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RESEARCH ARTICLE

Design, Optimization and Characterization of Nanostructured Lipid Carriers of Raloxifene Hydrochloride for Transdermal Delivery

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Abstract: ***Introduction*:** Raloxifene Hydrochloride (RXL), a BCS class II drug, is used for the treatment of invasive breast cancer and osteoporosis in post menopausal women. Even though the drug is highly efficient, it shows poor bioavailability of 2% when administered orally. The aim of the study was to develop, statistically optimize, and characterize Raloxifene Hydrochloride loaded Nanostructured Lipid Carriers (NLC) for transdermal delivery to overcome the bioavailability issue.

***Methods*:** The RXL-NLC's were developed using glyceryl behenate (Compritol® 888 ATO), glyceryl monostearate (GMS), and capric triglyceride (Miglyol® 810) as solid and liquid lipids, and Polysorbate 80 (Tween 80) and cremophor EL were used as surfactants and co-surfactant. A response surface methodology was applied for the optimization of NLC, using Box-Behnken experimental design. Amount of the drug, tween 80 and polyethoxylated castor oil (cremophor EL), each at three levels, were selected as independent variables, while particle size and polydispersity index were identified as dependent variables. The optimized batch was characterized for Particle size (79.8 nm±3), Polydispersity index (0.229±0.05), Zeta potential (-12.3±5) and Entrapment efficiency (79.14%±5). Surface morphology of the NLC's were studied using Transmission Electron microscopy (TEM) and the shift in the endotherm of Differential scanning calorimetry confirmed the entrapment of the drug within NLC. *In vitro* drug release studies were performed using dialysis bag (12000-14000 Da) method. The optimized NLC dispersion was then incorporated into gel and characterized for gel uniformity, spreadability, pH, viscosity and drug content.

***Results*:** *In vivo* skin penetration study was carried out by tape stripping method, which showed increase in penetration when incorporated into nanogel as compared to plain drug gel.

***Conclusion*:** Based on the above result it can be concluded that transdermal delivery of NLC's can be a superior alternative for orally low bioavailable drugs such as RXL which undergoes rapid first pass metabolism.

Keywords: Transdermal, Nanostructured Lipid Carriers (NLC), optimization, bioavailability, skeletal disease, osteoporosis.

1. INTRODUCTION

Osteoporosis is a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture [1]. Inadequate new bone formation and excessive bone resorption lead to the development of fragile bone tissue. Hormonal factors strongly determine the rate of bone resorption. Estrogen has been the mainstay of treatment for postmenopausal women, leading to the use of hormone therapy [2]. However, this combined hormone therapy *i.e.* estrogen and progestin led to a significant increase in the incidence of breast cancer. Accordingly, Chlebowski *et al.* carried out a cohort study on women's health initiative observational trial which substantially supported the above findings [3]. In addition there was a considerable reluctance among women to undertake this treatment owing to adverse effects namely vaginal bleeding, and the discomfort associated with procedures used to monitor therapy such as endometrial biopsy. As a result of these problems
associated with hormone therapy the compliance rate among women using Estrogen Replacement Therapy (ERT) or estrogen/progesterone therapy (HRT) is low [4-6].

An increased awareness of the pharmacology of estrogen and its receptors led to the design of Selective Estrogen Receptors Modulators (SERM) [7]. These are a structurally diverse group of compounds that bind to the Estrogen

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Receptor (ER) and confer mixed functional ER agonist or antagonist activity depending on the target tissue [8, 9]. Raloxifene, second-generation SERM (formally called keoxifene) is a chemically distinct polyhydroxy phenol benzothiophene series SERM that has different tissue-specific effects compared to tamoxifen (which belongs to triphenylethylene class and is widely used in the treatment of breast cancer). It was proven to reduce the incidence of invasive breast cancer during major trials: Multiple Outcomes of Raloxifene Evaluation [MORE], Continuing Outcomes Relevant to Evista [CORE], and Study of Tamoxifen and Raloxifene [STAR] [8]. Following the evidence generated by these large-scale trials, raloxifene was approved by the US Food and Drug Administration in 2007 for breast cancer reduction in postmenopausal women with osteoporosis and for those at high risk for invasive breast cancer [9-13].

Raloxifene hydrochloride pertains to class II of the Biopharmaceutics Drug Disposition Classification System (BDDCS), characterized by poor solubility, high permeability and high metabolism. It is marketed under brand name Evista® manufactured by Eli lilly and company as a 60 mg daily dose tablet. However, the major obstacle for oral delivery of raloxifene is its poor systemic exposure, with only 2% absolute bioavailability. Poor bioavailability of such drugs is not only attributed to low solubility and dissolution but also to high presystemic clearance. Raloxifene is a dual substrate for Uridine 5′-diphospho-glucuronosyltransferases (UGTs) and permeability glycoprotein (P-gp) in the intestine. Furthermore, the drug is subjected to extensive phase II metabolism (extensive first-pass effect) in the liver [14]. Additionally, the commonly-reported adverse events associated with high and regular oral dosing of raloxifene hydrochloride are: hot flushes (flashes), leg cramps, and peripheral edema. An alternative route of administration can be utilized to circumvent the first pass effect. The drug can be delivered through transdermal route to improve the bioavailability and avoid the commonly occurring adverse events associated with oral medication. This will also reduce the drug dose without compromising therapeutic efficacy [15].

Owing to the studies being carried out in recent years, nanosized drug delivery systems have been intensively investigated for transdermal delivery as a reduction in size facilitates high penetration of the drug. Moreover, it has been reported that nanoencapsulation of drugs (nano-medicines) increases their efficacy, specificity, tolerability and therapeutic index. These nano-formulations are reported to be superior to traditional medicine with respect to controlled release, targeted delivery and therapeutic impact [16-20]. Many attempts have been made for transdermal delivery of raloxifene hydrochloride in nanosized carriers fabricated using both lipids and polymers. Robert *et al*. [21] developed a micellar nanoparticle formulation with 3%w/w of RXL, which on evaluation for *in vitro* drug release showed a fourfold increase in the transdermal flux when compared to the control (same amount of drug loading using 50%w/w ethanol in 4% w/w HPMC). Similarly, Mahmood *et al*. [22] formulated RXL loaded transfe-rosomes, which are lipid-based nanocarriers. Evaluation of these transferosomes significantly proved them to be superior in terms of the amount of the drug permeated and deposited in the skin (*in vitro* study using Hanson cell diffusion assembly and rat skin as barrier medium).

Likewise, a great deal of interest has been focused on lipid-based carriers represented by Solid Lipid Nanoparticles (SLN) and Nanostructured Lipid Carriers (NLC) since they show many favorable features such as adhesiveness, occlusion, skin hydration, skin penetration enhancement, modified release, and offering protection of actives against degradation [23, 24].

Literature shows a vast comparison between NLC and SLN to establish once superiority over other. Lopez-gracia *et al*. [25] compared these lipid carriers for their occlusive effect and penetration ability. In conclusion, it was found that SLN and NLC had a similar magnitude of occlusive effect but the penetration study revealed that, NLC penetrated into a deeper layer of stratum corneum compared to that of SLN. Moreover, NLC tends be more stable over SLN since they minimize the expulsion of the active compound during storage [22]. Owing to their intrinsic property to improve the bioavailability of lipophilic drugs with low aqueous solubility, NLC's were selected as the lipid carriers for this study (Fig. **1**).

2. MATERIALS AND METHODS

2.1. Materials

Raloxifene Hydrochloride was obtained as a generous gift from Aarti Industries Limited, Thane, India. Gift sample of Compritol® 888 ATO was provided by Gattefosse, Mumbai, India and Miglyol® 810 by Subhash Chemicals, Pune. Glyceryl monostearate, olive oil, tween 80, span 80, and surplus chemicals like solvents, both general grade and HPLC grade, were purchased from S.D Fine chemical Limited, Mumbai, India.

2.2. Formulation of Nanostructured Lipid Carriers

2.2.1. Method of Preparation

The NLC's were prepared by melt emulsification method. Briefly, compritol® 888 ATO, glyceryl monostearate, miglyol® 810 and cremophor EL were heated at 70°C to get a homogenous lipid phase. The drug was added to the above described mixture with constant stirring to ensure proper distribution. The aqueous phase consisting of water and tween 80 was heated at 75°C. Then the lipid phase was added to a hot external aqueous phase to get an emulsion. The emulsion was mechanically stirred by using a magnetic stirrer (Bio lab BL223-B) and Teflon coated beads till the temperature was lowered down to room temperature to yield uniform NLC dispersion [25, 26].

2.2.2. Optimization of the Formulation

A three-factor, three-level, Box-Behnken Design (BBD) was applied to optimize the NLC formulation, using response surface methodology (RSM). Amount of drug (A), tween 80 (B), and cremophor EL (C) were selected as independent variables, considering their overall effects on the formulation's stability. Accordingly, particle size (Y1) and polydispersity index (Y2) were selected as dependent variables for the optimization (Table **1**). Seventeen trial formulations of various combinations, including five center points, were prepared according to the BBD, as shown in (Table **2**). All other formulations and processing variables were kept

Table 1. Variables for box behnken design for drug loaded NLC formulation.

Factors	Factors Levels		
	Low (-1)	Medium (0)	High (1)
A Amount of drug loading (mg/ml)	6	7	8
B Tween 80 (µl)	200	300	400
C Cremophor EL (µl)	100	200	300
Y1 Particle size, Y2 Polydispersity index	-		

Table 2. DOE batches of the formulation.

Batch No.	Amount of Drug (mg/ml)	Tween 80 (%w/v)	Cremophor EL (%w/v)	Particle Size (nM)	PDI
D1	0	1	1	47.6±5	0.353±0.05
D2	0	1	-1	125.6±3	0.460±0.02
D3	0	0	0	85.5±4	0.324±0.03
D4	0	0	0	84.3±4	0.308±0.04
D5	-1	0	-1	47.8±4	0.331±0.02
D6	0	0	0	80.9±3	0.298±0.03
D7	1	1	0	149±2	0.576±0.02
D8	0	0	0	79.5±3	0.286±0.05
D9	1	0	-1	123.7±3	0.697±0.02
D10	-1	0	1	42.1±4	0.532±0.02
D11	0	0	0	95.5±3	0.354±0.04
D12	-1	-1	0	32.6±4	0.521±0.02
D13	0	-1	1	132.8±2	0.532±0.03
D14	1	0	1	127.9±3	0.678±0.01
D15	0	0	0	81.6±4	0.290±0.03
D16	-1	1	0	49.6±5	0.598±0.03
D17	1	-1	0	137.8±3	0.598±0.03
D18	0	-1	-1	164±4	0.257±0.02

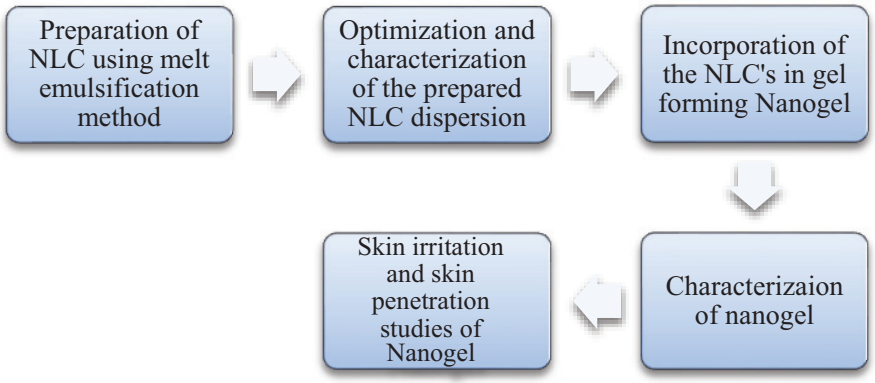

Fig. (1). Flow chart showing the work trend.

constant throughout the study and the batch was optimized based on the result from (Figs. **2** and **3**). Particle size, polydispersity index, drug content, entrapment efficiency and zeta potential of all the batches were determined.

2.3. Evaluation of Optimized NLC Dispersion

2.3.1. Particle Size Analysis and Size Distribution

Particle size and size distribution are important for characterization of lipid carrier. They predominantly confirm the desired colloidal size range and homogeneity of the dispersion obtained during preparation. Particle size and polydispersity index was measured for all the batches in triplicate using Malvern Zetasizer (Nano ZS 90, Malvern Instruments, UK), applying the dynamic light scattering technique. The Polydispersity Index (PDI) was used as a measure for size distribution, and PDI less than 0.5 rendered a homogenous dispersion. The result for the optimized batch is shown in Fig. (**4**).

2.3.2. Zeta Potential

Colloidal particles generally possess a surface charge due to the presence of ionized groups on the surface or because of adsorption of ions from the dispersion medium. These surface charges play an important role in the mutual repulsion of nanoparticles and determine their stability against aggregation. The samples were suitably diluted with distilled water and a dip cell electrode was employed to measure the zeta potential values for each sample. Measurements for all the batches were carried out in triplicate using Malvern Zetasizer (Nano ZS 90, Malvern Instruments, and UK). The result for the optimized batch is shown in Fig. (**4**).

2.3.3. Surface Morphology

Electron microscopic techniques have been employed to characterize the structure and shape of lipid carriers. Transmission Electron Microscopy (TEM) (Philips, CM200) was employed to study ultrastructure and particle morphology of the dispersion. TEM images provide valuable information on particle size, shape, and presence of various structures in the dispersion. Optimized NLC dispersion was examined by the negative staining method. A drop of the vesicular system was placed on to a film-coated copper grid of 300 mesh size, with a diameter of 3.05 mm, to form a thin film, before the specimen was dried on the grid. Then, the film was negatively stained with a drop of 1% w/v phosphotungstic acid. Excess solution was removed with filter paper. The grid was allowed to air-dry thoroughly and magnification of 20000x was used to obtain the TEM image. The result for the optimized batch is depicted in Fig. (**5**).

2.3.4. Drug Content by HPLC

A quantity of the NLC dispersion corresponding to 5 mg of RXL was dissolved in 50ml methanol to facilitate disruption of the vesicles and release the entrapped drug in the solvent. Further dilutions were made and the solution was filtered through 0.22 µm membrane filter and analyzed by HPLC method for estimation of drug content. The chromatographic system used was Agilent 1260 infinity model with UV detector. The column consisted of Qualisil C18, 5 micron, 4.6mm x 250mm. The flow rate was kept at 0.5ml/-min with the injection volume of 20 µl. The mobile phase comprised of an isocratic mixture of Acetonitrile: Isopropyl alcohol: water (45:45:10) along with 2 mM of triethylamine and pH was adjusted to 6.0 with orthophosphoric acid. The analysis was carried out at 287 nm at 25°C. A seven-point standard curve in the range of 0.5 - 10 ppm was prepared, taking peak area of drug versus the concentration and using linear least squares regression as the mathematical model. The prepared standard curve was linear, with a correlation coefficient of 0.9977 [27, 28].

2.3.5. Entrapment Efficiency

Apart from particle size, the other most important parameter is the percentage of drug that has been entrapped in the carrier under the experimental conditions. To measure the percentage of entrapped drug, methanol and water in a ratio of 20:80 was added to the volume of the dispersion corresponding to 10 mg of RXL. The specific amount of methanol was used in order to dissolve free drug without disrupting the vesicles. The solution was centrifuged at 12000 rpm using a laboratory centrifuge (Remi elektrotechnik limited) and the supernatant was analyzed for drug using above HPLC method [29]. The % entrapment efficiency was found using the equation:

$$EE\ (\%) = \frac{[\text{Total drug}] - [\text{Free drug}]}{[\text{Total drug}]} * 100$$

2.3.6. Differential Scanning Calorimetry (DSC) Studies

Differential Scanning Calorimetry studies were carried out for determining whether a molecularly dispersed state of the incorporated drug in the lipid carriers was achieved. Plain drug, placebo batch and optimized NLC batch were analyzed. About 10 mg of sample was added in a 40 µl hermetic aluminum pan which was sealed and heated in the range of 30-300°C at a heating rate of 10°C /min. Further the samples were analyzed using DSC (DSC1; Mettler-Toledo LLC, Columbus, OH, USA) to obtain the thermograms Fig. (**6**).

2.3.7. In Vitro Drug Release Studies

In vitro release of the RXL from the prepared NLC dispersion was determined using dialysis bag method comprising molecular porous membrane (Himedia dialysis membrane 12,000-14,000 Da). An accurately measured amount of the preparation, equivalent to 2 mg of drug was filled into dialysis membrane (pre-soaked in release medium overnight) and was suspended in the dissolution flask of the USP Dissolution Apparatus Type 2 (Electrolab, India) containing 250 ml of dissolution medium. Dissolution apparatus was adjusted to a constant speed (50 rpm) and temperature of 37°C±0.5°C. Samples were collected over a period of 24 hours and assayed by developed HPLC method for drug content. Graphical representation of % drug released as a function of time is depicted in Fig (**7**).

2.4. Preparation of Nanogel

The optimized NLC dispersion was further incorporated into the gel to facilitate proper transdermal administration of the drug through the skin. Gels were prepared using HPMC (Vivapur®) and Carbopol® 934 NF with different concentra-

tion ranging from 0.5%-1.5% w/v. Based on the compatibility with the NLC dispersion, the aesthetic appeal and the ease of spreadability, 1% w/v concentration of Carbopol® 934 NF was selected as the gelling agent. The carbopol dispersion was neutralized using triethanolamine and pH of the gel was adjusted. The prepared gels were evaluated.

2.5. Evaluation of the Nanogel

2.5.1. Physical Characterization

All the batches of the Nanogel were visually inspected for any gritty particles and lumps. They were also viewed under an optical microscope at 45X for aggregates at minute level. pH of topically applied products is an important consideration as topical formulations with extreme pH values is likely to cause skin irritation. pH of the NLC-gel was determined using a calibrated pH meter. Spreadability of the gel was determined using modified wooden block apparatus to figure out its ease of application on skin.

2.5.2. Viscosity

Rheological characteristics of optimized batch GC2 nanogel were measured using Brookfield Viscometer (Model R/S – CPS +). The viscosity of the gel affects the drug diffusion through the gel matrix, spreadability and as well as stability.

2.5.3. Drug Content

The drug content in the optimized batch GC2 nanogel was determined by taking accurately weighed quantity of the formulation corresponding to 2 mg of RXL and was dissolved in 100ml methanol in a volumetric flask and sonicated to facilitate complete dissolution of the drug. This solution was further diluted and was filtered through 0.22 µm membrane filter and analyzed by HPLC method for estimation of drug content.

2.5.4. In Vitro Diffusion Studies

In this study, an accurately weighed quantity of the gel containing 2 mg of RXL was applied onto a membrane (Whatman® nitrocellulose membrane filters, 0.45 µm) and placed between the donor and receptor compartment of Franz diffusion cell. Receptor chamber of the cell was filled to capacity with a medium consisting of phosphate buffer of pH 7.4 and methanol in the ratio 90: 10 and maintained at a temperature of 37°C ± 2°C. At selected time intervals, a quantity of 2 ml of the medium was withdrawn and replaced with fresh medium to maintain sink conditions. Samples were analyzed for drug content by HPLC method [30]. Results are expressed as Flux (J) across the stated membrane which was calculated using following equation:

$$J = \frac{\text{Amount Permeated (mg)}}{\text{Area (cm}^2\text{) X Time (hr)}}$$

2.5.5. Primary SKIN Irritation Studies

Skin irritation studies were performed on Wistar rats in accordance with guidelines for consumer product safety commission under the protocol number of CUSCP/IAEC/09/2016. The formulation was applied on the marked area of the dorsal side (depilated skin) and observations were made at the end of 24 and 72 hours for erythema and edema as per standard scoring system [26].

2.5.6. Skin Penetration Studies

Skin penetration study was carried out by using Tape stripping method using Wistar rat animal model. This test is employed for quantification of the drug crossing the stratum corneum to reach deeper layers of skin. The study was carried out by depilating the animal 24 hours prior to study and applying formulation to the depilated marked skin area. The tape stripping procedure for investigating penetration of drug was performed using an adhesive tape (Transpore, 3M). Adequate numbers of tapes were removed from the region of application at various time intervals after application. These tapes were then analyzed for drug content by developed HPLC method [29, 31].

2.6. Stability Studies

The optimized formulations of NLC gel (GC2) and NLC dispersion (D8) were then prepared in larger batch sizes and suitably packed in lacquered aluminum collapsible tubes and glass vials respectively. Over the time period of the study, samples were withdrawn and assessed for any changes representing instability. The preparation is intended for storage in refrigerator and stability was investigated at conditions of 2-8°C and 25°C ± 2°C; 60%RH ± 5%RH. Samples were withdrawn at three-time points 1, 3 and 6 months from the date of preparation and evaluated for various parameters.

3. RESULTS AND DISCUSSION

As the objective of this study, NLC dispersion was prepared and was statistically optimized using Box-Behnken design. Further, the NLCs were characterized and then incorporated into the gel for ease of application as a formulation.

3.1. Preparation of Drug Loaded NLC

Melt emulsification technique was selected as the method of preparation as batches prepared by this method were found to yield smooth, uniform dispersions and required shorter processing time. The basic composition of the NLC comprised of glyceryl behenate (compritol® 888 ATO) and glyceryl monostearate as solid lipid and capric triglyceride (miglyol® 810) as liquid lipid. These spatially different lipids provide imperfections in the crystal order of the lipid nanoparticle to accommodate the drug in the amorphous cluster. Addition of tween 80 (HLB 15) and Cremophor EL (HLB 13) had a considerable effect on particle size as well as on the stability of NLC dispersion. The intrinsic property of the surfactant to inhibit coalescence helps in stabilizing the dispersion and leads to the reduction in particle size. Additionally, cremophor EL helps in the solubilisation of drug ensuring proper distribution throughout the dispersion. The amount of both the surfactants was optimized using Box-Behnken design. The formulation was optimized at 2% w/v of lipid as it was found to provide a stable dispersion and required particle size. Several process variables were optimised including time of stirring (half an hour with cooling at room temperature was found to be sufficient); and speed of stirring (800 rpm on a magnetic stirrer using Teflon coated beads yielded best dispersions). The therapeutic transdermal daily dose of the drug was calculated according to the equation given by Vaddi *et al*. [32] Considering a 60 mg oral

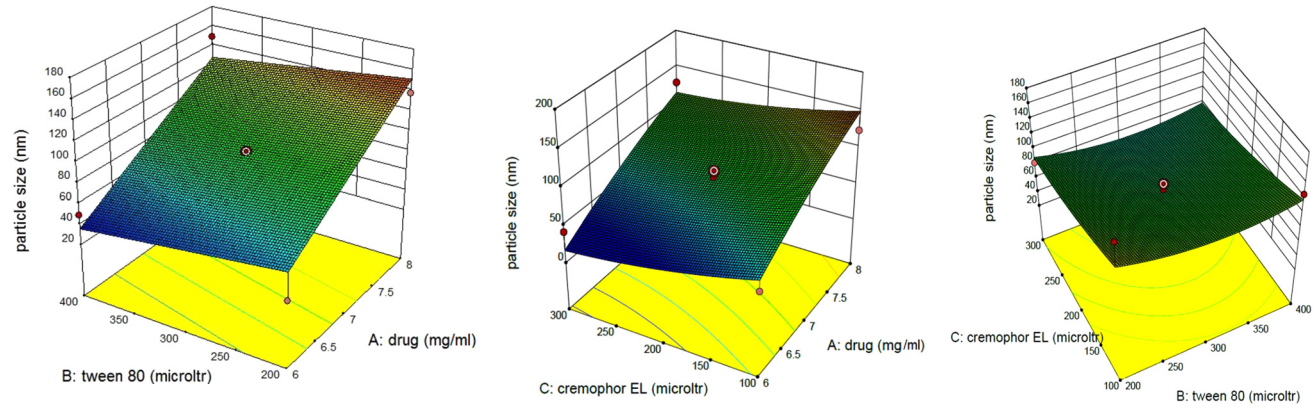

Fig. (2). 3D Response Surface plots for Particle size.

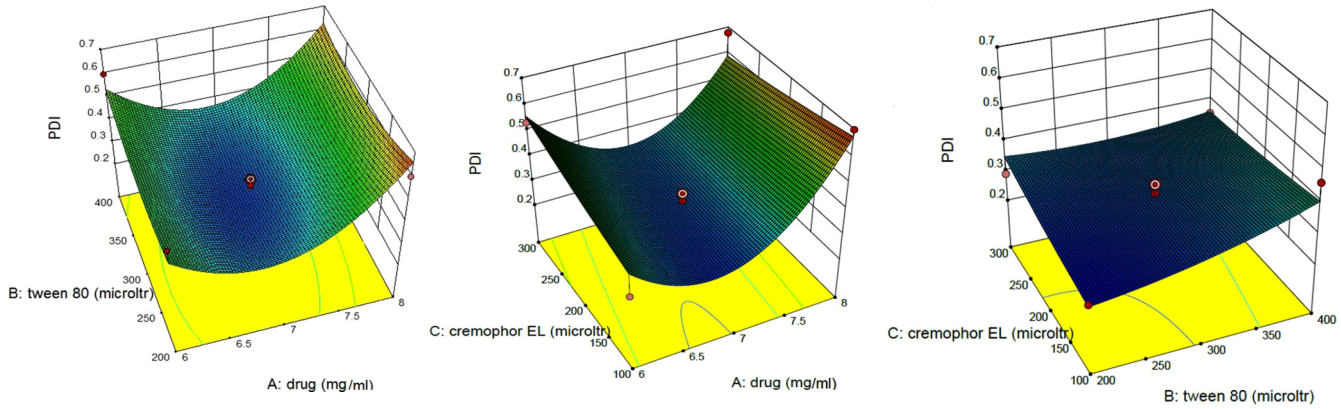

Fig. (3). 3D Response Surface plots for Polydispersity index.

dose of Raloxifene hydrochloride tablet, a systemic dose of 1.5 mg was calculated. Considering the absorption capacity of the skin, maximum amount of 8 mg/ml of the drug was loaded into NLC.

3.2. Optimization of the Formulation

In order to optimize the formulation, relationships among independent variables, such as amount of drug loading (**A**), tween 80 (**B**), and cremophor EL (**C**), at three levels (-1, 0, +1), with dependent variables, such as particle size (Y1) and polydispersity index (Y2), were evaluated by BBD, using Design Expert® software. The obtained response values of the individual trial formulations were fitted into the model matrix, in order to find the best-fitted model. For estimation of the significance of the model, analysis of variance was performed using Design Expert® software (Table **1**). Using a 5% significance level, a model is considered significant if the *P*-value (significance probability value) is less than 0.05. Both the models were found to be significant.

3.2.1. Effect of Independent Factor on Particle Size

The effects of the independent variables on entrapment efficiency are represented by three-dimensional response surface graphs and their corresponding contour plots are shown in the figure. The sizes of the NLC's were less than 200 nm, which is the optimum range for effective transdermal application. The variations in vesicle size were statistically significant (P>0.05). The vesicles size is significantly influenced by the amount of the drug incorporated as seen in plots. Due to the increased particle size sedimentation was observed after 8 hours. Increasing amount of tween 80 and cremophor EL has shown a decrease in particle size Fig. (**2**).

3.2.2. Effect of an Independent Factor on Polydispersity Index

Polydispersity index seems to be increasing with the increase in the drug amount. This could be because of the inefficient entrapment of drug, which could have led to the nonhomogenous distribution. Increasing amount of surfactant *i.e.* tween 80 and cremophor EL shows a decrease in the PDI indicating a narrow range of distribution and homogeneity in distribution Fig. (**3**).

3.2.3. Selection of Optimized Formulation

The point prediction method of Design Expert® software was utilized to select the optimized formulation from 17 trial formulations, according to the BBD experimental design. This selection was based on the criteria of attaining minimum values of particle size and polydispersity index. After a thorough evaluation by Design Expert® software, it was found that formulation D8 fulfilled the requisites of an optimum formulation with drug content 96.35%±2% and entrapment efficiency of 79.14%±5%.

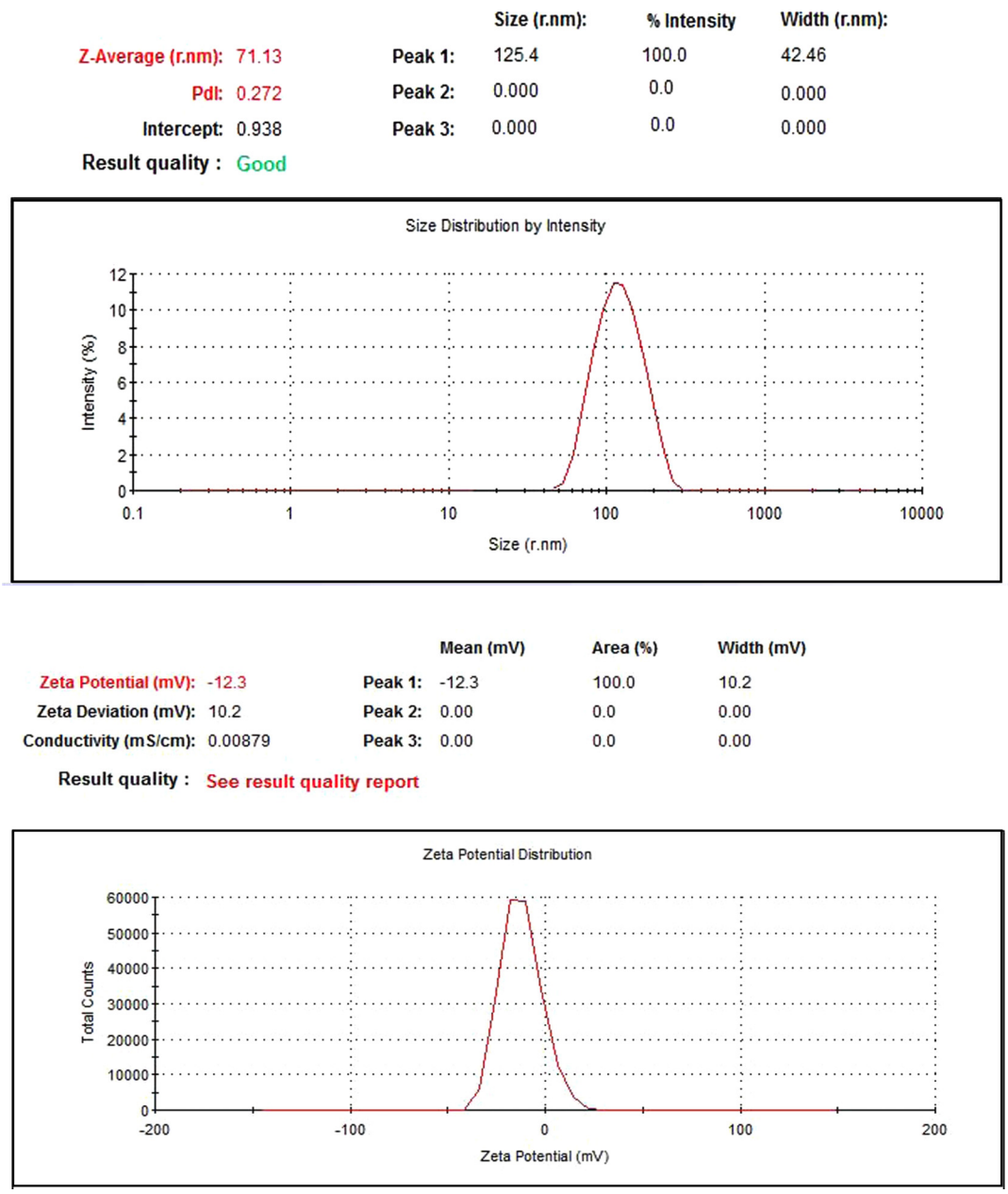

Fig. (4). Particle size and zeta potential of batch D8.

3.3. Evaluation of the Optimized Formulation

3.3.1. Particle Size Analysis, Polydispersity Index and Zeta Potential

The particle size of the lipid carriers is the most important characteristic which facilitates its passage through subcutaneous layers of skin. The particle size of the vesicles for the NLC dispersion ranged from 42 nm to 164 nm for the batches D1 to D18. Initially, higher lipid concentration resulted in increased particle size due to the formation of aggregates and so lipid concentration was optimized at 2% w/v. Increase in drug amount exhibited increase in the particle size which was optimized using BBD. The particle size of the optimized batch D8 was found to be 79.8±3 nm. Polydispersity index and Zeta Potential also play a major role in stability of the NLC dispersion. The polydipersity index indicates the homogeneity of the particles in the dispersion. Lower the value of polydispersity index; more homogenously the particles are distributed. Polydispersity index and zeta potential of the optimised batch D8 was found to be 0.229±0.05 and 12.3±5 mV respectively. The results are depicted in (Fig. **4**).

3.3.2. Surface Morphology

The optimized batch D8 was evaluated for surface morphology using TEM. NLC were examined by negative staining with 1% phosphotungstic acid. Usually in negative staining, the particle is surrounded with electron dense material and the surface is revealed by contrasting between the stain and the specimen. TEM images of NLC dispersion revealed the presence of spherical structures in dispersion with the particle range of 100-200 nm Fig. (**5**).

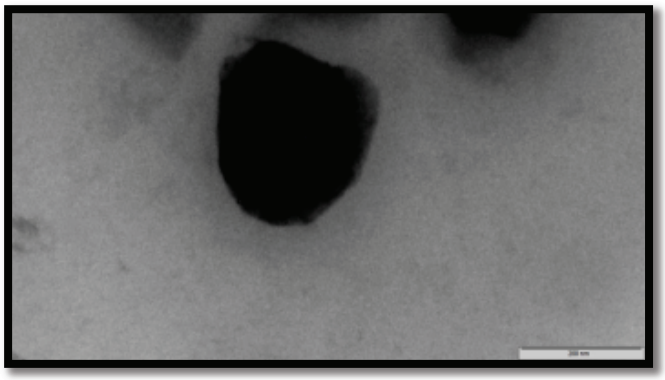

Fig. (5). TEM image (20000x magnification) of batch D8.

3.4.3. Drug Content and Entrapment Efficiency

The drug content in the NLC dispersion was found to be 96.35%±0.5 and entrapment efficiency was 79.14%±5.

3.4.4. Differential Scanning Calorimetry

DSC was carried out to confirm the entrapment of drug in the lipid lattice of NLC. DSC of pure drug, placebo batch, and optimized NLC batch was performed. Overlay of the drug, placebo batch and optimized batch exhibited endotherms at different temperatures. Pure drug endotherm shows melting at 274.3°C, whereas disappearance of this peak in formulation endotherm confirmed entrapment of drug within the carrier. The overlay is shown in the Fig. (**6**).

3.4.5. In Vitro Drug Release

In vitro drug release studies of optimized batch D8 was carried out. The dissolution medium selected was phosphate buffer pH 7.4 + methanol (10%) based on the solubility of the drug. Drug release from the dispersions at the end of 24 hours was found to be 90.41%. The release data was assimilated into various release models. The data was found to fit Korsmeyer Peppas model (R^2=0.9591) indicating that the drug release is controlled by more than one process, *i.e.* superposition of both phenomenon, the diffusion controlled as well as swelling controlled release Fig. (**7**).

3.5. Evaluation of NLC Based Gel

Rationale for incorporating NLC batch D8 into gel was ease of application and to improve its stability. Moreover, gel provides adherence to the skin and consequently increases the contact time. HPMC and Carbopol®934 were used in different ratios for preparation of gel. The batch was optimized with 1% carbopol® 934 since the gels obtained with it were smooth, elegant and completely free from grittiness. On contrary, gels with HPMC showed aggregation and were stiff. The optimized batch GC2 nanogel was characterized for various parameters and was found to have drug content of 96%, flux of 0.01423 mg/hr/cm^2 and percent drug diffusion of 70%

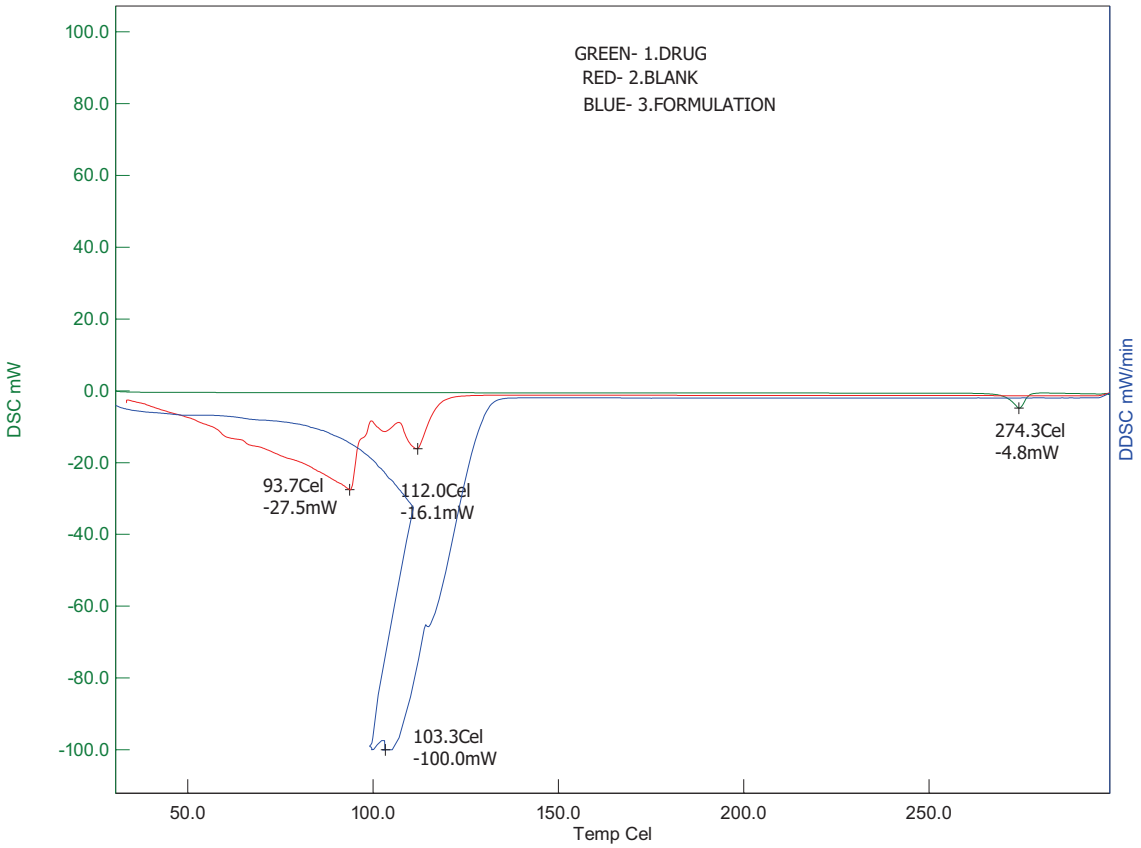

Fig. (6). DSC Thermogram of drug, blank batch and drug loaded batch D8.

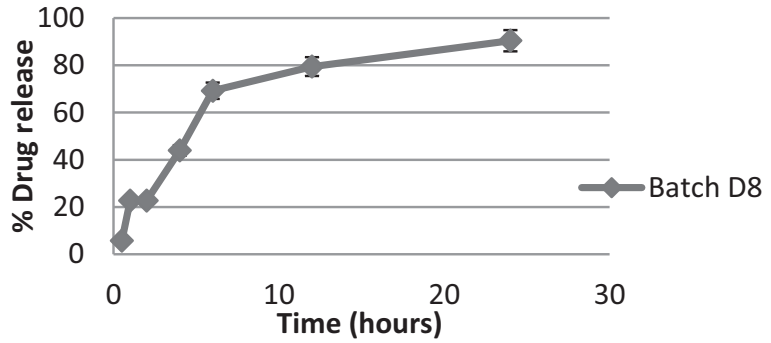

Fig. (7). *In vitro* drug release studies of plain drug and batch D8.

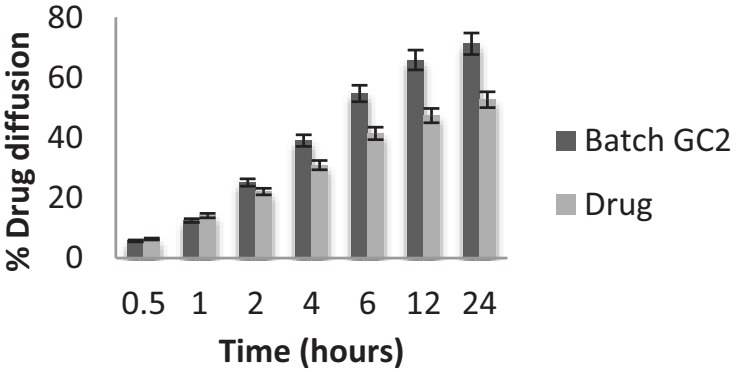

Fig. (8). *In vitro* diffusion studies of batch GC2 using franz diffusion cell.

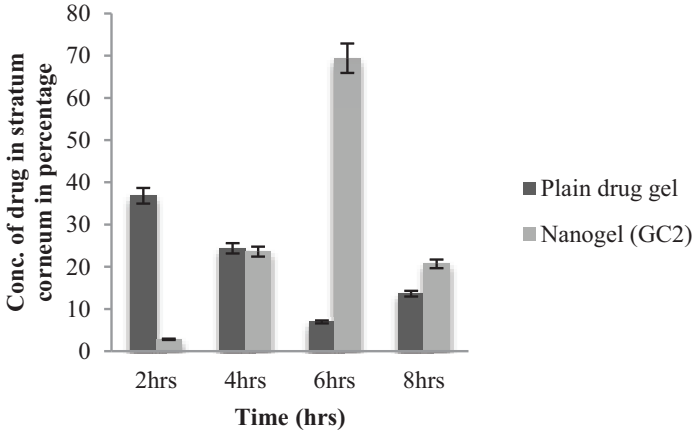

Fig. (9). Penetration studies of nanogel *vs.* plain drug gel.

as compared with the plain drug gel Fig. (**8**). Spreadability of the gel was evaluated on a Modified Wooden Block Spreadability determination apparatus and was found to be 6.1g/sec. The pH was found to be in the range of 6-7 and viscosity as determined using Brookfield Viscometer was found to be 26.70 Pas. The gel exhibited non-Newtonian flow. The diffusion studies carried out for batch GC2 showed 70% of drug diffusion within 24hrs.

3.5.1. Skin Irritation and Penetration Studies

Skin irritation studies were performed on the Wistar rat skin in accordance with the Federal Hazardous Substances Act (FHSA) under the protocol no. CUSCP/IAEC/09/2016. The Primary Irritation Index for the developed formulation was found to be 0.0. Thus on the basis of the score and the PII the developed NLC gel was classified as non-irritant to the skin. The skin penetration study was carried out on the NLC gel (GC2) and on plain drug gel. The concentration of drug in stratum corneum as a percent of applied amount of NLC gel GC2 after 2, 4, 6, and 8 hours were found to be 2.82%, 23.60%, 69.38% and 20.70% respectively. The concentration of drug in stratum corneum as a percent of the applied amount of plain drug formulation after 2, 4, 6 and 8 hours were found to be 36.81%, 24.37%, 6.95% and 13.63% respectively. The amount of drug found in the stratum corneum as a percent of the applied amount of formulation was more in case of NLC gel as compared to the plain drug gel Fig. (**9**). The optimized NLC gel showed more penetrability through the skin compared to the plain drug and student t-test ($t=1.639$, $p=0.02$) was found to be significant.

3.6. Stability Studies

The optimized batches of NLC dispersion (D8) as well as nanogel (GC2) were kept at 2-8ºC and 25ºC ± 2ºC; 60%RH ± 5%RH according to the ICH guidelines and samples were withdrawn at the time points of 1, 3 and 6 months from the date of preparation and evaluated for various parameters such as appearance, particle size, drug content and drug diffusion studies. At accelerated conditions, noticeable drop in the drug diffusion in the gel was seen after 3 months which could be due to the aggregation of NLC at a higher temperature in gel matrices. Both dispersion and gel formulations did not show appreciable variation in drug content upon storage. This indicates good stability of the drug in the formulation, and confirms the compatibility of the selected packaging system and the product.

CONCLUSION

Nanostructured lipid carriers have been extensively studied for transdermal delivery. They exhibit low systemic toxicity as they consist of physiological and biodegradable lipids. In addition, the small size of the lipid particles ensures close contact to stratum corneum and has shown to increase the amount of drug penetrating into mucosa or skin. In the current study, NLC loaded with Raloxifene hydrochloride were formulated by using a modified melt emulsification method. The results revealed that a stable NLC dispersion can be prepared by using a combination of lipids like Compritol® 888 ATO, Glyceryl monostearate and Miglyol® 810 with an average particle size of 79.8 nm±3, a polydispersity index of 0.229±0.05, Zeta potential of -12.3±5 and Entrapment efficiency 79.14%±5. Box Behnken experimental design was used to evaluate the effect on the physical properties of RXL loaded NLC. The observed responses were close to predicted values for the optimized formulation. The DSC and surface morphology studies confirmed the entrapment of drug and NLC formation respectively. The korsmeyer-peppas model gave the highest value of the R2, indicating that this model was a fit for RXL release profiles from NLC's. Penetration studies illustrated the increase in penetration of drug when in nanogel as compared to the plain drug gel. The Nanogel was tested on the membrane which mimics the human skin but further studies are required to examine penetration of the formulation in human cadaver. In conclusion, the studies confirm the potential of transdermal delivery of nanostructured lipid carriers consisting of poorly bioavailable drugs which undergo extensive first-pass metabolism.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Institutional Animal Ethical Committee (IAEC), India (Approval no. USCP/IAEC 09/2016).

HUMAN AND ANIMAL RIGHTS

No humans were used for studies that are the basis of this research. All the animals procedures were used in accordance with the US Public Health Service's "Policy on Humane Care and Use of Laboratory Animals," and "Guide for the Care and Use of Laboratory Animals."

CONSENT FOR PUBLICATION

Not applicable.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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