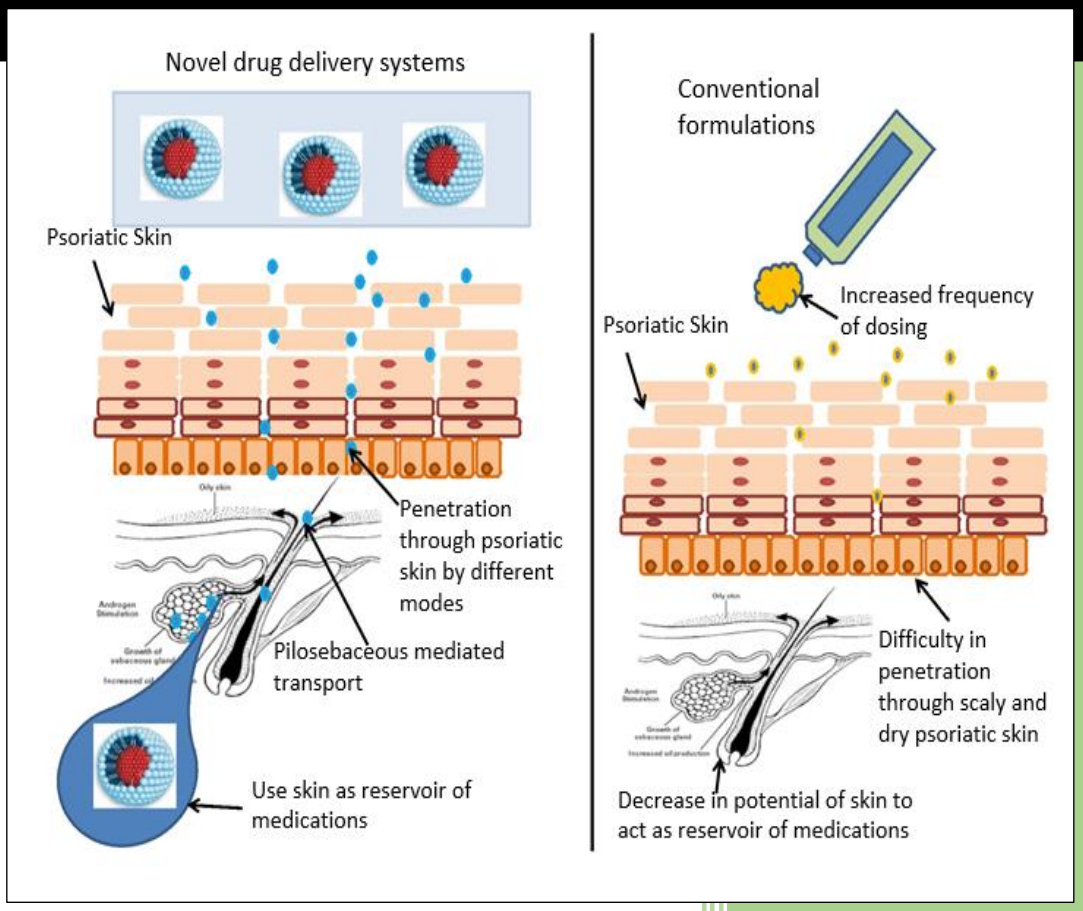
CHAPTER 4: EXPERIMENTAL (MATERIAL AND METHODS



CHAPTER 4:
EXPERIMENTAL (MATERIALS AND METHODS)

4.1 CHEMICALS AND EXCIPIENTS

Various chemicals used in the present work are enlisted in Table 4.1.

Table 4.1: List of chemicals and excipients

Chemical	**Manufacturing Company**
1-Octyl sulfonic acid	Lobachemie, Mumbai
Absolute alcohol	Merck Ltd., India
Acetone	SDFCL Mumbai
Acetonitrile	CDH Ananlytical reagents
Acetonitrile (HPLC)	CDH Analytical Reagents New Delhi
Carbopol (carbomer) 934 p, 974 p	Sigma Aldrich, USA
Chloroform	SDFCL, Mumbai
Clobetasol propionate	Helios Pharmaceuticals Pvt. Ltd.
Dialysis membrane	Himedia Pvt. Ltd
Eosin	SDFCL, Mumbai
Glyceryl monostearte (gms)	Lobachemie, Mumbai
Hematoxylin	SDFCL, Mumbai
Hydrocortisone	SDFCL, Mumbai
Hydrogenatedsoy phosphotidycholine	Avanti
Imiquimod (IMQ)	Imiquad, Glenmark Pharmaceuticals 5% w/w
L- tyrosine	Lobachemie, Mumbai
Methanol	SDFCL, Mumbai
Methanol (hplc grade)	SDFCL, India
NaOH	CDH, India
N-Octanol	CDH, India
Potassium dihydrogen phosphate	CDH Analytical Reagents, New Delhi

Soy phosphatidyl choline	Lobachemie, Mumbai
Squalene	Sigma Aldrich, Mumbai
egg-yolk phosphatidylcholine (EPC)	SDFCL, Mumbai
sodium cholate	SDFCL, Mumbai
α-tocoferol	SDFCL, Mumbai
Ethanol	SDFCL, Mumbai

Various types of equipment employed in the present study are enlisted in Table 4.2. Table 4.3 enlists various softwares used.

Table 4.2: List of different instruments

S. No.	Instrument	Model number	Manufacturer
1.	Brookfield viscometer	R/S plus	Brook-field Engineering Laboratories, Inc, Middleboro, MA
2.	Bath Sonicator	40050	SterylMedi Equip system
3.	Centrifuge machine	R-24C	Remi Motors, India
4.	Confocal microscope	LSM 510 META,	Zeiss, Germany
5.	Franz Diffusion cell	4G- 01- 00-09-05	Prefit, India
6.	FTIR	380	Thermo Scientific, USA
7.	High-Performance Liquid Chromatography	515	Waters, USA
8.	Homogenizer	-	Heidolph, Germany
9.	Hot air oven	PMTC-3040	PERFIT, India
10.	Incubator	1005RE	Daihan, Korea

11.	Magnetic mixer	122	Remi work, India
12.	Melting point apparatus	31354	Decibels, India
13.	Micropipette	(100-1000 µl)	Tarsons, India
14.	Microtome	HM 325	Thermo Scientific, USA
15.	Motic digital microscope	BA 310	Motic Incorporation, Hong Kong
16.	pH meter	ME 963-P	Remi, India
17.	Rota evaporator	4011	Heidolph, Germany
18.	Sonicator	40050	Lark, USA
19.	Spinner	3020	Tarsons, India
20.	Stability chamber	400G	Thermolab, India
21.	Tissue homogenizer	WiseMix HG-15D	Dathan Scientific, New Delhi, India
22.	Transmission electron Microscope	H-7000	Hitachi Ltd., Japan
23.	Ultracentrifuge	3K- 30	Sigma, USA
24.	UV-VIS Spectrophotometer	UV-1700	Shimadzu, Japan
25.	Vacuum pump	65	Welch, USA
26.	Water Purifier	Milli-Q	Millipore, USA
27.	Weighing balance	AB265	Mettler Toledo, UK
28.	Zeta-sizer	Nano 4 C	Beckman Coulter, USA

Table 4.3: List of various computer software used in the present research

Software	Manufacturer	Use
End note		Reference management and generate bibliographies
Graph Pad Instat 3	GraphPad Software Inc., USA	Statistical Analysis
MS-Office 2016	Microsoft Corp., USA	Thesis Writing

4.2 PREFORMULATION STUDIES OF BIOACTIVE (CLOBETASOL PROPIONATE)

Before putting a drug into a formulation, a preformulation research is a crucial tool for determining its physical and chemical properties. The nature of the medication has a significant impact on processing characteristics such as technique of preparation, entrapment efficiency, compatibility, and the formulation's in vitro response. Preformulation studies are an essential technique for developing a dosage form that is safe, effective, and stale. Preformulation experiments were carried out in order to establish the best conditions for a therapeutically beneficial delivery system. To obtain preliminary information, several of the preformulation parameters given in Fig 4.1 were used.

4.2.1 Drug identification

Clobetasol propionate was obtained from Helios Pharmaceuticals Pvt. Ltd., Baddi as gift sample. Clobetasol propionate was confirmed by FT-IR analysis according to the standard procedure.

4.2.2 Physical appearance

Physical appearance of the procured drugs (Clobetasol propionate) was noted by

visual observation.

4.2.3 Melting point

The melting point of a substance is a criterion for its purity and identification. The melting point of Clobetasol propionate was determined using a capillary melting point instrument. The melting point of benzoic acid was used to calibrate the thermometer before determining the melting point. The melting point of benzoic acid was tested when a small amount of the acid was placed in the capillary tube. The temperature at which benzoic acid melted was measured. The melting point of Clobetasol propionate was measured after a little amount of the compound was placed in the capillary tube (Crovini *et al.*, 1981).

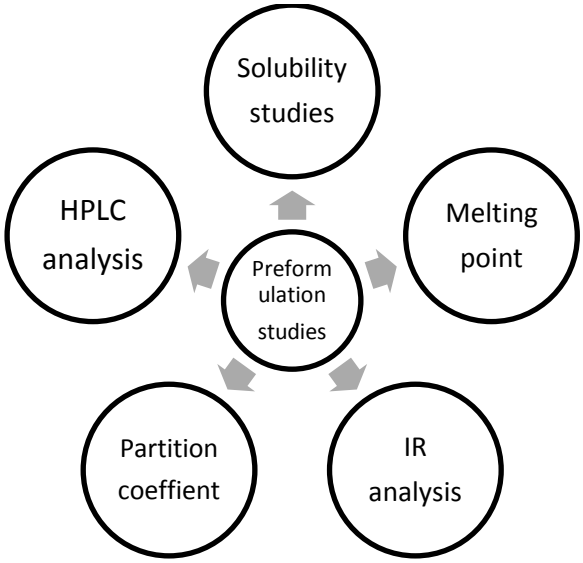


Figure 4. 1 Screening techniques for preformulation

4.2.4 Solubility studies

The extent of a drug's solubility in a certain solvent is measured as saturation solubility, which is defined as the point at which adding any additional solute does not raise the concentration of the solution(Alsenz and Kansy, 2007). Clobetasol

propionate's solubility in different organic solvents was tested by dissolving the medication in a certain volume of solvents until complete dissolution was observed.

4.2.5 FT-IR determination

The `MeExIR spectrum is an important record that provides enough information about a compound's structure and allows for its purity to be checked. A FTIR spectrophotometer was used to get the FTIR transmission spectrum of Clobetasol propionate (Thermo Nicolet 310i). In comparison to potassium bromide-KBr, a total of 2% (w/w) of sample was combined with dry KBr. An agate mortar was used to grind the mixture into a fine powder. After that, a hydraulic press at 10,000 pressure was used to compress the powder combination into a KBr disc. Over a wave number range of 400–4000 cm-1, each KBr disc was scanned 32 times at 4 mm/s with a resolution of 2 cm^{-1}.

4.2.6 Partition coefficient

The drug was added with n-octanol and water in a specified amount and maintained in a separating funnel. Layers were separated after 24 hours. The drug concentration was then determined using a spectrophotometer in n-octanol and water. The following equation was used to compute the partition coefficient

$$\textbf{Partition coefficient} = \frac{\text{Concentration in organic phase (n-octanol)}}{\text{Concentration in aqueous phase (water)}}$$

4.2.7 Standard curve of clobetasol propionate in Octanol

Because of its lipophilic nature, clobetasol propionate was estimated for calculating partition coefficient by plotting its calibration curve in octanol. Clobetasol propionate stock solution of 1 mg/ml was freshly made by dissolving 10 mg of pure medication in 10 ml of octanol. To produce a concentration of 100 g/ml, the prepared solution

was further diluted using n-octanol. Aliquots of 0.4, 0.6, 0.8, 1.0, 1.2, and 1.4 ml were taken from the stock solution and deposited in separate 10 ml volumetric flasks, with the volume increased to 10 ml with octanol to yield working solutions of 4, 6, 8, 10, 12, and 14 g/ml. Using a UV-visible spectrophotometer, the absorbance of these solutions was measured against a blank at a maximum of 239 nm.

4.2.8 Standard curve of clobetasol propionate in 10% methanolic phosphate buffer (PBS) (pH 5.5)

Clobetasol propionate stock solution of 1 mg/ml was freshly made by dissolving 10 mg of pure drug in 10 ml of 10% methanolic PBS pH 5.5. Aliquots of 0.4, 0.6, 0.8, 1.0, 1.2, and 1.4 ml were taken from the stock solution and placed in separate 10 ml volumetric flasks with methanolic phosphate buffer pH 5.5 to make up to 10 ml. To obtain working solutions of 4, 6, 8, 10, 12, and 14 g/ml, the absorbance of these solutions was measured against a blank using a UV-visible spectrophotometer at a maximum of 239 nm.

4.3 METHODOLOGY

Homogenization method is used for the formulation of nanoemulgel. There are three steps involved in the formulation of nanoemulgel which are given below in Figure 4.2.

1. Preparation of nanoemulsion,

2. Preparation of hydrogel

3. Finally nanoemulgel will be produced by the incorporation of nanoemulsion into gel with continuous stirring.

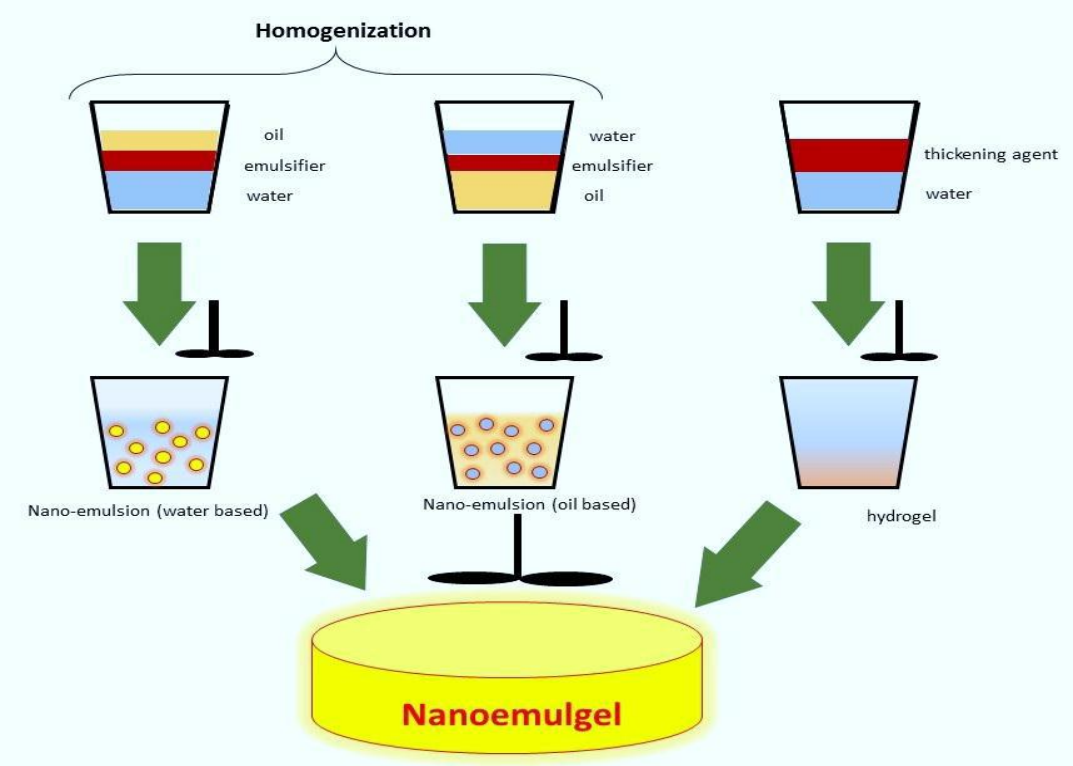


Figure 4.2 Preparation of nanoemulgel

4.3.1 Method of preparation of nanoemulsion

Nanoemulsion preparation process is depicted in Fig 4.3, in which the aqueous and lipid phases of the nanoemulsions were created separately. Double-distilled water and PF68 (3.2 percent w/v) were used in the aqueous phase. Different ratios of squalene and SPC were used in the lipid phase. Both phases were heated to 85°C for 15 minutes each. The aqueous phase was then added to the lipid phase and homogenised for 20 minutes at 12,000 rpm. After that, the mixture was treated for 15 minutes with a probe-type sonicator set at a power of 25 W. For each batch, a 10-ml amount was created. (Aljuffali *et al.*, 2014)

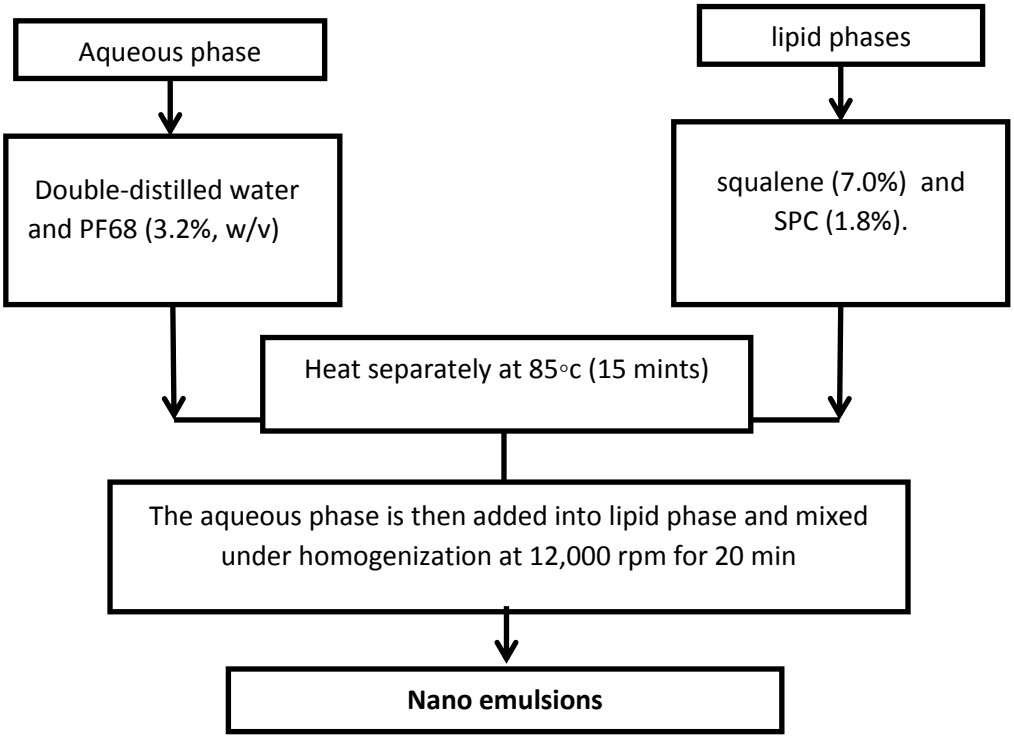


Figure 4.3 Preparation of nanoemulsion

4.3.2 Method of preparation of nanostructured lipid carrier

Alijuffali technology was used to make nanostructured lipid carriers using the homogenization procedure. Fig 4.4 shows a schematic view of the NLC preparation process. Squarticles (NLCs) were manufactured in two phases: aqueous and lipid. Double-distilled water (88 percent, v/v) and PF68 (3.5 percent, w/v) made up the aqueous phase. Squalene (3.5%), Glyceryl monostearate (3.5%), and SPC (3.5%) made up the lipid phase (1.8 percent). Both phases were heated to 85°C for 15 minutes each. After that, the aqueous phase was added to the lipid phase and homogenised for 20 minutes at 12,000 rpm (RM-06, Decibels Instruments, India). After that, the mixture was treated for 15 minutes with a probe-type sonicator (VCX600, Sonics and Materials, Newtown, CT, USA) set at a power of 25 W.A 10 ml volume was prepared for each batch (Aljuffali *et al.*, 2014)

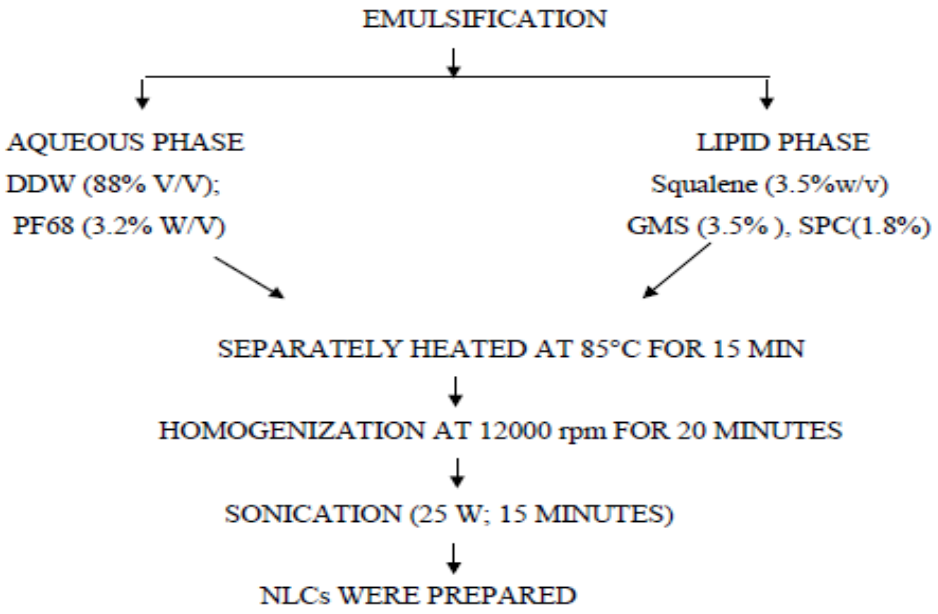


Fig 4.4 Schematic view for preparation of NLCs

4.3.3 Method of preparation of deformable liposomes

The thin film hydration method was used to make deformable liposomes, and the sodium cholate content was optimised previously. To summarise, an ethanol solution of the antioxidant tocoferol (16 M of final concentration) was mixed with an ethanol solution of egg-yolk phosphatidylcholine (EPC) and sodium cholate blended in a proportion of 86:14 percent w/w (5 percent w:v of final lipid concentration). The lipid film was created by evaporating the organic solvent at ambient temperature, at a pressure of 40 mbar, and at position 4 of the rotating speed control in a rotary evaporator. The lipid film was placed under high vacuum (Vaccuu brand GMBH+CO, VSP 3000-Germany) for at least 3 hours to ensure that any residual residues of ethanol were removed. The dried lipid film was hydrated with 7 % v/v ethanol in ultra-pure water, and then vortexed for 15 minutes at room temperature at 2400 rpm and above the phospholipid phase transition.

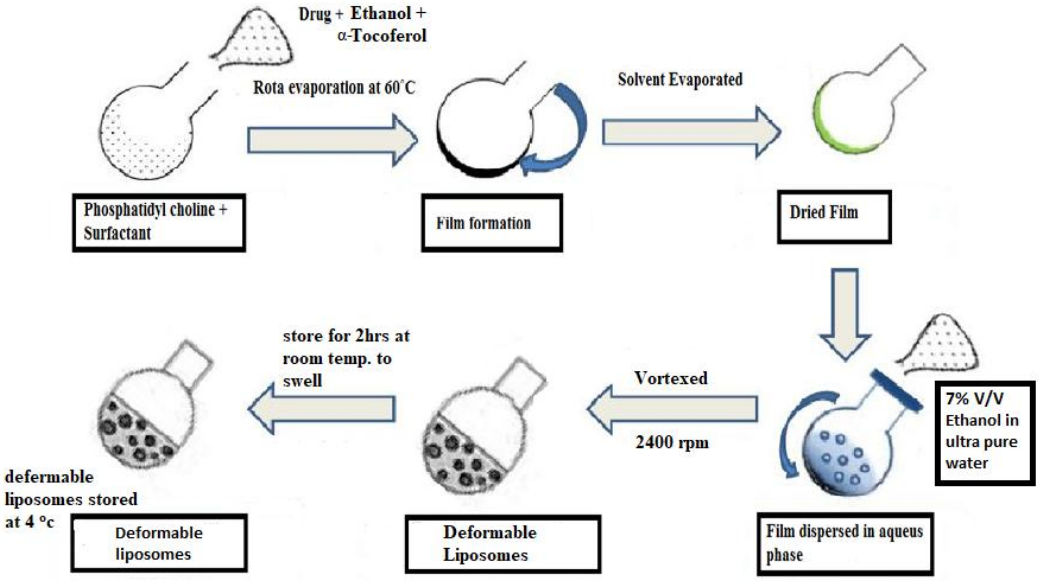


Figure: 4.5 Preparation of deformable liposomes

After that, the vesicles were allowed to swell for 2 hours at room temperature before being extruded through polycarbonate filters with consecutive pore sizes of 0.2 and 0.1 microns (Whatman Int. Ltd) to generate a homogeneous suspension. In terms of size distribution, increasing the extrusion number did not improve the qualities of the lipidic suspension(Ferreira *et al.*, 2015). (Fig 4.5)

4.4 CHARACTERIZATION OF NANOEMULSIONS, NANOSTRUCTURED LIPID CARRIER AND DEFORMABLE LIPOSOMES

4.4.1 Determination of particle size, polydispersity index and zeta potential

A particle size analyzer was used to determine the mean particle size of the prepared NLC (Beckman Coulter, Delsa Nano C, USA). This equipment used the rate of fluctuation in the laser light intensity scattered by particles as they diffused through a

fluid to determine particle size. A 3 mL sample of NLC dispersion was deposited in a cuvette and analysed using software included with the equipment to determine particle size and polydispersity index. The size was determined by using an average of three measurements. The resulting formulations' zeta potential was assessed by depositing 3 mL NLCs dispersion in a transparent disposable zeta potential specific cuvette and using the built software to take a reading. (Sinha *et al.,* 2019)

4.4.2 Entrapment Efficiency of NLCs and Deformable liposomes.

The centrifugation method was used to assess entrapment efficiency. The free drug was extracted from the nanostructured lipid carrier and deformable liposome encapsulated drug using the centrifugation process. Centrifugation at 7000 rpm for 30 minutes was used to separate the dispersion. NLCs and deformable liposomes were isolated from clear supernatant liquid. The amount of entrapped drug in the supernatant was calculated using UV at 238 nm.(Mishra *et al.,* 2017)

The entrapment efficiency of the NLC formulation was calculated from the following equation.

% Entrapment efficiency = DL - DF / DL x 100

Where:

DL = Initial drug loaded (mg)

DF = Free drug (mg)

4.4.3 Percentage drug loading in NLCs and Deformable liposomes.

The centrifugation method was also used to assess loading efficiency. The free drug was extracted from the nanostructured lipid carrier and deformable liposome encapsulated drug using the centrifugation process. Centrifugation at 7000 rpm for 30 minutes was used to separate the dispersion. NLCs and deformable liposomes were isolated from the clear supernatant liquid. (Mishra *et al.*, 2017)

Supernatant was analysed by using UV at 238 nm to calculate the amount of entrapped drug. The drug loading efficiency of the NLC and deformable liposome formulation was calculated from the following equation.

DL% = W (Total) × W (Free) / W (Lipid) × 100

W_{total}, W_{free} and W_{lipids} are the weight of drug added in system, analysed weight of drug in supernatant and weight of lipid added in system, respectively.

4.5 PREPARATION OF GEL

Gel was chosen as the delivery vehicle for nanosystems for skin delivery. Carbopol 940 (0.25 g) was disseminated in distilled water (100 ml) after 60 minutes of stirring at 800 rpm. The mixture was neutralised by adding triethanolamine drop by drop. The mixing was continued until a translucent gel developed, and the amount of base was adjusted to produce a pH of 5.5 in the gel.

4.6 INCORPORATION OF NANOEMULSION OR NLCS OR DEFORMABLE LIPOSOMES INTO CARBOPOL 940 SOLUTION

Carbopol 940 (0.4 percent w/v) was disseminated in distilled water for 60 minutes by stirring at 800 rpm. The mixture was neutralised by adding triethanolamine drop by drop. The colloidal suspension was added to the mixture and continuously mixed until a transparent gel developed; the pH of the gel was adjusted to 5.5 with the help of a base (triethanolamine), and the finished gel was kept overnight for swelling.(Srivastava *et al.*, 2018)

4.7 CHARACTERIZATION OF GEL FORMULATION

4.7.1 Physical examination

The prepared gel formulations were inspected visually for their colour, homogeneity and consistency.

4.7.2 pH

The concentration of hydrogen ions in a solution is measured by pH. In terms of numbers, it's the negative logarithm of that concentration in moles per litre (M). A pH (Pico) metre was used to determine the pH of the prepared gels (Lab India instruments Pvt. ltd.).

4.7.3 Viscosity

The Brookfield rheometer was used to measure viscosity at room temperature (25-27 C). For each treatment, the spindle (R3-C75 -1) was used, and formulations were monitored at shear rates ranging from 10 to 100 per sec. (Jones *et al.*, 1997).

4.7.4 Drug content

100 gm. gel was dissolved in 10 ml of dichloromethane. Then on 20 ml methanol was added into it to precipitate the polymer. The solution obtained was filtered and volume was made to 100 ml. the solution obtained was diluted suitably with methanolic PBS and peak height was measured by HPLC method at 215nm (Reddy *et al.*, 2006).

4.7.5 Spreadability

It was determined by wooden block and glass slide apparatus. Weights about 20g were added to the pan and the time were noted for upper slide (movable) to separate completely from the fixed slides.

Spreadability was then calculated by using the formula:

$S = M.L / T$

Where,

S = Spreadability

M = Weight tide to upper slide

L = Length of glass slide

T = Time taken to separate the slide completely from each other.

4.8 *IN VITRO* DRUG RELEASE STUDY

4.8.1 *In vitro* drug release study of nanoemulsion

In vitro release study of marketed gel, and nanoemulgel was carried out using dialysis bag method. In this method 1ml formulation was added to donor compartment after centrifugation and resuspension. Receptor compartment was taken into 100 ml of phosphate buffer saline pH 5.5 in a conical flask. This flask was taken in incubator shaker and speed of shaker was maintained at 60 rpm at 37°C. At specific time intervals, samples (2 ml) were withdrawn and filtered. Same volume (2 ml) was replaced after each sampling. The drug content in the sample was determined by HPLC method being developed at 215 nm.

4.8.2 *In vitro* drug release study of NLCs

In vitro release study of gel, NLCs dispersion and NLCs gel was carried out using dialysis bag method (De Andrade *et al* 2015). In this method formulation 10 ml was added to donor compartment after centrifugation and resuspension. Receptor compartment was taken into 100 ml of phosphate buffer saline pH 5.5 in a conical flask. This flask was taken in incubator shaker and speed of shaker was maintained at 60 rpm at 37°C. At specific time intervals, samples (2 ml) were withdrawn and filtered. Same volume (2 ml) was replaced after each sampling. The drug content in the sample was determined by UV method being developed at 238 nm.

4.8.3 *In vitro* drug release study of Deformable liposomes

In vitro release study of gel, Deformable liposomes dispersion and Deformable liposomes loaded gel was carried out using dialysis bag method (Dar *et al.*, 2020). In this method formulation 10 ml was added to donor compartment after centrifugation and resuspension. Receptor compartment was taken into 100 ml of phosphate buffer

saline pH 5.5 in a conical flask. This flask was taken in incubator shaker and speed of shaker was maintained at 60 rpm at 37°C. At specific time intervals, samples (2 ml) were withdrawn and filtered. Same volume (2 ml) was replaced after each sampling. The drug content in the sample was determined by HPLC method being developed at 238 nm.

4.9 PHYSICAL STORAGE STABILITY STUDIES

The stability of vesicles to retain the drug (i.e. drug retentive behaviour) was assessed by keeping the nanoemulsions at different temperature conditions, i.e. 4±2 ^{0}C (Refrigerator; RF), 25 ± 2 ^{0}C (Room temperature; RT) and 45 ± 2 ^{0}C for a period of 6 months as per ICH guidelines for drug and formulation. The optimised formulations were kept in sealed vials (20 ml capacity). Samples were withdrawn periodically and analysed for entrapment efficiency, PDI and particle size. Further, the stability of active ingredient in gel formulation was determined using HPLC.(Rai *et al.*, 2014)

4.10 *IN-VIVO* STUDIES

Wistar rats (200- 250gm) were used as an animal model for the present study. All animal experiments were carried out as per the approved Reg. no. ISFCP/IAEC/CPCSEA/Meeting No.23/2018/ Protocol No. 390 by Institutional Animal Ethical Committee (IAEC) formed as per the norms of Committee for prevention, Control and supervision of Experiments on Animals (CPCSEA). Healthy wistar female/male rats (200-250gm) were procured from animal house of ISF College of Pharmacy, Moga for animal study.

4.10.1 Experimental protocol:

The experimental protocol consists of 6 group Total number of animals: 36 (n=6)

a) **Animal model**: Imiquimod-induced Psoriasis Model

b) **Drug used**: clobetasol propionate

Standard drug: Topinate gel.

c) **Experimental Grouping is** shown in Table 4.4.

4.10.2 Disease induction

Topical application of IMQ to animals resulted in psoriasis-like skin lesions, which were generated following the procedure reported elsewhere with some alterations.(Johnston *et al.*, 2011) From the 3rd day to the 3rd day, cyclophosphamide was given i.p. into the purchased BALB/c female mice at a dose of 3 mg/kg BW. On the 0th day of the trial, hair on the dorsal side of the mice was removed with depilatory cream. After hair removal, the mice were given a betadine solution and left for 24 hours. Except in normal control mice, imiquimod (62.5 mg of 5% Imiquad cream) was applied once daily to the skin surface for 7 days, resulting in a daily imiquimod dose of 3.125 mg. (Sun *et al.*, 2013)

4.10.3 Antipsoriatic activity.

Antipsoriatic potential of the formulations was evaluated on basis of scoring to check severity as well as histopathology of skin. The severity of psoriasis was decided based on three expressions viz. erythema, scaling and thickening and score was assigned independently as 0, none; 1, slight; 2, moderate; 3, marked; 4, very marked. After completion of treatment, animals were sacrificed and affected skin samples were taken and stored (10% v/v formalin). Samples were sliced transversely, and paraffin embedded for light microscopic evaluation. Samples were stained with haematoxylin and eosin (H&E) and observed under a light microscope. Histopathological changes (orthokeratosis, hyperkeratosis, and parakeratosis) in these tissues were assessed. At least 25 randomly selected tissue sections from each group were studied. (Sun *et al.*, 2013)

4.10.4 Route of administration and withdrawal of blood samples

Plasma drug concentration–time profile and pharmacokinetic parameters were

obtained from the topical application of marketed and prepared formulations. After the psoriasis has been induced 1 mg of the nanoformulations and marketed is applied on the psoriasis induced area (dorsal skin) with the help of spatula. Pharmacokinetic parameters were calculated by noncompartmental analysis also called as model independent analysis using Kinetica software version 5.0. Two groups of rats were taken for pharmacokinetic studies. Each group had six rats. Mean of these rats was taken for pharmacokinetic studies.

Table 4.4. Experimental Grouping of the animals.

S. No.	Groups	Animal Species	No. of animals		Blood sampling time and volume
1.	Control group	Swiss albino Mice	6	G1	2- 1hr (0.3ml)
				G2	2 - 8hr (0.3ml)
				G3	2- 16hr (0.3ml)
				G1	2- 24 hr (0.3ml)
2.	Imiquimod-induced plaque psoriasis.		6	G1	2- 1hr (0.3ml)
				G2	2 - 8hr (0.3ml)
				G3	2- 16hr (0.3ml)
				G1	2- 24 hr (0.3ml)
3.	Disease + Marketed formulation (clobetasol ointment) 0.5mg/gm (Topical)		6	G1	2- 1hr (0.3ml)
				G2	2 - 8hr (0.3ml)
				G3	2- 16hr (0.3ml)
				G1	2- 24 hr (0.3ml)
4.	Disease + Test drug (clobetasol Loaded Nanoemulsion (gel)) 0.5mg/gm (Topical)		6	G1	2- 1hr (0.3ml)
				G2	2 - 8hr (0.3ml)
				G3	2- 16hr (0.3ml)
				G1	2- 24 hr (0.3ml)
5.	Disease + Test drug (clobetasol Loaded NLCs (gel)) 0.5mg/gm (Topical)		6	G1	2- 1hr (0.3ml)
				G2	2 - 8hr (0.3ml)
				G3	2- 16hr (0.3ml)
				G1	2- 24 hr (0.3ml)
6.	Disease + Test drug (clobetasol Loaded Deformable liposomes (gel)) 0.5mg/gm (Topical)		6	G1	2- 1hr (0.3ml)
				G2	2 - 8hr (0.3ml)
				G3	2- 16hr (0.3ml)
				G1	2- 24 hr (0.3ml)

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4.11 EX-VIVO SKIN PERMEATION STUDIES

In vitro skin permeation studies of the prepared formulations were carried out using Franz diffusion cell. Penetration was studied using rat abdomen skin. The dermal side of skin was carefully cleaned (Satturwar *et al.*, 2005). Dermis part of the skin was washed with a cotton swab soaked in isopropyl alcohol for the removal of fatty material. Later on, skin samples were washed with normal saline and were cut into appropriate sizes. The skin was placed on Franz diffusion cell, maintained at 37^0C with continuous stirring and 3 g of the gel was spread on donor side using a spatula. 10% methanolic Saline phosphate buffer (pH 5.5) was used as receptor compartment. The sample (2 ml) was removed from the receptor compartment. At the regular time interval for a period of 24 h and replaced with same amount of buffer to maintain sink condition. The same procedure was used for the drug loaded nanoemulgel and marketed gel. Sample was filtered through 0.45 l nylon membrane filters and analysed by HPLC at 239 nm using UV detector. We had also compared our drug with marketed formulation which shows least retention in the skin which also prove that with squalene its affinity increased towards pilosebaceous gland hence provided depot effect. The cumulative percentage of drug retained in the skin of clobetasol propionate loaded nanoemulgel was 62 ± 1.28% which was more than the marketed formulation (23.12% ±0.54).

4.12 EX-VIVO SKIN DEPOSITION STUDIES

After performing skin permeation studies, the skin was carefully removed from the Franz diffusion cell. Formulation of the skin was scraped using a spatula. It was dissolved in sufficient amount of dichloromethane and methanol to extract out the surfactant. After extraction, the resulting solution was filtered via nylon membrane filter and amount of drug content in the filtrate was determined using HPLC at 239 nm and the amount in the receptor compartment was also determined and sum of

these two is subtracted from total amount of drug (Agarwal *et al.*, 2001). The remaining dispersion adhering to the skin was scraped off carefully with a spatula and skin pieces were washed three to four times with distilled water and dried using cotton swab. Subsequently, the skin tissue was cut into small pieces and homogenized with 10 mL of the methanol for extraction of drug. Homogenate dispersion thus obtained was centrifuged for 5 minutes at 5000 rpm. The supernatant was filtered using 0.45 µ nylon membrane filter and quantified for drug content.

4.12.1 Preparation of sebum removed skin

After sacrifice, full-thickness skin from the dorsal region was removed. To prepare sebum-free skin, the stratum corneum (SC) side of the skin was gently washed five times with cold hexane (4 ^{0}C) to remove sebum from the skin surface (Campbell et al. 2012). In vitro skin delivery experiments used both intact and desebum skins as diffusion barriers.

4.13 SKIN IRRITATION STUDY

Animal was acclimatised for at least 7 days before experimentation. The dorsal abdominal skin of rats was shaved 24 h before the study. Nanoformulations was applied and site of the application was occluded with gauze and covered with a nonsensitising microporous tapes. The formulation was removed after 24 h and score of erythema was recorded and compared with the standard marketed formulation. Same observation of the sites was repeated at 48 and 72 h also, A score of erythema is read and recorded as Score 0 for no erythema; Score 1 for mild erythema (barely perceptible-light pink); Score 2 for Moderate erythema (dark pink); Score 3 for severe erythema (Extreme redness). The conducted study work was approved by ISFCP/ IAEC/CPCSEA/Meeting No 17/2016/Protocol No.310.

4.14 HISTOPATHOLOGY

The histopathological examination was performed at the conclusion of the experiment to determine the histological alterations that happened during the development of the psoriatic model. On the day of sacrifice, mice's irritated skin samples were obtained from various treatment groups and kept in 10% formalin buffer. After being fixed in 10% formalin, the skin samples were imbedded in paraffin wax (60-62 °C) and sliced into 5m pieces using a microtome. Furthermore, these slices were stained with Hematoxylin and Eosin dye according to conventional protocols and examined under a light microscope Leica DM 750 at a magnification of 40X. (Sinha *et al.*, 2019)

4.15 HPLC BIOANALYTICAL METHOD DEVELOPMENT

Optimized analytical method was already developed which is a selective and sensitive method for analysis of clobetasol propionate on HPLC using UV detection by PDA detector. Mobile phase used was a mixture of methanol and acetonitrile.

4.15.1 Instrumentation and Conditions

HPLC analysis was performed using Waters 515 Series pumps combined with a Waters PDA 2998 series photo diode array detector. The column used was Agilent® C_{18} bonded silica (5 µm, 4.6 x 150 mm). The mobile phase used was a mixture of methanol and acetonitrile and flow rate of 1.6 ml/min. The elution was monitored at λ_{max} 239 nm. Analyst weighing, for preparation of calibration standards and quality controls, was done on a microbalance, Mettler-Toledo AB 204-S. All mobile phase solutions were filtered by Rocker 410 vacuum filter assembly using 0.2 micron membrane filters and degassed ultrasonically by Steryl 40050 bath sonicator before use.

4.15.2 Method development for the determination of clobetasol propionate in plasma

0.1 ml animal plasma was taken in which 0.1 ml of drug solution of known concentration was spiked to get a linearity range from 0.6 to 1.6 µg/ml and 10ml of methanol as ppt agent was added to get total volume 10 ml. This solution was vortexed 4-5 min and then centrifuge at 10000 rpm for 10min at 4°C. 0.2ml of supernatant liquid was taken and diluted with methanol phase up to 2 ml this solution was injected to get response for linearity.