ABSTRACT

Objective: Formulation and characterization of clobetasol propionate (CP) and pramoxine hydrochloride (PH) loaded nanostructured lipid carriers (NLC) offering improved performance in terms of drug loading and long-term stability for topical drug delivery.

Methods: Drug-loaded NLC formulation was designed by melt-emulsification ultrasonication technique, by fluctuating the concentration of stearic acid and oleic acid. Poloxamer F68 and tween 80 were used as surfactants in the formulation and soya lecithin was used as stabilizer and co-surfactant. Differential scanning calorimetry (DLS), scanning electron microscopic studies (SEM), transmission electron microscopy (TEM), fourier transform infrared spectroscopy (FT-IR), differential scanning calorimetry (DSC), x-ray diffraction (XRD), are the techniques used to characterize the preparations. Optimized drug-loaded formulations were evaluated for particle size, zeta potential, entrapment efficiency, in vitro drug release, hemocompatibility assay and cytotoxicity screening.

Results: For drug loaded formulation the particle size was found in nanometric range. In vitro drug release was carried out using dialysis membrane and drug release after 24h was found to be 90.98±1.89 for CP and 79.81±4.20 for PH.

Conclusion: The formulated NLC is a potential approach for sustained release of drug which may reduce systemic side effects, increase skin retention time and duration of action. Further in vivo studies will confirm the effect of NLC to increase skin retention time, decreases systemic absorption of the corticosteroid thereby avoiding side effects.

Keywords: Clobetasol propionate, Pramoxine hydrochloride, NLC, Melt-emulsification ultrasonication method, Atopic dermatitis

INTRODUCTION

Atopic dermatitis (AD) is a highly pruritic chronic inflammatory skin disease that commonly presents during early childhood [1] and is often thought as synonymous with eczema. In several cases, atopic dermatitis is the first manifestation of atopy and is frequently associated with personal and family history of respiratory allergy and have profound effects on patient's lives, career, choices and social interactions [2]. The prevalence of AD has increased exponentially over the past few decades in industrialized nations affecting 15% to 30% and 2% to 10% of children and adults, respectively [3]. Among individuals infected with AD, up to 60% of males and 55% of females are diagnosed under the age of 1 and less than 2% of new cases occur after the age of 20 [4].

Nanostructured lipid carriers (NLC) are the second generation SLN composed of solid lipid matrix which are incorporated with liquid lipids [5]. The small size of the lipid particles ensures close contact to stratum corneum (SC) and can increase the amount of drug penetrating into mucosa or skin. Due to their solid lipid matrix, a controlled release from these carriers is possible. This becomes an important tool when it is necessary to supply the drug over a prolonged period of time, to reduce systemic absorption, and when drug produces irritation in high concentrations [6]. NLCs have a higher loading capacity for a number of active compounds, and the potential expulsion of active compounds during storage is minimized [7]. Because of the small size of NLCs, close contact with the SC is guaranteed and the amount of the drug penetrated into the skin can be increased [8].

Corticosteroids have been successfully employed for the treatment of several inflammatory skin conditions. Their clinical efficacy is associated with drug skin retention; however, they can permeate the SC, reaching deeper skin layers, which may cause local and systemic side effects. CP is a super-potent topical corticosteroid. Skin atrophy, skin infections and hypothalamic-pituitary-adrenal (HPA) axis suppression are some of the possible side effects of topical corticosteroids. Improvements in the safety of topical corticosteroid therapy may be achieved by drug nanoencapsulation, which can increase the drug’s accumulation in the skin while minimizing deep drug penetration and adverse systemic effects [9]. In the present work, CP and PH were encapsulated in NLCs to increase drug retention in the outer skin layers and avoid systemic absorption. PH is a local anaesthetic that will help to reduce the itching sensation, currently, there are no combinational dosage form of these two drugs available in the market.

MATERIALS AND METHODS

Materials

CP was gifted by mahima life sciences, haryana, India. PH was purchased from sigma-aldrich Co. LLC. Stearic acid was obtained from nice chemicals; kochi. Oleic acid is obtained from loba chem. Pvt. Ltd. mumbai. Poloxamer F68 was shipped from research-lab fine chem industries mumbai, India and Tween 80 was procured from nice chemicals, kochi. Soya lecithin is obtained from hi media laboratories Pvt. Ltd. mumbai, India. Millipore water was used during the course of the study.

Preformulation studies

The melting point of CP and PH (both are API) were determined by open capillary tube method. Methanol, dimethyl sulphoxide (DMSO), diethyl ether and ethanol are the organic solvents used to determine the solubility of pure drugs by the shake flask method reported as [10]. Solution of both drugs ranging from 2-10 µg/ml were scanned from 200-400 nm using a UV-visible spectrophotometer, support to define the absorption maxima (\(\lambda_{max}\)) Partition coefficient was done by equal millilitre of N-octanol and water in separating funnel. 100 mg of the drug was added to it, which is then equilibrated for 2h at a constant temperature with intermittent shaking at regular intervals and separate the aqueous layer and organic layer. Collect 1 ml of the aqueous layer and the concentration of drug in it were determined at 240 nm by UV spectrophotometer. The same procedure was repeated for PH and the concentration of drug in it was determined at 224 nm by UV Spectrophotometer [10].
Preparation of clobetasol and pramoxine loaded NLC

Nine different batches of formulations were prepared by melt-emulsification ultra-sonication method (fig. 1) by varying the concentration of lipids and surfactants (table 1). In brief, the required amount of solid lipid and liquid lipid was occupied in lipid phase and amount of surfactants, co-surfactants and stabilizer were taken in the aqueous phase. Accurately weighed the amount of drug is added to lipid phase and both phases were heated separately at 80 °C for 20 min. Then the aqueous phase was added drop by drop to the lipid phase upon magnetic stirring at 750 rpm, maintaining a temperature above 80 °C. This primary emulsion is then probe sonicated for 15 min under 70% amplitude and 6-2 pulse immediately cooled in ice water bath for the formation of NLC [11].

Table 1: Composition of ingredients used for formulating the nano lipid carrier

<table>
<thead>
<tr>
<th>Trials</th>
<th>Stearic acid (mg)</th>
<th>Oleic acid (ml)</th>
<th>Poloxamer F 68 (mg)</th>
<th>Tween 80 (ml)</th>
<th>Soya lecithin (mg)</th>
<th>Distilled water (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>280</td>
<td>120</td>
<td>150</td>
<td>-</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>700</td>
<td>300</td>
<td>150</td>
<td>1.5</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>500</td>
<td>300</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>20</td>
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<tr>
<td>4</td>
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<tr>
<td>5</td>
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<td>2</td>
<td>160</td>
<td>1</td>
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<td>40</td>
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<td>6</td>
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<td>3</td>
<td>380</td>
<td>0.4</td>
<td>375</td>
<td>50</td>
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<tr>
<td>7</td>
<td>1750</td>
<td>3</td>
<td>375</td>
<td>0.4</td>
<td>375</td>
<td>50</td>
</tr>
<tr>
<td>8</td>
<td>1750</td>
<td>3</td>
<td>500</td>
<td>0.5</td>
<td>375</td>
<td>50</td>
</tr>
<tr>
<td>9</td>
<td>1750</td>
<td>3</td>
<td>500</td>
<td>0.5</td>
<td>425</td>
<td>50</td>
</tr>
</tbody>
</table>

Characterization of optimized drug loaded NLC

Dynamic light scattering (DLS)

For determining the particle size and zeta potential, DLS method with a computerized system of nano zeta sizer was employed [12].

Scanning electron microscopy (SEM)

SEM was utilized to analyze the morphological characteristics of the drug carrier system and the formulations using scanning electron microscope (VEGA3 TESCAN) at an accelerated voltage of 20KV at x1000 and x10000 magnifications [12].

Transmission electron microscopy (TEM)

Morphological characters were analysed with transmission electron microscopy technique by using negative staining method. About 5 µl of the stained sample was placed on the grid crusted with copper followed by removing the spare amount of stains, air dried in room temperature and images were taken [13].

Fourier transform infrared spectroscopy (FTIR)

FTIR, a qualitative analyzer used to characterize the properties of each agent in the formulation. It was conducted by using a perkin elmer FTIR (USA) instrument and ranging from 4000-500 cm⁻¹ the dried sample which was placed in sample holder were scanned [13].

Differential scanning colorimetry (DSC)

The DSC thermogram of various samples were carried out by weighing the sample about 5 mg, sealed in an aluminum pan of 40 µl capacity and equilibrated at 25 °C were subjected to DSC run over a temperature range 10-325 °C for heating rate of 10 °C/minute in an inert nitrogen gas atmosphere [14].

X-ray diffraction study (XRD)

The X-ray diffraction patterns were recorded, under CuKα monochromatised radiation, voltage 40 KV and current of 20 mA at ambient temperature. The data of pure drugs and drug-loaded nano lipid carriers were collected in the continuous scan mode from 2°–4° (2θ) at an angular increment of 0.02°/sec and count time of 1 sec/step [15].

Entrapment efficiency (EE)

Entrapment efficiency gives an idea about the percentage of the drug that is successfully entrapped/adsorbed into nanoparticles. For ultracentrifugation method [16], 500 µl of drug contained sample was filled in the ultra-centrifugal filter unit’s Hermle labortecnik GmbH (Germany). It was running at 10,000 rpm for 30 min in a centrifuge, the liquid in the topmost layer was diluted with methanol and scanned in shimadzu uv-spectrophotometer for the quantification of the drug.
**In vitro release study**

Dialysis membrane technique is used for in vitro drug release study. The prepared NLC (2-5 ml) is introduced dialysis membrane (pore size 0.2 µm) which is hermetically sealed in one end of the open-end tube. This tube was then immersed in the 30 ml solution of phosphate buffer solution (PBS) 5.5 and placed in the orbital shaking incubator set at temperature 37 °C (to simulate the human skin condition) and at 50 rpm. At predetermined intervals of 1, 2, 4, 6, 10, 12 and 24 h, aliquots of 1 ml were withdrawn and replaced with an equal volume of fresh buffer solution. These samples were made up to 10 ml using PBS 5.5 and the absorbance was measured using UV spectrophotometer at 240 and 224 nm. The amount of drug released was calculated and the percentage drug released was plotted against time [17].

**Kinetic modeling of drug release**

The data obtained from the drug release studies of the NLC formulations were fitted to various models such as Zero order, First order, Higuchi–peppas model and Hixson-crowell cube root law to ascertain the kinetic modelling of drug release [18]. The plots were drawn using the computational system and the regression equations were obtained for each plot. The correlation coefficient values (r^2) of the plots were obtained. The model with the highest correlation coefficient (r^2 value approaching unity) was chosen as the best fit model.

**In vitro haemocompatibility study**

5 ml of blood was immediately collected in an EDTA coated vacutainer (to prevent clotting), then centrifuged at 15,000 rpm for 15 min to separate RBC and plasma. After that RBC were diluted with normal saline in ratio 1:9 for further studies. The different concentration of drug loaded NLC ranging from 20-80µg/ml were added to 500 µl diluted blood and made up to 1 ml with normal saline, incubated for 24h. 0.1% Triton treated blood sample was used as positive control and normal saline as negative control. After incubation, centrifuged at 3500 rpm for 10 min at 4 °C. From the above solution, 100 µl supernatant was collected and absorbance measured at 540 nm [19].

**MTT assay**

MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium] assay was used to access the cytocompatibility and cytotoxicity of the prepared NLC formulation. 1029 (fibroblast) cell line was purchased from the national centre for cell science (NCCS) pune the prepared NLC formulation. L929 (fibroblast) cell line was cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% fetal bovine serum, L-glutamine, sodium bicarbonate and an antibiotic solution containing Penicillin (100U/ml), streptomycin (100µg/ml) and amphotericin B (2.5µg/ml). Cultured cell lines were kept at 37 °C in a humidified 5% CO2 incubator (NBS Eppendorf, Germany). The viability of cells was evaluated by direct observation of cells by inverted phase contrast microscope and followed by MTT assay method [20].

**In vitro skin permeation studies**

Porcine ear skin obtained from a local abattoir was used to conduct in vitro skin permeation procedure. Raw skin was cleaned in cold running water followed by removing the hair and fats by using scalpels. The portion corresponding to the penetration region was stamped out, frozen at -20 °C and kept for a period up to 3 m. The experiment was conducted by Franz-diffusion cell [21], the skin sample were sandwiched between receptor slot containing 7 ml of phosphate buffer (pH 5.5) and an optimized formulation containing donor compartment. It was uniformly stirred at 100 rpm maintained at 33±2 °C. Withdraw the sample from the receptor compartment at predetermined intervals and replaced the same amount with buffer and triplicate the experiment.

**Stability studies**

The optimized NLC formulation was stored at room temperature (at 25 °C), refrigerator temperature (4-8 °c) and accelerated condition (40±2 °C, 75±5% RH) for a period of 3 m. The colour, appearance and odor were examined in every 30 d for determining physical stability. Then the formulation was estimated using for particle size (PS) and zeta potential [22].

**RESULTS AND DISCUSSION**

**Preformulation studies**

Preformulation studies were done for confirming the identity, purity and to establish a suitable drug profile. The melting point of the CP (A) and PH (B) was found to be 195 °C and 182 °C respectively, equivalent with the monograph value. The solubility of the drugs was determined in different solvents such as methanol, DMSO, diethyl ether, ethanol (table 2). From the result, it is clearly evident that both drugs are freely soluble [96.662 mg/ml and 93 mg/ml] in DMSO. CP showed less solubility in water whereas PH showed more solubility as compared with CP.

The X_max of the drug A and B was determined in methanol and phosphate buffer having pH 5.5 was found to be 240 nm and 224 nm respectively was in accordance with official standards. Log P value of both drugs was found to be nearly 3, any compounds with a log p-value greater than 2 indicate the lipophilicity of chemical agents.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Amount of drug (A) dissolved (mg/ml)</th>
<th>Amount of drug (B) dissolved (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>0.70</td>
<td>66</td>
</tr>
<tr>
<td>Ethanol</td>
<td>11.31</td>
<td>66</td>
</tr>
<tr>
<td>Methanol</td>
<td>20.64</td>
<td>66</td>
</tr>
<tr>
<td>DMSO</td>
<td>96.62</td>
<td>93</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>3.26</td>
<td>66 mg/ml</td>
</tr>
<tr>
<td>pH buffer 5.5</td>
<td>2.64</td>
<td>53 mg/ml</td>
</tr>
</tbody>
</table>

**Table 2: Solubility profile of CP (A) and PH (B)**

**Characterization of optimized formulation**

**Particle size, particle size distribution, and zeta potential**

The plain NLC formulation shows lowest particle size (68.49± 17.59 nm) and the drug-loaded NLC formulation shows a particle size of 86.7±7.48 nm, a single narrow peak can be seen in the fig 2A and 2C. The value of polydispersity index (PDI) value was found to be 0.473 which is below 1.0, suggesting that the optimized NLC formulation having particle size in nano range which remains stable and will not convert macro range particles and suggesting that the NLC was monodispersed PDI is a measure of particle homogeneity and it varies from 0.0 to 1.0. When the value of polydispersity index is closer to zero, more homogenous the particles are. Many papers have shown that zeta potential (fig: 2B and 2D) influences the stability of NLC, the high value of zeta potential indicates electrostatic repulsion between two particles of the optimized formulation [23].

DLVO theory states that electric double layer repulsion will stabilize NLC and aggregation is not expected to take place, due to the highly negative charge of particles. In this study, by increasing the concentration of lipids helps to achieve the desired particle size ie.,<100 nm with single narrow peak and stable zeta value.
Scanning electron microscopic studies

SEM analysis was also performed to obtain more information about the particle size and shape. The SEM microphotographs of pure drugs CP, PH and drugs loaded NLC are shown in fig. 3A, 3B and 3C respectively. Fig. 3A and 3B disclose the irregular crystalline nature of pure drugs, fig. 3C shows spherical morphology and the size of the NLCs was found within the nanometer range. SEM also revealed the agglomeration of nanoparticles which might be due to the lipid nature of the carrier and the drying process during sample preparation prior to SEM analysis.

Fig. 2(A) and (C): Particle size of optimized plain and drug-loaded NLC (B) and (D) zeta potential of optimized plain and drug-loaded NLC

Fig. 3A: SEM image of CP

Fig. 3B: SEM image of PH

Fig. 3C: SEM image of drug loaded NLC
Transmission electron microscopy (TEM)
The surface morphology and mean diameter of optimized NLC formulation was visualized using TEM. The observed diameters of particles were found to be consistent with the particle size obtained in DLS i.e. 96.97 nm (fig. 4). Developed lipid nanoparticles possessed spherical and smooth surface. By using the TEM result the particle size was confirmed.

Fourier transform infrared (FTIR) spectroscopy
The peaks were found in accordance with the monograph (I. P). The principle absorption peaks of CP were at 3304 cm\(^{-1}\) (aromatic C-H), 2954 cm\(^{-1}\) (aliphatic C-H), 1675 cm\(^{-1}\) (aldehyde C=O stretch), 1570 cm\(^{-1}\) (C=C stretch). PH was at 2937 cm\(^{-1}\) (aliphatic C-H stretch) 3258 cm\(^{-1}\) (aromatic C-H stretch). The FTIR Spectrum of clobetasol-pramoxine-loaded NLC is shown in fig. 5, the IR spectra showed characteristic peaks at 3462.89 cm\(^{-1}\) (aromatic C-H stretching), above 3000 cm\(^{-1}\) broad peak (O-H stretch), 2855.23 cm\(^{-1}\), 2923.42 cm\(^{-1}\) (aliphatic C-H–stretching), 1639.42 cm\(^{-1}\) (C=O–stretching), 1099.12 cm\(^{-1}\) (C-O stretch), 582.92 cm\(^{-1}\) (C-F-stretching). All the characteristic peaks for both the drugs were found to be present in the NLC indicating compatibility of the drug with excipients.
Differential scanning calorimetry (DSC) analysis

The results of the DSC analysis of CP, PH, plain NLC and the drug-loaded NLC formulation are shown in fig. 6. The DSC thermogram of CP showed an exothermic peak at 196 °C, which is the reported melting point of the CP (fig. 6A) and the DSC thermogram of PH showed an exothermic peak at 171-181 °C, which is the reported melting point of the PH (fig. 6B). Drug-loaded NLC showed a large endothermic peak at 91.19 °C. It is observed from the DSC thermogram that the exothermic peak of pure drugs at about 196 °C and 181 °C no longer exists in the DSC traces of the drug-loaded NLCs. Taking into consideration the drug-crystal-free particle surface, it is apparent that CP and PH is amorphously dispersed within the nanoparticles, which is preferable for a sustained release system. Furthermore, the inclusion of drug molecules in the lipid is normally accompanied by a depression in the lipid's melting point.

X-ray diffraction study

X-ray diffractograms of CP, PH, plain NLC and the drug-loaded NLC are presented in fig. 7. The XRD patterns of CP and PH showed numerous diffraction peaks at diffraction angles (2θ) 26.69 °, 20.55 °, 14.61 °, 12.84 °, 10.27 °, 34.07 °, 47.16 °, 15.32 °, 12.20 °, 25.43 °, 28.09 °, 31.51 °, 6.12 ° and 9.10 ° corresponding to its crystalline pattern. Such characteristic patterns were absent in the diffraction peaks of plain NLC and drug-loaded NLC, indicating the presence of the drug in the amorphous form, confirming the DSC data.

Entrapment efficiency

The average entrapment efficiency of the optimized formulation was found to be: CP 78%±3.26 and PH 89.76%±2.66. It might be due to the incorporation of liquid lipids into solid lipids which have led to massive crystal order disturbance [24]. Greater imperfections in the crystal lattice leave enough space to accommodate drug molecules, which ultimately improved drug-loading capacity and drug entrapment efficiency. The percentage entrapment efficiency is mainly depending on the nature of the drug and lipids. As CP and PH are lipophilic drugs with Log P of 3.5 to 3.3 respectively their entrapment efficiency is high.

In vitro drug release studies

In vitro drug release study was performed for NLC formulation revealing a biphasic release pattern of drugs i.e. a burst release in the initial stage followed by a sustained release. About 15-20 % of loaded drugs were released within first 2 h, followed by the release of 90.98-79.81% within 24 h of the assay. The initial burst release might be due to the presence of the unentrapped drug in the NLC dispersion, another reason might be due to most of the liquid lipid being located in the outer shell of the nanoparticles, which lead to a drug-enriched shell that is related to burst release at the initial stage [25]. The oleic acid enriched outer layers possessed a soft and considerably higher solubility for lipophilic drugs, which ultimately increased the loading of the drug and could be easily released by diffusion or matrix erosion. The release time profile data of CP and PH from drug loaded NLC are represented in the fig. 8.

Kinetic modelling of in vitro drug release

The release kinetics was determined by fitting the data obtained from in vitro release studies of NLC into standard release equation, i.e. zero order, first order, higuchi and korsmeyer-peppas and represented in table 4. The model that fits best release data was selected based on correlation coefficient value of various models. The results suggested that release of drug from NLC follows first order release model and as indicated by the higher R² value of 0.9807 and 0.9788 for CP and PH respectively. This type of kinetics indicates that the drug release is dependent of drug concentration. When the release data were analysed using the korsmeyer peppas equation, the value of release exponent ‘n’ was found to be 0.7232 for CP and 0.8587 for PH, which indicates that the mechanism of drug release from NLC follows non-fickian diffusion.
**Fig. 8: In vitro** drug release of CP and PH from optimized NLC. The values are expressed as mean±SD; n=3

### Table 4: Kinetic modelling of A and B release from NLC

<table>
<thead>
<tr>
<th>Optimized formulation</th>
<th>R² value</th>
<th>Zero order model</th>
<th>First order model</th>
<th>Higuchi model</th>
<th>Peppas model</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.8871</td>
<td>0.9807</td>
<td>0.9801</td>
<td>0.9809</td>
<td>0.7202</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.8101</td>
<td>0.9788</td>
<td>0.9788</td>
<td>0.9433</td>
<td>0.8587</td>
<td></td>
</tr>
</tbody>
</table>

**In vitro haemolysis assay**

Haemocompatibility test was carried out to check the compatibility of the drug and formulation with blood, since it may come in contact with the blood even on topical administration. The degree of haemolysis is a sensitive indicator of the extent of damage to RBC. In the present study, different concentrations of optimized formulations were tested and showed no evidence of haemolysis (<0.5%) after incubation for the specified time period. The obtained results were compared with standard normal saline and the positive control Triton X. As per the results obtained the haemolytic ratios of the NLCs were below 5% which is the safe haemolytic ratio for biomaterials in accordance with ISO/TR 7406. It confirms that the NLCs are free from the risk of haemolysis.

**MTT assay: L929 Normal cell line**

In vitro cytotoxicity screening was carried by MTT assay on L929 normal fibroblastic cell line. Here absorbance rate or percentage cell viability of the experimental samples were less than the control group which indicates reduced cell proliferated rate or decreased overall cell viability rate. Prepared NLC formulation was tested for cytotoxicity in different concentrations like 100µg/ml, 50µg/ml, 25µg/ml, 12.5µg/ml, 6.25µg/ml. Percentage viability was found to be 96.95 to 64.20 (minimum to maximum dose) which indicates that prepared NLC formulation is not toxic towards the normal cell line. The percentage viability of prepared NLC on L929 cell lines are shown in fig. 9.

**Fig. 9: Percentage viability of L929 normal cell line on treating with prepared NLC (A) control (B) 6.25µg/ml (C) 12.5 µg/ml (D) 25 µg/ml (E) 50 µg/ml (F) 100 µg/ml. The values are expressed as mean±SD; n=3, p<0.05**

**In vitro skin permeation studies**

The in vitro permeation study for CP-PH NLC was carried out. The results are shown in table 5 and found out that 75.85 µg/cm² CP and 65.588 µg/cm² PH permeated through 2.54 cm² area of skin. The major parameter influencing the drug permeability through the skin is the particle size which is in nanometric range ie; <100 nm can easily penetrate via SC [26]. Moreover, lipids are skin friendly and compatible to the epidermis region so that interaction between keratinocytes enriched topical area and NLC would be higher through vanderwaal force.

Oleic acid act as liquid lipid as well as permeation enhancer able to disrupt the lipids and corneocytes cause an increase in diffusion coefficient.

<table>
<thead>
<tr>
<th>Time in h</th>
<th>CP</th>
<th>PH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>4.392±0.035</td>
<td>3.913±0.063</td>
</tr>
<tr>
<td>4</td>
<td>10.984±0.092</td>
<td>8.9670±0.033</td>
</tr>
<tr>
<td>6</td>
<td>19.378±0.061</td>
<td>14.715±0.052</td>
</tr>
<tr>
<td>8</td>
<td>30.666±0.955</td>
<td>23.622±0.096</td>
</tr>
<tr>
<td>10</td>
<td>42.915±1.221</td>
<td>33.802±0.939</td>
</tr>
<tr>
<td>12</td>
<td>58.715±1.221</td>
<td>48.612±1.766</td>
</tr>
<tr>
<td>24</td>
<td>75.856±1.109</td>
<td>65.588±1.491</td>
</tr>
</tbody>
</table>

The values are expressed as mean±SD; n=3

### Table 5: Amount of drugs permeated per cm² of skin vs. time

The values are expressed as mean±SD; n=3
Stability studies

The stability studies of the optimized formulation were carried out at room temperature (30±2 °C), at refrigerator temperature (4±2 °C) and at accelerated condition (40±2 °C, 75±5% RH) for 3 mo. Fig. 10A and 10B portrayed the particle size and zeta potential of the CP-PH loaded NLC in three dissimilar storage conditions. The size distribution varies from 86±0.09 nm to 114±0.29 nm after one month and further increased to 154±1.56 nm. In fact, it showed a relevant increase in particle size (p<0.05) at an accelerated temperature as compared with refrigerated temperature in the matching period. Upon storage, at room temperature and accelerated temperature, the formulation shows an increase in the particle size and decrease in zeta potential. In-room temperature and accelerated condition, there was a significantly visible particle aggregation resulted in increasing particle size.

Zeta value decreased from -39±0.04 to -19±0.83 and -39±0.03 to -12±1.20 in accelerated and room condition respectively indicates less stability. But here observed no drastic changes that will affect the particle size and zeta potential at 4±2 °C, so the stability of NLC stored at refrigerated temperature.

Fig. 10: A. Particle size of optimized NLC after storage at different temperatures. Each value represents mean±SD (n=3)

Fig. 10: B. Zeta potential of optimized NLC after storage at different temperatures. Each value represents mean±SD (n=3)

CONCLUSION

Drug-loaded NLC was prepared by melt emulsification ultrasonication method, prior to which preformulation studies of both the drugs were carried out. NLC was optimized by altering the composition of various ingredients of the formulation until the NLC were obtained of the desired size range. Drug-loaded NLC were characterized by particle size analysis, zeta potential measurement, FT-IR, SEM, XRD, DSC and TEM. DSC and XRD studies have indicated the presence of CP and PH in the amorphous or molecularly dispersed state within the NLC and the NLCs were found to be spherical in shape having a narrow size distribution and were stable. The optimized drug loaded formulation was assessed for in vitro drug release, ex vivo skin permeation studies, in vitro haemolysis and MTT assay. In vitro studies showed a sustained release behaviour of both the drugs following non fickian release pattern and the ex vivo skin permeation studies have showed good permeation of both the drugs. In vitro haemolysis assay was done to evaluate the haemocompatibility of the formulation, which showed that the formulations are haemocompatible with haemolytic percentage<5%. MTT assay results showed that the prepared NLC is not toxic towards the normal cell line.

The stability studies conducted revealed the ideal storage conditions for the NLC are refrigeration condition.

AUTHORS CONTRIBUTIONS

All the author have contributed equally

CONFLICT OF INTERESTS

Authors have no conflict of interest

REFERENCES