¹, and O-H stretching vibration observed at 3446.16 cm⁻¹. The FT-IR spectrum of HSPC, the stretching vibrations at 3399 cm⁻¹ is corresponding to the O-H group. In addition to, the bands at 2850 cm⁻¹ and 2917 cm⁻¹ are represented the symmetric and antisymmetric vibrations in the CH₂ groups of alkyl chains, 1740 cm⁻¹ and 1468 cm⁻¹ are identified as stretching vibrations of the ester carbonyl groups and scissoring vibrations of the CH₂ groups. The FT-IR spectrum of tristearin, 3465 cm⁻¹ is O-H stretching, 2956 cm⁻¹ is C-H stretching, 1741 cm⁻¹ is C-H bending, 1466 cm⁻¹ C-H bending, 1380 cm⁻¹ C-H bending, 1266 cm⁻¹ C-O stretching, 1174 cm⁻¹ C=O stretching, 735 cm⁻¹ CH₃ rocking mode. All the characteristic peaks of the drug corresponding to the C-H aliphatic stretching, C-H or C=C-H aromatic stretching, NH stretching of a secondary amine, C=C aromatic stretching, C=O amide
Suresh Reddy KVN et al., Int. J. Res. Pharm. Sci., 10(1), 77-89

Figure 7: XRD spectra of a) Ritonavir b) Tristearin c) SSLN, DSC thermograms of d) Ritonavir e) Tristearin f) SSLN

Figure 8: FT-IR spectra of a) Ritonavir b) tristearin c) HSPC d) DSPE-m-Peg 2000 e) Ritonavir SLNs
Figure 9: Comparative plasma drug concentration profile of the test products

All the pharmacokinetic parameters were expressed as mean ± SD. Statistical analysis was performed using Prism 5.0 software trial version (Graph pad Inc. USA). All the statistical tests were performed at a significance level p < 0.05.

The three treatments were very well tolerated by the healthy rat subjects as any adverse effect was not found during the study. No any signs of gastric tract disturbances or allergic reactions were observed in any of the subjects during the study. The average plasma concentration-time profile is shown in Figure 9. Several factors have been influenced the in vivo rate of the SLN after intravenous dosings such as size, %EE, charge, a method of preparation and lipid/composition and their thermal properties.

**DISCUSSION**

Nanoparticles are preferentially useful in drug delivery as well as imaging and diagnosis. SLNs are biodegradable and bioabsorbable dosage forms which alter the pharmacokinetic parameters of the poorly water drug with lower doses by various routes of administration. SLN’s can encapsulate and scale up the lipophilic, hydrophilic and amphiphilic active ingredients without using organic solvents.

In the current study, ritonavir loaded SLNs were developed using tristearin, HSPC, Poloxamer 188 and DSPE-m-PEG-2000 by full factorial design. Tristearin is a triglyceride available as solid form. It contains three fatty acids linked by glycerol bridge due to this reason. Tristearin isn’t able to form lipid bilayers. HSPC is a saturated phospholipid. It has phosphoric acid as a head group as hydrophilic phase instead of one fatty acid among three fatty acids and linked with two saturated fatty acids by Glycerol Bridge. These hydrophilic and lipophilic portions of HSPC makes to form bilayers in the presence of water.

The physicochemical properties of ritonavir loaded SLNs are tremendously influenced the elected independent variables, i.e. HSPC and Poloxamer 188. While developing formulation, ritonavir was uniformly miscible with lipid melt which may increase the ritonavir solubilized in the lipid matrix. HSPC predominantly influenced the physicochemical properties of SLNs over poloxamer 188. At the middle concentration of HSPC, SLNs shows optimum physicochemical properties of SLNs compared to the lower and higher level of HSPC. At middle level of HSPC promotes the adequate stability to the formulation by covering overall surface of ritonavir solid lipid nanoparticles. At a lower level of HSPC makes lipid nanoparticle aggregation leads to increase the size. At higher concentration of HSPC may cause to increase the size and decrease encapsulation efficiency due to layered forming nature. SLNs showed optimum zeta potential (< 30 mV) which enables them to provide better stability.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Units</th>
<th>Pure Ritonavir solution</th>
<th>Conventional SLN</th>
<th>SSLN</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_{max}</td>
<td>ng/mL</td>
<td>1126.5±1.1</td>
<td>950.2±1.22</td>
<td>958.6±1.36</td>
</tr>
<tr>
<td>T_{max}</td>
<td>hr</td>
<td>0.25±0.00</td>
<td>0.25±0.00</td>
<td>0.25±0.00</td>
</tr>
<tr>
<td>K_{el}</td>
<td>hr⁻¹</td>
<td>0.2309±1.3</td>
<td>0.1460±1.4</td>
<td>0.0625±1.1</td>
</tr>
<tr>
<td>t½</td>
<td>hr</td>
<td>3.00±1.31</td>
<td>4.74±1.2</td>
<td>11.08±1.1</td>
</tr>
<tr>
<td>AUC_{0-24hr}</td>
<td>ng.hr/mL</td>
<td>3598.5±1.26</td>
<td>2963.2±1.31</td>
<td>9269.9±1.33</td>
</tr>
<tr>
<td>AUC_{0-∞}</td>
<td>ng.hr/mL</td>
<td>3846.2±1.22</td>
<td>3408.3±1.31</td>
<td>10884.9±1.22</td>
</tr>
<tr>
<td>AUMC_{0-∞}</td>
<td>ng.hr/mL</td>
<td>16133.5±1.41</td>
<td>19110.2±1.23</td>
<td>165260.7±1.16</td>
</tr>
<tr>
<td>MRT</td>
<td>hr</td>
<td>3.44±1.1</td>
<td>4.85±1.22</td>
<td>14.43±1.14</td>
</tr>
<tr>
<td>F_rel(%)</td>
<td>%</td>
<td>100%±0.9</td>
<td>88.6±1.1</td>
<td>283.0±3.1</td>
</tr>
</tbody>
</table>

*HSPC is a saturated phospholipid.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Units</th>
<th>Pure Ritonavir solution</th>
<th>Conventional SLN</th>
<th>SSLN</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_{max}</td>
<td>ng/mL</td>
<td>1126.5±1.1</td>
<td>950.2±1.22</td>
<td>958.6±1.36</td>
</tr>
<tr>
<td>T_{max}</td>
<td>hr</td>
<td>0.25±0.00</td>
<td>0.25±0.00</td>
<td>0.25±0.00</td>
</tr>
<tr>
<td>K_{el}</td>
<td>hr⁻¹</td>
<td>0.2309±1.3</td>
<td>0.1460±1.4</td>
<td>0.0625±1.1</td>
</tr>
<tr>
<td>t½</td>
<td>hr</td>
<td>3.00±1.31</td>
<td>4.74±1.2</td>
<td>11.08±1.1</td>
</tr>
<tr>
<td>AUC_{0-24hr}</td>
<td>ng.hr/mL</td>
<td>3598.5±1.26</td>
<td>2963.2±1.31</td>
<td>9269.9±1.33</td>
</tr>
<tr>
<td>AUC_{0-∞}</td>
<td>ng.hr/mL</td>
<td>3846.2±1.22</td>
<td>3408.3±1.31</td>
<td>10884.9±1.22</td>
</tr>
<tr>
<td>AUMC_{0-∞}</td>
<td>ng.hr/mL</td>
<td>16133.5±1.41</td>
<td>19110.2±1.23</td>
<td>165260.7±1.16</td>
</tr>
<tr>
<td>MRT</td>
<td>hr</td>
<td>3.44±1.1</td>
<td>4.85±1.22</td>
<td>14.43±1.14</td>
</tr>
<tr>
<td>F_rel(%)</td>
<td>%</td>
<td>100%±0.9</td>
<td>88.6±1.1</td>
<td>283.0±3.1</td>
</tr>
</tbody>
</table>

*HSPC is a saturated phospholipid.
In vitro drug release of ritonavir from SLNs was influenced by selected dependent parameters such as particle size and % EE. The lower particles size of SLNs provides a larger surface area, provides maximum drug release. Ritonavir was uniformly mixed in the melted lipid matrix which provides higher encapsulation efficiency of ritonavir; this could be due to the maximum carbon chain length of tristearin and saturation state of HSPC which extends the drug release up to 30 hrs. On other side, factors contributing to a controlled release are the large surface area, a low diffusion coefficient due to high molecular size, uniform distribution of ritonavir in lipid matrix and a larger diffusion distance (i.e., release from inner lipid matrix region of the SLN. A possible explanation is a larger diffusion path due to intercalation of the drug in the melted lipid matrix of the SLNs (Zur Muhlen et al., 1998).

The particle size and surface charges are the critical factors for delivery systems which may influence the biodistribution and long circulation of the nanoparticles by parenteral delivery. If the particle size of nanoparticles (around 50 to 100 nm) more favourable to reach the target site by extravasates leaky trough vasculature and easily permeates through lymphatic and accumulates in tissue whereas the particles size below 8 nm is cleared by the kidney (US2014/0328759). Polyethene glycol (PEG) is a polymer of choice for the development of stealth formulation to overcome the opsonization. The USFDA also approved as injectable excipients due to which can adsorb on the surface of the particles by the covalent reaction. The DSPE-PEG-2000 is lipid-polymer conjugated by methylation process. The DSPE-mPEG-2000 inhibits non-specific and specific protein interaction on the carrier surface, it is extensively fabricated to exert the stealth property which is a flexible polymer, has an inherent long-circulating property by avoiding opsonization (Barenholz et al., 2012). PEGylation process extends the ritonavir release time from 30hr to 35hrs. The drug and excipients compatible studies (DSC, FTIR and XRD) conform good compatibility among the drug and excipients. Two-step sterilization techniques were utilized successfully to sterilize selected formulations for in vivo pharmacokinetic study on rats.

Sterilization is essential to step for injectable formulations. Thermolabile formulations are usually sterilized by the aseptic filtration process. Sterilizing filtration is a process of removing microorganisms from a fluid stream without adversely affecting product. Sterile grade filtration designations is not dependent on the filter pore size. It defined by qualification (ability to remove of a standard test organism (Brevundimonas diminuta at minimum concentrations of 10⁷ cfu/cm²) (www.pda.org, 2017). In this study, selected products were sterilized by two-step sterilization technique. Initially, selected products were subjected for filtration using 0.22μm EDF filter for SLN formulations whereas 0.22μm PVDF filter for pure ritonavir solution and followed the lyophilization cycle.

Lyophilization cycle was carried out for 24 hrs. 10% w/v sucrose was used as a cryoprotecting agent who helps to avoid stress while developing during cycles and protect the physicochemical properties of selected products. 10% sucrose help to form hydrogen bonding among SLNs particles while removing unbounded and bonded water from formulations. This hydrogen bonding helps to avoid the formation of hard cake of formulations. The lyophilized products in half sealed vials were found to be free flowing and fluidly without any cake formation. The Phosphate buffer saline of pH 7.4 sterilized by autoclaving was used for the reconstitution of the lyophilized vesicular systems for parenteral administration to Wistar rats. The reconstitution time was found to be 40-50 sec. Lyophilization process was didn’t alters the physicochemical properties of reconstitution products.

The pharmacokinetic study results state that SSLNs are promising drug delivery systems to alter the properties of ritonavir with a lower dose and improves the patient compliance by reducing the repeatability of administration of ritonavir. The PEGylation process (using DSPE-mPEG-2000) hindered the opsonins molecules and improved the circulation time of the drug by overcome opsonization process.

CONCLUSION

Ritonavir loaded solid lipid nanoparticles were successfully developed by full factorial design using tristearin, HSPC and poloxamer 188. Solid lipid nanoparticles are proved as successful drug delivery tools and overcome the disadvantages of ritonavir by increasing solubility and controlled release up to 34 hrs. The stealth SLNs have changed the pharmacokinetic profile of ritonavir resulting in reduced plasma clearance and improved systemic circulation time of the drug comparatively conventional SLN and pure ritonavir solution. The pharmacokinetic study reveals that SSLN improved the systemic circulation time (MRT) of ritonavir (14.43 hrs) and provides the steady-state concentration (958.6±1.36 ng/mL) over a period of time at the targeted site, which may decrease the HIV propagation and increase the CD4 cells to improve the immunity. So the stealth solid lipid nanoparticles proved as a tool for antiretroviral therapy by reducing the frequent administration and increased the patient compliance.

Conflict of Interest: None
Acknowledgements

Authors are thankful to Dr. A. Muralidhar and management of Srinivasa Institute of Pharmaceutical Sciences, Proddatur, Kadapa, AP, India for providing the facilities for animal studies.

Human and Animal Rights

The European Commission guidelines are considered for the experiments on animals. Ethical committee clearance was obtained from IAE (Institutional Animal Ethics Committee) of CPCSEA, India.

REFERENCES


Muller, R.H., Mader, K., Gohla, S., 2000, Solid lipid nanoparticles (SLN) for controlled drug delivery - a review of state of the art. European Journal of Pharmaceutics and Biopharmaceutics, 50, 161-177.


Sudhakar, B., Krishna, M.C., Murthy, K.V.R., 2016, Factorial design studies of antiretroviral drug-loaded stealth liposomal injectable PEGylation, lyophilization and pharmacokinetic studies. Applied Nanoscience, 6, 43-60.

Uner M; Yener G. 2007, Importance of solid lipid nanoparticles (SLN) in various administration