Rosuvastatin calcium-loaded Solid Lipid Nanoparticles (SLN) using design of experiment approach for oral delivery

Anjali Beniwal1,a and Hema Choudhary2

1G & W Labs, Department of Product Development, South Plainfield, New Jersey, USA-07080
2PDM college of Pharmacy affiliated by Pandit Bhagwat Dayal Sharma Post Graduate Institute of Medical Sciences, Haryana, India-124001

Received: 4/16/2017; Accepted: 4/27/2017

Abstract: This investigation utilizes quality-by-design approach to develop the Rosuvastatin Calcium (Rst)-loaded solid lipid nanoparticles (SLN). Effect of formulation variables such as amount of lipid (200-500 mg stearic acid) and surfactant concentration (0.5-2.0% PVA) were studied. Design of Experiment (DoE) was used to quantify the extent of impact of lipid amount and surfactant concentration on the physicochemical properties of the SLN and to identify optimized SLN formulation. It was observed that interplay of formulation variables had significant effect on particle size (198.25 to 622.36 nm), %EE (28.82 to 35.87%) and In vivo release (44.87 to 64.29%). Based on the results, point optimization was carried out to obtain the SLN with minimum particle size (202.5 ± 9.29 nm), maximum %EE (34.78 ± 0.37 %) and sustained In vivo release (57.3 ± 2.6 % at 36 hours) within the design space. In vitro drug release data fitted well in Korsmeyer-peppas model indicating the fickian diffusion mechanism. Ex vivo studies indicated sustained permeation of the Rst compared to the control. Furthermore, stability studies indicated Rst formulation exhibited no significant physical or chemical change under accelerated conditions.

Keywords: Solid Lipid nanoparticle, Rosuvastatin, Controlled Release, Nanoparticles, Lipid based delivery, DoE, Hyperlipidemia, Drug Delivery, Quality by design (QbD), Sustained Drug Delivery

Introduction

Hyperlipidemia is a condition caused by elevated lipid levels, especially of cholesterol and triglycerides, in the blood circulation. High levels of these lipids in the blood circulation can cause formation of the plaques on the surface of artery that can subsequently result in atherosclerosis. This can also increase the risk of heart disease, stroke, and other vascular diseases [1]. In this regard, statin drugs are most commonly prescribed to reduce endogenous synthesis of these lipids (cholesterol and triglycerides) and prevent the onset and development of atherosclerosis [2]. Rosuvastatin Calcium (Rst) is a semi-synthetic lipid lowering statin that inhibits HMG-CoA (3-hydroxy-3-methyl glutaryl CoA) reductase, which plays a central role in the synthesis of cholesterol [3]. Oral route is the most preferred route for delivery of statins like Rst. However, oral delivery of Rosuvastatin itself is challenging, as it is a BCS (Biopharmaceutica Classification System) Class II drug with extremely low water solubility [4]. Furthermore, it exhibits excessive first pass metabolism with only a small proportion of the Rst recovers as metabolites (predominantly N-desmethyl Rst), which possess only one-sixth of HMG-CoA reductase inhibitory activity compared to the Rst. This is evident for the fact that the most marketed brand formulation of Rst called Crestor® tablets, exhibits an absolute oral bioavailability of merely 20%. Therefore, researchers are looking into new avenues for delivery of Rst via oral route considering the low drug solubility, poor gastro-intestinal absorption, rapid metabolism, and limitations of traditional dosage form.

Increasing the solubility and by passing the first pass metabolism of Rst are some of the approaches researchers are working in order to develop the formulations to improve bioavailability of Rst. Dissolution enhancement of Rosuvastatin Calcium by liquisolid compact technique and development of microemulsion of Rosuvastatin Calcium are reported in the literature [5,6]. In recent past, colloidal lipid based nano delivery system has been successfully utilized to deliver poorly bioavailable drug via oral route [7–11]. However, based on the literature review, only limited studies had been performed with Rst [12,13]. Amongst them, most of the studies are performed using self-nanoemulsifying drug delivery system with limited success in improving the oral bioavailability of Rst [3,13]. Recently, Solid Lipid Nanoparticles (SLN) has emerged as a most successful lipid based delivery system for oral drug delivery [14]. SLN are composed of biodegradable lipids, which forms a solid lipophilic matrix at the room temperature in which hydrophilic or lipophilic drug can be incorporated. SLN provide flexibility in modulating the drug release, higher drug loading of lipophilic moieties, and enhance drug stability by protecting the drugs from chemical degradation, oxidation, light degradation, and moisture. Furthermore, SLN
can induce stimulation of chylomicron formation by enterocytes, which promote the absorption of lipid matrix through intestinal lymphatics by passing the portal circulation and consequently hepatic first pass metabolism [15]. Carvedilol loaded solid lipid nanoparticles have demonstrated the increased intestinal lymphatic uptake using in-vitro cell culture model [7,10]. Despite the obvious advantages of the SLN, very limited studies had been performed using Rst [12].

Therefore, present investigation was undertaken to utilize quality by design (QbD) approach to develop the Rst-loaded SLN and to understand the effect of interaction of formulation variables in improving the oral delivery of Rst in a defined design space. QbD approach emphasizes the understanding of various components of the system for improved control over desired output. Design of experiments (DoE) and multivariate statistical data analysis are essential elements of QbD, recognized by recent International Conference of Harmonization Q8 guideline. These tools facilitate varying all the formulation variables simultaneously, allowing quantification and prioritizing the effects produced by these variables, along with any possible interaction between them, in the defined design space. Following the fabrication of Rst-loaded SLN formulations, effect of formulation variables, amount of lipid and concentration of surfactant on particle size, zeta potential, entrapment efficiency and drug release were studied. Mechanism of drug release from the SLN was also evaluated. The point prediction was utilized to obtain optimized formulation with minimum particle size, maximum entrapment efficiency and sustained drug release. Ex-vivo permeation of optimized Rst-loaded SLN along with drug solution (as control) was also studied using small intestine of the Wistar rat. Finally, accelerated stability studies were conducted using ICH stability guideline to evaluate the suitability of optimized formulation during shelf life.

Materials and Methods
Rosuvastatin calcium (Rst) was obtained from Biocon (Mumbai, India). Dichloromethane (DCM), Tween 80, Polyvinyl alcohol (PVA), ethanol, sodium hydroxide, hydrochloride acid, sodium chloride, potassium chloride, magnesium sulphate, glucose and calcium chloride were procured from Central Drug House Ltd. (Delhi, India). Analytical grade methanol (MeOH) was obtained from Qualigens Fine Chemicals (Mumbai, India). Potassium dihydrogen Orthophosphate, sodium bicarbonate, Ethylene Diamine Tetra Acetate (EDTA) were obtained from Thomas Baker Private Ltd. (Mumbai, India).

Preparation of Rst-loaded SLN
Rst-loaded SLN was prepared using the double emulsion solvent evaporation homogenization technique (DESEH) In brief, the required quantity of Rst was added in the aqueous solution of 1.0% w/v Tween 80. The lipid (stearic acid) was dissolved in mixture of MeOH: DCM (3:2). Both the solutions were then mixed and sonicated for approximately 2 min until the primary emulsion was formed. Primary emulsion was then transferred into the PVA solution at room temperature and stirred for 3 h at 1600 rpm to ensure completely evaporation of organic solvent. The formed secondary emulsion was then cooled at 4°C ± 0.5°C which was further homogenized by using Ultraturrax Homogenizer for 10 minutes at 20,000 rpm. The preparation technique including the time and speed were optimized as a preliminary experiment (data not shown).

Characterization of Rst-loaded SLN
Entrapment Efficiency
Entrapment efficiency (%EE) is defined as the amount of drug encapsulated in the nanoparticles. The %EE was determined by ultra-centrifugation using centrisart®, a centrifugation tube that consists of filter membrane at the base of the sample recovery chamber. In this method, a specified volume of the sample was taken in the outer chamber of the centrisart® and the sample recovery chamber was then placed on the top of the outer chamber. The centrisart® unit was then centrifuged at 16,000 rpm so that the encapsulated drug remained in the outer chamber, and aqueous phase with free drug can be collected from the sample recovery chamber. From the obtained supernatant (aqueous phase), the amount of free drug was determined using analytical method developed for Rst and entrapped drug was calculated using below equation:

\[
%EE = \frac{\text{Total Drug - Free Drug}}{\text{Total Drug}} \times 100 \quad (1)
\]

The measurement was performed in triplicates.

Particle Size and Zeta Potential
The mean particle size, polydispersity index (PDI), and zeta potential of the SLN were measured using a Nano ZS90 Zetasizer (Malvern Instruments Ltd). Lower particle size and narrow PDI is essential for lymphatic uptake of the SLN [16,17]. Zeta potential, on the other hand, is a key factor to evaluate the stability of colloidal dispersion. High zeta potential values should be achieved to ensure higher energy barrier and consequently superior stability [18]. The SLN samples were diluted with distilled water to determine the particle size (volume distribution) and zeta potential. The measurements were performed in triplicates.
In vitro Drug Release Studies

In vitro release studies were performed in phosphate buffer pH 6.8 using dialysis bag (Hi-Media, Mumbai, India) of molecular weight cut-off of 12,000 Da. Briefly, 10 ml of Rst-loaded SLN was added in dialysis bag and placed in 150 ml of the receptor media (phosphate buffer pH 6.8). The temperature of the receptor medium was maintained at 37°C ± 1°C. The receptor medium was stirred at 300 rpm using magnetic stirrer and 2 ml of the samples were withdrawn at predetermined time intervals. Fresh receptor media was replaced at each time interval to maintain the sink conditions. The concentration of drug released from the SLN was calculated by using the standard curve obtained from UV-Visible Spectro-photometer. Rst drug solution was used as a control.

Design of Experiment (DoE) Approach

Preliminary experiments were performed using One Factor at a Time (OFAT) approach to select suitable process as well as excipients (lipid and surfactant) and their levels (or range) for further Design of Experiment (DoE) study. These preliminary data are not shown here. After the selection of suitable excipients and their working range, DoE approach was utilized to comprehensively and systematically study the effect of formulation variables as well as to obtain optimized formulation composition [7,19,20].

In the present investigation, response surface factorial design was used to quantify the extent of impact of lipid amount (at a fixed drug amount) and surfactant concentration on particle size, entrapment efficiency and drug release from the SLN and to further identify optimized formulation. For this purpose, a face-centered central composite design was used. According to the face-centered design (alpha value of ± 1), the total number of experimental combinations is \(2^k + 2k + n_0\), where \(k\) is the number of independent variables and \(n_0\) is the number of repetitions of the experiments at the center point [21]. Based on preliminary evaluation using OFAT approach, lipid amount and surfactant concentration were found to impact the physicochemical properties of the Rst-loaded SLN. For number of independent variables being 2 with 5 repetitions of the experiments at the center point, the design containing 13 runs \(4\) (i.e., \(2^3\)) factorial points and \(4\) (i.e., \(2\times2\)) star points plus \(5\) center points) was generated. Based on this experiment design, various formulations were prepared and particle size, %EE and In vitro drug release were determined. The obtained data was used as input to design of experiments and was then further analyzed by the statistical software package Design Expert software (Stat-Ease Inc., Minneapolis, MN). Each run based on the face-centered central composite design was carried out in triplicate.

For the statistical analysis, the experimental variables \(X_i\) have been coded as \(x_i\) according to the following transformation equation:

\[
x_i = \frac{x_i - X_o}{\delta x}
\]

where \(x_i\) is the dimensionless coded value of the variable \(X_i\), \(X_o\) is the value of \(X_i\) at the center point and \(\delta x\) is the step change. This conversion of different levels of independent variables into coded level helps to determine the relative magnitude of the independent variables impacting the response parameters.

The response surface of response variables \((Y_1, Y_2 \text{ and } Y_3)\) as a function of independent variables \((X_1, \text{ and } X_2)\) can be expressed as \(Y = f(X_1, X_2)\). Response variables were analyzed by multiple regressions through the least squares method to fit the following polynomial equation:

\[
Y = b_0 + b_1X_1 + b_2X_2 + b_12X_1X_2 + b_11X_1^2 + b_22X_2^2
\]  

where \(Y\) is %EE, particle size, or % In vitro release; \(b_0\) is the intercept; \(b_1\) to \(b_{22}\) are the regression coefficients computed from the observed values of \(Y\); and \(X_1\) and \(X_2\) are the coded levels of independent variables- lipid amount and surfactant concentration, respectively. The magnitude of regression coefficient represents the extent of impact of the corresponding independent variable. The positive and negative signs of the regression coefficient value in the equations represent the agonist and antagonist effect of the independent variables, respectively. The terms \(XX_i (i, j = 1, \text{ or } 2)\) and \(X_i^2 (i = 1, \text{ or } 2)\) representing the interaction and quadratic terms, respectively, are used to simulate the curvature of the design space.

Furthermore, all the response variables were statistically analyzed by applying ANOVA at 0.05 level to determine the significance and the magnitude of the effects of independent variables in Design-Expert software. Predictor equations for measured response variables containing only the significant quadratic terms were generated using backward elimination procedure. The quadratic terms statistically found non-significant (\(p > 0.05\)) were removed from the initial polynomial equation and the observed data were refitted until the final polynomial equation with reduced quadratic terms was acquired [22]. The effect of lipid amount and surfactant concentration on the response parameters of the SLNs was presented using response surface plots to illustrate the effect of lipid amount and surfactant concentration simultaneously. Response surfaces and Countour plots were used to evaluate the relationship between the independent variables and the responses. The resulting observed responses were compared with the predicted responses and a linear regression plots between actual and predicted responses was plotted [20,23].
Validation and Optimization of Data

Based on the results and response surfaces obtained, point optimization was carried out to obtain the Rst-loaded SLN with maximum %EE, minimum particle size and sustained % In vitro release within the studied design space. Formulations with different levels of lipid amount and surfactant concentrations were generated by software in the decreasing order of desirability based on the input. Formulations were generated based on the equations obtained by fitting various input of independent variables. The reliability of optimized formulation derived based on the quadratic equation was evaluated. Optimized Rst-loaded SLN (ROpt) was evaluated for %EE, particle size, zeta potential, and In vitro drug release. Release profiles were fitted in various release kinetics models such as zero order model, first order model, Higuchi model and Korsmeyer-peppas model in order to understand the release mechanisms.

Ex-vivo Studies

Ex-vivo studies were performed in accordance to approved protocol from animal ethical committee of PDM College of Pharmacy, Bahadurgarh. Ex-vivo permeation studies were carried out using male wistar rat on Ropt formulation and drug solution as control. The upper part of small intestine of sacrificed rat was taken and thoroughly cleaned using cold Krebs solution. The cleaned upper part of intestine was filled either with drug solution or SLN formulation containing 1 mg of Rst and was immersed in the specified amount of phosphate buffer pH 6.8 and stirred at 400-500 rpm and 37°C temperature for 24 h. At predetermined time intervals, 5 ml sample was withdrawn and replaced it with phosphate buffer pH 6.8 to maintain the sink condition.

Stability Studies

Major disadvantage attributed to SLN based formulations is drug expulsion during prolonged storage [11]. Therefore, accelerated stability studies were performed for ROpt in accordance to FDA Guidance for Industry Q1A (R2) Stability Testing of New Drug Substances and Products. According to the guideline, the accelerated condition intended for storage under refrigerator is 25°C ± 2°C/60% RH ± 5% RH. Therefore, optimized formulation was stored under accelerated condition and physical changes, entrapment efficiency and drug loading was evaluated at 0, 15, 30, 60 and 90 days.

Results and Discussion

Design of Experiment (DoE) Approach

In the present investigation, the levels of 200-400 mg for lipid amount and 0.5-1.5 % for surfactant concentration (selected from OFAT study) were included as defined design space of the formulation. Major disadvantage attributed to SLN based systems is drug expulsion during prolonged storage. Therefore, accelerated stability studies were performed for Ropt in accordance to FDA Guidance for Industry Q1A (R2) Stability Testing of New Drug Substances and Products. According to the guideline, the accelerated condition intended for storage under refrigerator is 25°C ± 2°C/60% RH ± 5% RH. Therefore, optimized formulation was stored under accelerated condition and physical changes, entrapment efficiency and drug release was evaluated at 0, 15, 30, 60 and 90 days.

Table 1. Summary of independent and dependent variables utilized for design of experiment.

<table>
<thead>
<tr>
<th>Independent variables</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>X1: Lipid amount (mg)</td>
<td>Minimum (1)</td>
</tr>
<tr>
<td>X2: Surfactant Concentration (%)</td>
<td>200</td>
</tr>
<tr>
<td>Dependent variables</td>
<td></td>
</tr>
<tr>
<td>Y1: Entrapment efficiency (%EE)</td>
<td>Goals</td>
</tr>
<tr>
<td>Y2: Particle Size (nm)</td>
<td>Maximize</td>
</tr>
<tr>
<td>Y3: In vitro drug release for 36 hrs (% release)</td>
<td>Maximize</td>
</tr>
</tbody>
</table>

Table 2. Summary of entrapment efficiency (%EE), particle size and in-vitro drug release (% In vitro release) for various formulations obtained from design of experiments.

<table>
<thead>
<tr>
<th>Formulation Runs</th>
<th>Lipid amount (mg)</th>
<th>Surfactant concentration (%/w/w)</th>
<th>%EE ± S.D*</th>
<th>Particle Size (nm) ± S.D*</th>
<th>% In vitro Release ± S.D*</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>300 (0)</td>
<td>0.5 (-1)</td>
<td>35.54 ± 0.28</td>
<td>201.51 ± 9.65</td>
<td>44.87 ± 0.36</td>
</tr>
<tr>
<td>R2</td>
<td>400 (1)</td>
<td>1.5 (1)</td>
<td>28.82 ± 0.56</td>
<td>622.36 ± 9.12</td>
<td>50.36 ± 0.50</td>
</tr>
<tr>
<td>R3</td>
<td>200 (-1)</td>
<td>0.5 (-1)</td>
<td>30.16 ± 0.72</td>
<td>348.51 ± 8.51</td>
<td>56.45 ± 0.22</td>
</tr>
<tr>
<td>R4</td>
<td>300 (0)</td>
<td>1.0 (0)</td>
<td>34.05 ± 0.78</td>
<td>198.25 ± 6.34</td>
<td>53.50 ± 0.35</td>
</tr>
<tr>
<td>R5</td>
<td>300 (0)</td>
<td>1.0 (0)</td>
<td>34.55 ± 1.02</td>
<td>210.34 ± 10.12</td>
<td>52.98 ± 1.07</td>
</tr>
<tr>
<td>R6</td>
<td>400 (1)</td>
<td>0.5 (-1)</td>
<td>35.87 ± 0.67</td>
<td>520.85 ± 9.01</td>
<td>45.33 ± 0.62</td>
</tr>
<tr>
<td>R7</td>
<td>300 (0)</td>
<td>1.0 (0)</td>
<td>34.78 ± 0.37</td>
<td>202.51 ± 9.29</td>
<td>52.96 ± 0.43</td>
</tr>
<tr>
<td>R8</td>
<td>200 (-1)</td>
<td>1.0 (0)</td>
<td>31.24 ± 0.60</td>
<td>329.30 ± 6.65</td>
<td>63.16 ± 0.54</td>
</tr>
<tr>
<td>R9</td>
<td>300 (0)</td>
<td>1.0 (0)</td>
<td>35.02 ± 0.60</td>
<td>205.88 ± 8.07</td>
<td>53.15 ± 0.76</td>
</tr>
<tr>
<td>R10</td>
<td>200 (-1)</td>
<td>1.5 (1)</td>
<td>28.87 ± 0.33</td>
<td>344.98 ± 7.60</td>
<td>64.29 ± 0.47</td>
</tr>
<tr>
<td>R11</td>
<td>300 (0)</td>
<td>1.0 (0)</td>
<td>34.45 ± 0.44</td>
<td>202.78 ± 12.11</td>
<td>53.02 ± 0.98</td>
</tr>
<tr>
<td>R12</td>
<td>400 (1)</td>
<td>1.0 (0)</td>
<td>34.31 ± 0.46</td>
<td>552.38 ± 7.5</td>
<td>49.66 ± 0.64</td>
</tr>
<tr>
<td>R13</td>
<td>300 (0)</td>
<td>1.5 (1)</td>
<td>32.89 ± 0.25</td>
<td>230.65 ± 7.63</td>
<td>53.77 ± 0.19</td>
</tr>
</tbody>
</table>

It was observed that amount of lipid and surfactant concentration significantly affected SLN parameters viz % EE (28.82 ± 0.56% to 35.87 ± 0.67%), particle size (198.25 ± 6.34 nm to 622.36 ± 9.12 nm), and % In vitro drug release (44.87 ± 0.36% to 64.29 ± 0.47%). Thus, modulation of these

DOI: http://dx.doi.org/10.21746/ijclsi.2017.5.1

2032
independent variables would allow formulators to achieve SLNs with desired parameters within these ranges. To further understand the effect of formulations variables and their interaction on the particle size, %EE and % in vitro drug release of Rst-loaded SLN, regression analysis was performed.

Table 3. Comparative statistics for selection of model

<table>
<thead>
<tr>
<th>Models</th>
<th>%EE (p value)</th>
<th>Particle size (p value)</th>
<th>%Release (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear</td>
<td>0.0555 0.3269</td>
<td>-0.3136 0.1910</td>
<td>0.1382 &lt;0.0001 0.8183</td>
</tr>
<tr>
<td>2FI</td>
<td>0.1706 0.3999</td>
<td>-1.4990 0.7303</td>
<td>0.0557 -2.0123 0.5881 0.8049</td>
</tr>
<tr>
<td>Quadratic</td>
<td>0.0002 0.9346</td>
<td>-0.7426 &lt;0.0001 0.9986</td>
<td>0.9942 0.0001 0.9818 0.9100</td>
</tr>
<tr>
<td>Cubic</td>
<td>0.4449 0.9338</td>
<td>-1.3896 0.1229</td>
<td>0.9991 0.9900 0.1193 0.9891 0.5336</td>
</tr>
</tbody>
</table>

Based on the data reported in Table 3, quadratic model fitting showed lowest p value, highest adjusted $r^2$ value and comparative coefficients of adjusted $r^2$ and predicted $r^2$ for all three response variables viz. particle size, %EE and % in vitro drug release. Based on this information, quadratic models suggested the sufficiency of quadratic equation for further analysis.

Effect on the %EE (Y1) of Rst-loaded SLN

The polynomial equation representing the % EE (Y1) of the Rst-loaded SLN is shown below:

**Uncoded Equation:** $Y_1 = -1.49 + 0.19X_1 + 13.57X_2 - 0.03X_1X_2 + 2.51X_1^2 + 4.30X_2^2$

**Coded Equation:** $Y_1 = 34.78 + 1.45X_1 - 1.83X_2 - 1.44X_1X_2 + 2.31X_1^2 - 1.07X_2^2$

Based on the "prob>F Value" less than 0.05, the model terms $X_1$, $X_2$, $X_1X_2$, $X_1^2$, $X_2^2$ were found to be statistically significant. The equation indicates that $X_1$ (lipid amount) has positive effect while other coefficients $X_2$ (surfactant concentration), $X_1X_2$, $X_1^2$, and $X_2^2$ has negative effect on $Y_1$. The Model F-value of 35.31 implies the model is significant and there is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. The "Pred R-Squared" of 0.7426 is in reasonable agreement with the "Adj R-Squared" of 0.9346 indicating the adequacy of model to predict the response of %EE (Table 4).

Table 4. Summary of regression analysis

<table>
<thead>
<tr>
<th>Regression Statistics</th>
<th>Y1 (%EE)</th>
<th>Y2 (particle size)</th>
<th>Y3 (In-vitro drug release)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>Quadratic</td>
<td>Quadratic</td>
<td>Quadratic</td>
</tr>
<tr>
<td>F value</td>
<td>35.31</td>
<td>1662.75</td>
<td>130.39</td>
</tr>
<tr>
<td>P value Prob&gt;F Value</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Lack of Fit F Value</td>
<td>5.80</td>
<td>2.65</td>
<td>25.28</td>
</tr>
<tr>
<td>Lack of fit p-value Prob&gt;F</td>
<td>0.0610</td>
<td>0.1845</td>
<td>0.0046</td>
</tr>
<tr>
<td>Predicted $r^2$</td>
<td>0.7426</td>
<td>0.9942</td>
<td>0.9100</td>
</tr>
<tr>
<td>Adjusted $r^2$</td>
<td>0.9346</td>
<td>0.9986</td>
<td>0.9818</td>
</tr>
<tr>
<td>Adequate Precision</td>
<td>15.158</td>
<td>107.328</td>
<td>38.721</td>
</tr>
</tbody>
</table>

Equations for response*

$Y_1 = 34.78 + 1.45X_1 - 1.83X_2 - 1.44X_1X_2 + 2.31X_1^2 - 1.07X_2^2$

$Y_2 = 202.68 + 112.15X_1 + 21.19X_2 + 26.26 X_1X_2 + 240.00 X_1^2 + 15.29 X_2^2$

$Y_3 = 52.91 + 6.44X_1 + 3.63X_2 + 4.01X_1X_2 + 0.68X_1^2 + 3.08X_2^2$

*X1, and X2 are the coded levels of lipid amount and surfactant concentration, respectively. The terms $X_1X_1$ (i, j = 1, or 2) and $X_2^2$ (i = 1, or 2) represents the interaction and quadratic terms, respectively.

Figure 1 illustrates the effect of the amount of lipid and surfactant concentration on the %EE of the Rst-loaded SLN (Y1). Within the design space of lipid and surfactant levels, overall %EE ranged from 28.82% to 35.87%. It was also observed that %EE increased with increase in lipid amount, with more prominent effect observed at the lower surfactant concentrations compared to higher surfactant concentrations. On the other hand, increase in surfactant concentration resulted in the decrease of %EE. Increase in the lipid amount (at the fixed amount of drug) facilitated drug partitioning between lipid and aqueous phase resulting in higher %EE. Furthermore, increase in amount of lipid resulted in increase in particle size, providing an extra space for drug to get entrapped.

To select suitable model, independent data of particle size, % EE and % in vitro drug release were fitted in linear, 2FI, quadratic and cubic equations. Lower p value for F statistics, high adjusted $r^2$ value, and comparative coefficients of adjusted $r^2$ and predicted $r^2$ were the criteria for model selection.
Effect of the amount of lipid \( (X_1) \) and surfactant concentration \( (X_2) \) on the (a) %EE, (b) particle size and (c) % In vitro release of Rst-loaded SLN.

Researchers have also reported that increase in the lipid amount would increase the viscosity of the medium, which could result in faster solidification of the nanoparticles \([7,24]\). This would prevent drug diffusion to the external phase of the medium and consequently result in higher %EE. However, it is important to acknowledge that formulations with higher lipid amount and higher surfactant concentrations showed lower %EE. This could be due to leaching of drug from the SLN at the higher concentration of surfactant resulting in lower %EE. Additionally, higher surfactant concentration increases the aqueous solubility of drug in external aqueous phase, which leads to low %EE.

**Effect on the Particle Size \( (Y_2) \) of Rst-loaded SLN**

The polynomial equation representing the particle size \( (Y_2) \) of the Rst-loaded SLN is shown below:

**Uncoded Equation:**  
\[
Y_2 = 2202.73 - 13.80 X_1 - 237.49 X_1^2 + 0.53X_1X_2 + 0.02 X_2^2 + 61.15 X_2^3
\]

**Coded Equation:**  
\[
Y_2 = 202.86 + 112.15 X_1 + 21.19 X_1^2 + 26.26 X_1X_2 + 240.00 X_2^2 + 15.29 X_2^3
\]

Based on the “prob>F Value” less than 0.05, the model terms \( X_1, X_2, X_1X_2, X_1^2, X_2^2 \) were found to be statistically significant. The equation indicates that \( X_1 \) (lipid amount), \( X_2 \) (surfactant concentration) and all other coefficients had positive effect on the \( Y_2 \) (particle size). The Model F-value of 1716.73 implies the model is significant and there is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. The "Pred R-Squared" of 0.9942 is in reasonable agreement with the "Adj R-Squared" of 0.9986 indicating the adequacy of model to predict the response of particle size (Table 4).

Figure 1 illustrates the effect of the amount of lipid and surfactant concentration on the particle size of the Rst-loaded SLN \( (Y_2) \). It was observed that particle size of the SLN increased with increase in lipid amount, with more prominent effect at the higher surfactant concentrations compared to the lower surfactant concentrations. Furthermore, increase in surfactant concentration resulted in increase in particle size of the SLN, however, the coefficient of surfactant concentration \( (X_1^2) \) was much lower than coefficient of lipid \( (X_1) \) and \( X_1^2 \) amount indicating that amount of lipid plays a more dominant effect in influencing the particle size of the SLN. The positive influence of amount of stearic acid on the particle size of the SLN could be attributed to the increase in viscosity of the dispersion medium at higher lipid amount. Higher viscosity of the dispersion medium could negatively affect the shearing efficiency of the homogenizer that could result in higher particle size of the SLN \([25,26]\). Furthermore, at higher amount of lipid, relatively less amount of surfactant is available to cover the SLN causing higher particle size \([27]\). It has also been reported that once lipid concentration exceeded a critical concentration, a concentration dependent increase in particle size of the SLN can be observed \([28]\). Furthermore, relatively lower influence of surfactant concentration on the particle size of the SLN, in comparison to the lipid amount was observed which is in alignment to the previously published reports \([29,30]\).

**Effect on the In vitro release \( (Y_3) \) of Rst-loaded SLN**

The polynomial equation representing the In vitro release \( (Y_3) \) of the Rst-loaded SLN is shown below:

**Uncoded Equation:**  
\[
Y_3 = 52.91 - 6.43X_1 + 3.63X_1^2 + 4.01X_2^2 - 3.08X_2^3
\]

**Coded Equation:**  
\[
Y_3 = 84.54 - 0.29X_1 + 36.08X_1^2 + 0.04X_2^2 - 12.30X_2^3
\]
Based on the “prob>F Value” less than 0.05, the model terms $X_1$, $X_2$, $X_1^2$, and $X_2^2$ were found to be statistically significant. The equation indicates that $X_1$ (lipid amount) had negative effect on $Y_3$ (In vitro release) and $X_2$ (surfactant concentration) had positive effect on $Y_3$ (In vitro release). The Model F-value of 130.39 implies the model is significant and there is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. The "Pred R-Squared" of 0.9100 is in reasonable agreement with the "Adj R-Squared" of 0.9818 indicating the adequacy of model to predict the response of In vitro release (Table 4).

Figure 1 illustrates the effect of the amount of lipid and surfactant concentration on the drug release of the Rst-loaded SLN ($Y_3$). It was observed that % In vitro drug release decreased with increase in lipid amount as well as with decrease in surfactant concentration. However, the negative impact of lipid amount (coefficient of $X_1$: 6.43) was higher compare to positive impact of surfactant concentration (coefficient of $X_2$: 3.63). This could be attributed to the fact that with increase in lipid amount, the particle size of the SLN increases that resulting into less surface area available for contact with release medium causing slower drug release. Whereas, increase in surfactant concentration could have helped in better solubilization of drug resulting in improved release.

Validation and Formulation Optimization
The correlation plots between observed and predicted physicochemical data based on the quadratic equation are shown in Figure 2. Equations derived were adequately predicted the response parameters of the SLN were compared with observed values. Thus, SLN parameters can be adequately estimated with these equations at various inputs of independent factors. However, estimating the mean response parameters by extrapolating the equations beyond the design space should be handled carefully. It is very much possible that a model that fits well within the region (defined space) of the original data will no longer fit well outside the region [31,32]. It is important to note that if particle size, %EE or % In vitro Release beyond design space is desired, then defined independent variables and/or levels used for the design space should be adjusted, including the usage of alternate lipids and surfactants. With new defined experimental variables and conditions; new design space can be defined and regression model can be achieved in the similar manner.

Numerical optimization was carried to maximize %EE with sustained % In vitro drug release, and minimize particle size of SLNs, within the studied design space. The overall desirability is based on the compromise between the aforementioned individual target goals. Solution 1 with lipid amount of 257.9 mg and surfactant concentration of 1.03% showed the highest overall desirability of 0.74 with predicted %EE of 33.63, particle size of 198.8 nm, and the % In vitro drug release of 56.6 %. Detailed desirability plot is shown in Figure 3.

Figure 2: Actual versus predicted plots for model generated from analysis of experimental design for (a) %EE, (b) particle size and (c) % In vitro release of Rst-loaded SLN.

Figure 3: Desirability plot for optimized Rst-loaded SLN
Optimized formulation obtained from numerical optimization was prepared to validate predicted results obtained from the quadratic model. The %EE of the R_opt formulation was 34.78 ± 0.37 (in comparison to 33.63%). The particle size of R_opt was 202.5 ± 9.29 (in comparison to predicted 198.8 nm) which is suitable for lymphatic absorption of SLNs by oral route. The PDI of R_opt was found to be 0.085, which indicates that the SLNs had narrow particle size range and homogenous distribution of the SLN [33]. Furthermore, the zeta potential of R opt was -13.85 ± 6.52 mV due to the presence of negatively charged stearic acid in the SLN. The SLN formulation with high negative surface charge are considered relatively stable as the Coulombic repulsion forces arising from their surface charge can overcome the Van der Waals attractive forces between them, which can consequently prevent aggregation on ageing. The drug release profile of R_opt showed that there was an initial burst release (approximately 30% of drug release within four hours) while the remaining drug was released in a controlled manner by sustaining the drug release up to 36 hours (drug release of 57.3 ± 2.6 % at 36 hours). The initial burst release of the drug could be attributed to the presence of adsorbed drug on the surface of the SLN, and sustained release of drug could be attributed to the increased diffusional distance of entrapped drug (in the SLN) as well as hindering effects by the surrounding solid lipid shell. Fitting of release profiles in release kinetics models revealed highest r² value of 0.9798 by fitting in Higuchi modes in comparison to lower r² values of 0.7626 and 0.8410 by fitting in zero and first order models, respectively. Fitting the data in Korsmeyer-peppas model resulted in r² of 0.9658 and n value of 0.45, also indicating the fickian mechanism.

### Ex-vivo Permeation Study

Ex-vivo permeation studies were performed on R_opt formulation and drug solution (control) using small intestine of male wistar rat. Rst-loaded SLN showed sustained permeation across the membrane in comparison to the drug solution (Figure 4).

![Figure 4: Ex-vivo permeation profile for optimized Rst-loaded SLN (R_opt) and drug solution (control).](image)

It has been reported that SLN mostly forms a matrix structure that releases the drug in the controlled fashion [11,34] In some cases, SLN can increase the solubilization capacity of the drug allowing maintaining increased concentration gradient for longer time. It is widely reported that SLNs are taken up by the M cells of Peyer’s patches in the gut and get absorbed by lymphatic absorption [15]. Based on this Rst-loaded SLN permeation profile, SLNs would be able to release the drug in controlled manner once taken up by lymphatic pathway. This would help to reduce the first pass metabolism of Rst and potentially improve oral bioavailability of the Rst.

### Accelerated Stability Studies

The stability studies were carried out as per ICH Q1A (R2) guidelines of the R_opt. The formulation was stored at 25° ± 2°C/60±5% RH for 3 months. The result of physical and chemical changes at the accelerated condition (25 ± 2°C/60±5% RH) is summarized in Table 5.

Table 5. Physical and chemical stability of R_opt under accelerated conditions (25 ± 2°C/60±5% RH)

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Redispersibility</th>
<th>Color</th>
<th>%EE</th>
<th>Total drug content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Yes</td>
<td>White</td>
<td>34.5</td>
<td>100</td>
</tr>
<tr>
<td>15</td>
<td>Yes</td>
<td>White</td>
<td>34.3</td>
<td>99.4</td>
</tr>
<tr>
<td>30</td>
<td>Yes</td>
<td>White</td>
<td>33.7</td>
<td>99.3</td>
</tr>
<tr>
<td>60</td>
<td>Yes</td>
<td>White</td>
<td>33.3</td>
<td>98.9</td>
</tr>
<tr>
<td>90</td>
<td>Yes</td>
<td>White</td>
<td>32.6</td>
<td>98.3</td>
</tr>
</tbody>
</table>

The result indicates that no significant physical or chemical change indicating that the R_opt was stable at accelerated conditions.

### Conclusion

This investigation utilizes quality-by-design approach to develop the Rst-loaded SLN. Based on the preliminary studies, design of experiment was used to quantify the extent of impact of lipid amount and surfactant concentration on the physicochemical properties of the SLN. It was observed that interplay of formulation variables have significant effect on the particle size, % EE and % In vitro release. The optimized formulation showed sustained drug permeation compared to the controls and was stable under accelerated condition. These findings indicate the feasibility of the SLN to successfully delivery Rst.

DOI: [http://dx.doi.org/10.21746/iijels.2017.5.1](http://dx.doi.org/10.21746/iijels.2017.5.1)
Acknowledgements
The authors acknowledge PDM College of pharmacy and Post Graduate Institute of Medical Sciences for providing research facilities to carry out this research.

Declaration of Interest
The authors declare no conflict of interest (monetary or otherwise) in conducting this research. The authors alone are responsible for the content and writing of the paper.

References


19. Jain S, Patel N, Madan P, Lin S. Quality by design approach for formulation, evaluation and statistical optimization of diclofenac-loaded ethosomes via transdermal route. Pharm Dev Technol [Internet].


Source of support: Nil
Conflict of interest: None Declared

DOI: http://dx.doi.org/10.21746/iijels.2017.5.1 2038