FORMULATION AND CHARACTERISATION OF LIPID NANOPARTICLES BEARING TAZAROTENE FOR ENHANCED TOPICAL DELIVERY IN PSORIASIS

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By

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1. TITLE OF THESIS AND ABSTRACT

TITLE OF THESIS
Formulation and Characterisation of Lipid Nanoparticles bearing Tazarotene for enhanced topical delivery in Psoriasis.

ABSTRACT

The present investigation dealt with the formulation development, optimization, in vitro and in vivo study of tazarotene loaded nanocarriers for the treatment of psoriasis. The lipid based nanocarriers i.e. SLN and NLCs were prepared by melt emulsification-sonication. Process variables were optimized by one variance at time (OVAT) approach. Experimental design - Box Behnken design and Artificial Neural Network were used for optimization of various formulation parameters of Tazarotene loaded NLCs and SLNs. Various physicochemical parameters such as particle size, zeta potential, assay and % entrapment efficiency were measured. Further drug loaded SLNs and NLCs were loaded in Carbopol 980 gel. The gel was characterized for pH, viscosity, drug content uniformity, homogeneity, stability study and occlusion test. Drug loaded NLCs, SLNs with or without gel base was evaluated for in-vitro drug diffusion study, cellular uptake study, MTT assay, and cytotoxicity study using A431 skin cancer epithelial cell line. The particle size range of the optimized formulation was $215.2 \text{ nm} \pm 4.5 \text{ nm}$ with zeta potential $-37.8 \text{ mV} \pm 3.4 \text{ mV}$, and entrapment efficiency was $57\% \pm 3\%$ for SLNs while in case of NLCs, the particle size was around $235.22 \text{ nm} \pm 6.8 \text{ nm}$ with zeta potential of $-39.8 \text{ mV} \pm 3.7 \text{ mV}$, and entrapment efficiency of $60-65\%$ for NLC. The cell viability study also indicated that formulation was non-toxic to cells. The cellular uptake study was done using confocal microscopy which indicated that the drug loaded SLNs and NLCs were taken up by the cells in peripheral region. Ex vivo studies indicates more amount of drug retains in the skin while delivered through lipid nanoparticles compared to conventional formulations. Histopathological studies confirms that formulation do not have acute dermal toxicities. Imiquomod induced psoriasis model was utilised for In Vivo Pharmacodynamic studies which indicates better efficacy for drug delivered in Lipid nanoparticles bearing Gel.

2. BRIEF DESCRIPTION ON THE STATE OF THE ART OF THE RESEARCH TOPIC

Psoriasis is a common chronic, systemic, inflammatory disease commonly manifested by painful and pruritic skin lesions on the elbows, knees, scalp, genitals, and trunk that has been estimated to affect 1% to 3% of the population worldwide. Psoriasis is classified according to morphological appearance and includes plaque, inverse, erythrodermic, pustular, and guttate forms, as well as nail manifestations. It is estimated that around 125 million people worldwide are suffering from Psoriasis. For each patient, it causes a considerable, quantifiable reduction in quality of life. Given this great burden of illness, psoriasis warrants significant clinical and research attention. Over the past 20 years, there have been many developments in the understanding genetic, molecular and cellular mechanisms that underlie these inflammatory processes and many new and effective treatments have been developed. The negative impact of the disease on health-related quality of life (QoL) is comparable to that of ischaemic heart disease, diabetes, depression and cancer [1].

Biologic, phototherapy, topical and systemic treatment can induce reduction of psoriasis for months to years, treatments mainly depend on the severity of disease. Most
patients with psoriasis have limited disease (less than 20% of their body surface area), so topical therapy [2]. Topical therapies remain the mainstay of treatment for mild psoriasis. Patients with severe psoriasis often use topical therapies. The main groups of topical therapies for psoriasis are emollients, vitamin D and its analogues, topical corticosteroids, coal tar preparations, dithranol, and tazarotene (a topical retinoid) [3, 4]. Novel formulations based on nanocarriers are promising prospect to overcome the limitation of conventional formulations by offering a reduction in dose, dosing frequency, dose-dependent, side effect with enhanced efficacy [5]. Different lipid nanocarriers such as solid lipid nanoparticles, nanostructured lipid carriers, liposomes, ethososomes, microemulsions and lipid Nanocapules are studied as a topical drug delivery systems [5, 6].

Lipid nanoparticles like SLNs and NLCs show many advantages over other colloidal carriers as they showed close contact interaction with the stratum corneum resulting in enhanced occlusion and skin hydration [7]. Topical preparations were used for the localized effects at the site of their application by virtue of drug penetration into the underlying layers of skin or mucous membranes [8]. The main advantage of topical drug delivery system is to bypass first pass metabolism. Avoidance of the risks and inconveniences of intravenous therapy and of the varied conditions of absorption, like pH changes, presence of enzymes, gastric emptying time are other advantage of topical preparations [8].

Tazarotene is the first receptor-selective retinoid for the topical treatment of plaque psoriasis [3]. On application, tazarotene is rapidly hydrolyzed to its main metabolite, tazarotenic acid, which binds to retinoic acid receptors (RARs) in the nucleus [9]. Tazarotenic acid selectively binds to RARs and exhibits little affinity for retinoid X receptors [10]. The predominant type of RAR expressed in the human epidermis is RAR, indicating that it may be an important mediator of retinoid action in skin [10, 11]. By regulating gene transcription, tazarotene normalizes abnormal keratinocyte differentiation, reduces epidermal hyperproliferation, and decreases inflammation, the 3 pathogenic factors in psoriasis, thereby producing a more normal expression of skin differentiation in psoriatic lesions [4, 12]. Based on the above facts, it was hypothesized that Tazarotene loaded SLNs and NLCs will showed enhanced anti psoriatic activity as compared to marketed formulation like

The current research focuses on formulation development of SLN and NLCs and its optimization by OVAT, response surface design and Artificial Neural Network approach as well. In vitro characterization and in vivo studies was carried out to identify potential efficacy of developed formulation.

### 3. DEFINITION OF THE PROBLEM

Topical therapy remains the mainstay of treatment for mild psoriasis. Patients with severe psoriasis often use topical therapies (at least for selected body areas). Oral Therapy have serious systemic adverse Drug Reactions. Topical therapy offers the possibility of achieving local therapeutic benefit while reducing or eliminating the risk of systemic side effects. Conventional Topical preparations like creams, gels andointments covers major part of the formulations available in the market. However Conventional Gels have some limitations like,

- Short duration of action.
- Penetration Enhancers used to increase permeation in most conventional topicals are toxic to skin.
- No control on release characteristics
- Skin reaction/ Colouration.
Lipid nanoparticles like SLNs and NLCs show many advantages of colloidal carriers which makes them ideal formulation for topical drug delivery for treatment of psoriasis. Specifically they are the colloidal carrier of the choice because it shows,

- Close contact/Interaction with the SC.
- Increase of skin Occlusion
- Increase of skin hydration and elasticity.
- Enhancement of skin permeation.
- Enhancement of Chemical Stability of the chemically labile compounds.
- Physical stability in topical formulations

4. OBJECTIVE AND SCOPE OF WORK
The objective of the present work includes,

- Formulation and characterization of the NLC/SLN bearing Tazarotene for enhanced topical delivery for psoriasis.
- To incorporate NLC/SLN in Hydrogel and characterize it.
- To Perform In vitro, Ex vivo and In Vivo studies to characterise safety and efficacy of the formulations

Scope of the research work:

- Prepared Formulations by virtue of lipidic nature would be able to increase the permeable fraction through skin and Increased skin retention. The skin permeation can be enhanced by occlusion property of NLC/SLN and their interaction with Stratum corneum.
- Toxicity associated with the conventional topical preparations can be overcome by using lipid particles as lipids are generally non toxic and possess GRAS status.

5. ORIGINAL CONTRIBUTION BY THE THESIS
The entire work in this synopsis, as well as thesis is original. Extensive literature review was done to identify the challenges associated with treatment of Psoriasis. Although multitude of researchers have work on development of topical formulations for treatment of psoriasis, the idea of Formulation of lipid nanoparticles (like Solid Lipid nanoparticles and Nanostructured Lipid carriers) incorporated hydrogels by systematic approach of design of experiments for optimisation of various parameters and InVitro, ExVivo and InVivo characterisation for topical drug delivery of Tazarotene for therapy of psoriasis was probably yet not investigated by any other researcher.

6. METHODOLOGY OF RESEARCH
Experimental Work – I
Preparation of NLC and SLN: The NLCs were prepared by melt emulsification – sonication method [13]. Using the same method, SLN was also prepared by replacing the liquid lipid part with solid lipid to compare with NLC for the topical application. The solid lipid (Cutina GMS), and liquid lipid (Cremophor EL) were selected based on drug solubility study from various lipids. Lipid and drug were taken in a beaker and heated up to 60° C. In another beaker, hydrophilic surfactant dissolved in dist. water was heated to same temperature as of lipid melt. The lipid melt was added to surfactant solution and was stirred by Ultraturrax T25 basic at 19000 rpm for 5 min with continued heating. The prepared emulsion was sonicated using probe sonicator for 5 min. The emulsion was allowed to cool naturally and the NLCs were evaluated.
**Factors identification and optimization**: FishBone diagram was generated to understand which variables affect, or the stable formulation development of Tazarotene NLCs as shown above. [16]. During formulation optimization batch size (10ml) and temperature of processing (60° C) were kept constant. From the Fish Bone diagram, the variables were identified. Those variables which affected less to the final quality product of tazarotene NLCs were kept constant based on preliminary batches formulation results and literature survey. Those variables which had medium risk were optimized by one variable at a time (OVAT) approach.

**Statistical optimization**: Based on the results of single – factor experiments, three significant parameters were confirmed as follow: solid Lipid : Liquid Lipid ratio, Lipid : Drug ratio, and Surfactant concentration. These factors were optimized by a statistical approach of design of experiment. Box–Behnken Design (BBD0 was performed using Stat-Ease Design Expert Software ver.9.0.4 (Stat-Ease Inc. Minneapolis, MN, USA) by Response Surface Methodology augmented with 5 center points for optimization of parameters. The range and the levels of experiment variable investigated are presented in Table 1. The responses were as size, zeta potential and % entrapment efficiency. The design matrix generated by software is shown in Table 10. The result obtained practically were compared with the predicted value.

**Table 1: Factors and levels of variables**

<table>
<thead>
<tr>
<th>Factors (Variables)</th>
<th>Code of variables</th>
<th>Actual levels of the variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solid lipid: Liquid lipid ratio</td>
<td>X1</td>
<td>-1 0 1</td>
</tr>
<tr>
<td>Lipid: Drug ratio</td>
<td>X2</td>
<td>9:1 11:1 13:1</td>
</tr>
<tr>
<td>Surfactant Concentration (w/w)</td>
<td>X3</td>
<td>1% 1.5% 2%</td>
</tr>
</tbody>
</table>

To find out appropriate range of the solid lipid, liquid lipid and surfactant the screening trials were run. First screening trials were taken to identify the range within which the design space needs to be generated. For initial trial, In the lipid part the ratio of solid lipid: liquid lipid was taken as 7:3. Three different surfactants Poloxamer F 127, Tween 80 and PVA were used. The emulsion was evaluated for globule size, and entrapment efficiency. Process variables like speed of ultra turrax homogenization (11000, 13000 and 19000 rpm),stirring time (1min, 3 min,5 min and 7 min), Sonication parameters such as amplitude (10%, 30%, 50% and 70% ) and time of sonication (1, 3, 5 and 7 min ) were optimized. The cycle for sonication was kept constant at 0.5

**Preparation of SLNs or NLCs loaded gel**: The NLCs or SLNs dispersion was incorporated in the carbopol 980 NF. 0.8%w/w carbopol gel was prepared by pH change method. Triethanolamine (0.23%w/v) was used as the alkylating agent. The Carbopol 980 NF was allowed to swell for 3 hr. The gel was neutralized by dilute solution of triethanolamine. The NLC or SLN dispersion was added to the gel and mixed properly by using high speed stirrer at 500 rpm for 2 min. The gel was evaluated for parameters like appearance, viscosity and pH [17].

**Characterization of Nanostructured lipid carriers**: **Physicochemical characterization**: The NLC dispersions were characterized for their physicochemical properties such as colour, odour and stability after centrifugation. Centrifugation was performed at 200 g for 20 min using centrifuge
**Particle size and Zeta potential:** The particles size and zeta potential analysis of nanoparticles was performed Malvern Zetasizer Nano ZS (Malvern, UK). Each sample was suitably diluted with filtered distilled water (10 times) to avoid multi scattering phenomena and placed in a small disposable size cell. The analysis was carried out at 25°C.

**Assay:** For determination of assay, 0.1 ml of formulation was measured and diluted with chloroform: methanol (3:7) solution and volume was made up to 100 ml. The diluted solution was analyzed by UV spectrophotometer by validated method.

**% Drug entrapment:** Free drug and entrapped drug were measured for mass balance. The free (unentrapped drug) was separated using centrifugation at low speed of 3000 rpm for 5 min and supernant was taken which was NLCs dispersion. Pellet was dissolved in chloroform: methanol (3:7) and absorbance was taken in UV Spectrophotometer using validated method, which gave unentrapped drug. Then, NLCs dispersion (supernatant) was diluted with chloroform: methanol (3:7) and absorbance was taken at 351 nm against blank to determine entrapped drug. Then %EE (Percent Drug Entrapped, PDE) was calculated by equation (2).

\[
\%DE = \frac{\text{Drug entrapped}}{\text{Total drug added}} \times 100 \quad \text{Equation (2)}
\]

** Occlusion test :** The occlusion test was performed as following[18].50 ml of distilled water was taken in 125 ml beaker. All beakers were covered with filter paper. NLC/SLN dispersion, NLC/SLN gels or plain gel was applied uniformly on the filter paper. One beaker was covered with filter paper but without sample served as a reference. The Area of the filter paper was 23.74 cm². The Initial weight of all beakers was recorded and the samples were weighed after 6, 24 and 48 h, giving the water loss due to evaporation at each time (water flux through the filter paper). The Occlusion factor was calculated according to the following equation

\[
F = \frac{\text{A} - \text{B}}{\text{A}} \times 100 \quad \text{Equation (3)}
\]

Where A is the water loss without sample (Control) and B is the Water loss with sample.

(An occlusion factor of Zero means no Occlusive effect compared with the reference, and 100 is the maximum occlusion factor.)

**Characterization of Gel**

**pH:** The pH of the gel was measured by digital pH meter.

**Drug Content:** 1 gm of the gel was taken and extracted with chloroform: methanol mixture (3:7). The mixture was diluted sufficiently and analyzed by UV Spectrophotometer by validated analytical method.

**Homogeneity:** Gel was tested for homogeneity by visual inspection after the gel was set in the container. It was tested for appearance and presence of any aggregates. For determination of the homogeneity three samples of 1 gm gel were taken from the different sites from the gel container. The gels were extracted with chloroform: methanol mixture (3:7). The mixture was diluted sufficiently and analyzed by UV spectrophotometer.

**Viscosity:** Viscosity of the formulation was determined using Brookfield cone and plate Rheometer (Model LVDV III) using CPE spindle at 25°C. The speed utilized is 20 rpm.

**In Vitro drug diffusion study:** It was performed using modified Franz diffusion cell to evaluate the drug release profile form the formulation. Artificial Dialysis Membrane 70 (Himedia, Mumbai India) having pore size 2.4 nm and molecular weight cut-off of 12000 Dalton was used [20]. The formulation equivalent to 10 mg drug was applied to the donor compartment and the receptor Compartment was filled with Phosphate buffer pH 6.8 (100 ml). The area of the diffusion is 3.79 cm². During the experiments, the solution in the receptor side was maintained at 37°C and stirred at 100 rpm with Teflon-coated magnetic stirring bars. At fixed time intervals, 5 ml aliquot was withdrawn and analyzed by UV spectroscopy after suitable dilution. To Find out flux of the drug from the membrane quantity of the drug released is divided by area of drug release (Co./A). The plot of Co/A vs time was plotted. The slope of the plot indicates the flux.
**Stability study**: The stability of the developed SLNs & NLCs loaded gel was conducted for 6 months as per International Conference on Harmonization ICH Q1A (R2) guidelines [21]. The optimized formulation was selected for the stability study. Briefly, samples were stored in the sealed amber colored glass vials at 25°C ± 2°C, 60% RH ± 5% RH. After 1, 2, 3 and 6 months, the samples were characterized with respect to physical appearance, pH, viscosity and drug content.

**Experimental Work – II**

**Cytotoxicity Study (MTT assay)**: The cytotoxicity of formulation was carried out on A 431 (Skin cancer) Epithelial cell line procured from NCCS, Pune, India. Dulbecco’s Modified Eagle Medium (HiMedia) with added 10% Fetal Bovine Serum and 0.5% antibiotic solution was used as media for culturing cells at 37°C. The cells of A 431 were maintained in 5% CO₂ incubator. After 70 – 80% confluency of cells in culture flask, subculturing was carried out. The complete media from the flask was removed aseptically in laminar air flow hood. After gentle wash with PBS 7.4, the trypsin – EDTA treatment for NMT 2 min was carried out. The trypsin – EDTA treatment detaches the adherent cells from the flask and the roundup of the cells was visible under microscope. The cell suspension was then transferred to centrifuge tube. After centrifuge for 2 min at 1000 rpm, the supernatant was removed and the cells were resuspended in complete media. The cells were counted using Neubauer’s chamber under microscope after staining with tryphan blue. Accordingly, the cells were subcultured in other flask after appropriate cell density was obtained. After counting the cells, 10⁴ cells were seeded in each well of 96 well plate with 200 µl of complete media. After the cells get adhered at around 24 h, the formulation treatment was given for 6 h using 1000 µg/mL, 500 µg/mL, 100 µg/mL and 10 µg/mL concentration. After the treatment, the formulation was removed and 10 µL of MTT dye (5 mg/mL) was added to each well containing 100 µL of complete media. The cells were further incubated for 4 hr after MTT treatment. Then 100 µL of DMSO was added to each well for solubilization of Formazan crystal. Then the plate was incubated for 2 hr to allow the solubilization of formazan crystal and absorbance was read at 570 nm using ELISA plate reader. Based on comparative absorption of control cells, the % viability was calculated.

**Cellular uptake using confocal microscopy**: Qualitative cellular uptake was carried out after proliferating the cells on a coverslip in a 6 well plate. The cells were treated with formulations prepared by replacing the drug with Coumarin-6 dye for 4 hr. After treatment, the coverslips were washed with phosphate buffer saline pH 7.4 thrice. DAPI was used to stain nuclei. Following staining, the coverslips were mounted using glycerin. The slides were observed using confocal microscopy (ZEISS LSM, Germany) and images were processed for color channeling using ZEN lite – Blue edition software

**Animal study**: All the protocols of animals study was approved by the Institutional Animal Ethics Committee (IAEC) accordance to the guidelines of committee for the purpose of control and supervision of experiments on animals (CPCSEA), Ministry of social justice and empowerment, government of India, New Delhi, India.

**Acute dermal toxicity study**: The acute dermal toxicity performed on mice (n=18) according to the Organization for Economic Co-operation and Development guidelines No. 402 [13, 14]. Approximately 24 hr before the test, 10% hairs of the body were removed from the dorsal area of the test animal by using sterile shaving blade. Animals were divided into three groups
(Normal control group, Formulation 1 treated group and formulation 2 treated group) having 6 animals in each groups study. All animals were well observed during study

**Anti-psoriatic activity evaluation (Imiquimod Induced psoriatic model)**: Topical application of imiquimod (MQ) can induce and exacerbate psoriasis.[14, 15] 8 – 11 week old mice (n=36) having weight 25g to 45 gm of either sex were kept under specific pathogen-free conditions and provided with food and water ad libitum. Animals divided into six group having six animals in each group. Animals except normal group’s received a daily topical dose of 62.5 mg of commercially available IMQ cream (5%) (Aldara; 3M Pharmaceuticals) on the shaved back and the right ear for 5 or 6 consecutive days, translating in a daily dose of 3.125 mg of the active compound. Control mice were treated similarly with a control vehicle cream (Vaseline Lanette cream; Fagron). To score the severity of inflammation of the back skin, an objective scoring system was developed based on the clinical Psoriasis Area and Severity Index (PASI), except that for the mouse model the affected skin area is not taken into account in the overall score. Erythema, scaling, and thickening were scored independently on a scale from 0 to 4: 0, none; 1, slight; 2, moderate; 3, marked; 4, very marked. The level of erythema was scored using a scoring table with red taints. The cumulative score (erythema plus scaling plus thickening) served as a measure of the severity of inflammation (scale 0–12). At the days indicated, the ear thickness of the right ear was measured in duplicate using a micrometer (Mitutoyo). The increase in ear thickness was used to indicate the extent of inflammation[16]. At the end of the study, all animals were euthanized.

**Table 2: Animal group**

<table>
<thead>
<tr>
<th>Groups (n=6)</th>
<th>Samples applied</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>Placebo gel (In-house developed)</td>
</tr>
<tr>
<td>Disease control</td>
<td>Imiquimod (3.125 mg/mice)</td>
</tr>
<tr>
<td>Drug Gel (In-house developed)</td>
<td>Imiquimod (3.125 mg/mice) + 0.1%w/w gel</td>
</tr>
<tr>
<td>Zorotene Gel (Marketed sample)</td>
<td>Imiquimod (3.125 mg/mice) + 0.1%w/w gel</td>
</tr>
<tr>
<td>NLC gel</td>
<td>Imiquimod (3.125 mg/mice) + 0.1%w/w gel</td>
</tr>
<tr>
<td>SLN gel</td>
<td>Imiquimod (3.125 mg/mice) + 0.1%w/w gel</td>
</tr>
</tbody>
</table>

Imiquimod applied topically for first 7 days. Later on treatment continued up to 21 days.

**Biochemical estimation**: Excise tissues were stored in formaline solution at minus 20° C until used for the estimation of hydroxyproline content. The tissue samples were hydrolyzed with 6N HCL for 3 hr at 130° C then after neutralized to pH 7.0. After that, tissue was subjected to chloramines-T oxidation for 20 min. after 5 min 2.5 ml each to ehrlich reagent was added[18].
And samples tube immersed to in a water bath at 60°C for 25 min. tubes were transferred to ice bath for cooling. Samples were stirred after addition of 6.6 ml Isopropyl alcohol [18]. At last samples were analyzed in 1 cm cuvettes by UV spectrophotometer at a wavelength 557 nm against control in which the solution being analyzed was replaced by distilled water [17, 18].

7. RESULTS AND DISCUSSION

Experimental Work – I

Optimization by Box Behnken Design:
There were 17 experimental run for optimizing the three parameters through BBD. The dressing of experiment and results were shown in Table 10. The variables Cutina GMS VPH : Cremophor EL (X1), Cutina GMS VPH : Drug ratio (X2) and PVA concentration (X3) were used as input to produce stable NLCs having optimized size (Y1), zeta potential (Y2) and %EE (Y3). Three parameters taken as dependent variables as shown in table 7 are important for storage and its performance after applying on skin [25]. Lesser the size (Y1) of formulation, it provides better contact time because of its larger surface area. Another advantage of occlusiveness due to lesser size of nanoparticles also increases its effectiveness. Higher value of zeta potential (Y2) provides stability. With PVA as surfactant, the zeta potential resulted in negative value. So more negative value is desirable for optimization. %EE is important parameter because the more the %EE, lesser amount of lipid and other excipients would be needed which can lead to cost cutting.

Table 3: Results of Box–Behnken design for optimization

<table>
<thead>
<tr>
<th>Sr No</th>
<th>Solid : Liquid Lipid</th>
<th>Lipid : Drug</th>
<th>% Surfactant</th>
<th>Size ± SD (nm)</th>
<th>Zeta Potential ± SD (mV)</th>
<th>%EE ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>11:1</td>
<td>1</td>
<td>295±09.41</td>
<td>-22.53±2.96</td>
<td>69.43±5.12</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>9:1</td>
<td>1.5</td>
<td>273±11.24</td>
<td>-40.14±4.26</td>
<td>60.25±6.43</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>13:1</td>
<td>1.5</td>
<td>266±05.49</td>
<td>-39.13±4.28</td>
<td>70.68±2.34</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>11:1</td>
<td>2</td>
<td>260±08.54</td>
<td>-50.11±4.62</td>
<td>65.13±6.27</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>11:1</td>
<td>1.5</td>
<td>225±05.49</td>
<td>-40.10±4.28</td>
<td>65.20±2.34</td>
</tr>
<tr>
<td>6</td>
<td>5.5</td>
<td>9:1</td>
<td>1</td>
<td>273±09.86</td>
<td>-28.14±2.75</td>
<td>58.21±2.65</td>
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<tr>
<td>7</td>
<td>5.5</td>
<td>13:1</td>
<td>1</td>
<td>285±05.49</td>
<td>-30.03±4.28</td>
<td>70.23±2.34</td>
</tr>
<tr>
<td>8</td>
<td>5.5</td>
<td>11:1</td>
<td>1.5</td>
<td>225±08.75</td>
<td>-40.10±2.65</td>
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<td>9</td>
<td>5.5</td>
<td>11:1</td>
<td>1.5</td>
<td>225±08.75</td>
<td>-40.10±2.65</td>
<td>65.20±7.53</td>
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<tr>
<td>10</td>
<td>5.5</td>
<td>11:1</td>
<td>1.5</td>
<td>225±08.75</td>
<td>-40.10±2.65</td>
<td>65.20±7.53</td>
</tr>
</tbody>
</table>
For all the dependent variables, summary is shown in Table. The selected design of experiment is Box-Behnken design which is a type of surface response design. The resulted 17 runs were randomized in order to remove any bias error of the model. The design model for the three factors was quadratic model which indicates complex relationship between input and the output variables.

**Design summary**

**Table 4: Summary of Design**

<table>
<thead>
<tr>
<th>Response</th>
<th>Units</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Mean</th>
<th>Std. Dev.</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size, R1</td>
<td>nm</td>
<td>219</td>
<td>295</td>
<td>246.941</td>
<td>25.0511</td>
<td>Quadratic</td>
</tr>
<tr>
<td>Zeta Potential, R2</td>
<td>mV</td>
<td>-50.11</td>
<td>-22.53</td>
<td>-39.4135</td>
<td>7.64596</td>
<td>Quadratic</td>
</tr>
<tr>
<td>Entrapment, R3</td>
<td>%</td>
<td>55.07</td>
<td>70.68</td>
<td>63.7571</td>
<td>4.46096</td>
<td>Quadratic</td>
</tr>
</tbody>
</table>

Fraction of design space (FDS) graph was generated at process sigma, $s = 1$ and $\alpha$ risk level of 0.05 as shown in Fig. 3. It displayed the area/volume of the design space having a mean std error less than or equal to a specified value. The ratio of the area/volume to total area/volume is the fraction of design space. This figure indicated that 37% of design space has a relative std error of less than 0.5 from the graph. It suggested that only 37% design space is precise enough to predict the response.
Figure 1: FDS graph
Mathematical model for Particle size: In terms of actual components, the inter-relation between independent and dependent variables was derived.

\[ Y_1, \text{Size} = +937.83 - 16.28 X_1 - 79.13 X_2 - 253.08 X_3 + 0.29 X_1X_2 + 0.57 X_1X_3 - 7.25 X_2X_3 + 0.55 X_1^2 + 3.99 X_2^2 + 98.81 X_3^2 \]

\[ \text{Equation (i)} \]

The equation indicated the complexity between the input and output variables. From the quadratic equation or its co-efficient one cannot draw a conclusion whether the effect of any independent variable is a positive or negative on the response. The interrelation between the dependent and independent variables is shown in 2D contour plot and 3D response surface curves.
**Figure 2: Response surface quadratic model for the Y1 (Size) Response(2D contour plot) and 3D Response surface curves**

From this 2D graphs for response Y1 – size, the effect of inter-relation between the independent variables can be seen. The effect of variable Solid:Liquid Lipid (X₁) and Lipid:Drug Ratio (X₂) on size (Y₁) can be interpreted from the contour lines as well as its 3D replica. From both the graphs, it is shown that the individual components may have statistically significant effect on Y1 but the interaction of X1 and X2 is not much as the colour coding for Y1 is varying between green to blue which is lower middle range for colour coding. But when the surfactant (X3) is changed either with Solid:Liquid Lipid (X1) or Lipid:Drug Ratio (X2), there is large variation in size as seen from the colour coding of contours for the selected input range. The contours are varying from the lower range of size (Y₁) 219 nm – blue colour to highest size (Y₁) – 295 nm. Thus we can conclude that the effect of X1 is highest for the response Y1 as seen from the ANOVA table and from the interaction terms highest effect is of X2X3.

**Statistical analysis for zeta potential (Y2):**

Mathematical model for Zeta Potential: In terms of actual components, the inter-relation between independent and dependent variables was derived.

Zeta Potential = 23.06 + 1.19 X₁ + 6.38 X₂ – 108.60 X₃ – 0.66 X₁X₂ + 5.35 X₁X₃ + 1.60 X₂X₃ – 0.21 X₁² – 0.16 X₂² + 9.25 X₃²  ……………………………Equation (ii)

The interrelation between the dependent and independent variables is shown in 2D contour plot and 3D response surface curves.
From this 2D graphs for response Y2 – zeta potential, the effect of interrelation between the independent variables can be seen.

The effect of variable Solid:Liquid Lipid (X1) and Lipid:Drug Ratio (X2) on zeta potential (Y2) can be interpreted from the contour lines as well as its 3D replica. From both the graphs, it is shown that the individual components may have statistically significant effect on Y2 but the interaction of X1 and X2 is not much as the colour coding for Y2 is varying between green to blue which is lower middle range for colour coding. But when the surfactant (X3) is changed either with Solid:Liquid Lipid (X1) or Lipid:Drug Ratio (X2), there is large variation in size as seen from the colour coding of contours for the selected input range. The contours are varying from the lower range of zeta to higher range and it is showing significant change. Thus, we can conclude that the effect of X3 is highest for the response Y2 as seen from the ANOVA table and even for the interaction terms where X3 is there i.e. X1X3 and X2X3, only that is showing major effect on zeta (Y2). So the change in zeta is the solitary effect of surfactant concentration.
Statistical analysis for %EE (Y3):

Mathematical model for %EE
In terms of actual components, the inter-relation between independent and dependent variables was derived.

\[
% \text{EE} = -63.40 - 1.49 X_1 + 20.23 X_2 + 17.42 X_3 - 0.051 X_1X_2 + 0.463 X_1X_3 - 1.24 X_2X_3 + 0.06X_1^2 - 0.71 X_2^2 - 3.48 X_3^2 \]

Equation (iii)

The interrelation between the dependent and independent variables is shown in 2D contour plot and 3D response surface curves.

Figure 4: 2D contour plot and 3D response surface curve for %EE

From this for response Y3 – %EE, the effect of interrelation between the independent variables can be seen. The effect of variable Solid: Liquid Lipid (X1) and Lipid: Drug Ratio (X2) on %EE (Y3) can be interpreted from the contour lines as well as its 3D replica. From both the graphs, it is shown that the interaction of X1X2 and X2X3 is having more effect than X1X3. When the X2 is changed either with Solid: Liquid Lipid (X1) or Surfactant (X3), there is large variation
in size as seen from the colour coding of contours for the selected input range. The contours are varying from the lower range of zet to higher range and it is showing significant change. Thus, we can conclude that the effect of X2 is highest for the response Y3 as seen from the ANOVA table and even for the interaction terms.

**Optimization using Desirability function**

To optimize the formulation variables, mathematical numerical approach was used rather than graphical. For numerical approach desirability function was used. The value of desirability varies between 0 to 1. The formulation should be prepared for which input parameters, highest desirability is obtained. There are no any standards stating that desirability should be near to 1. Because the value of desirability depends on the constraints applied to input and/or output variables. The more the constraints are applied the desirability value decreases. But on the contrary we are getting more optimized formulation having the desired characteristics. Because of this the design space generated may get constrained. But if the obtained design space is robust enough then it won’t affect the desired output. The constrained applied to the input and output variables are shown below in table 18.

**Table 5: Constraints applied on the variables**

<table>
<thead>
<tr>
<th>Name</th>
<th>Goal</th>
<th>Lower Limit</th>
<th>Upper Limit</th>
<th>Lower Weight</th>
<th>Upper Weight</th>
<th>Importance</th>
</tr>
</thead>
<tbody>
<tr>
<td>X1: Solid:Liquid Lipid</td>
<td>minimize</td>
<td>2</td>
<td>9</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>X2: Lipid:Drug</td>
<td>minimize</td>
<td>9</td>
<td>13</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>X3: %Surfactant</td>
<td>minimize</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Y1: Size</td>
<td>minimize</td>
<td>219</td>
<td>295</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Y2: Zeta Potential</td>
<td>minimize</td>
<td>-50.11</td>
<td>-22.53</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Y3: Entrapment</td>
<td>maximize</td>
<td>55.07</td>
<td>70.68</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

More is the liquid lipid, the structure of NLC will be more fluidic in nature leading to increase permeation and increased partition through the biological barrier. But as observed from the contour plots for size, higher is the solid lipid amount the lesser is the size. This is due to when more liquid lipid is added, it leads to distortedness in the final structure of NLCs leading to variation in Brownian motion of the NLCs in dispersing media. This leads to increase in size along with large values of PDI. But for its biological desired action lesser size with high permeation is desired. So, X1 was kept as minimize so as to incorporate less amount of solid lipid and Y1 was also kept minimize so as to get lesser size. The Factor X2 was kept as minimum, which will reduce the amount of lipid used for formulation. The X3 was kept low, because surfactant causes health hazard generally. Even though the range selected herein as per the GRAS limit for human consumption, but keeping patient health as priority, the X3 was kept minimal. Response Y1 (size) was kept minimum, as lower size is important for the increase surface area leading to increase contact to skin so the formulation can permeate. Response Y2 (zeta) was also kept minimum because it’s on negative side. Lesser it is, more stable is the formulation. The higher %EE (Y3) is obviously desired so as to incorporate as
much drug as possible. Based on the solution for the selected constraints the formulations were prepared (table 6).

**Table 6: Solution selected for the applied constraints.**

<table>
<thead>
<tr>
<th></th>
<th>Solid: Lipid</th>
<th>Liquid Lipid X1</th>
<th>Lipid: Drug Ratio X2</th>
<th>%Surfactant X3</th>
<th>Size Y1</th>
<th>Zeta Potential Y2</th>
<th>Entrapment Y3</th>
<th>Desirability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suggested</td>
<td>6.839</td>
<td>12.240</td>
<td>1.678</td>
<td>219.00</td>
<td>-43.981</td>
<td>65.136</td>
<td>0.795</td>
<td></td>
</tr>
<tr>
<td>Prepared</td>
<td>6.839</td>
<td>12.240</td>
<td>1.678</td>
<td>235.22±6.8</td>
<td>-40.53±3.7</td>
<td>62.72±6.7</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

*n=3

**Process variables optimization**

**Optimization of ultra turrax homogenization parameter:** As the Ultraturrex plays the important role in size reduction of NLCs, the stirring speed of the homogenization are the important [22]. The effect of RPM (11000, 13000 and 19000 rpm) and time (3, 5, 7 and 10 min) is shown in the table 17. As seen from the Table 20, as the ultraturrax speed has been increased the size of NLCs decreases, but there is no much difference in %EE of drug. The homogenization speed affects size only. As there is no significant difference when rpm were increased from 13000 to 19000, the speed was fixed to 13000 rpm. At constant rpm of 13000, the time was varied for homogenization. There is no much change in size after increasing the time from 5 to 7 min. So, based on this OVAT approach for optimization of ultraturrax speed and time, 13000 rpm and 5 min were selected.

**Table 7: Optimization of homogenization rpm and time of rotation.**

<table>
<thead>
<tr>
<th>Batch no.</th>
<th>Ultraturrex rpm</th>
<th>Time (min)</th>
<th>Size (nm) ±S.D*</th>
<th>PDI±S.D*</th>
<th>%EE±S.D*</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>11,000</td>
<td>5</td>
<td>271±0.332</td>
<td>0.854±0.03</td>
<td>60.34±0.34</td>
</tr>
<tr>
<td>02</td>
<td>13,000</td>
<td>5</td>
<td>238±0.543</td>
<td>0.342±0.01</td>
<td>62.83±0.13</td>
</tr>
<tr>
<td>03</td>
<td>19,000</td>
<td>5</td>
<td>235±0.21</td>
<td>0.432±0.03</td>
<td>63.60±0.52</td>
</tr>
<tr>
<td>04</td>
<td>13,000</td>
<td>3</td>
<td>aggregated particles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>05</td>
<td>13,000</td>
<td>5</td>
<td>237±0.414</td>
<td>0.332±0.01</td>
<td>63.13±0.13</td>
</tr>
<tr>
<td>06</td>
<td>13,000</td>
<td>7</td>
<td>234±0.212</td>
<td>0.413±0.08</td>
<td>67.11±0.84</td>
</tr>
<tr>
<td>07</td>
<td>13,000</td>
<td>10</td>
<td>231±0.887</td>
<td>0.321±0.05</td>
<td>66.88±1.45</td>
</tr>
</tbody>
</table>

**Optimization of sonication parameter:**

Sonication parameters such as amplitude and time of sonication were optimized at constant input variables as obtained from BBD. Different amplitude 10%, 30%, 50% and 70% were tried for 1, 3, 5 and 7 min. The cycle for sonication was kept constant at 0.5. The Table 21 shows optimization of sonication by OVAT approach. At constant input variables, at 30% amplitude, the time required for desired size is 5 min sonication as shown in Table 18. Increasing amplitude to 50% gives size reduction but comparatively it is not showing significant difference from 30%. Increasing it to 70% leads to decreased size at the cost of %EE. So, 30% amplitude was optimized [23,24]. The sonication time was found to be 5 min as optimized as it gives less size with higher %EE.
Table 8: Optimization of Sonication parameter

<table>
<thead>
<tr>
<th>Amplitude</th>
<th>Time</th>
<th>Size (nm) ±S.D*</th>
<th>PDI±S.D*</th>
<th>% EE±S.D*</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>5</td>
<td>330±1.542</td>
<td>0.942±0.30</td>
<td>67.31±0.12</td>
</tr>
<tr>
<td>30</td>
<td>5</td>
<td>239±0.112</td>
<td>0.491±0.01</td>
<td>61.73±0.13</td>
</tr>
<tr>
<td>50</td>
<td>5</td>
<td>237±0.744</td>
<td>0.422±0.08</td>
<td>62.02±0.64</td>
</tr>
<tr>
<td>70</td>
<td>5</td>
<td>232±0.645</td>
<td>0.442±0.63</td>
<td>58.33±0.15</td>
</tr>
<tr>
<td>30</td>
<td>1</td>
<td>visible aggregates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>3</td>
<td>310±0.611</td>
<td>0.512±0.00</td>
<td>62.83±0.64</td>
</tr>
<tr>
<td>30</td>
<td>5</td>
<td>231±0.543</td>
<td>0.112±0.01</td>
<td>64.1±0.13</td>
</tr>
<tr>
<td>30</td>
<td>7</td>
<td>229±1.343</td>
<td>0.521±0.02</td>
<td>66.49±0.54</td>
</tr>
</tbody>
</table>

Table 9: Physicochemical property of the NLCs/SLNS

<table>
<thead>
<tr>
<th>NLC DISPERSION</th>
<th>PROPERTY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colour</td>
<td>White dispersion</td>
</tr>
<tr>
<td>Odour</td>
<td>Odourless</td>
</tr>
<tr>
<td>Stability on centrifugation (200 g for 20 minutes)</td>
<td>Stable on centrifugation</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SLN DISPERSION</th>
<th>PROPERTY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colour</td>
<td>White dispersion</td>
</tr>
<tr>
<td>Odour</td>
<td>Odourless</td>
</tr>
<tr>
<td>Stability on centrifugation (200 g for 20 minutes)</td>
<td>Stable on centrifugation</td>
</tr>
</tbody>
</table>

Particle Size Analysis: Result of particle size analysis of SLN and NLCs as measured by the zeta sizer, it is observed that size of NLCs is comparatively more than SLNs. The liquid lipid present in NLCs may have contributed to the larger size but the size is in nano scale. Here both SLNs and NLCs are in nano range which will provide better occlusion and interaction with skin for increased penetration. The PDI in the range of 0.2 – 0.3 indicated that the samples were monodispersed in nature.

Table 10: Particle Size and Zeta Potential of SLN and NLCs as measured by the Zeta Sizer.

<table>
<thead>
<tr>
<th>Mean Particle size of SLN</th>
<th>Mean Particle size of NLCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-average (nm)</td>
<td>Z-average (nm)</td>
</tr>
<tr>
<td>PDI</td>
<td>PDI</td>
</tr>
<tr>
<td>Zeta potential (mV)</td>
<td>Zeta potential (mV)</td>
</tr>
<tr>
<td>215.2±4.5</td>
<td>235.22±6.8</td>
</tr>
<tr>
<td>0.272±0.08</td>
<td>0.242±0.09</td>
</tr>
<tr>
<td>-37.8±3.4</td>
<td>-39.8±3.7</td>
</tr>
</tbody>
</table>
Zeta Potential: Zeta potential is a key factor to evaluate the stability of colloidal dispersion. In general, zeta values near to ±30 mV indicate that the SLNs give good stability in dispersion due to less chance of cohesion force between particles. As shown in Fig 16 and 17, zeta potential of optimized batch for SLNs and NLCs were -37.8 ± 3.2 mV and -39.8 ± 2.4 mV respectively, nearer to ±30mV. This indicates that the dispersions of NLC and SLN will remain deflocculated and would be physically stable [23].
Assay and % Drug Entrapment
Assay for either the SLNs or NLCs was found to be in the range of 97 to 98%. The loss may be due to processing steps involving transfer of the samples from one to another beaker or stirring.

% Drug entrapment for SLNentrts was in the range of 55 – 63 % whereas for NLCs it was 60 - 65%. For SLNs less % EE was observed. The drug shows more solubility in liquid lipid than solid lipid. This might be the reason for high % EE in NLC than SLN.
**Occlusion Test**

The occlusion effect is one of the major effects responsible for penetration enhancement by lipid based nanoparticles [25]. The occlusion will increase the hydration of the skin and due to hydration, the penetration enhancement is achieved [18]. NLC have recently been investigated as carriers for enhanced skin delivery of sunscreens, vitamins A and E, triptolide and glucocorticoids [16]. It is thought their enhanced skin penetration is primarily due to an increase in skin hydration caused by the occlusive film formed on the skin surface by the NLC/SLN. A 31% increase in skin hydration has been reported following 4 weeks application of lipid nanoparticles enriched cream.

The Occlusion Factor for NLC Dispersion SLN Dispersion, Gel incorporating plain Drug and Gel incorporating NLC/SLN dispersion is shown in Table 2. An occlusion factor of zero means no occlusive effect compared with the control, and 100 is the maximum occlusion factor [18].

![Occlusion Test Graph](image)

**Figure 3: Occlusion Test**

As we can observe from results that the Gel incorporating NLC dispersion showed highest occlusion factor. The gel incorporating plain Drug showed significantly less occlusion factor therefore with comparison to Gel incorporating NLC/SLN dispersion, it showed less hydration and less penetration enhancement.

**Evaluation of Gel**

The pH of the gel containing either SLN or NLC formulation was found to be in the range of 6.3 to 6.8 which is much related to skin pH. So, the applied gel would not cause any irritation or discomfort to patient. Initially the gel formed with Carbopol showed acidic pH around 4 – 4.5. This is not suitable for application to skin. The consistency of that swelled dispersion was less rigid having less viscosity. After neutralization with triethanolamine the gel become rigid and pH of the gel is near to neutral. The hydration of Carbopol molecule when dispersed in water leads to partial uncoiling of every molecule. When this acidic Carbopol molecules were neutralized with basic TEA, it converted to salt from its acidic property. This leads to thickening of the Carbopol and provided the desired pH for skin application. The assay of the drug in gel was found to be in the range of 96 – 98%, which is in acceptable limit. To draw a
conclusion on homogeneity of the gel sample, appearance and assay was checked. For assay three samples taken from different area of container were compared. The sample was homogenous in nature and non-gritty. The assay for all samples were in the acceptable range.

The viscosity of the NLC Gels and SLN Gels was found 29,235 cp and 29,789 cps at 25° C respectively. This viscosity favors the extrusion of the gel from the collapsible tube container as well as easy handling by patient during use.

**In – vitro Drug Release**

![Graph showing drug release over time](image)

**Figure 10: In-vitro Drug Release**

The In vitro release is the important parameter which can be utilize to compare the release characteristic of the product in vivo by finding appropriate correlation and also to study batch to batch uniformity. The release characteristics of the plain Drug, NLC formulation, Gel containing plain Drug and the Gel containing NLCs are shown in Table 25. The plain Drug suspension shows slower release as the drug is not having sufficient solubility in dissolution media. The drug being BCS class II in nature, the dissolution is the rate limiting process for its absorption. Increasing solubility in dissolution media using this lipid based formulation technique either SLN or its modified version of NLCs led to increase in vitro dissolution as can be seen from Fig. 19. The increased dissolution rate may be due to increase solubility of drug by lipid and surfactant used. The added advantage of nano-sizing led to increased solubility. When the Drug / SLN / NLC is incorporated in the carbopol 980 NF Gel, it will hinder the release of the Drug as it has to diffuse through the Gel matrix [17]. So, the drug release in gel at the end of 24 h is lesser than the pure drug or formulation in SLN/NLC form. Initial high release from SLN/NLC may be due to burst release or due to the drug present on peripheral portion or entrapped drug[20]. But when added in gel this burst release is also controlled and the diffusion of drug occurred on controlled manner for up to 24 h [17].

**Stability study**:

Stability result are shown in Table 27. There was not significant change in Appearance, pH, viscosity and assay observed during 6 months study period. Thus, it can be concluded that
formulation have good physical stability when stored at LT stability study (25° C ± 2° C, 60% RH ± 5% RH) for 6 month study period.

Table 11 Effect of storage time and conditions on Physicochemical parameters of NLCs and SLNs loaded gel

<table>
<thead>
<tr>
<th>Sampling time: NLCs loaded gel</th>
<th>Storage condition LT stability study (25° C ± 2° C, 60% RH ± 5% RH)</th>
<th>Appearance</th>
<th>pH</th>
<th>Viscosity</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td></td>
<td>Smooth, off white, odourless</td>
<td>6.4 ±0.3</td>
<td>29,235 cps</td>
<td>98% ± 1.2%</td>
</tr>
<tr>
<td>After 1 Month</td>
<td></td>
<td>Smooth, off white, odourless</td>
<td>6.3 ±0.1</td>
<td>28,652 cps</td>
<td>97.5% ± 1.2%</td>
</tr>
<tr>
<td>After 2 Month</td>
<td></td>
<td>Smooth, off white, odourless</td>
<td>6.5± 0.2</td>
<td>31,974 cps</td>
<td>98% ± 1.2%</td>
</tr>
<tr>
<td>After 3 Month</td>
<td></td>
<td>Smooth, off white, odourless</td>
<td>6.3±0.2</td>
<td>27,168 cps</td>
<td>97% ± 1.2%</td>
</tr>
<tr>
<td>After 6 Month</td>
<td></td>
<td>Smooth, off white, odourless</td>
<td>6.6 ±0.2</td>
<td>28,634 5 cps</td>
<td>98% ± 1.2%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SLNs loaded gel</th>
<th>Appearance</th>
<th>pH</th>
<th>Viscosity</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>Smooth, off white, odourless</td>
<td>6.5 ±0.1</td>
<td>28,789 cps</td>
<td>98% ± 0.8%</td>
</tr>
<tr>
<td>After 1 Month</td>
<td>Smooth, off white, odourless</td>
<td>6.4±0.2</td>
<td>31,435 cps</td>
<td>97.5% ± 1.5%</td>
</tr>
<tr>
<td>After 2 Month</td>
<td>Smooth, off white, odourless</td>
<td>6.5±0.3</td>
<td>29,219 cps</td>
<td>98.1% ± 0.5%</td>
</tr>
<tr>
<td>After 3 Month</td>
<td>Smooth, off white, odourless</td>
<td>6.4±0.1</td>
<td>28,391 cps</td>
<td>97.6% ± 1.1%</td>
</tr>
<tr>
<td>After 6 Month</td>
<td>Smooth, off white, odourless</td>
<td>6.5±0.2</td>
<td>31,614 cps</td>
<td>96.9% ± 0.6%</td>
</tr>
</tbody>
</table>
Cyto-toxicity Assay:

As shown in Fig. 1, the cell viability was in the range of 93-100%. At higher concentration of SLN/NLC, the cell viability was less. This result when compared with pure drug, the only reason for decrease in % viability may be presence of surfactant. This surfactant molecules at higher concentration may have led to irreversible changes in cell that lead to apoptosis of cells. This in vitro cell toxicity study develops basis for in vivo or ex vivo study. From this %viability study, it was concluded that the formulation is safe for external use. On this basis, the formulation was carried forward for the ex-vivo and in-vivo study.

Animal studies:
In Acute dermal toxicity study, sample topically applied at a dose of 1% w/w gel. After 14 days observation, all animals were normal and there were no changes in fur, eyes, and behavior of treated animals. There was no toxic reactions like inflammation redness, erthyma observed on skin. Hence, formulation was found to be safe for topical application[14].

The result of the topical formulation on right ear thickness are given in Fig. 3. The maximum increase in ear thickness in the IMQ-treated group on day 7. After 7 day all group were daily treated as per group and further ear thickness was measured using micrometer regularly[14]. The maximum increase in ear thickness in the test groups was significant decrease on day 24 compare to other group II, III, IV showed no significant changes during the experiment.
The result of the topical formulations on Hydroxyproline content are shown in figure. The biochemical evaluation revelled no significant reduction in hydroxyproline content in treated group. The hydroxyproline content of the test group V and VI was found to be almost equal which was higher than that of control group and standard groups tissues. An increase in collagen catabolism and regeneration is an important link in the pathogenesis of many diseases[21]. Twelve fourteen percent of all amino acid residues of collagen are hydroxyproline[22]. Collagen is major constituent of the dermis, and is therefore involved in many facets of skin disease including psoriasis as well as the recovery process[36].
The results of macroscopic observation in animals after topical application of formulations are shown in below given Table. Severity index was calculated by observing the psoriatic lesion by naked eye[24]. Result of %Relative epidermal thickness in both test group V (NLC gel) and Group VI (SLN gel) were 56.38% and 51.28% respectively which was higher compare to Group III (36.82%), group IV (40.24%) but lesser that that of Normal control group (96.34%) which indicate good anti-psoriatic strength of the formulation. There was also less severity index observed in test (Group V and Group VI) compare to Drug Gel formulation and marketed formulation treated animal group.

Table No. 12: Macroscopic observation in animals

<table>
<thead>
<tr>
<th>Group</th>
<th>% Relative Epidermal thickness (μm)#</th>
<th>Psoriasis Area and Severity Index (PASI)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Erythema</td>
</tr>
<tr>
<td>Group I (Normal control)</td>
<td>96.34</td>
<td>0</td>
</tr>
<tr>
<td>Group II (Disease control)</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>Group III (Drug Gel-In-house developed)</td>
<td>38.62</td>
<td>3</td>
</tr>
<tr>
<td>Group IV (Zorotene Gel)</td>
<td>40.24</td>
<td>3</td>
</tr>
<tr>
<td>Group V (NLC gel)</td>
<td>56.38</td>
<td>2</td>
</tr>
<tr>
<td>Group VI (SLN gel)</td>
<td>51.28</td>
<td>2</td>
</tr>
</tbody>
</table>

#The % are calculated with reference to Disease control group.
*PASI: 0 – none, 1 – slight, 2 – moderate, 3 – marked, 4 – very marked

8. ACHIEVEMENTS WITH RESPECT TO OBJECTIVES
Dry nasal powder and thermoreversible in situ gel with mucoadhesive polymer was increased drug residence time in nasal cavity so increase absorption of drug. By selection of intranasal route of delivery, the first pass metabolism of drug was avoided. Dry nasal powder was improved the stability of peptide hormone desmopressin acetate due to its dry form. By selecting intranasal route, drug was targeted directly to the brain through the olfactory region.

9. CONCLUSION
From the experimental data it can be concluded that the developed formulation of tazarotine is effective in treatment of psoriasis. The lipid nanocarriers incorporated in the gel show retention at the site of application due to enhanced interaction with the skin with sustained release at the site of application with negligible toxicity which is desirable characteristic of the formulation. The future aspect of the developed formulation should be commercial scale up once the safety and efficacy is confirmed from clinical trials.
10. REFERENCES


35. Mahant, S., R. Rao, and S. Nanda, Chapter 3 - Nanostructured lipid carriers: Revolutionizing skin care and topical therapeutics, in Design of Nanostructures for