DEVELOPMENT AND PHARMACODYNAMIC EVALUATION OF ROSUVASTATIN-LOADED NANOSTRUCTURED LIPID CARRIERS FOR ORAL ADMINISTRATION

Rajshekhar Agarwal*, 1, H. Padmalatha Malthar2, CH. Madhumathi1 and B. Chaitanya Reddy1

1Department of Pharmaceutics, Vijaya College of Pharmacy, Munaganoor, Hyderabad-501511, R.R. Dist., India.
2Principal & HOD, Gyana Jyothi College of Pharmacy, Uppal, Hyderabad, India.

ABSTRACT

Rosuvastatin calcium (ROS) is the choice of anti-hyperlipidemic agent having 18-20% oral bioavailability because of its poor aqueous solubility (log P 5.7) followed by extensive first pass metabolism. The prime motivation for this work was to develop first time nanostructured lipid carriers (NLCs) to enhance oral bioavailability of ROS via targeting through intestinal lymphatic transport system. Amongst various lipids stearic (solid lipid) and oleic acid (liquid lipid) were screened to prepare five different batches of ROS loaded NLCs by employing hot homogenization technique. It was observed from obtained results, as content of oleic acid increased from 0 to 30 % there was decrease in particle size with increase in zeta potential, EE % and DL %. Optimized batch of ROS-NLCs (F4) showed excellent results for all evaluated parameters. In vitro dissolution study proven the biphasic release pattern of ROS-NLCs. Surface morphology of ROS-NLCs was assessed by scanning electron microscope which revealed non spherical shape of particles. Fourier transform infrared (FTIR) studies concluded that there were no interactions between ROS and the lipids used for study. Differential scanning calorimetry, powder X-ray diffractometry studies confirmed that ROS was entrapped in the NLCs and crystalline form converted to amorphous form. Triton induced hyperlipidemic model was used for examing in vivo pharmacodynamic activity of ROS-NLCs, results of which was found to be highly significant than plain ROS suspension, making NLCs as a promising perspective for oral delivery of ROS. Accelerated stability
studies showed that there was no significant change in the mean particle size, PDI and EE % after storage at 25 ± 2 °C / 60 ± 5 % RH for the period of three months.

**KEYWORDS:** Lipoidal nanoparticles, Intestinal lymphatic targeting, Stearic acid, Oral bioavailability, High pressure homogenisation, Triton induced hyperlipidemic model.

**1. INTRODUCTION**

In the present scenario, oral drug delivery is continuously looking into newer avenues and since the last two decade, the oral drug delivery has taken a new dimension with the increasing application of lipid as a carrier for the delivery of poorly water soluble, lipophilic drugs. The unique properties of lipids reported earlier made them very attractive candidates as carriers for oral formulations. The emerging field of lipid-based oral drug delivery systems is expected as promising carriers because of their potential to increase the solubility and improve oral bioavailability of poorly water-soluble, lipophilic drugs and has attracted considerable academic attention.

Gastrointestinal tract is richly supplied with blood and lymphatic vessels. Since rate of fluid flow in portal blood is about 500 fold higher than that in intestinal lymph, the majority of the dietary compounds are transported to portal blood. It is now well established that many lipophilic compounds are absorbed, to a certain extent, via the lymphatic route following oral administration. Various mechanisms of targeting drugs to intestinal lymphatics include paracellular mechanism, transport through M cells of Peyer patches and transcellular mechanism. Among these, transcellular mechanism is the most relevant for the transport of lipid carriers.

Solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs) are colloidal systems; NLCs are considered smarter, type of submicron particulate drug delivery system (approximately in the range of 10-1000 nm) and second generation of SLNs, produced from a blend of solid lipid with spatially incompatible liquid lipid. The resulting matrix of the lipid particles shows a melting point depression compared to the original solid lipid; however, the matrix remains solid at body temperature. In contrast, SLN being yielded from solid lipid or blends of solid lipids, the incorporation of liquid lipids to solid lipids (NLCs) leads to massive crystal order disturbance and yields NLCs. The resulting matrix shows great imperfections in the crystal lattice and leaves enough space to accommodate drug molecules, leading to improved drug loading capacity, preventing risk of gelation and avoiding drug
expulsion or leakage during storage caused by lipid polymorphism are major potential advantages of NLCs over SLNs\textsuperscript{[5]} while some common advantages are as high oral bioavailability, use of physiologically tolerated lipids exhibiting low systemic toxicity, large scale production, protection of drugs from degradation, avoidance of organic solvents, suitable for sterilization, controlled release characteristics and no problems with multiple routes of administration, such as oral, intravenous, pulmonary and transdermal administration.\textsuperscript{[6,7,8]} NLCs can be employed as an alternative to liposomes, microemulsions, self-emulsifying drug delivery systems, SLNs and polymeric nanoparticles.\textsuperscript{[3]} Due to the lipophilic nature of the matrix produced, NLCs are considered particularly useful for the oral administration of lipophilic drugs via intestinal lymphatic transport system.\textsuperscript{[9]}

Rosuvastatin calcium (ROS) is a selective and competitive inhibitor of hydroxyl methyl glutaryl-coenzyme A reductase (HMG-CoA reductase). HMG-CoA reductase catalyzes the reduction of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) to mevalonate, which is rate limiting step in hepatic cholesterol biosynthesis. Inhibition of the enzyme decreases \textit{de novo} cholesterol synthesis, increasing expression of low density lipoprotein receptors (LDL-receptors) on hepatocytes. This increases LDL uptake by the hepatocytes, decreasing the amount of LDL-cholesterol in the blood. It is used in the treatment of hyperlipidaemias, including hypercholesterolaemias and combined (mixed) hyperlipidaemia (type IIa or IIb hyperlipoproteinaemias), hypertriglyceridaemia (type IV), and dysbetalipoproteinaemia (type III). ROS can be effective as adjunctive therapy in patients with homozygous familial hypercholesterolaemia who have some LDL-receptor function. It is also used for primary and secondary prophylaxis of cardiovascular events in patients with multiple risk factors, including diabetes mellitus. ROS is rapidly absorbed from the gastrointestinal tract. However, the bioavailability of ROS is about 20 % due to extensive first pass metabolism in the liver, its primary site of action and it belongs to Biopharmaceutical Classification System (BCS) “Class II” drugs (i.e., low solubility and high permeability) having log $P$ value and mean plasma elimination half life of 5.7 and 14 h respectively. All above mentioned points makes ROS promising drug for formulation in to NLCs for enhancement of its oral bioavailability. An alternative parenteral route of administration could have provided greater bioavailability, however, till date for ROS not a single parenteral formulation is reported in pharmaceutical market. Delivering the existing drug molecules, by using advanced technology will be more preferred strategy so as to improve its therapeutic efficiency.
Literature survey revealed that ROS loaded NLCs (ROS-NLCs) have not been reported so far, so first time study was undertaken to formulate ROS-NLCs and targeting them via oral route to overcome its extensive first pass metabolism. They were prepared by high pressure homogenization technique because of its prominent advantages such as its simplicity, ability for production at industrial scale up, avoids the use of organic solvents and it also produces narrow and uniform size of nanoparticles.\[^7\] Furthermore it was also reported that high pressure homogenization is a more effective method for the production of submicron sized dispersions of solid lipids compared to high shear mixing or ultrasound and SLN dispersions produced by this method have low microparticle content.\[^10\] Stearic acid (solid lipid) and oleic acid (liquid lipid) were successfully applied to prepare NLCs with high pressure homogenization by\[^11\] and also ROS shown highest solubility in them, thus they were chosen for this study. Physicochemical characteristics of ROS-NLCs were investigated in detail. The role played by the oily component of the NLC was also judged by comparing the particle size, zeta potential, entrapment efficiency (EE %) and in vitro release study. The surface morphology of the ROS-NLCs was assessed by using scanning electron microscopy. The study was further continued to evaluate the pharmacodynamic activity of ROS-NLCs in male Albino Wistar rats.

2. MATERIALS AND METHODS

2.1. Materials

ROS was a generous gift from Shashun Pharmaceutical Industries Ltd., Pondicherry, India. Stearic acid and poloxamer 188 were procured from HiMedia Lab. Pvt. Ltd., Mumbai, India. Oleic acid was purchased from S.D. Finechem Ltd., Mumbai, India. Soya lecithin was received as a kind gift from Phospholipid GmbH, Germany. Methanol and other reagents used were of analytical grade. Water used in all the studies was double distilled and filtered through 0.22 µm nylon filter paper before use.

2.2. Screening of components (solubility studies)

Selection of components for development of ROS-NLCs was based on liquid lipid, solid lipid, and surfactant screening. The solubility of ROS was determined in different solid lipids, liquid lipids and surfactants.\[^12\]

An excess of ROS was added individually to liquid lipid, and surfactant listed in Table 1 (5 ml each) in screw capped tubes. After 24 h, each sample was centrifuged and 0.5 ml of the
clear supernatant layer was diluted suitably with methanol, and analyzed at 252 nm by UV-visible spectrophotometer (UV 1700, Shimadzu, Japan).

One of the most important factors that determine the loading capacity of the drug in the lipid is the solubility of drug in melted lipid. However, equilibrium solubility studies cannot be carried out in this case.

Table 1: Solubility of ROS in different liquid lipids and surfactants

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Solubility (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquid lipids</td>
<td></td>
</tr>
<tr>
<td>Oleic acid</td>
<td>29.23 ± 3.4</td>
</tr>
<tr>
<td>Castor oil</td>
<td>24.14 ± 2.5</td>
</tr>
<tr>
<td>Soya bean oil</td>
<td>19.06 ± 2.7</td>
</tr>
<tr>
<td>Surfactants</td>
<td></td>
</tr>
<tr>
<td>Poloxamer</td>
<td>284.74 ± 20.11</td>
</tr>
<tr>
<td>Tween 80</td>
<td>259.27 ± 11.34</td>
</tr>
<tr>
<td>Tween 60</td>
<td>243.45 ± 9.16</td>
</tr>
<tr>
<td>Tween 20</td>
<td>231.15 ± 18.96</td>
</tr>
</tbody>
</table>

Data represented as mean ± SD (n = 3).

Hence a modified method was used to select the solid lipid having better solubilization potential for ROS.\textsuperscript{[12]} For studying the solubility in solid lipids (Table 2), accurately weighed 40 mg of the ROS was taken in a test tube; the solid lipid was added in increments of 0.5 g, and the test tube was heated in a controlled temperature water bath kept at 80 °C till the clear melt was achieved. By this way the quantity of lipid required to solubilize 40 mg of ROS was estimated.

Table 2: Amount of solid lipid required to solubilize 50 mg of ROS.

<table>
<thead>
<tr>
<th>Solid lipid</th>
<th>Amount (mg)</th>
</tr>
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<tbody>
<tr>
<td>Stearic acid</td>
<td>73.5 ± 0.83</td>
</tr>
<tr>
<td>GMS</td>
<td>118.9 ± 0.39</td>
</tr>
<tr>
<td>Precirol ATO 5</td>
<td>136 ± 0.48</td>
</tr>
<tr>
<td>Bees wax</td>
<td>184 ± 1.56</td>
</tr>
</tbody>
</table>

Data represented as mean ± SD (n = 3)

2.3. Preparation of ROS–loaded NLCs

ROS–loaded NLCs were prepared by hot high pressure homogenization technique according to previously reported method.\textsuperscript{[10,22]} All the five formulations as shown in Table 3 were prepared.
Stearic acid (F1) or mixture of stearic acid with oleic acid (F2 to F5) were prepared by adding oleic acid to stearic acid (Table 3) and then heated together in water bath at 80 °C to form a uniform and clear oil phase, ROS was incorporated in it. Meanwhile, the aqueous phase was also prepared by dispersing Poloxamer 188 and soya lecithin in double distilled water and heated to the above temperature \(^{(11)}\). Then the hot aqueous phase was added drop wise to the lipid phase maintained at 80 °C under magnetic stirring (Remi Instruments Ltd., Mumbai, India) at 600 rpm to form pre-emulsion. Then the final ROS-NLC dispersion was produced by passing this hot pre-emulsion for four homogenisation cycles through a high pressure homogenizer (Panda 2K, Niro Soavi, Italy) at pressure of 500 bar and temperature of about 80 °C. Subsequently the dispersion was cooled at room temperature to solidify ROS-NLCs. The formulations were stored at 4 °C.

### Table 3: Composition of different ROS loaded NLCs formulations (% , w/v).

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Total lipid (g)</th>
<th>Ratio of liquid lipid to solid lipid</th>
<th>Surfactant</th>
<th>Co-surfactant</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>2</td>
<td>0:100</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>F2</td>
<td>2</td>
<td>5:95</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>F3</td>
<td>2</td>
<td>15:85</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>F4</td>
<td>2</td>
<td>30:70</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>F5</td>
<td>2</td>
<td>45:55</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

In each formulation ROS 40 mg/g was used.

#### 2.3.1. Determination of particle size, PDI and zeta potential of the ROS-NLCs

Mean particle size of ROS-NLCs and PDI as a measure of the width of particle size distribution was determined by photon correlation spectroscopy (PCS) using a Zetasizer (Nano ZS 90, Malvern Instruments, UK). ROS-NLC formulations were diluted with double distilled water to get optimum 50–200 kilo counts per second (kcps) for measurements. Based on the Smoluchowski equation, the surface charge of the NLCs was determined by measuring the zeta potential of NLCs using the same equipment. Zeta potential measurements were run at 25°C with electric field strength of 23 V/m.\(^{[13]}\)

#### 2.3.2. Determination of percent entrapment efficiency (EE %) and drug loading capacity (DL %) of ROS-NLCs

Percent EE of ROS-NLCs was calculated by measuring the concentration of the unentrapped or free drug in suspension.\(^{[14]}\) About 2 ml of the dispersion was placed in the Eppendorf tubes and centrifuged at 15,000 rpm for 45 minutes at 4 °C (Remi Instruments Ltd., Mumbai,
India). The amount of ROS in the aqueous phase was estimated spectrophotometrically at \( \lambda_{\text{max}} \) 252 nm. The EE % and DL % of all formulated batches were calculated using the following Eqs. (1) and (2), respectively.

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

\[
\text{DL} (\%) = \frac{W_{\text{Total}} - W_{\text{Free}}}{W_{\text{Lipid}}} \times 100 \quad (2)
\]

Where \( W_{\text{Total}}, W_{\text{Free}} \) and \( W_{\text{Lipid}} \) were the weight of total drug in NLC, the weight of free drug in aqueous phase and the weight of lipid used in system, respectively.

2.4. Physicochemical characterization of ROS-NLCs

2.4.1. Fourier transform infrared spectroscopy (FTIR) studies

Fourier transform infrared (FTIR) spectra of pure ROS, stearic acid, physical mixture of ROS and stearic acid and optimized ROS-NLC formulation were obtained on a FTIR (8400S, Shimadzu, Japan) using the KBr disk method (2 mg of sample in 200 mg of KBr). The scanning range was 400–4000 cm\(^{-1}\).

2.4.2. Differential scanning calorimetry (DSC) studies

DSC analysis was performed of pure ROS, bulk stearic acid, physical mixture of ROS and stearic acid in 1:1 ratio and freeze dried ROS-NLCs of the optimised batch using differential scanning calorimeter (DSC 1 STAR\(\text{®} \) System, Mettler-Toledo, Greifensee, Switzerland). Accurately weighed 2 mg of each sample was placed in aluminum pans and sealed with a lid. In the scanning process, a heating rate of 10 \( ^{\circ} \text{C}/\text{min} \) was applied in the temperature range from 35 to 300 \( ^{\circ} \text{C} \) (for stearic acid because of its low melting point temperature range was kept from 35 to 100 \( ^{\circ} \text{C} \)) with a nitrogen flow of 5 ml/min.

2.4.3. Powder X-ray diffraction studies (PXRD)

Powder X-ray diffraction (PXRD) was performed to analyze crystalline or amorphous nature of ROS loaded NLCs. PXRD studies were performed by powder X-ray diffractometer (Brucker Axs, D8 Advance, Germany) using Cu-K\(\alpha \) line as a source of radiation. The samples were scanned over a 2\( \theta \) range of 10–600 at a scan rate of 3\(^{\circ} \text{min}/\text{min}. \) Samples used for study were pure ROS, stearic acid, physical mixture of ROS and stearic acid and lyophilized ROS–NLCs.
2.4.4. Scanning electron microscopy (SEM)

The surface morphology of NLCs was visualized by scanning electron microscopy (LEO 440i, Leo Electron Microscopy Ltd., Cambridge, UK). The pure ROS and optimized freeze dried ROS-NLC formulation was kept onto metal plate and dried under vacuum to form a dry film which was then observed under the scanning electron microscope.

2.5. Determination of *in vitro* drug release from ROS NLCs

The *in vitro* drug release from ROS-NLC was carried out in phosphate buffer saline (PBS) solution (pH 6.8) by using the dialysis bag diffusion technique. As reported earlier,[15] dialysis bag (molecular weight cut off 12–14 kDa) was equilibrated in PBS medium for 12 h prior to study. Two ml of NLCs dispersion was poured into the dialysis bag and tightly sealed at both ends. Then it was placed in a beaker containing 200 ml PBS media at 37 ± 2 °C and magnetically stirred at 50 rpm. At predetermined time intervals of 60 min for 14 h, 5 ml of samples were withdrawn by filtration through 0.22 µm filter (Millipore, USA) and sink condition was maintained by replacing with fresh pre-warmed PBS solution of same temperature. The filtrate was suitably diluted if necessary and analyzed by using UV spectrophotometer at λmax 252 nm.

2.6. *In vivo* pharmacodynamic studies

The animal experiment was carried out in full compliance with the protocol approved by institutional animal ethical committee (Registration no. IAEC/1230/a/08 under the (CPCSEA) Committee for the Purpose of Control and Supervision of Experiments on Animals, India). The *in vivo* study was carried out according to a previously reported method (16,17). Male Wistar rats, weighing 150–200 g, were divided into four groups such as control, test, standard and normal group, each containing six rats. The rats were fasted overnight and then (except normal group) intraperitoneally injected with 250 mg/kg of Triton WR 1339 (isoctyl-polyoxyethylene phenol) (tyloxapol; Sigma Chemical Co, St Louis, MO, US) dissolved in 0.9% saline. Control group of rats were given with vehicle (saline solution containing high fat diet), test groups of rats were treated with optimized ROS-NLCs formulation (equivalent to 25 mg/kg ROS), standard groups of rats were treated with plain ROS (25 mg/kg bodyweight) and normal groups of rat were given with normal diet. Blood samples were collected in Eppendorf tubes at 18 and 24 h post oral dose. Serum was separated by centrifugation (Remi Instruments Ltd., Mumbai, India) at 16770 g/min and used for biochemical analysis. Statistical analysis of the collected data was performed using non
parametric one way analysis of variance (Kruskal-Wallis test followed by Dunnett’s-test) to evaluate the individual differences between the treatment groups.

2.7. Stability testing
The purpose of stability study is to provide evidence on the quality of a drug substance or drug product which varies with time under the influence of a variety of environmental factors such as temperature, humidity & light. The final optimized ROS-NLC batch (F4) was lyophilized and utilized for carrying out accelerated stability studies according to International Conference on Harmonization (ICH) Q1A (R2) guidelines and previously reported method (3). Accelerated stability study was performed with the prime aim to assess the stability of ROS-NLCs at 25 ± 2°C/60 ± 5% RH (relative humidity) with respect to particle size, PDI and EE. Freshly prepared freeze dried powder of ROS-NLCs was filled in 3 different amber colored glass vials, sealed and placed in stability chamber (CHM-10S, Remi Instruments Ltd., Mumbai, India) maintained at 25 ± 2°C/60 ± 5% RH for a period of total 3 months. The dried powder samples subjected for stability test were re-dispersed in double distilled water and analyzed with a sampling interval of 1 month for particle size, PDI and EE of the ROS-NLCs over 3 months period.

3. RESULTS AND DISCUSSION

3.1. Screening of components (solubility study)
Among the liquid lipids that were screened (Table 1), maximum solubility of ROS was found in oleic acid followed by castor oil and soya bean oil. Among the surfactants (Table 1), poloxamer 188 proved to be the best followed by Tween 80, Tween 60 and Tween 20. Thus the saturation solubility studies helped to streamline the choice of liquid lipid and surfactant. Of the solid lipids screened, ROS showed maximum solubility in stearic acid as compared to GMS, precirol ATO 5 and bees wax (Table 2). Thus stearic acid, oleic acid and poloxamer 188 as the solid lipid, liquid lipid and surfactant respectively were chosen for this study.

3.2. Preparation and characterization of ROS loaded-NLCs
Five different batches of ROS-NLCs were successfully developed by hot high pressure homogenization technique. The homogenizer pressure was optimised to 500 bar for 4 cycles. Indeed, it has been reported by that lipid nanoparticles stabilized with surfactant mixtures such as Poloxamer 188 and soya phospholipid have lower particle size and higher storage stability compared to formulations prepared with alone surfactant (10,18). The mixture of a lipophilic (soya lecithin) and hydrophilic (Poloxamer 188) surfactants in a molar ratio (1:1)
was helpful in promoting the stabilization of the system hence in the development of ROS-NLCs the same combination with same concentration was selected (19). Results clearly suggested that 1% of Poloxamer was sufficient to cover the surface of nanoparticles and thereby preventing agglomeration during the homogenization process. The mean particle size, PDI, zeta potential, EE % and DL % of the resulted ROS-NLCs are tabulated in Table 4. The formulated ROS loaded NLCs showed decrease in mean particle size with increase in EE % and DL % with an increase in oleic acid content from 0 up to 30% while beyond this level there was slight decrease in EE %, DL % and also in mean particle size (F5). These results were in consistent with the previous studies reported by Agrawal et al. (6). This might be due to incorporation of liquid lipids in to solid lipids, which leads to massive crystal order disturbance, and the resulting matrix of lipid particles indicates great imperfections in the crystal lattice and leaves enough space to accommodate drug molecules, thus, leading to improved DL % and EE %. A similar kind of results was reported by Jenning et al., 2001 and Souto et al., 2004(19, 20). The EE % is mainly depend on the nature of the drug and lipids. As ROS is a lipophilic drug (Log P 5.7) and has a greater solubility in the lipids used (conclusion drawn from solubility study), the EE obviously was found to be high. Thus, it might ultimately decrease the dose of ROS in the formulation and attain higher plasma concentration through the lymphatic transport system by avoiding first pass metabolism.

If we compare on the basis of composition (Table 3) F1 can be called as SLNs while F2 to F5 were NLCs. Once again it was proved that NLCs were performed best than SLNs.

Table 4: The mean particle size, PDI, zeta potential, EE % and DL % of ROS–NLCs formulations.

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Mean particle size (nm)</th>
<th>PDI</th>
<th>Zeta potential (mV)</th>
<th>EE %</th>
<th>DL %</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>372.8 ± 12.5*</td>
<td>0.278 ± 0.034</td>
<td>-7.48 ± 1.37</td>
<td>46.67 ± 1.59</td>
<td>11.5 ± 0.40</td>
</tr>
<tr>
<td>F2</td>
<td>289.0 ± 8.3*</td>
<td>0.357 ± 0.058</td>
<td>-12.5 ± 2.32</td>
<td>61.22 ± 1.53</td>
<td>15.3 ± 0.35</td>
</tr>
<tr>
<td>F3</td>
<td>221.6 ± 10.1***</td>
<td>0.221 ± 0.064</td>
<td>-27.6 ± 3.72</td>
<td>67.53 ± 2.31</td>
<td>16.88 ± 0.14</td>
</tr>
<tr>
<td>F4</td>
<td>147.8 ± 7.4***</td>
<td>0.211 ± 0.050</td>
<td>-42.5 ± 3.64</td>
<td>76.34 ± 0.58</td>
<td>19.08 ± 0.22</td>
</tr>
<tr>
<td>F5</td>
<td>152.21 ± 8.2**</td>
<td>0.363 ± 0.062</td>
<td>-40.1 ± 4.25</td>
<td>75.47 ± 1.73</td>
<td>18.12 ± 0.30</td>
</tr>
</tbody>
</table>

Data represented as mean ± SD (n = 3).

*P > 0.05: no significant difference between 0, 5 and 15 wt% OA incorporated nanoparticles using Student’s t-test.

**P > 0.05: no significant difference between 30 and 45 wt% OA incorporated nanoparticles using Student’s t-test.
***$P < 0.05$: significant difference between 15 and 30 wt% OA incorporated nanoparticles using Student’s t–test.

3.3. Fourier transform infrared (FTIR) spectroscopy

Results of FTIR study showed characteristic peaks of ROS between 3700 and 3000 cm$^{-1}$, specifically at 3670, 3363, 3055, 1728 cm$^{-1}$ in Figure 1. A. The peak at 3670 cm$^{-1}$ indicated free O-H stretching, other peaks at 3363 cm$^{-1}$ (N-H stretching), 3055 cm$^{-1}$ (symmetrical O-H stretching) were seen in the ROS spectra. All the above characteristic peaks of ROS appeared in the spectra of the physical mixture and ROS-NLCs at the same wave number indicating no interaction between the drug and lipids. The intensity of characteristic peaks of ROS was found to be reduced in the physical mixture and ROS-NLCs as seen in Figure 1. C and D which was mainly due to the molecular dispersion of crystalline ROS in stearic acid.

![Figure 1: FTIR spectra of A) pure ROS, B) stearic acid, C) physical mixture of ROS and stearic acid, D) ROS loaded NLCs](image)

3.4. Differential scanning calorimetry

The DSC thermograms of bulk ROS, physical mixture of ROS and stearic acid, bulk stearic acid, and lyophilized ROS-NLCs are depicted in Figure 2. ROS showed a characteristic endothermic peak at 185.15 °C and the DSC thermogram of the stearic acid showed endothermic peak at 53.36 °C. The lyophilized ROS-NLCs showed a small endothermic peak around 167.83 °C, which indicates the conversion of crystalline form of the drug to the amorphous form i.e., complete entrapment of ROS in the lipid matrix. It was also concluded that ROS is molecularly dispersed in lipid matrix, indicating its reduction in crystallinity as the peak intensity of the drug was found to be reduced as shown in Fig. 2.
3.5. Powder X-ray diffraction studies

X-ray diffractograms of pure ROS, stearic acid, physical mixture of ROS and stearic acid and freeze dried ROS-NLCs are presented in Fig. 3. The X-ray diffractogram of ROS has sharp peaks at diffraction angles (2θ) 6.24°, 9.26°, 10.36°, 11.92°, 15.38°, 17.10°, 19.54°, 21.70°, 22.75° and 23.38° showing a typical crystalline pattern. All major characteristic crystalline peaks appear in the diffractogram of ROS-NLCs and physical mixture, but with low intensity. This indicates that the drug was converted crystalline to amorphous form. DSC studies already supported the same hypothesis, which was further confirmed by X-ray diffractometry.

![DSC thermogram of Pure ROS, physical mixture of stearic acid and ROS, stearic acid, Freeze dried ROS-NLCs](image)

**Figure 2:** DSC thermogram of Pure ROS, physical mixture of stearic acid and ROS, stearic acid, Freeze dried ROS-NLCs

![PXRD diffractogram](image)

**Figure 3:** PXRD diffractogram of A) pure ROS, B) stearic acid, C) physical mixture of ROS and stearic acid, D) Freeze dried ROS-NLCs.
3.6. Scanning electron microscopy

Scanning electron photomicrographs showed long platy crystals (Fig. 4 A), where as the ROS NLCs showed a smooth surface in which drug and lipid get completely fused to form a uniform molecular component indicating conversion of crystalline to amorphous form (Fig. 4 B)

![Scanning electron micrographs](image)

Figure 4: Scanning electron micrographs of A) pure ROS, B) optimized lyophilized batch (F4) of ROS-NLCs.

3.7. In vitro drug release study

The drug release data obtained for formulations F1, F2, F3, F4 and F5 is depicted in Fig. 5. The in vitro drug release of all the five batches of ROS-NLCs showed an interesting biphasic release with an initial burst effect followed by controlled release. However, during the production of lipid particles, surfactants were also incorporated into the lipid phase which could affects over the drug release, such as initial burst (act as loading dose) release of ROS \(^{(20,21)}\). The second supportive reason for the burst release in the first hour can be attributed to the drug absorbed over the surface of NLCs. The third supportive reason for the burst release is reported by Hu et al., 2005. He reported that if NLCs are prepared with hot high pressure homogenization (i.e. at high pressure, temperature and high concentration of surfactant), it is believed that these NLCs will produce burst release \(^{(11,22)}\). And thus at the end of first hour, drug release was high 26.11, 21.98, 26.4, 32.77 and 28.71 % for batch F1, F2, F3, F4 and F5 respectively. Afterwards the drug release followed a steady pattern of controlled release (act as maintenance dose) for the next 13 h.
3.8. In vivo Pharmacodynamic studies

The effect of treatments on serum lipid levels after Triton treatment in phase I and II (at the end of 18 and 24 h) are shown in Table 5. Plain ROS (in standard group) in phase I produced a fall in serum triglyceride, cholesterol, LDL and rise in protective HDL levels as compared to control group. The ROS loaded-NLCs (in test group), as expected, performed better than plain ROS and found significant ($p < 0.05$) reduction in serum triglyceride, cholesterol, LDL and rise in protective HDL levels as compared to standard group. It has been reported that there is a natural tapering in cholesterol and triglyceride values in phase II of the Triton test\(^{(16,17)}\). However, this normal clearance of serum lipid in phase II of the Triton test can also be triggered by the presence of ROS in the circulation, as ROS has long biological half life of 14 h thus a longer duration of action is guaranteed provided. In phase II of the Triton test, as seen from Table 5, plain ROS (in standard group) again lowered serum triglyceride, cholesterol, LDL and rise in protective HDL levels as compared to control group while ROS loaded-NLCs (in test group), again performed better than plain ROS and found significant ($p < 0.05$) reduction in serum triglyceride, cholesterol, LDL and rise in protective HDL levels as compared to standard group. Thus, the greater lipid lowering activity of the ROS loaded-NLCs in both phases I and II of the Triton test might be the probable reason for faster dissolution of ROS and observed differences in pharmacodynamic activity of ROS-NLCs.
could suggest enhancement of solubility and thereby oral bioavailability. Earlier low bioavailability of ROS was attributed because of its poor aqueous solubility.

**Table 5: Pharmacodynamic evaluation (in vivo) of optimized batch (F4) of ROS loaded-NLCs**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Triglyceride (mg/dl)</th>
<th>Cholesterol (mg/dl)</th>
<th>HDL (mg/dl)</th>
<th>LDL (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase I (18 h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>254.9 ± 4.42</td>
<td>329.2 ± 6.45</td>
<td>44.59 ± 1.29</td>
<td>183.63 ± 5.62</td>
</tr>
<tr>
<td>Test</td>
<td>123.53 ± 7.14</td>
<td>212.1 ± 8.60</td>
<td>73.39 ± 3.97</td>
<td>120.80 ± 8.63</td>
</tr>
<tr>
<td>Standard</td>
<td>157.79 ± 4.82*</td>
<td>266.3 ± 7.19*</td>
<td>66.82 ± 8.16*</td>
<td>161.32 ± 8.39*</td>
</tr>
<tr>
<td>Normal</td>
<td>90.17 ± 8.14</td>
<td>151.9 ± 8.37</td>
<td>62.33 ± 1.03</td>
<td>71.54 ± 8.03</td>
</tr>
<tr>
<td>Phase II (24 h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>310 ± 10.51</td>
<td>449.34 ± 5.64</td>
<td>32.17 ± 1.26</td>
<td>335.17 ± 4.63</td>
</tr>
<tr>
<td>Test</td>
<td>223.8 ± 3.05</td>
<td>314.9 ± 9.19</td>
<td>67.54 ± 1.79</td>
<td>202.62 ± 9.59</td>
</tr>
<tr>
<td>Standard</td>
<td>264.9 ± 7.28*</td>
<td>368.5 ± 5.48*</td>
<td>48.68 ± 2.60*</td>
<td>266.84 ± 4.89*</td>
</tr>
<tr>
<td>Normal</td>
<td>194 ± 1.44</td>
<td>121.5 ± 3.78</td>
<td>54.43 ± 2.36</td>
<td>148.27 ± 3.32</td>
</tr>
</tbody>
</table>

Data represented as mean ± SD (n = 6). * indicates significant test

**3.9. Stability studies**

Accelerated stability studies were carried out on optimized ROS-NLCs (F4) by considering particle size, zeta potential and EE % as the prime parameters for 3 months. There was a slight increase in the particle size while slight decrease in zeta potential and EE % with time as reported in Table 6. Results of EE % revealed that even up to 3 months storage there was no more drug expulsion from ROS-NLCs. Thus, they were found to be stable at 25 ± 2°C/60 ± 5 % RH for a total period of 3 months.

**Table 6: Stability study data of optimized batch (F4) of ROS loaded NLCs.**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>0 month</th>
<th>Test period</th>
<th>2 months</th>
<th>3 months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 months</td>
<td>2 months</td>
<td>3 months</td>
</tr>
<tr>
<td>Particle size</td>
<td>147.80 ± 2.6</td>
<td>153.45 ± 3.56</td>
<td>156.44 ± 1.44</td>
<td>157.98 ± 3.85</td>
</tr>
<tr>
<td>Zeta potential</td>
<td>−42.5 ± 4.69</td>
<td>−40.14 ± 3.11</td>
<td>−39.65 ± 2.86</td>
<td>−38.29 ± 2.54</td>
</tr>
<tr>
<td>% EE</td>
<td>76.34 ± 0.58</td>
<td>75.84 ± 1.94</td>
<td>75.13 ± 2.67</td>
<td>73.96 ± 1.54</td>
</tr>
</tbody>
</table>

**CONCLUSIONS**

In the present study, ROS-NLCs for oral administration were successfully prepared by hot high pressure homogenization method and targeted via intestinal lymphatic transport system. The prepared ROS–NLCs found to be narrow and homogeneous in particle size with high EE %. *In vitro* drug release study of ROS-NLCs indicated biphasic release pattern of initial burst release followed by controlled release profile of ROS for desired time period. *In vivo*...
pharmacodynamic study showed significant ($p < 0.05$) reduction in serum triglyceride, cholesterol, LDL and rise in protective HDL levels for both phases as compared to standard group in Albino Wistar rats after oral administration. After comparison of both SLN and NLCs of ROS, ROS-NLCs showed better result. The NLCs could improve the gastrointestinal absorption of ROS and which could be the probable reason for remarkable increase in solubility, dissolution rate and there by enhancement in oral bioavailability of ROS. The explored nanostructured lipid carriers offered a potential and promising approach in improving oral delivery of poorly water soluble drug like ROS. Further studies are needed to prove the efficacy in clinical situation.

**Conflict of interest**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

**REFERENCES**


