Nanostructured lipid carriers for oral bioavailability enhancement of raloxifene: Design and in vivo study

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ABSTRACT

The objective of present work was to utilize potential of nanostructured lipid carriers (NLCs) for improvement in oral bioavailability of raloxifene hydrochloride (RLX). RLX loaded NLCs were prepared by solvent diffusion method using glyceryl monostearate and Capmul MCM C8 as solid lipid and liquid lipid, respectively. A full 3² factorial design was utilized to study the effect of two independent parameters namely solid lipid to liquid lipid ratio and concentration of stabilizer on the entrapment efficiency of prepared NLCs. The statistical evaluation
Keywords:
Poor solubility
Lipid carrier
Bioavailability
Pharmacokinetic parameters
Transmission electron microscopy
Amorphous nature

Introduction

The oral route is the most imperative route for administering varieties of drugs. It has been extensively used for both conventional and novel drug delivery systems. In spite of the wide success with some other routes for drug administration, the oral route is still most preferred route for its vast qualities.

Raloxifene hydrochloride (RLX) is a selective estrogen receptor modulator (SERM) with a proven estrogen agonist action on bone that leads to an improvement in bone mass [1] and a reduction in vertebral fractures [2]. RLX is poorly soluble drug as it belongs to class II category according to BCS classification. RLX has oral bioavailability of only 2% owing to extensive first pass metabolism. Therefore, it is necessary to increase the solubility and dissolution rate of RLX which lead to improvement in oral bioavailability [3].

Enhancement in oral bioavailability can be achieved by reducing the hepatic first pass metabolism. Such problem with conventional dosage form can be minimized by any suitable novel drug delivery system such as prodrug concept or by the use of novel lipid based system such as lipid nanoparticles, microemulsion [4] and Self emulsifying microemulsion drug delivery system [5].

Since last decade, various techniques have been studied to formulate nanoparticulate carrier systems [6]. Polymeric and solid lipid nanoparticles (SLNs) are two varieties of such nano carrier systems. Polymeric nanoparticles suffered with some drawbacks such as toxicity and unavailability of some good techniques for production of nanoparticles at large scale. Compared to polymeric nanoparticles, SLNs gain some advantages in terms of less toxicological risk because of natural origin lipids. Despite SLNs being good carriers, less capacity of drug loading and expulsion of the drug during storage may require to think of some good technique to overcome such problems. As an effect, nanostructured lipid carriers (NLCs) have been developed, which in some extent can avoid the aforementioned limitations. NLCs can be defined as a second generation of SLNs having solid lipid and liquid lipid (oil) matrix that create a less ordered or imperfect structure which helps in improving drug loading and decreasing the drug expulsion from the matrix during storage period [7,8]. In the present work, RLX loaded NLCs were developed by solvent diffusion method as this method has remarkable advantages such as use of simple equipment accessories, easiness in handling and quick manufacturing [9].

The aim of present research work was to develop stable RLX loaded NLCs formulation using solvent diffusion method and to evaluate in vitro characteristics and in vivo pharmacokinetic parameters of prepared formulation.

Material and methods

Materials

RLX was gifted from Aarti drugs Pvt Ltd, Mumbai, India. Dynasen 114 (Trimyristin) and Dynasen 118 (Tristearin) were gifted from Cremer Oleo GmbH & Co. KG, Germany. Glycerol monostearate (GMS), Isopropyl myristate, oleic acid, polyvinyl alcohol (PVA) and stearic acid were purchased from Loba Chemie, Mumbai, India. Capmul MCM C8, Labrafil ICM 1944 CS and Labraflex CC were gifted from Abitec Corporation, Janesville, USA. All other reagents used in research work were of analytical grade.

Methods

Selection of solid lipid

Solid lipid was selected by checking the solubility of the drug in melted solid lipid by means of visible observation with the naked eyes under normal light [10–13]. Lipids used for this study were Dynasan 114, Dynasan 118, stearic acid and GMS. Weighed quantity of drug (50 mg) separately with various lipids (5 g each) was heated above the melting point of lipid in a temperature regulated water bath (Macro Scientific Work Pvt Ltd, Delhi, India) in 10 mL glass vials. After melting of lipid, the solubility of RLX in each lipid was observed visually under normal light [14,15].

Partition behavior of RLX in various solid lipids

Weighed quantity of drug (25 mg) was added into the blend of melted solid lipid (5 g) and hot water (5 g). Mixture was shaken on an isothermal orbital shaker (MSW-132, Macro Scientific Work Pvt Ltd, Delhi, India) at 70 ± 2.0 °C for 24 h to reach equilibrium followed by separation of aqueous phase through centrifugation at 5000 rpm for 5 min using cooling centrifuge (C-24 BL, Remi Instrument Pvt Ltd, Mumbai, India). Drug content was analyzed spectrophotometrically at 288 nm using UV visible spectrophotometer (UV-1800, Shimadzu, Japan) [13,16].

Selection of liquid lipid

Liquid lipid was selected based on the maximum solubility of the drug in different liquid lipids. Lipids used for this study
were Capmul MCM C8, Isopropyl myristate, oleic acid, Labrafil ILM 1944 CS and Lebrafec CC. Excess amount of drug was taken in stopper vials containing 5 g of liquid lipids and mixing was carried out on a vortex mixer for 10 min. Thereafter, vials were kept in an isothermal orbital shaker at 25 ± 2.0 °C for 24 h to reach equilibrium. Supernatant was separated by centrifugation at 5000 rpm for 15 min and analyzed spectroscopically at 289 nm [17–19].

**Formulation of RLX loaded NLCs**

**Design of the experiment**

A complete $3^2$ factorial design was utilized to study the effect of two independent variables namely solid lipid to liquid lipid concentration and stabilizer concentration on entrapment efficiency of drug in prepared formulations. Variables and levels used for optimization of RLX loaded NLCs are shown in Table 1. Based on preformulation studies discussed earlier GMS, Capmul MCM C8 and PVA were selected as solid lipid, liquid lipid and stabilizer, respectively.

**Preparation of NLCs**

NLCs loaded with RLX were developed using solvent diffusion method in aqueous system with some modification [20]. Drug (5% w/w to the total weight of drug and lipids) and Capmul MCM C8 were mixed in a 10 mL solvent mixture of ethanol and acetone (1:1 v/v) followed by bath sonication (SW-4, Toshniwal Instruments Pvt Ltd, Ajmer, India) for 10 min [13]. The obtained mixture was kept on a water bath maintained at 70 °C followed by addition of GMS to make clear solution of lipids and drug in organic solvent system. The resultant organic mixture was hastily added into 100 mL of an aqueous phase comprising of PVA as stabilizer kept on water bath maintained at 70 °C under mechanical agitation of 500 rpm for 10 min using mechanical stirrer (RQ-121/D, Remi Instrument Pvt Ltd, Mumbai, India). The obtained RLX loaded NLCs dispersion was cooled at room temperature for 20 min on magnetic stirrer (SW-4, Toshniwal Instruments Pvt Ltd, Ajmer, India) for 10 min [13]. The obtained mixture was kept on a water bath maintained at 60 °C followed by addition of GMS to make clear solution of lipids and drug in organic solvent system. The resultant organic mixture was hastily added into 100 mL of an aqueous phase comprising of PVA as stabilizer kept on water bath maintained at 70 °C under mechanical agitation of 500 rpm for 10 min using mechanical stirrer (RQ-121/D, Remi Instrument Pvt Ltd, Mumbai, India). The obtained RLX loaded NLCs dispersion was cooled at room temperature for 20 min on magnetic stirrer for the liberation of organic solvent [20–23]. The prepared NLCs dispersion was transferred to centrifuge tubes equipped with cooling centrifuge and centrifugation was carried out for 17,000 rpm and 1 h at −10 °C [11,13,21] to separate precipitated NLCs. NLCs were collected and lyophilized using freeze dryer (MSW-137, Macro Scientific Work Pvt Ltd, Delhi, India). Composition of prepared NLCs formulations is shown in Table 2.

**Evaluation of RLX loaded NLCs**

**Percentage yield**

The percentage yield was determined by dividing the weight of recovered nanoparticles with the weight of drug and lipids used for the preparation of nanoparticles.

\[
\text{Percentage Yield} = \frac{\text{Weight of recovered nanoparticles}}{\text{Theoretical weight (drug + lipids)}} \times 100
\]

**Drug loading and entrapment efficiency**

The prepared NLCs dispersion was centrifuged by aforementioned experimental parameters. Supernatant was separated, diluted and determined for RLX content spectroscopically at 288 nm.

Entrapment efficiency of drug was calculated as follows [11,24]:

\[
\text{% Entrapment efficiency} = \frac{[\text{RLX}]_{\text{total}} - [\text{RLX}]_{\text{supernatant}}}{[\text{RLX}]_{\text{total}}} \times 100
\]

where “[RLX]_{total}” is the weight of total incorporated drug and the “[RLX]_{supernatant}” is the weight of free drug analyzed in supernatant layer.

Loading capacity of drug was calculated as follows [11,25]:

\[
\text{% Drug loading} = \frac{\text{Amount of RLX entrapped in NLCs}}{\text{Amount of RLX and lipids added}} \times 100
\]

**Optimization of formulation**

The optimization of prepared formulations was done by considering percentage drug entrapment and studying interaction between factors as discussed underneath.

**Interaction between the factors**

The statistical evaluation of all the obtained results data was carried out by analysis of variance (ANOVA) using Microsoft excel version 2007. The ANOVA results ($P$ value) showed the effect of various independent variables on dependent parameter such as percentage drug entrapment. After regression analysis of all formulations, full polynomial model was obtained followed by omission of non-significant terms ($P > 0.05$) to obtain reduced model for the analysis. This equation represents effect of independent formulation variables on entrapment efficiency.

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### Table 1 Variables and levels used in $3^2$ factorial design for RLX loaded NLCs.

<table>
<thead>
<tr>
<th>Factors</th>
<th>Levels</th>
<th>X₁</th>
<th>X₂</th>
</tr>
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<tbody>
<tr>
<td>–1</td>
<td>95:5</td>
<td>0.5</td>
<td>1.0</td>
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<tr>
<td>0</td>
<td>90:10</td>
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<td>1</td>
<td>85:15</td>
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\[X_1 = \text{Solid:liquid lipid ratio (}% w/w\text{)}, \ X_2 = \text{Concentration of stabilizer (}% w/v\text{).}\]
Construction of contour and response surface plots
Both plots were constructed from reduced polynomial equation using sigma plot version 11.0 by keeping one parameter stationary and varying others.

Evaluation of model/check point analysis
Checkpoint analysis was carried out to evaluate the dependability of the model through comparison between experimental and predicted values of the responses.

In vitro drug release studies
In vitro drug release of plain drug suspension and prepared NLCs was carried out using the dialysis sac method [10] (Himedia-Dialysis membrane 135, Mol. cut off 12,000–14,000 Da, Mumbai, India). An accurately measured amount of plain drug suspension and NLCs formulations equivalent to 5 mg of RLX were introduced into sac and both ends of the sac were tied with the help of thread. The sac was hanged with the assistance of thread in beaker comprising of 200 mL of Citro phosphate buffer pH 7.6 with 1% of polysorbate 80 kept on magnetic stirrer [10,26,27]. The temperature of the receptor compartment was maintained at 37 ± 1 °C. Aliquots of 5 mL were withdrawn at predefined time interval with a pipette and replaced with fresh buffer at each time. The filtered samples (0.45 µm membrane filter) were analyzed spectrophotometrically at 288 nm. Blank formulations were prepared and treated in same manner as discussed above. Blank formulations were taken for base correction by suitable dilution with buffer system in UV–Visible spectrophotometer to nullify any effect of ingredients used in formulation other than drug. Each test was carried out in triplicate.

Characterization of optimized RLX loaded NLCs
Fourier transform infrared (FTIR) spectroscopy
FTIR spectra were recorded by FTIR spectrometer (IRAffinity-1, Shimadzu, Japan) to study any interaction between drug and excipients. Samples were mixed with KBr in a ratio of 1:300 and spectrum was recorded in the range of 4000–400 cm⁻¹.

Characterization of particle size and zeta potential
The particle size and zeta potential of optimize formulation NLC-8 were measured by Malvern zeta sizer (Nano ZS, Malvern Instruments, Worcestershire, UK) after suitable dilution with distilled water.

Differential scanning calorimetry (DSC) analysis
Thermogram of samples was recorded by Differential scanning calorimeter (DSC TA – 60, Shimadzu, Japan). Samples were weighed directly in aluminum pan and scanned at 50–300 °C temperature under dry nitrogen atmosphere at the heating rate of 10 °C/min.

X-ray diffraction (XRD) study
XRD study of samples was performed by Panalytical Xpert PRO X-ray Diffractometer (Xpert Pro MPD, Panalytical, Netherlands) where CuKα radiation wavelength of 1.5405 Å was used as X-ray source. For the measurements, samples were kept in the glass sample holders followed by scanning from 2° to 60° with scan angular speed (2θ/min) of 2°/min, 40 kV working voltage and 30 mA current.

Surface morphology study
Surface morphology of optimized formulation NLC-8 was studied by Transmission Electron Microscope (TEM) (Philips Tecnai – 20, USA). NLCs were dispersed in distilled water and a drop of dispersion was placed on carbon coated copper grid followed by drying. This grid was mounted in the instrument and photographs were taken at various magnifications.

Stability study
Freeze-dried optimized formulation was subjected to stability studies as per ICH guidelines. The samples were placed in vials and kept at 25 ± 2 °C/60 ± 5% RH and 40 ± 2 °C/75 ± 5% RH atmospheric conditions using stability chamber (Macro scientific work Pvt Ltd, Delhi, India) over period of six months. The samples were analyzed for entrapment efficiency and physical appearance at specified time intervals (0, 15, 30, 60, 120 and 180 days of storage). Cumulative drug release study was also carried out at the end of stability study for both storage conditions.

In vivo pharmacokinetic study
To study the bioavailability of RLX, in vivo pharmacokinetic study was carried out for optimized formulation (NLC-8) and plain drug suspension as per the below discussed protocol.

Experimental animals
The experimental protocol in the present study was approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and the Institutional Animal Ethics Committee (IAEC) of SBKS medical college and research institute, Sumandeep Vidyapeeth, Vadodara, India with clearance No. SVU/DP/IAEC/2014/03/18. The experiment was carried out on healthy female Wistar rats with weight range 200 to 250 g [10]. Rats were kept in polypropylene cages, under standard situation (12 h light/dark cycle, 24 °C, 35–60% humidity) with free access to diet (Nav Maharashtra oil mills Ltd, Pune, India) and drinking water ad libitum [28,29].

Bioanalytical method
In the present work, chromatographic separation was carried out by previously validated chromatographic method [28] using HPLC (UFLC, Shimadzu Corporation, Japan) prominence liquid chromatographic system which is controlled by LC solution software (Version 1.24 Sp1, Shimadzu Corporation, Japan). The system was equipped with Binary pump (LC 20AD version 1.10, Shimadzu corporation, Japan), a manual injector, a column (C18 250 mm x 4.6 mm, 5 µm) (Luna, Phenominax, USA) and a photo diode array (PDA) detector (SPD 20A version 1.08, Shimadzu corporation, Japan). Freshly prepared, sonicated and filtered (0.45 µm membrane filter) mobile phase consisted of a 67% 0.05 M ammonium acetate buffer (pH was adjusted to 4.0 with glacial
acetic acid) and 33% acetonitrile was used at a flow rate of 1 mL min\(^{-1}\) to elute the drug [26,28,30]. The samples were injected at 20 μL volume and analyzed at 288 nm.

**Preparation of standard solution**

A series of standard solutions of RLX ranging from 20 to 1000 ng/mL were prepared in methanol. Samples were prepared by addition of 50 μL of standard solution and 200 μL of acetonitrile to the eppendorf tube containing 100 μL of blank plasma. The mixture was then processed according to the sample treatment procedure described below [28]. Final RLX concentrations in plasma were 10–500 ng/mL.

**Sample treatment procedure**

The eppendorf tube consisting of an aforementioned mixture was meticulously vortex-mixed (Macro Scientific Work Pvt Ltd, Delhi, India) for 30 s followed by centrifugation at 15,000 rpm for 10 min at \(-6^\circ C\) to separate denatured protein. After centrifugation, 20 μL of the filtered supernatant (0.45 μm membrane filter) was injected into HPLC system and analyzed at 288 nm [28,30].

**Experimental design**

Before dosing, the animals were fasted for the period of 12 h prior and 4 h post with free access to water. Animals were divided into two groups consisting of six animals in each. Control group received an suspension of RLX (drug suspended in 0.5% w/v sodium CMC [10,12]) and the test group received the optimized formulation (NLC-8) at a dose of 15 mg/kg body weight, p.o [26,28].

Serial blood samples (0.5 mL) were withdrawn through capillary inserted into retro-orbital plexus under mild ether anesthesia at a time interval of predose, 0.25, 1, 2, 4, 6, 8, 10, 12, 16, 20 and 24 h post dose as described in Table 3. The samples were transferred into micro centrifuge tubes containing anticoagulant (3.8% w/v sodium citrate [26]). The plasma samples were collected immediately from aforementioned samples by centrifugation at 5,000 rpm for 10 min at 4 °C and stored in micro centrifuge tubes at \(-20^\circ C\) until analysis [10,31]. Samples were analyzed by standard HPLC method after sample treatment procedure as discussed earlier.

**Pharmacokinetic data analysis**

PK solver add-in program for Microsoft excel (version 1.0, China) was used for the estimation of Pharmacokinetic parameters. The maximum plasma concentration (\(C_{\text{max}}\)) and the time to reach maximum plasma concentration (\(T_{\text{max}}\)) were obtained directly from the graph between plasma concentration and time. Area under curve [AUC]\(_{0-24}\) was considered up to last point of measurement. Relative bioavailability (\(F\)) was calculated by dividing [AUC]\(_{0-24}\) of formulation with plain drug suspension [12]. Each experiment was carried out in triplicate.

**Statistical analysis**

The obtained data were statistical analyzed by one way analysis of variance (ANOVA) using student’s \(t\)-test. Graph Pad Instat program version 3.01 (Graph Pad Software, Inc. CA, USA) was utilized to determine the significance difference between formulations. The level of statistically significance was selected as \(P < 0.05\).

**Results and discussion**

**Selection of solid lipid**

A selection of suitable lipids and other excipients is significant to develop NLCs for poorly soluble RLX. To keep the drug in solubilization form, it is of prime importance that drug has...
higher solubility in solid lipid. It was found from the study that drug solubility in Dynasan 114, Dynasan 118 and stearic acid was indistinct but found fairly visible in GMS.

**Partition behavior of RLX in various solid lipids**

Determination of partition behavior of drug in lipid is important criterion in controlling two parameters namely drug entrapment efficiency and drug release profile. Therefore, the success of development of NLCs is depending on selection of proper lipid for formulation of nanoparticles. From the result shown in Table 4, it was found that RLX had higher partitioning in GMS compared to other lipids. This finding also supported the high solubility of drug in GMS as discussed earlier. Therefore, GMS was chosen as solid lipid for development of NLCs owing to its high potential for solubilization of RLX and thereby entrapment of more amount of drug in NLCs formulation.

**Selection of liquid lipid**

As discussed earlier for solid lipid, a variety of short chain liquid lipids is also playing major role in entrapment of more amount of drug in case of NLCs formulation. It was found from the result that Capmul MCM C8 has maximum drug solubility. Furthermore, it was reported that Capmul MCM C8 from 5 to 15% w/w results in increased drug entrapment significantly (P < 0.05) compared to 5% w/w and 10% w/w liquid lipid concentration. The observed difference may be because of stabilizer concentration. It was noted from Fig. 1A that percentage yield of NLCs was increasing significantly with stabilizer concentration increased from 0.5% to 1% w/v (P < 0.05) but non-significant increment observed with concentration from 1% to 1.5% w/v. Hence, it can be conclude that formulation with optimum 1.0% w/v PVA concentration may achieve maximum nanoparticles yield with good stability.

The drug entrapment efficiency and loading capability of NLCs were remarkably increased from 30.83 ± 2.39 to 74.78 ± 3.34% and from 1.92 ± 0.12 to 4.02 ± 0.17%, respectively with increasing the proportion of Capmul MCM C8 from 5 to 15% w/w. Furthermore, it was reported that Capmul MCM C8 being a Mono glycerides of caprylic acid form unstructured matrix with many imperfections providing a space to incorporate more amount of drug [32–35]. As shown in Fig. 1B, it was observed that 15% w/w liquid lipid content in formulation improves drug entrapment significantly (P < 0.05) compared to 5% w/w and 10% w/w liquid lipid content. High proportion of liquid lipid may help in increasing drug solubility in lipid matrix followed by high entrapment efficiency.

**Evaluation of RLX loaded NLCs**

**Percentage yield, drug loading and entrapment efficiency**

The Percentage yield of NLCs formulations was found with significant differences ranging from 80.15 ± 3.54 to 93.85 ± 2.17%. The observed difference may be because of stabilizer concentration. It was noted from Fig. 1A that percentage yield of NLCs was increasing significantly with stabilizer concentration increased from 0.5% to 1% w/v (P < 0.05) but non-significant increment observed with concentration from 1% to 1.5% w/v. Hence, it can be conclude that formulation with optimum 1.0% w/v PVA concentration may achieve maximum nanoparticles yield with good stability.

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**Optimization of formulation**

**Interaction between the factors**

A 3² full factorial design was employed in optimizing the formula. The concentration of GMS: Capmul MCM C8 (X₁) and concentration of PVA solution (X₂) were taken as the independent variables and the entrapment efficiency as the dependent variable. The maximum percent entrapment (74.78%) was found at 1 level of X₁ and 0 level of X₂ as shown in Fig. 2A. The entrapment efficiency was obtained by conducting systematic experiments at various levels and was subjected to regression analysis to obtain a polynomial equation of the full model as follows:

\[
Y = 58.39 + 17.07X_1 + 5.21X_2 - 1.11X_1^2 - 6.52X_2^2 + 0.56X_1X_2
\]

Non-significant terms were rejected (P > 0.05) to obtain reduced model as follows:

\[
Y = 58.39 + 17.07X_1 + 5.21X_2 - 6.52X_2^2
\]

Based on the P value, X₁, X₂ and X₂² factors were found to be significant and all other factors were found to be insignificant. For the given model, calculated F value was found very low than the tabular F value (α = 0.05, 2) so it can be confirmed that the omitted terms do not significantly contribute.
to the prediction of the entrapment efficiency. High coefficient value of $X_1$ reveals that it can affect the maximum entrapment efficiency. However, at the same time the value of $X_2$ was also found to be significant. Therefore, the concentration of surfactant can also be considered as critical factor in formulation along with concentration of solid lipid to liquid lipid.

**Contour/response surface plots**

Contour and response surface plot were drawn at the selected values of the independent variables. The plots shown in Fig. 2B were found to be nonlinear and having curved segment for each prefixed values that signify nonlinear relationship between the selected variables.

**Check point analysis**

Check point analysis was performed to verify the effectiveness of established contour plot and reduced polynomial equation in development of drug loaded NLCs. The percent error for entrapment efficiency in the check point analysis was found to be very less between theoretical value and experimental value. This finding signifies the role of the reduced model, contour plots and the check point analysis in the mathematical

![Fig. 2](A) Contour plot and (B) 3D surface response plot for levels of solid lipid:liquid lipid and concentration of stabilizer with % entrapment of prepared NLCs.

![Fig. 3](Comparison of in-vitro drug release profile between NLCs and plain drug suspension.)
modeling. By studying full $3^2$ factorial design, it was notified that formulation NLC-8 showing maximum entrapment efficiency of $74.78 \pm 3.34\%$ and may be optimized for further characterization but that can be confirmed only after performing in vitro release study of all prepared NLCs formulations.

In vitro drug release

In vitro release profile of RLX from all NLCs formulations portrayed in Fig. 3 showed burst drug release for initial 8 h followed by slow and sustained release up to 36 h. However from the data, it was found that drug release profile of RLX was improving from formulations NLC-1 to NLC-9 as the concentration of liquid lipid in formulations increases. The formulation containing 15% w/w Capmul MCM (NLC-8) showed considerable improvement in release profile ($90.82 \pm 2.4\%$) compared to other NLCs formulations. Therefore, NLC-8 formulation was selected for further characterization based on its improved drug release profile and maximum drug entrapment efficiency optimized by $3^2$ factorial design. The optimized formulation (NLC-8) also showed significant enhancement ($P < 0.05$) in drug release profile compared with plain drug suspension as shown in Fig. 3.

Such type of drug release pattern in NLCs was most likely related to allotment of liquid lipid in nanoparticles. It was reported in earlier study [20] that when NLCs were prepared by solvent diffusion method at 70°C, liquid lipid was not allotted equivalently with solid lipid matrix. In such cases, more amounts of liquid lipid remain at the external shell of nanoparticles and very less liquid lipid incorporated into the center during cool process [22]. Therefore, the external part of particles becomes soft and exhibited significantly more solubility for hydrophobic drugs which imparts initial burst effect in release profile [36].

![Fourier transform infrared spectra](image-url)

**Fig. 4** Fourier transform infrared spectra of (A) RLX, (B) physical mixture of GMS and RLX, (C) physical mixture of Capmul MCM C8 and RLX, (D) physical mixture of RLX, GMS and Capmul MCM C8 (E) optimized batch NLC-8.
Various release kinetic models were fitted to determine release pattern of optimized formulation. The release kinetics of optimized formulation calculated by the regression analysis ($R^2$ value) had higher linearity for zero order and Higuchi model. Therefore, it can be concluded that optimized formulation NLC-8 follows zero order kinetics with diffusion controlled release mechanism as per Higuchi model.

Characterization of optimized RLX loaded NLCs

**FTIR spectroscopy**
As shown in Fig. 4E of NLC-8, it was observed that characteristic peaks of drug (Benzene ring), $1458.18\, cm^{-1}$ (–S– benzothiophene) and $1600.92\, cm^{-1}$ (–C–O–C– stretching) were found to be similar with pure drug spectra as shown in Fig. 4A. This reveals no physicochemical interaction between drug and excipients in NLCs formulation.

**Particle size and zeta potential**
The particle size of NLC-8 showed considerably smaller mean size of $32.50 \pm 5.12\, nm$ with less polydispersive index that represents narrow distribution of nanoparticles within the system. Zeta potential is necessary for analyzing stability of colloidal dispersion during storage. The zeta potential of optimized formulation was found to be $-12.8 \pm 3.2\, mV$, which imparts good stability of NLCs dispersion.

**Differential scanning calorimetry**
Thermogram of RLX and GMS showed endothermic peaks at $272.92\, ^{\circ}C$ and $62.89\, ^{\circ}C$ corresponding to their melting points as depicted in Fig. 5A and B, respectively. DSC plot of physical mixture shown in Fig. 5C showed sharp peaks at $272.18\, ^{\circ}C$ and $61.94\, ^{\circ}C$ representing melting points of drug and GMS, respectively. Thermogram of NLC-8 (Fig. 5D) showed endothermic peak at $63.42\, ^{\circ}C$ representing the melting point of GMS but the absence of endothermic peak within the melting range of RLX indicates either solubilization or conversion of drug from crystalline to amorphous form in the solid and liquid matrix.

**X-ray diffraction study**
The XRD study was carried out with support of DSC to verify the reduction in crystalline nature of RLX in prepared formulation. The XRD spectrums of drug in Fig. 6A and physical mixture in Fig. 6C showed distinct and intense peaks at 20 scale indicate crystalline nature of drug. In contrast, there was a considerable decline in intensity of all peaks in XRD pattern of NLC-8 as shown in Fig. 6D. Therefore, it can be revealed that RLX drug is completely in amorphous state in optimized NLCs formulation with solid lipid and liquid lipid.

**Surface morphology study**
TEM study showed the discrete NLCs particles with spherical shape and smooth surface as shown in Fig. 7. The spherical shape of NLCs has been reported in previous findings [22,37]. In addition, TEM image also confirms nano size

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**Fig. 5** Differential scanning calorimetry thermograms of (A) RLX, (B) GMS, (C) physical mixture of RLX, GMS and Capmul MCM C8 (D) optimized batch NLC-8.

**Fig. 6** X-ray diffraction patterns of (A) RLX, (B) GMS, (C) physical mixture of RLX, GMS and Capmul MCM C8 (D) optimized batch NLC-8.

**Fig. 7** Transmission electron microscopy image of optimized batch NLC-8. The magnifications are 65,000×.
(< 50 nm) of prepared NLCs that support the result obtained with particle size measurement by zetasizer.

**Stability study**

The result of stability study is depicted in Table 5. At the end of study, no change was observed in physical appearance of formulation in both stability conditions but significant reduction was notified in entrapment efficiency at accelerated condition. The release rate for the formulation kept at room condition was satisfactory but showed significant reduction ($P < 0.05$, data not shown) at accelerated conditions. The result shown for accelerated condition may attribute small degradation of drug at this condition which supports the fact that accelerated temperature is not a suitable storage condition for lipid based formulation. Therefore, it can be concluded that the room condition (25 $\pm$ 2°C/60% $\pm$ 5% RH) is a more favorable storage condition than the accelerated condition for NLCs formulation for a longer period of time.

**In vivo pharmacokinetic study**

RLX was found to be well separated under used HPLC conditions. Retention time of drug was found to be 5.346 ± 0.21 min. Standard curve of RLX for estimation in rat blood plasma showed linearity in the concentration range of 10–500 ng/mL with equation $Y = 21.22 X + 904.5$ and regression coefficient of 0.982 at $\lambda_{max}$ 288 nm.

The oral bioavailability of RLX is very much limited due to its poor water solubility and extensive first pass metabolism. Therefore, an attempt was made to improve bioavailability of RLX using the concept of novel drug delivery system. In the present work, plain drug suspension and optimized NLCs were administered orally to female Wistar rats for estimation of various pharmacokinetic parameters.

Fig. 8 illustrates the higher $C_{max}$ for NLC-8 formulation (207.63 ± 15.81 ng/mL) with respect to plain drug suspension (37.88 ± 3.99 ng/mL). The [AUC]$_{0-24}$ that denote the extent of absorption was found 3.75-fold significantly higher ($P < 0.05$) in NLC-8 formulation (1817.72 ± 81.42 ng h/mL) compared to plain drug suspension (484.83 ± 32.16 ng h/mL) as shown in Table 6. This significance increase in [AUC]$_{0-24}$ for NLCs may be due to its nano size and the avoidance of first pass metabolism through lymphatic transport pathway.

Many attempts have been made to improve the oral bioavailability of poorly soluble RLX by using conventional carriers such as solid dispersion and inclusion complex or by utilizing the potential of SLNs. However drug encapsulated in NLCs has proven more superior over the others as far as the oral bioavailability is concerned. In case of SLNs, they suffered with some issues of low drug loading capacity and

**Table 5** Stability study data for optimized formulation (NLC-8).

<table>
<thead>
<tr>
<th>Sr. no.</th>
<th>Time (days)</th>
<th>Physical appearance</th>
<th>Entrapment efficiency ± SD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>Yellow free flowing Powder</td>
<td>74.78 ± 3.34</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>74.71 ± 3.18</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>74.63 ± 2.10</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>60</td>
<td>73.88 ± 3.52</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>120</td>
<td>73.92 ± 2.47</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>180</td>
<td>73.85 ± 1.18*</td>
<td></td>
</tr>
</tbody>
</table>

Physical appearance | Entrapment efficiency ± SD (%)

| 1       | 0           | Yellow free flowing Powder | 74.78 ± 3.34                     |
| 2       | 15          | 74.44 ± 2.25               |
| 3       | 30          | 74.07 ± 2.44               |
| 4       | 60          | 73.11 ± 0.85               |
| 5       | 120         | 71.81 ± 1.64               |
| 6       | 180         | 70.05 ± 1.26**             |

Value are expressed as mean ± SD; $n = 3$.

* $P > 0.05$.

**Table 6** Comparative study of the pharmacokinetic parameters of optimized batch NLC-8 and plain drug suspension.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Pharmacokinetic parameters</th>
<th>C$_{max}$ ± SD (ng/mL)</th>
<th>$T_{max}$ (h)</th>
<th>[AUC]$_{0-24}$ ± SD (ng h/mL)</th>
<th>$t_{1/2}$ (h) ± SD</th>
<th>$F$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plain RLX suspension</td>
<td></td>
<td>37.88 ± 3.99</td>
<td>8</td>
<td>484.83 ± 32.16</td>
<td>16.01 ± 1.91</td>
<td>–</td>
</tr>
<tr>
<td>NLC-8</td>
<td></td>
<td>207.63 ± 15.81*</td>
<td>4</td>
<td>1817.72 ± 81.42*</td>
<td>9.93 ± 2.12</td>
<td>3.75</td>
</tr>
</tbody>
</table>

Value are expressed as mean ± SD; $n = 3$, $F$ – Relative bioavailability.

* $P < 0.05$ compared with plain RLX suspension.
potential expulsion of drug due to crystallization of pure solid lipid into perfect lattice during manufacturing process and due to time dependant restructuring process of lipid molecules during storage period, respectively that might ultimately lower the performance of SLNs in terms of bioavailability [38]. Such issues can be conquered by the formulation of NLCs due to its unstructured imperfect matrix formed between solid and liquid lipid and this may lead to improvement in drug loading with reduction in expulsion of drug during storage condition which eventually played a role in enhancement of bioavailability of drug. In case of conventional techniques, they suffered with problem of hepatic metabolism of drug at some extent which can be avoided by large margin through the encapsulation of drug in nano carriers such as NLCs. NLCs can transport the drug by lymphatic delivery through thoracic lymph ducts to the systemic circulation [39,40]. Some other mechanism such as reduction in efflux of drug from intestinal membrane due to modulation of p-glycoprotein inhibitory function might be also responsible for enhancement of bioavailability of RLX by formulating NLCs.

Conclusion

In the present study, an attempt was made to improve bioavailability of poorly soluble RLX by preparing nanostructured lipid carrier. NLCs were prepared by solvent diffusion method at 70 ºC which exhibit high entrapment efficiency with sustained release of drug up to the period of 36 h. DSC and XRD confirm the transformation of crystal nature of drug into amorphous nature that plays an important role in enhancement of absorption rate followed by bioavailability. Particle size and TEM study confirms nano sized discrete spherical globules with smooth surface area. Stability study of optimized formulation at room condition shows extremely stable formulation for the period of six months that support the fact that dried lyophilized nanocarriers may remain stable for longer period of time.

Result of pharmacokinetic study shows pronounced improvement in pharmacokinetic parameters (Cmax, Tmax and [AUC]0−t) which are responsible for enhanced absorption and bioavailability of drug from NLCs. The pharmacokinetic study of RLX loaded NLCs showed 3.75-fold significant improvement in bioavailability of poorly soluble RLX than plain drug suspension which bestows its potential role as suitable carrier system for oral delivery of RLX in the treatment of osteoporosis. For the commercial purpose, this dried NLCs product can be used orally either by incorporating into capsule or by making dispersion of powder in distilled water.

Conflict of Interests

The authors have declared no conflict of interest.

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References


