Research Article

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Formulation, Design and Development of Niosome Based Topical Gel for Skin Cancer

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Abstract

Melanoma is the most dangerous type of skin cancer in which mostly damaged unpaired DNA starts mutating abnormally and staged an unprecedented proliferation of epithelial skin to form a malignant tumor. In epidemics of skin, pigment-forming melanocytes of basal cells start depleting and form uneven black or brown moles. Melanoma can further spread all over the body parts and could become hard to detect. In USA Melanoma kills an estimated 10,130 people annually. This challenge can be succumbed by using the certain anti-cancer drug. In this study design, cyclophosphamide were used as a model drug. But it has own limitation like mild to moderate use may cause severe cytopenia, hemorrhagic cystitis, neutropenia, alopecia and GI disturbance. This is a promising challenge, which is caused due to the increasing in plasma drug concentration above therapeutic level and due to no rate limiting steps involved in formulation design. In this study, we tried to modify drug release up to three-fold and extended the release of drug by preparing and designing niosome based topical gel. In the presence of Dichloromethane, Span60 and cholesterol, the initial niosomes were prepared using vacuum evaporator. The optimum percentage drug entrapment efficacy, zeta potential, particle size was found to be 72.16%, 6.19mV, 1.67µm.Prepared niosomes were further characterized using TEM analyzer. The optimum batch of niosomes was selected and incorporated into topical gel preparation. Cold inversion method and Poloxamer -188 and HPMC as core polymers, were used to prepare cyclophosphamide niosome based topical gel. The formula was designed using Design expert 7.0.0 software and Box-Behnken Design model was selected. Almost all the evaluation parameters were studied and reported. The MTT shows good % cell growth inhibition by prepared niosome based gel against of A375 cell line. The drug release was extended up to 20th hours. Further as per ICH Q1A (R2), guideline 6 month stability studies were performed. The results were satisfactory and indicating a good formulation approach design was achieved for Melanoma treatment.

Keywords: Melanoma, Cyclophosphamide, Box-Behnken Design, Topical Gel, Niosome

Introduction

For cancer treatment chemotherapy is a cure for an inevitable curse, as chemotherapy not only targets cancerous cells but also any growing cells of a patient. Due to this alopecia, anima and other complications are frequent. The main cause of those side effects is inappropriate drug delivery and non-specific targeting. If any chemotherapeutic drug delivery controlled by polymeric modifications, that could succumb maximum toxicity of chemotherapy by minimizing systematic side effect, increasing the drug solubility, consistent drug delivery within melanoma or carcinoma cells, improving intrinsic bioavailability, can improve the overall survival and quality of life of any cancerous patient[1-10]. In modern research on cancer, tumor targeting has improved by constantly implicating solid lipid nanoparticles, biodegradable nanoparticles, liposomes, niosomes, dendrimers, gold nanoparticles, and carbon nanotube concepts widely. This nanocarrier can be targeted by passive diffusion within the leaky tumor vesicles. The nanomedicine then targets the over-expressed cells of tumor outer surface, like folic acid receptors, monoclonal antibody receptors etc. Local stimulation such as alteration of formulation pH, temperature changes were needed to increase the payload of the medications. These nanomedicines are liable for intravenous administrations and they promise the cancerous tissue targeting by the accumulation of the drug on the surface of the cancerous tissue. But main challenges is removal and sequestering of nanomaterials by reticuloendothelial system [11]. Which significantly decrease the drug concentration and retention of nono medicine within the cancer outer surface. An additional challenge like witnessing cyto cellular toxicity also an important issue to monitor. To circumvent all those associated problems with the nano medicines, non-invasive biodegradable polymer concept was recognized by scientists. Among all those nano drug delivery concepts, niosome has maximum drug entrapment efficacy, good

physical stability, and drug release profile, hence prepared niosomes can be administered within the topical gel formulation to improve viability against cancer and maintain prolong release effect of the drug on cancerous tissue. Melanoma is the most dangerous type of skin cancer in which mostly damaged unpaired DNA starts mutating abnormally and staged an unprecedented proliferation of epithelial skin to form a malignant tumour. In epidemics of skin, pigment-forming melanocytes of basal cells start depleting and form uneven black or brown moles. Melanoma can further spread all over the body parts and could become hard to detect. In USA Melanoma kills an estimated 10,130 people annually. Eventually, we design our concept against Melanoma treatment and successfully incorporated cyclophosphamide (model drug) loaded niosome into topical gel formulations prepared from Poloxamer-188 and HPMC as polymers. The results were astonishing and promising. Hence, more such research is a warrant to improve melanoma treatment.

Compatibility studies: IR and DSC studies were performed on a physical mixture of drug and prepared formulations.

The method of preparation of niosomes using cyclophosphamide as a model drug

Cyclophosphamide (Gift samples from emcure pharmaceuticals, Gandhinagar), Span60 (Sigma eldritch, Mumbai) and cholesterol (Krishna-Chem industry, Vadodara) was dissolved in 30ml of 2ml Dimethyl Sulphoxide Extra pure(Sisco research Laboratory, New Mumbai) [DMSO], and 28 ml of Dichloromethane (Krishna-Chem industry, Vadodara) in one 250ml round bottomed flask. The flask was further connected with rotatory evaporator and vacuum pump. The solvent system was evaporated using 20-25 °C for 30 minutes under 250mmHg vacuum pressure. After 30 minutes a surface film was formed. This film was hydrated with 10ml distilled water for 1 hour 30 minutes at 20-25 °C using rotatory flask evaporator. The hydrated flask was kept in the refrigerator for 2 hours for the sealing of vesicles. The unentrapped drug was removed by using Refrigerator centrifuge at 10000 rpm at 10°C for 10 minutes. The supernatant was removed and noisome pellets were collected. Sonication was done in a bath sonicator for 10minutes.

Transmission electron microscopic study

Transmission electron microscopy of an optimized batch of niosomal dispersion was performed and analyzed. It was found that particles were in spherical shape and in very narrow size distribution. The size of particles from TEM is almost accordance with the mean particle size distribution curve.

Evaluation parameters of freshly prepared niosomes

Particle size, zeta potential, % drug entrapment efficacy, *in-vitro* drug release study, hydration time, hydration volume, annealing time, film forming the time of prepared optimized niosome formulation were measured and reported.

Results and Discussion

Transmission electron microscopic study

Transmission electron microscopy of an optimized batch of niosomal dispersion **(Figure 1)** was performed and analyzed. It was found that particles were in spherical shape and in very narrow size distribution. The size of particles from TEM is almost accordance with the mean particle size distribution curve.

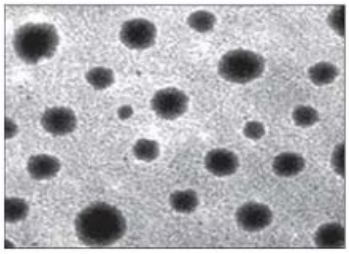


Figure 1: TEM analysis of optimized batch N33

FTIR and DSC studies have shown no specific chAlmost all the formulations (N1-N33) possessed the good quality of drug release. But after comprehensive trials, it can be concluded that N33 batch was shown good spiral shape in TEM studies and results were satisfactory enough to consider it as optimized batch **(Table no:1)**.

Table 1: Optimize process and formulation parameters of N33 batch

Parameters	Optimized value
Surfactant	Span 60
Drug: surfactant: cholesterol ratio	3:1
Hydration volume	12ml for N16 batch
Hydration temperature	35 °C for N24 batch
Hydration time	65 minutes for N28 batch
Annealing time	2hours for N31 batch
Film formation time	10 minutes
%Drug entrapment	72.12 % for N28
Mean diameter	1.67 micrometre
Zeta potential	-40.56mV
% Cumulative drug release at 18th hour	101.23±1.34

Preparation of cyclophosphamide niosome based topical gel formulation

Here we used Poloxamer 188 as a principal polymer. As it has less viscosity and maximum retention. It has the capability to form a thin layer over the skin and by which maximum bioavailability could be achieved. Poloxamer 188 containing topical gel were prepared by cold method [12 & 13], as Poloxamer 188 possessed reverse thermal gelling property. As per factorial design weigh accurately Poloxamer 188 and transfer it into cold (0-10°C) distilled water with persistent stirring. Simultaneously add HPMC premixed cold distilled water solution and stirred. Slowly add three to four drops of triethanolamine to neutralize the gel solution. The mechanical agitator (Teknik: P-P2) RPM slowly increased up to 3000.The transparent gel was formed. The pH of the gel dispersion was maintained within 5-6.

Incorporation of cyclophosphamide niosomal optimized batch into poloxamer 188 gel

Prepared and optimized niosomes were incorporated into poloxamer 188 gel formulation. Further, this mixture was stirred using BIOBASE electrical mixture (Model number: D2004W) at 50 rpm for 10 minutes. The amount of niosomes incorporated into gels must have 2% w/w cyclophosphamide drug concentration. On the other had controlled also get the same treatment, but direct 2%w/w of cyclophosphamide was introduced into it. The volume was made up to 50 gm using double distilled water. This mixture was kept in a refrigerator at 5°C for 24 hours. After 24 hours of refrigeration, the

gelling formation completed and a semi-transparent gel was formed. This gel was used to perform further evaluations.

Experimental design

3 level factorial design was incepted for this study. The fundamental effects on variation of two formulation variables concentration of poloxamer 188 and HPMC in various outcomes of topical gels, such as mucoadhesives, viscosity and *in–vitro* drug release studies has been recorded. The various concentrations of polymers considered as dependent variables (X) and outcomes were considered as independent variables(Y). Dependent and independent variables were listed in the following the table.

Table 2: Experimental design for the preparation of topical gel

A. Dependent variables (X):

Polymers	Coded value			Actual concentration in %w/w		
Poloxamer 188(X1)	-1	0	+1	25	30	35
HPMC (X2)	-1	0	+1	4	6	8

B. Independent variables (Y):

a. Mucoadhesive ness in dyne/cm^2 (**Y1**)
b. Viscosity in CPS (**Y2**)
c. % CDR at 4th hour (**Y3**)
d. % CDR at 8th hour (**Y4**)
e. % CDR at 12th hour (**Y5**)

Table 3: Box-Behnken Design for 2 factor

		Coded value		Actual concentration in %w/w	
Standard	Run	% w/w poloxamer 188 (X1)	%w/w HPMC (X2)	% w/w poloxamer 188 (X1)	%w/w HPMC (X2)
3	1	-1	1	25.00	8.00
9	2	0	-1	30.00	4.00
7	3	-1	0	25.00	6.00
6	4	1	0	35.00	6.00
10	5	0	1	30.00	8.00
4	6	1	1	35.00	8.00
1	7	-1	-1	25.00	4.00
12	8	0	1	30.00	8.00
11	9	0	-1	30.00	4.00
5	10	-1	0	25.00	6.00
13	11	0	0	30.00	6.00
8	12	1	0	35.00	6.00
2	13	1	-1	35.00	4.00

Evaluation parameters:

Physical examination: The prepared gels batches were inspected for their homogeneity, color, odor, by visual appearance.

pH: The pH of the prepared niosomal topical gel can be determined by using Zeal-Tech digital pH meter (model number 09112A). Two gram of prepared gel was incorporated into 40 ml of distilled water. The pH was determined for all the 13 batches and triplicate readings were recorded.

Content uniformity: Accurately weight 100 mg of prepared gel and dissolved it in 10 ml of double distilled water using ultrasonicator bath (Leela electronics; model number: LeelaSonic-60).Further filter the slurry using Whatman® qualitative filter paper, Grade 1. After filtration, 0.5ml of the filtrate was diluted with 5 ml of double distilled water and triplicate measurement was performed using SHIMADZU-1880UV-VIS Spectrophotometer at 722nm.

Viscosity: Using T-bar spindle (no-94) of DV-II model (Brookfield viscometer, USA) was used for determining viscosity. The spindle was placed perpendicularly towards lowered viscous gel. The spindle should not touch the bottom of the beaker. The spindle should rotate at such a speed that it should generate torque >30%. The viscosity of the gel was then finally obtained by multiplying with multiplication factor given in Brookfield viscometer catalog and viscometer reading. Reading was taken thrice and mean was taken as final one.

Extrudability: It is basically a verifiable test to estimate force required to extrude the gel from the aluminum collapsible tube. The lacquered collapsible aluminum tube starts extruding the gel when applied weight (in gm) extruded at list 0.5 cm ribbon of gel in 10 seconds. The measurement of extrudability was finalized by taking the average of triplicate readings. The extrudability was calculated by using following this formula:

Eb = Wtgm/D Where, Eb=extrudability, Wtgm=applied weight to extrude gel from tube (in gm), D=area (in cm2).

Gel strength: TA.XTplus-Flagship Texture Analyser of stable microsystems was used for determining gel strength. The probe was fit and kept upwards the gels, which is place in a standard beaker. After incorporating probe into the gels, then TA.XTplus-Flagship Texture Analyser was set to "gelling strength test" mode or the comparison mode with a test speed of 1.0 mm/sec. The trigger force was selected as 5 gm and an acquisition rate of 50 points per second was set. An aluminum probe of 7.6 cm diameter was used for all the sample. At room temperature, the force required to penetrate the gel was measured as gel strength in terms of gram.

Spreadability Test

Mutimer, a prominent scientist suggested the spreadability test. Here in laboratory scale, we slightly modified the procedure. At first, we have taken two glass slides of same shape and size. Between these two sides, 2 g of prepared gel was added. Now, one-kilogram weight was placed on the top of the two slides, it helps to produce air bubble free thick surface of gels in-between the two slides. The excess gels which come out to the edge of the internal surface of this slide were scrapped off. The top side was then subjected to pull off 50 g weight. After applying weight, the time (second) required to separate or pull the first slide was noted. The spreadability was then calculated by using this following formula:

$S = (M \times L)/T$

Where, S=spreadability, M=weight tide to upper slide, L=length of glass slide, and

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

Bioadhesive Test

As per Mutimer procedure, modified two-arm balance method was used to determined bioadhesive strength. It is a very important parameter for evaluating the consistency of the prepared gel. In this method accurately weighed 0.8 g gel was placed in one glass side and this slide has been covered by same sized another sidle. Then the upper plate was placed over the lower plate and 50 g preload force (contact pressure) was applied for minimum 5 minutes (preload time). After the removal of preload force, the slides were attached with a siphoned. On the left-hand side of the balance, add sufficient water in a bottle, the water flow can be controlled by on/off switch which makes it like an infusion device. The flow rate of water maintained up to 10 ml/ minute, until in right-hand side of the modified balance the upper slide detached from the lower one. The weight of the water required to the detached upper plate from the lower was considered as the bioadhesive force of the applied gel [14]. The unit of the bio-adhesion is gram-force (gf).

***In vitro* drug release and kinetic study:** Dialysis membrane was used for diffusion study. This membrane (LA-393 -Mol. Wt. 12,000-14,000 Daltons, Hi-media, Average flat width 29.31mm and average diameter 17.5mm) before mounting in the USP apparatus type II (paddle), the membrane was soaked in ultra-pure boiling distilled water for at least 12 hours. The dissolution temperature has to be maintained 37±0.5°C The optimized conjugated Anti-EGFR-BSA-CYP-SLNs niosomal topical gel (each contents 0.1% w/w of cyclophosphamide) was kept in different bags of dialysis membrane. The dissolution medium was continuously stirred at a speed of around 50 rpm at 37°C ± 0.5°C. With every one-hour interval 5ml of the sample was withdrawn and 5ml of fresh phosphate buffer solution was placed inside in receptor compartment (Glass dissolution bowl).Withdrawn 5ml of each sample was analysed using SHIMADZU-1880UV-VIS Spectrophotometer at 722nm.

Concentration of drug (µg/ml) = (slope × absorbance) ± intercept

Y=0.0344X-0.0082 (As per linearity curve of cyclophosphamide)

R2= 0.9991

$$\text{Amount of drug release in (mg/ml)} = \frac{\text{Concentration} \times \text{dissolution bath volume} \times \text{dilution factor}}{1000}$$

$$\text{Cumulative percentage release (\%)} = \frac{\text{Volume of sample withdrawn (ml)} \times P(t-1) + Pt}{\text{Bath volume (v)}}$$

Where Pt = Percentage release at time t

Where P (t – 1) = Percentage release the previous to't'

Dissolution studies were determined by a best fitting method using Higuchi and Korsmeyer -Peppas plots. With the used of linear regression analysis using Microsoft 210, n and rate constant k were calculated. Co-efficient studies (R2) were used for evaluating the accuracy of the fit model (16, 17).

Ex-vivo permeability studies

In-house modified Franz-diffusion cell apparatus was used to study the *ex vivo* diffusion. The cyclophosphamide drug solution, the aqueous drug solution of Lyophilized SLNs, Cyclophosphamide nisomal topical gel (NGF8), were studied for the permeation through gout averted dermis part of the skin. The receptor area cross section was found to be 5.12 cm. Which is actually filled with double distilled water. The prepared gels placed uniformly on gout averted dermis part of the skin. Each 1 hour of interval 0.5 ml of the samples was removed and immediately replaced with equal volume of double distilled water. The amount of the drug diffused out to the receptor compartment can be determined by SHIMADZU-1880UV-VIS Spectrophotometer at 722nm.

Skin deposition study

Immediately after permeation study, the Franz diffusion cell was dismantled after a period of 720 minutes. The gout averted dermis part of the skin was carefully removed from the diffusion cell. The formulation which stacked into cell membrane mopped properly using phosphate buffer (pH 6.8) and methanol. This cleaning procedure was repeated thrice to ensure no traces of formulation particles left onto the skin surface. The skin was then chopped into pieces and extracted out with methanol for 48 hours. Then it was analyzed by SHIMADZU-1880UV-VIS Spectrophotometer at 722nm.The standard calibration curve equation was used to determine how much amount of drug is deposited in the skin.

Acute skin irritation study:

As per Draize et al. (1944) method, skin irritation test was performed in Deshpande laboratory, Bhopal (1410/c/11/CPCSEA). Prior one week of the experiment Wistar albino rats was acclimatized to laboratory conditions. The humidity of the room was maintained up to 40-45 % RH and temperature was monitored at 25°C. Approximately 5 cm of the dorsal part of the rat was trimmed and hairs have been removed. Now, animals were divided into three groups (n=9) and treated as follows:

Group I: Negative controlled (No treatment)

Group II: Test formulation (Applied niosomal gel)

Group III: Applied formalin-a standard irritant; 0.8% v/v)

The animals were treated daily with gels/formalin for consistent seven days. The treated skin was examined by visual observation for erythema and edema. The observations have been scored as per modified Draize et al. method.

In the vitro anticancer study

By using MTT assay method prepared niosome based topical gel formulation was studied for anticancer activity. The basal cell carcinoma cell line (A375) and African green monkey kidney cell line-Vero, were used for this study. The vero cell line and A375 cell lines were procured from Deshpande laboratory; Bhopal. Furthermore, characterisation of the cell lines was done by studying microbial contamination, % cell viability, cross contamination, population doubling time and pH.

Characterization of cell lines and culture media

Characterisation of the cell line is important before initiating anti-cancer studies. Especially when cell lines were procured from any cell bank or research laboratory. Before initiating experiments, cell lines were examined under an inverted phase microscope. Throughout the experimental period viability of the cell populations were checked.

Testing for microbial contamination

Microbial contamination is an essential part of characterization. As bacterial and fungal contamination can detour integrity and viability of the cell line. The various detecting media like gram stain, tryptone soya broth (TSB), thioglycolate media (TGM) were used to detect the microbial contamination. Contamination due to the yeast or fungi can be detected by increasing the turbidity of the medium or declining pH (Presence of yellowish shade in media containing phenol red as an indicator). The cell was monitored daily for presence or absence of microbial growth.

Protocol

1. By using 25 cm2 non-vented T-flask, cell lines were cultured in the obsolete of antibiotics
2. Using cell scraper, adherent cell lines were brings into suspension. These suspensions were tested directly
3. At first, two separate test tube were selected and each of it contains thioglycollate medium(TGM), and tryptone soya broth(TSB)
4. Two separating test tube were used and 0.1ml e.coli, 0.1ml b.subtilis and 0.1 ml c.sporogenes inoculated into an individual test tube containing TMG and TSB. These acted as positive controls. On the other hand, two separate test tube containing TGM and TSB without inoculation consider as negative controls.

Broths were incubated as follows:

1. For TBS one broth of each pair was incubated at 32ºC the other at 22ºC for 4 days.
2. For TGM, one broth of each pair was incubated at 32ºC the other at 22ºC for 4 days.
3. For the TGM inoculated with C. Sporogenes incubate at 32ºC for 4 days.

Note: Test and Control broths were examined for turbidity after 4 days.

Criteria for a Validity of results:

Control broths show evidence of bacteria and fungi within 4 days of incubation in all positive control broths and the negative control broths show no evidence of bacteria and fungi.

Criteria for a Positive Result: Test broths containing bacteria or fungi show turbidity.

Criteria for a Negative Result: Test broths should be clear and show no evidence of turbidity.

Preparation of media

Preparation of DMEM

10.7gm of DMEM powder was added into 1litre of distilled water and then it was stirred continuously until a clear solution formed. To this, $NaHCO_3$ was added to maintain pH 7.0-7.2 and then the solution was filtered using membrane filtration assembly. It was stored in reservoir bottle under room temperature.

Preparation of the Trypsin dilution

5ml of Trypsin solution was pipetted out into 50ml falcon tube containing 45ml of PBS using a 10ml pipette.

Determination of cell viability and population doubling time:

The quantification of cellular growth, including proliferation and viability, has become an essential tool for working on cell-based studies.

Cell viability by Trypan Blue Dye Exclusion Method:

The viability of cells was determined by the Trypan Blue dye exclusion method. It takes advantage of the ability of healthy cells with uncompromised cytoplasmic membrane integrity to exclude dyes such as trypan blue.

Haemocytometer Cell Count

1. Haemocytometer and coverslip were cleaned and wiped with 70% alcohol. Then cover slip was placed on a hemocytometer.
2. In separate 2ml centrifuge tube, a cell suspension (cells in culture media) was added. Then two-fold dilution of the reaction mixture was prepared by mixing aliquot of 0.1 ml cell suspensions with 0.1 ml trypan blue.
3. Afterward, 0.1ml of Cell suspension was then placed in the chamber of a hemocytometer.
4. By using a Lieca inverted microscope, numbers of cells were counted in 1mm2 area with the use of 10X objective.
5. Viable and non-viable cells were counted in both halves of the chamber.

Calculation:
(1) Total number of viable cells = A×B×C×104
(2) Total dead cell count = A×B×D×104
Where,
A = Volume of cell solution (ml)
B = Dilution factor in trypan blue
C = Mean number of unstained cells
D = Mean number of dead/stained cells
104 = Conversion of 0.1 mm3 to ml
(3) Total cell count = Viable cell count + dead cell count
% Cell Viability = (Viable cell count/Total cell count) × 100

Population doubling time (PDT):

It is the time expressed in hours, taken for cell No. to get double. Population doubling time can be determined as follows.
Population doubling time = (X/Y) x 24 hrs
Where,
X= (cell number at harvest/cell number initially plated)/2
Y= Total number of days

8.2.9.2 % Cell Growth Inhibition by MTT Assay Protocol:

1. Cells were pre-incubated at a concentration of 1× 106 cells/ml in culture medium for 3 h at 37°C and 6.5% CO_2 Cells were seeded at a concentration of 5× 104 cells/well in 100 µl culture
medium and various amounts of formulation (final concentration e.g. 100µM -0.005µM) were added into microplates (tissue culture grade, 96 wells, flat bottom).
2. Cell cultures were incubated for 24 h at 37°C and 6.5% CO_2.
3. 10 µl MTT labeling mixture was added and incubate for 4 h at 37°C and 6.5% CO_2.
4. 100 µl of solubilization solution was added to each well and incubate for overnight.
5. The absorbance of the samples was measured using a microplate (ELISA) reader.
6. From the absorbance, % cell growth inhibition was calculated using following formula.

$$\% \text{ Cell growth inhibition} = 100 - \left(\frac{\text{Mean absorption of individual test group}}{\text{Mean absorption of individual controlled group}}\right) \times 100$$

Accelerated stability study:

Stability study is an important parameter in which, it can be assumed, how a formulation would behave during in versatile humidity and temperature. It also gives us complete detail report on its self-life and storage condition, which has to be maintained. In this experiment, optimized niosome dispersion and optimized niosome gel formulations were exposed to different temperature and humidity condition as per ICH Q1A (R2) guideline for stability study.

Table 2: Product intended for general storage condition

Study	Storage condition	Minimum time period
Accelerated stability study (Intermediate)	30°C±2°C & 65%RH±5%RH	Minimum 6 month

Table 3: Formulations intended to store in refrigerator

Study	Storage condition	Minimum time period
Accelerated stability study (Moderate and Long term)	5°C±3°C	Minimum 6 month

As per specific period of time, charged niosomal sample instability chamber was collected and analyzed for various parameters such as mean particle size, zeta potential, drug entrapment efficacy. For niosomal gel dispersion, pH, viscosity, bioadhesive strength, extrudability, gel strength, drug content, *in-vitro* gelation study, & diffusional drug release study was performed and compared with the standard data.

Statistical data analysis for stability batches: Using one-way ANOVA general and refrigerator stability batch was analyzed and P value was determined.

Evaluation of niosomal gel formulation:

Physical examination: The prepared Anti-EGFR-BSA-CYP-SLNs based niosomal gel and without drug was light and Sami lucid in appearance. The color of the formulation was pale white.

PH: The pH value of prepared formulation were varied from 5.19±1.29 to 5.92±0.56

Content uniformity: The content of drug presence within the formulation was determined by the UV-Visible method. Content uniformity of the formulations was varied from 94.67±0.37 to 98.56±0.44%

Viscosity: The measured viscosity of the prepared topical gel was varied from 123±0.23 to 438±0.11CPS.

Extrudability: The variation of extrudability of niosomal gel formulation was varied from 22.34±0.38 to 32.53±0.03 gm/cm^2.

Gel strength: The gel strength of prepared formulations was within the range of 1.45±0.45 to 3.98±0.12 gm

Spreadability test: The spreadability was found to be in the range of 35.78±2.10 to 47.51±0.53 gm.cm/second

Bioadhesive strength: The bioadhesive strength of the prepared formulations was within the range of 12±2 to 97±5 gm.

Acute skin irritation study: Results of acute skin irritation test for topical gel formulation were shown below in table in terms of skin irritation score (erythema and edema). According to Draize et al., formulation producing a score of 2 or less are considered to be nonirritating.

Table 4: Draize score for skin irritation study

Group	Score in Erythema	Score in Edema
Negative controlled	0	0
Test controlled	0	0
Positive controlled	1.23±0.02	0.98±0.17

Erythema scale: 0-none; 1-Slight; 2-well defined; 3-moderate; 4-scare formation Edema scale: 0-none; 1-Slight; 2-well defined; 3-moderate; 4-severe Results show that in developed formulation, erythema and edema values are less than 2. Hence, the developed formulation is said to be free from skin irritation.

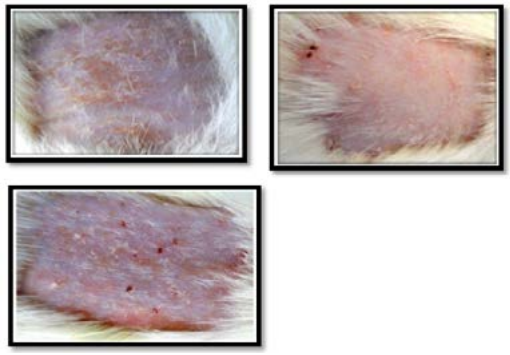


Figure 2: Negative, test & positive controlled

In-vitro anticancer study:

Characterization of the cell line and culture media: Characterization of the cell line was done to detect microbial cross contamination and microbial growth. Before starts experiments, the cell lines were double checked for any kind of cross contamination.

Table 5: Characterisation of cell line and cell culture

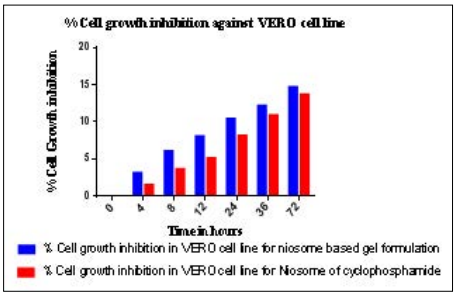
Cell line	% viability	PDT (hr)	Microbial contamination	Cross contamination	pH
VERO	76.15	31.2	NO	NO	7.6
A375	83.18	27.3	NO	NO	7.1

Culture media was also cross-checked for microbial contaminations. To cross check the presence of microbial contaminations, 2.5% Amphotericin B25 (µg/ml) was incorporated into the media, which acting as a working concentration. Bacterial contamination can be fragmented by adding 1% of antibiotics like 100X (100000U/ml Penicillin G, 10000µg/ml cephalosporin) into the culture medium. Using inverted microscope cross-contamination of cell lines were tested. From the viability studies and PDF studies it can be concluded that the cell line derived from Deshpande laboratory, Bhopal was initially free from cross contamination.

%Cell growth inhibition by MTT assay: Using MTT assay method in-vitro cytotoxicity studies were carried out for the freshly characterized A375 and VERO cell line. The various % cell growth inhibition at different time interval was recorded and reported.

Table 6: % cell growth inhibition against different cell line

Time in Hr	% Cell growth inhibition			
	Niosome based gel formulation		Niosome of cyclophosphamide	
	VERO	A375	VERO	A375
0	0	0	0	0
4	3.18	8.19	1.56	7.09
8	6.11	17.19	3.69	12.93
12	8.07	45.19	5.16	25.82
24	10.45	52.13	8.18	52.18
36	12.19	62.29	10.92	73.69
72	14.67	99.28	13.69	94.19



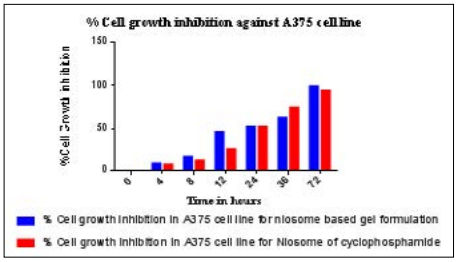


Figure 3: % cell growth inhibition studies using VERO & A375 cell line

Table 5: Responses of experimental design formulations

Batch number	Bio-adhesiveness (gf) (Y1) (mean±S.D)	Viscosity (CPS)(Y2) (mean±S.D)	%CDR 4th hour (Y3)	% CDR 8th hour (Y4) (mean±S.D)	%CDR 12th hour (Y5) (mean±S.D)
NGF1	74±1	343±0.23	9.38±0.11	23.38±0.18	52.53±1.89
NGF2	24±2	156±0.22	17.23±0.24	39.12±1.90	65.96±0.23
NGF3	42±2	221±0.18	14.58±0.26	32.73±0..11	62.87±0.12
NGF4	66±4	289±0.83	12.03±1.46	27.15±0.25	57.01±0.02
NGF5	86±1	404±0.14	7.481±0.22	19.87±0.03	49.38±0.13
NGF6	97±5	438±0.11	7.29±0.23	18.84±0.38	46.55±0.34
NGF7	12±2	123±0.23	18.89±0.14	43.78±0.45	68.43±0.34
NGF8	85±3	393±0.34	7.52±0.08	19.91±0.07	49.47±0.33
NGF9	23±2	164±0.11	17.013±0.87	38.66±0.35	65.72±0.29
NGF10	43±3	223±0.11	14.632±0.19	33.31±0.11	62.86±0.98
NGF11	51±1	268±0.34	12.368±0.03	29.18±0.98	59.94±0.31
NGF12	65±3	298±0.22	11.98±0.21	27.07±0.24	57.02±0.56
NGF13	37±2	170±0.11	16.681±0.03	36.16±0.34	63.72±0.11

Experimental design

For designing, we took 13 batches. The various dependent variables are bioadhesive strength (Y1), viscosity (Y2), %CDR at 4th hour (Y3), %CDR at 8th hour (Y4), %CDR at 12th hour (Y5), shows distinct results from 12-97gf, 123-438 CPS, 07.29–18.89 %, 18.84-39.12%, 46.55-68.43%.The multiple regression was performed, and shown in table number: Value of $p<0.05$ indicates models terms were significant itself.

Table 6: Design Summary

Study Type	Response Surface	**Runs**	13
Initial Design	**Box-Behnken**	**Blocks**	No Blocks
Design Model	Quadratic		

Factor	Name	Units	Type	Low Actual	High Actual	Low Coded	High Coded	Mean	Std. Dev.
A	Concentration of Poloxamer 188	%	Numeric	25.00	35.00	-1.000	1.000	30.000	3.922
B	Concentration of HPMC	%	Numeric	4.00	8.00	-1.000	1.000	6.000	1.569

Response	Name	Units	Obs	Analysis	Minimum	Maximum	Mean	Std.Dev.	Ratio	Trans	Model
Y1	Bio adhesive strength	gf	13	Polynomial	12.00	97.00	54.23	25.85	8.08	None	Quadratic
Y2	Viscosity	CPS	13	Polynomial	123.00	438.00	268.46	99.31	3.56	None	Quadratic
Y3	%CDR at 4th hour	%	13	Polynomial	7.29	18.89	12.85	3.88	2.59	None	Quadratic
Y4	%CDR at 8th hour	%	13	Polynomial	18.84	43.78	29.94	7.82	2.32	None	Quadratic
Y5	%CDR at 12thhour	%	13	Polynomial	46.55	68.43	58.57	6.91	1.47	None	Quadratic

Mathematical modeling

Using design expert statistical tool pack (Design expert® 7.0) it is possible to have good correlation between independent variables like polymers concentration and the dependent variables. At first, appropriate models need to be implemented for experimental data. The software itself selects a suitable model for experiments on the basis of individual parameters generates from regression analysis such as adjusted R^2, predicted R^2, Predicted Residual Sum of Square (PRESS) and p-value. At 5% level of significance, ANOVA was implicated. If more than one model is significant ($p<0.05$) then other parameters such as adjusted R^2 value and PRESS value was been compared to selected best model [14 & 15]. During mathematical model fitting the main focuses has to be given on higher adjusted R^2 value (<1) and lower PRESS value. The general quadric equation for two independent variables is as follow:

$$Y= \beta_0+X_1\beta_1+X_2\ \beta_2+X_1X_2\ \beta_3+X1^2\ \beta_4+X2^2\ \beta_5$$

β0 represent the arithmetic outcomes average of all the outcomes of experimentation-13 batches. **β1 to β5** represents the coefficient of observed experimental values of Y1 to Y5. On the other hand, X1 and X2 are the coded level of factors. X1 to Xn (n=any number) represent quadric terms and interaction respectively. The coefficient of one factor signifies the effect of particular factor and interaction of two-factor represents the quadric nature and effect between those two factors respectively. In front of factors if the negative sign was implicated, then it's indicating, it has an antagonistic effect on design, on the other hand, the positive sign represents the synergistic effect on design model.

Effect of formulations variables on bio-adhesion or bioadhesive strength

For bio-adhesion linear and the quadric model was found to be most appropriate and significant (Table no: 14).But further analysis reviled that quadric model has higher adjusted R^2 value and moderately lower PRESS value. PRESS model indicates best-fit method. F-value of the quadric model is significant 5.19. The quadric equation was found to be, as per software output:

$$\textbf{Bioadhesive strength (Y1)} = +52.43+11.75X_1+30.75X_2-0.50X_1X_2+1.21x1^2+1.71X2^2\ldots\ldots\ldots \textbf{(1)}$$

Equation 1 indicating that X_1 and X_2 factors significantly affecting the bio-adhesion of the topical gel. It has also revealed, the effect of a change in HPMC concentration seems more prominent than Poloxamer 188 concentration and can effect bioadhesive property of the topical gel. The coefficient of X^2 is larger (30.75) than X^1 (11.75) indicating the synergistic effect on bio-adhesion. The combined effect of X^1 and X^2 further interpreted by using surface and counter plots. The 3D surface plot is shown bio-adhesion (Y1) varies in a linear fashion with the polymer concentration. The descending portion of the curve towards HPMC (X_2) indicating the effect of HPMC is comparatively more prominent then Poloxamer 188 (X_2). From this conclusive evidence on can predict that alteration of polymers can change the quality of bio-adhesion. The linear plot with expected and predicted value indicates the perfect correlation of the model. From the Box-Cox plot of power transfer graph, it was observed that the blue color line was found to be within the red color line, indicating the model is in the optimized zone and no significant changes require for response transformation **(Figure 4)**.

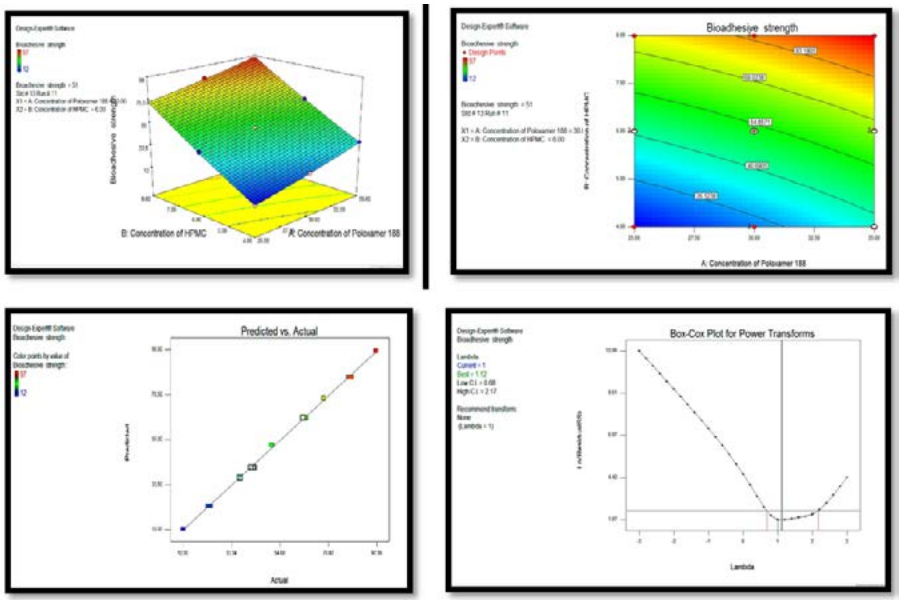


Figure 4: Various factorial output from design expert software on effect on formulation variables on bioadhesive strength (Y1)

Table 7: Regression analysis of bioadhesive strength (Y_1) with Poloxamer 188 (X_1) and HPMC (X_2)

Source	*df*	*SS*	*MS*	*F*	*Significance F*
Regression	2	8669	4334.5	2504.377778	3.14E-14
Residual	10	17.30769231	1.730769		
Total	12	8686.307692			

Effect of formulation variables on viscosity

Viscosity is an essential part of the topical gel integrity. But, the viscosity should not be too much as because Cyclophosphamide in prepared niosomal topical gel has several layers of coatings of cholesterols and span 60.Higher viscosity can cause less diffusion and permeation through the epidermis of the skin. Keeping those points in mind we prepared this gel with Poloxamer 188 and HPMC. Poloxamer 188 in higher concentration can produce a protective effect and can forms layer in skin, on the other hand, HPMC swells within the gel and forms several cross-linked surfaces to produce legitimate viscosity. Higher cross-linked and a high viscous polymer such as carbopol, tragacanth were rejected after series of initial trials, as these polymers produce a higher viscosity and drug might have leached out within the polymer matrix, due to this diffusion and permeation study and subordinately spreadability study failed.

After so many trials Poloxamer 188 and HPMC names were finalized and concentration of both the polymers was finalized. With the help of mathematical modeling, we are trying to figure out which model was the best fit for this experimental design. As per design expert software output, the quadric model has been selected. In the quadric model, maximum variables were been utilized. P value of quadric model was very minimal, that is 21.52 and f value was 0.0010, which makes it more significant. Further, adjusted R2 value was lesser than the linear and 2F model, and PRESS value was least as 420.71 compare to another model. Hence, it can be concluded that quadric model was the best fit for this design. The quadric equation was found to be:

$$\textbf{Viscosity (Y2)} = +268.29 + 35.62X_1 + 120.62X_2 + 12.00X_1X_2 - 10.61X1^2 + 10.89X2^2 \ldots\ldots\ldots (2)$$

From the equation 2, it can be postulated that HPMC has considerable amount of impact on viscosity, as the coefficient of X2 (HPMC) was more than the coefficient of X1 (Poloxamer-188).On the other hand, the combination of two X1 and X2 produces a synergistic effect on viscosity enhancement, as X1 X2 coefficient was positive. The X12 coefficient value was found to be in negative, indicating more increase of poloxamer-188 can have an antagonistic effect on viscosity, on the other hand, X22 higher coefficient than X12 and positive sign, indicating most significant effects on viscosity. From the 3D surface model plot, it can be clearly seen design leaner portion descending towards HPMC, showing the significance of HPMC in this design. The linear plot with expected and predicted value indicates the perfect correlation of the model. From the Box-Cox plot of power transfer graph, it was observed that the blue color line was found to be within the red color line, indicating the model is in the optimized zone and no significant changes require for response transformation **(Figure 5)**.

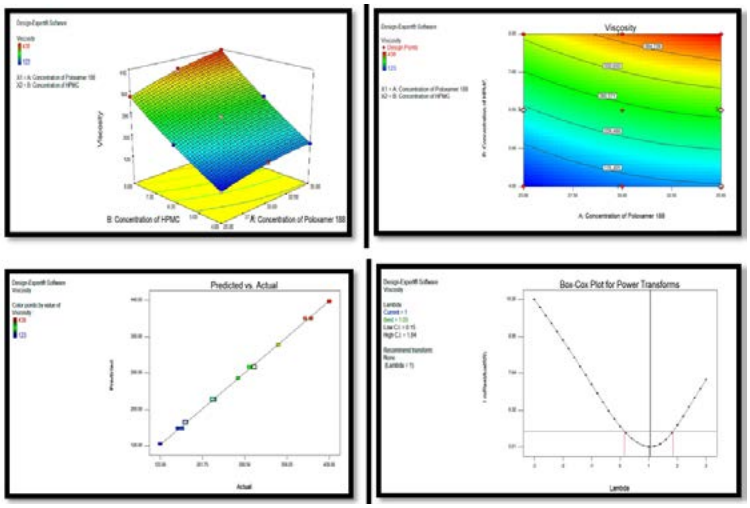


Figure 5: Various factorial output from design expert software on effect on formulation variables on viscosity (Y2)

Table 8: Regression analysis of viscosity (Y_2) with Poloxamer 188 (X_1) and HPMC (X_2)

Source	*dfs*	*SS*	*MS*	*F*	*Significance F*
Regression	2	126556.25	63278.13	383.2759665	3.54118E-10
Residual	10	1650.980769	165.0981		
Total	12	128207.2308			

Effect of formulation variables on cumulative percentage of drug released at 4th hour:
Except for 2FI model, the linear and quadric model shows significant in p-value (< 0.0001) indicating best-fit model. But again quadric model has less f value (50.93) as compared to the linear model, further adjusted R^2 value is higher, PRESS value is lesser for the quadric model, indicating best fit for designing this experiment. The quadric equation generates from the software is as follows:

%CDR at 4th hour (Y3) =+12.48-1.19X_1-4.77X_2+0.030X_1X_2+0.80$X1^2$-0.19$X2^2$ ……. (3)

This equation (number 3) clearly indicating non-linearity of the drug release with an increase of polymers concentration. The X_1 and X_2 negative coefficient value indicating antagonistic effect with drug release. On the other hand X_1X_2 positive coefficient value indicating a possible interaction between two polymers in a certain point of time. The mild increase of X^21 indicating reverse gelling properties of poloxamer 188. The negative sign of X^22 value signifies increase concentration of HPMC can cause a decrease in drug release. From the 3D model, it can easily estimate the non-linearity of the design with polymer concentrations. The linear plot with expected and predicted value indicates the perfect correlation of the model. From the Box-Cox plot of power transfer graph, it was observed that the blue color line was found to be within the red color line, indicating the model is in the optimized zone and no significant changes require for response transformation **(Figure 6)**.

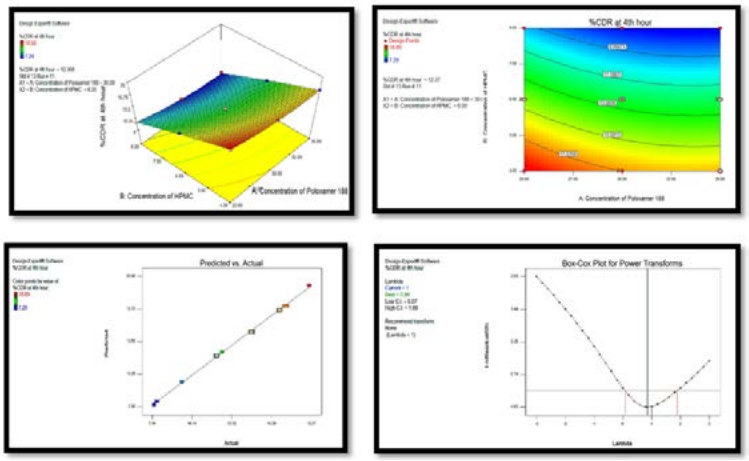


Figure 6: Various factorial output from design expert software on effect on formulation variables on % CDR at 4th hour (Y3)

Table 9: Regression analysis of %CDR at 4th hour (Y_3) with Poloxamer 188 (X_1) and HPMC (X_2)

Source	*df*	*SS*	*MS*	*F*	*Significance F*
Regression	2	193.1446813	96.57234	379.010887	3.74225E-10
Residual	10	2.548009673	0.254801		
Total	12	195.6926909			

Effect of formulation variables of cumulative percentage of drug released at 8th hour:

At particular 8th hour, drug release must be steady and controlled. Except for 2FI model, linear and quadric model possess lesser p-value (<0.0001) but compare to linear model f value of quadric model is lesser (34.16). The adjusted R^2 value of quadric model was found to be higher compared to other models, where else PRESS value (0.95) for the quadric model was found to be lesser than all the models. This is a conclusive evidence that quadric model was the best fit model for design experiment. The projected quadric equation was found to be:

%CDR at 8th hour (Y4) =+29.03-3.00X1-9.47X2+0.77X1X2+1.07X12+0.40X22 ……. (4)

The negative sign of X1 and X2 coefficient clearly indicating non-linearity of the model and persistence antagonistic effect with the drug release. The combined effect of X1 and X2 indicating an increase in drug release, due to the gelling effect and forming a thin layer at the certain point of time. At 30% of poloxamer -188 and 8% HPMC concentration design reaches its optimum point, and drug release was found to be lesser. Increased concentrations of polymers can be agonistic with drug release. 3D model indicating non-linearity of drug release with an increase in concentration. The linear plot with expected and predicted value indicates the perfect correlation of the model. From the Box-Cox plot of power transfer graph, it was observed that the blue color line was within the red color line, indicating the model is in the optimized zone and no significant changes require for the response transformation **(Figure 7)**.

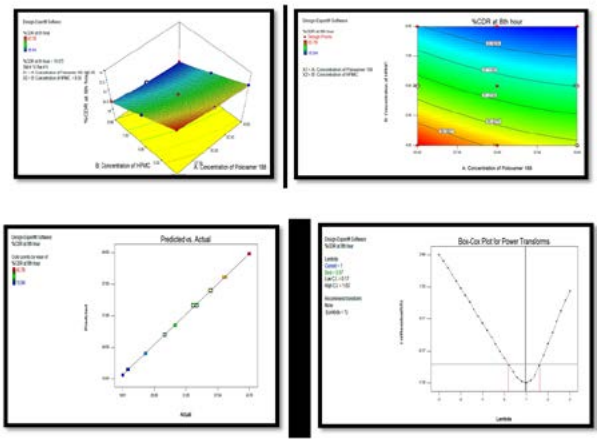


Figure 7: Various factorial output from design expert software on effect on formulation variableson % CDR at 8th hour (Y4)

Table 10: Regression analysis of %CDR at 8th hour (Y_4) with Poloxamer 188 (X_1) and HPMC(X_2)

Source	*df*	*SS*	*MS*	*F*	*Significance F*
Regression	2	788.56985	394.2849	661.1444322	2.38236E-11
Residual	10	5.963673077	0.596367		
Total	12	794.5335231			

Effect of formulation variables of cumulative percentage of drug released at the 12th hour:

Since this formulation comes under prolong drug delivery system, the drug release pattern must be steady and progressive. No unprecedented drug release can consider for design. From the mathematical modeling, it was revealed that p value of quadric model was significant, but F value was found to be larger. The adjusted R2 value was found to be maximum and PRESS value was been very minimum for the quadric model, hence quadric model was considered for experimental design. The quadric equation is as follows:

%CDR at 12th hour (Y_5) = +59.84-2.80X_1-8.24X_2-0.32X_1X_2+0.13$X1^2$-2.18$X2^2$ ……. (5)

Equation 5 clearly indicating that HPMC again has a dominating effect over Poloxamer 188. The negative sign of X1 and X2 coefficient indicates drug release can be decayed upon increase concentration of polymers. The mix responses of drug (X1, X2) signed negative; indicating a decrease in drug release. It was also revealed that multiple increases of X1 can increase drug release, due to the little surfactant property of Poloxamer 188, on the other hand, negative sign of multiple increase of X2 indicates a decrease in drug release patterns. 3D model indicating non-linearity of drug release with an increase in concentration. The linear plot with expected and predicted value indicates the perfect correlation of the model. From the Box-Cox plot of power transfer graph, it was observed that the blue color line was within the red color line, indicating the model is in the optimized zone and no significant changes require for the response transformation **(Figure 8).**

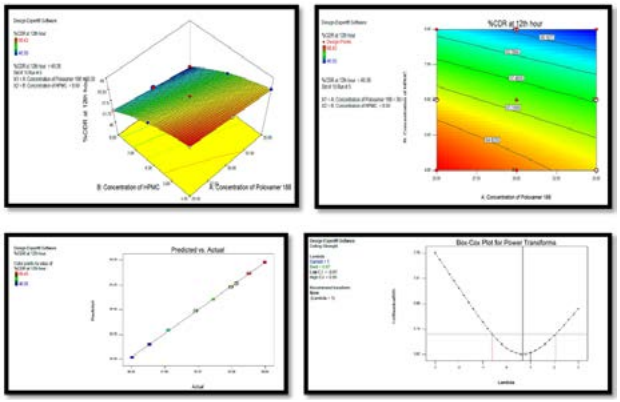


Figure 8: Various factorial output from design expert software on effect on formulation variables on % CDR at 8th hour (Y5)

Table 11: Regression analysis of %CDR at 12th hour (Y5) with Poloxamer 188 (X1) and HPMC(X2)

Source	*df*	*SS*	*MS*	*F*	*Significance F*
Regression	2	605.5152625	302.7576	191.6618755	1.0623E-08
Residual	10	15.79644519	1.579645		
Total	12	621.3117077			

Table 12: Polynomial coefficient for all the five responses

Coefficient	Bioadhesive strength (Y1)	Viscosity (Y2)	% CDR at 4th hour(Y3)	% CDR at 8th hour (Y4)	% CDR at 12th hour (Y5)
	Selected model: Quadratic	Selected model: Quadratic	Selected model: Quadratic	Selected model: Quadratic	Selected model: Quadratic
b_0	52.43	268.29	12.48	29.03	59.84
b_1	11.75	35.62	-1.19	-3.00	-2.80
b_2	30.75	120.62	-4.77	-9.47	-8.24
b_{12}	-0.50	12.00	0.030	0.77	-0.32
b_{11}	1.21	-10.61	0.80	1.07	0.13
b_{22}	1.71	10.89	-0.19	0.40	-2.18

Table 13: Fit summary of highest order polynomial measured responses of the independent variables of the reduced model

Source	Y1		Y2		Y3		Y3		Y5	
	f- value	p-value	f-value	p- value	f- value	p- value	f value	p- value	f value	p- value
Linear vs Mean	2504.38	<0.0001	383.28	<0.0001	379.01	<0.0001	663.57	<0.0001	191.26	< 0.0001
2FI vs Linear	0.55	0.4765	4.82	0.0557	0.013	0.9134	5.98	0.0371	0.24	0.6393
Quadratic vs 2FI	5.19	**0.0415**	21.52	**0.0010**	50.93	**<0.0001**	34.16	**0.0002**	288.46	**< 0.0001**

Table 14: Model Summary Statistics of response to select best model to fit data

Source		Linear			2FI			Quadratic		
		Adjusted R^2	Predicted R^2	PRESS	Adjusted R^2	Predicted R^2	PRESS	Adjusted R^2	Predicted R^2	PRESS
Response	**Y1**	0.9976	0.9971	25.50	0.9975	0.9962	32.98	**0.9987**	0.9969	**26.69**
	Y2	0.9845	0.9769	2963.97	0.9888	0.9866	1718.33	**0.9980**	0.9967	**420.71**
	Y3	0.9844	0.9793	4.06	0.9827	0.9753	4.83	**0.9986**	0.9949	**0.99**
	Y4	0.9910	0.9855	11.55	0.9940	0.9858	11.27	**0.9993**	0.9988	**0.95**
	Y5	0.9694	0.9583	25.92	0.9669	0.9465	33.22	**0.9995**	0.9982	**1.13**

Table 15: Analysis of variance (ANOVA) table for measured responses

Model/ model term	Bioadhesive strength (Y1)		Viscosity (Y2)		% CDR at 4th hour (Y3)		% CDR at 8th hour (Y4)		% CDR at 12th hour (Y5)	
	f-value	p-value	f-value	p-value	f-value	p-value	f-value	p-value	f-value	p-value
Model	1849.16	<0.0001	1192.08	<0.0001	1673.04	<0.0001	3350.41	<0.0001	4702.37	<0.0001
X_1	1176.53	<0.0001	472.57	<0.0001	482.74	<0.0001	1517.01	<0.0001	2372.43	<0.0001
X_2	8057.84	<0.0001	5417.96	<0.0001	7780.46	<0.0001	15116.68	<0.0001	20547.24	<0.0001
X_1X_2	1.07	0.3364	26.81	0.0013	0.15	0.7087	50.02	0.0002	15.26	0.0058
$X1^2$	4.40	0.0742	14.66	0.0065	77.25	<0.0001	67.93	<0.0001	1.68	0.2365
$X2^2$	8.77	0.0211	15.46	0.0057	4.39	0.0744	9.39	0.0182	4702.37	<0.0001

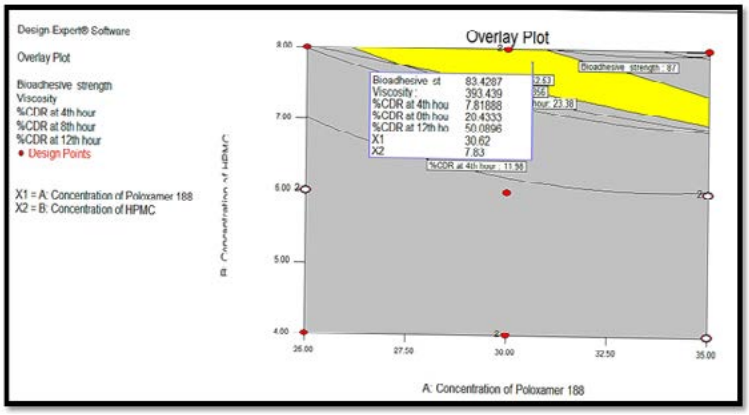


Figure 9: Overly plot for optimized batch of topical gel

Table 16: Results of checkpoint batch

Responses	Predicted value	Experimental value*	Percentage relative error
Bio-adhesive strength(gf)	83.248	86±2	3.305%
Viscosity(CPS)	393.439	392±0.23	0.365%
%CDR at 4th hour	7.818	7.39±1.07	5.476%
%CDR at 8th hour	20.433	21.90±0.28	7.241%
%CDR at 12thhour	50.089	49.81±0.92	0.557%

*All results were shown in mean ± S.D. (n=3)

Desirability function, used to determine optimized batch:

In order to produce the desired product, the formulations responses during optimization, has to combine. It gives us predicting optimum level for independent variables. In order to produce one desirability function, individual desirability has to be calculated. The optimized parameter to be consider was a Mucoadhesive strength, Viscosity, %CDR at the 4th hour, %CDR at the 8th hour, %CDR at the 12th hour. The best part of this study was, no need of specific requirement for gelling strength of the optimized formulation.

For mucoadhesive strength (d1):

Our target is to find desirability for maximum mucoadhesive strength, the formulation, hence the following equation to be followed:

d_1 = {(y-L)/ (T-L)} ------ (1) When, y<L, L≤ y ≤T, y>T

Where y=Individual mucoadhesive strength

L= Represents lower limit of mucoadhesive strength in experimental design batches (2710 dyne/cm2)

T= Targeted mucoadhesive strength as per controlled chart (4234.18 dynes/cm2)

For viscosity (d2):

We need higher viscosity to retain gels integrity, as we used Poloxamer 188 as a principal polymer.

Our target must be to find desirability for maximum viscosity, hence formula (1) to be considered for this experiment.

Where, L= lower limit for viscosity (123 CPS) y= individual viscosity T= targeted viscosity, as per controlled chart (394.49 CPS)

For %CDR at 4th hour (d3):

We need this time lesser drug release for maintaining prolong release action. Hence the following formula must be implemented.

d3= {(U-y) / (U-T)} ------- (2) when, y<T, T≤ y ≤U, y>U

Where U=Upper limit of all cumulative drug release at 4th hour (18.89%)

y=Individual formulations cumulative drug release at the 4th hour.

T=Targeted drug release as per control chart (7.930%).

For %CDR at 8th hour (d4):

To increase absorption in the blood and maintain proper bioavailability drug release has to increase. Hence, equation (1) is used for to target maximum cumulative drug release at the 8th hour.

Where L= Lower limit for cumulative drug release at 8th hour (18.84%)

y = Individual drug release at the 8th hour

T = Targeted drug release at 8th hour as per control chart (20.51%)

For % CDR at the 12th hour (d5):

To maintain steady-state absorption and sustainable drug release, almost maximum drug release is much needed from the formulation, hence again equation (1) was consider for this experiment.

Where, y= Individual cumulative drug release at 12th hour

L= lower limit for cumulative drug release at 12th hour (46.55%)

T= Targeted drug release at 12th hour as per control chart (50.10%)

The overall desirability of the prepared topical gel was calculated for all the 13 batches using following equation:

The overall desirability (D) = (d1×d2×d3……dm) $^{1/m}$ ….. (14)

Where m is the number of responses. The overall desirability value should be below 1 as the range is within 0-1, but the maximum value was to be considered for the optimizing batch. The optimized batch was found to be NGF8 as it produces maximum D value, that is 0.8801.Hence, optimized polymer concentration are: 30% Poloxamer 188 and 8% HPMC.

Table 17: Individual and overall desirability of prepared topical gel

Formulation code	d1	d2	d3	d4	d5	D
NGF1	0.8542	0.8101	0.8677	2.7185	1.6845	1.2634
NGF2	0.0190	0.1215	0.1514	12.1437	5.4676	0.4710
NGF3	0.3962	0.3609	0.3932	8.3173	4.5971	1.1654
NGF4	0.6462	0.6114	0.6259	0.6259	2.9464	1.2938
NGF5	0.9972	1.0350	1.0409	0.6167	0.7971	**0.8801**
NGF6	1.2334	1.1602	1.0583	0	0	0.0000
NGF7	0	0	0	14.9341	6.1633	0.0000
NGF8	**1.0084**	**0.9945**	**1.0374**	**0.6407**	**0.8225**	**0.8801**
NGF9	0.0328	0.1510	0.1712	11.8682	5.400	0.5585
NGF10	0.3877	0.3683	0.3885	11.6586	4.5943	1.2439
NGF11	0.5117	0.5340	0.5950	6.1916	3.7718	1.3058
NGF12	0.6423	0.6445	0.6304	4.9281	2.9492	1.2289
NGF13	0.1115	0.1731	0.2015	10.3812	4.8366	0.7213

Table 18: Results of experimental design batch

Formulation code	pH (mean±SD)	Viscosity In CPS (mean±SD)	Extrudability In gm/cm2 (mean±SD)	Gel strength In gm (mean±SD)	%drug content (mean±SD)	Spreadability In gm.cm/sec (mean±SD)	Bioadhesive strength Dyne/cm2 (mean±SD)	In-vitro gelation study
NGF1	5.23±0.24	343±0.23	24.11±1.01	3.23±0.56	95.89±0.04	38.17±0.11	74±1	+++
NGF2	5.32±0.11	156±0.22	31.59±0.56	1.72±1.63	98.34±1.09	41.46±0.73	24±2	+++
NGF3	5.64±0.78	221±0.18	28.62±1.07	2.14±2.89	97.88±0.34	40.91±0.29	42±2	+++
NGF4	5.78±1.08	289±0.83	26.28±0.78	2.97±0.78	96.59±0.99	39.69±1.56	66±4	+++
NGF5	5.98±0.13	404±0.14	23.89±0.28	3.98±0.12	95.13±1.86	36.16±1.09	86±1	+++
NGF6	5.23±0.34	438±0.11	22.34±0.38	4.12±0.36	94.67±0.37	35.78±2.10	97±5	+++
NGF7	5.19±1.29	123±0.23	32.53±0.03	1.45±0.45	98.78±0.82	42.18±0.95	12±2	+++
NGF8	5.28±0.23	393±0.34	24.03±0.38	3.78±0.29	98.56±0.44	36.91±0.44	85±3	+++
NGF9	5.92±0.56	164±0.11	30.83±0.16	1.83±0.52	98.19±0.30	41.36±0.18	23±2	+++
NGF10	5.34±0.23	223±0.11	28.57±1.78	2.23±0.39	97.34±0.38	47.51±0.53	43±3	+++
NGF11	5.81±0.58	268±0.34	27.29±0.07	2.70±0.33	96.98±0.32	40.68±0.73	51±1	+++
NGF12	5.23±0.03	298±0.22	25.19±1.45	3.04±0.34	96.11±0.28	39.37±0.30	65±3	+++
NGF13	5.51±0.49	170±0.11	30.57±0.34	1.98±1.09	97.88±0.65	41.11±1.25	37±2	+++

Table 19: *in-vitro* dissolution studies of prepared topical gel formulations F1 to F13

Hours	NGF1	NGF2	NGF3	NGF4	NGF5	NGF6	NGF7	NGF8	NGF9	NGF10	NGF11	NFG12	NGF13
0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.5	0.58	3.08	3.51	2.71	0.89	0.98	4.75	0.78	4.19	3.11	2.64	2.51	3.54
1	1.45	6.67	6.18	5.05	1.98	2.16	8.11	1.87	9.65	6.78	4.71	5.33	6.89
2	3.68	9.36	9.15	8.78	3.96	4.11	11.86	3.28	13.27	9.13	7.72	7.49	9.19
3	5.78	12.67	11.39	10.56	5.61	5.89	15.04	5.72	15.41	12.81	9.34	9.45	12.92
4	9.38	17.23	14.58	12.03	7.481	7.29	18.89	7.52	17.01	14.63	12.36	11.98	16.68
5	13.86	24.89	19.72	15.54	10.82	10.73	25.72	9.28	26.86	19.86	15.87	14.89	20.17
6	15.93	28.98	24.69	20.11	13.23	13.09	31.76	13.75	31.86	24.78	19.27	18.34	24.78
7	19.35	33.75	28.06	24.28	16.78	15.65	37.81	16.61	34.12	28.45	24.89	23.67	29.97
8	23.38	39.12	32.73	29.15	19.87	18.84	43.78	19.91	38.66	33.37	29.18	27.07	36.16
9	31.67	45.89	40.23	35.76	27.86	24.81	49.93	27.73	46.67	40.87	37.89	36.29	43.14
10	38.89	51.66	49.28	42.89	35.90	30.82	56.61	36.64	53.79	49.16	46.14	43.89	49.29
11	45.19	58.81	55.81	49.28	39.12	39.82	61.60	42.82	59.15	55.67	51.34	51.12	56.17
12	52.53	65.96	62.87	57.01	49.38	46.55	68.43	49.47	65.72	62.56	59.94	57.02	63.72
13	58.29	73.29	70.04	64.62	58.89	53.19	74.72	56.28	72.62	70.12	69.81	64.89	70.16
14	65.29	79.34	79.94	72.89	65.14	60.28	81.11	63.69	80.87	79.12	78.91	70.12	79.18
15	74.53	87.17	87.17	79.28	74.71	67.18	87.62	71.27	85.28	85.91	84.18	78.12	86.14
16	80.87	94.45	96.58	86.89	80.25	75.18	93.08	79.01	92.78	92.09	90.07	89.04	92.45
17	87.32	105.87	101.98	93.67	87.19	83.18	102.58	85.83	101.85	100.23	101.25	100.34	101.30
18	95.68	-	-	101.34	94.17	89.29	-	89.27	-	-	-	-	-
19	103.90	-	-	110.56	101.29	97.28	-	94.56	-	-	-	-	-
20	108.70	-	-	-	107.67	104.87	-	99.08	-	-	-	-	-

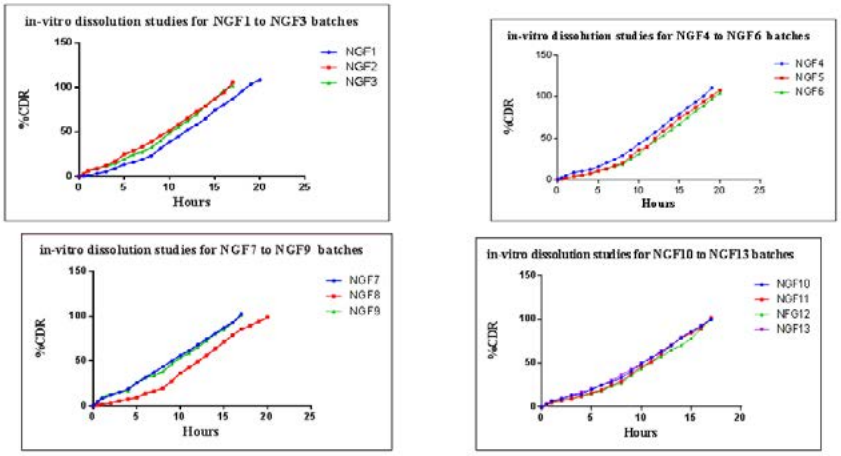


Figure 10: *in-vitro* dissolution studies of prepared topical gel formulations F1 to F13

Kinetics of drug release:

The obtained data from dissolution studies was fitted to various kinetic studies. The purpose of this study is to find the proper kinetic model for optimized batch (NGF8) and rest of the others.

Table 20: Model used in kinetics studies

S.No	Model name	Model equation	Graphs
1	Zero order	$Qt=Q_0-K0t$	Time vs Drug release
2	First order	$InQt=InQ_0-t$	Time vs Log% Drug remaining
3	Higuchi's	Qt=Kht1/2	SQRT Time vs Drug release
4	Korsmeyer –Peppas	Log Qt vs Log t	Log time vs Log% drug release

*N.B: Qt= Cumulative amount of the drug release at time t; Q_0= Initial amount of the drug present in the in-situ gel membrane; Ko = Zero order release rate constant K1= First order release rate constant Kh= Diffusion rate constant. The coefficient of regression and release rate constant values for zero, first order, Higuchi's and Korsmeyer-Pappas models were compared.

Table 21: Kinetics study of drug released profiles of formulation batch

Formulation code	Zero	First	Higuchi	Peppas	K_1	Best fit model
NGF1	0.9610	0.8803	0.8641	0.9943	0.082	**Peppas**
NGF2	0.9904	0.8844	0.9236	0.9786	0.119	**Zero**
NGF3	0.9700	0.8427	0.8805	0.9599	0.116	**Zero**
NGF4	0.9618	0.8684	0.8660	0.9520	0.094	**Zero**
NGF5	0.9309	0.8381	0.8177	0.9689	0.081	**Peppas**
NGF6	0.9344	0.8656	0.8234	0.9647	0.069	**Peppas**
NGF7	0.9952	0.9050	0.9375	0.9781	0.123	**Zero**
NGF8	0.9653	0.7168	0.8301	0.9804	0.077	**Peppas**
NGF9	0.9874	0.8949	0.9216	0.9526	0.115	**Zero**
NGF10	0.9731	0.8569	0.8868	0.9564	0.113	**Zero**
NGF11	0.9571	0.8435	0.8576	0.9547	0.110	**Zero**
NGF12	0.9619	0.8828	0.8659	0.9418	0.092	**Zero**
NGF13	0.9791	0.8650	0.8975	0.9644	0.114	**Zero**

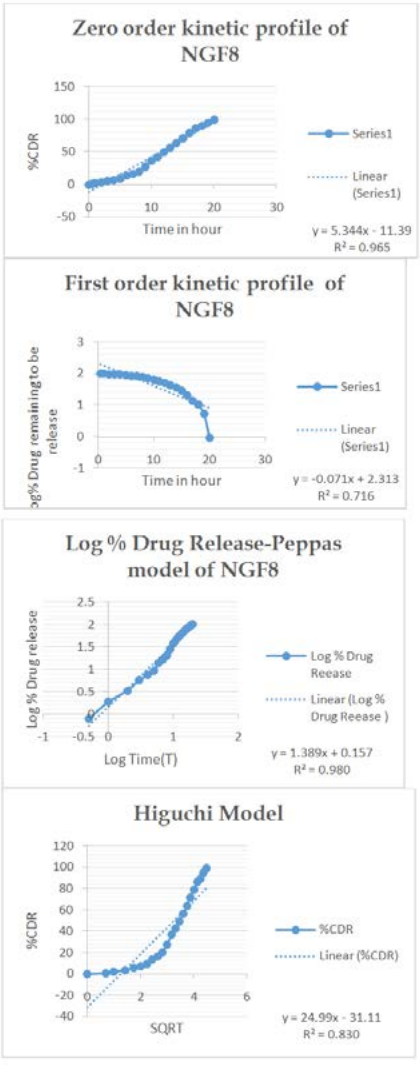


Figure 11: Kinetics study of drug released profiles of formulation batch

***Ex-vivo* permeability study:**

The best formulation should give minimum permeation and maximum skin deposition. After 720 minutes permeation study, it is concluded that Cyclophosphamide pure drug produces less permeation as it releases almost 101 % of drug within 480minutes.The niosome based topical gel of cyclophosphamide (NGF8) produces most minimum permeation of 4.92 % CDP after 720 minutes. Hence skin deposition study has to be performed.

Figure 12: a. *Ex-Vivo* permeability study on gout skin of prepared niosome based topical gel b. niosome based topical gel-optimized batch(F8)

Table 22: *Ex-vivo* permeability studies of optimized formulation

Time in Minute	Cyclophosphamide pure drug	Optimized solid lipid nanoparticle	Niosomal topical gel of cyclophosphamide (NGF8)
0	0.00	0.000	0.000
30	13.24±0.34	3.450±0.86	0.3122±1.97
60	23.46±1.07	5.520±0.23	0.6388±0.73
90	37.98±0.97	7.230±0.11	0.9528±0.93
120	49.56±0.05	8.134±0.09	1.2892±0.97
180	58.78±1.89	9.560±0.28	1.5821±0.86
240	69.23±0.08	10.343±0.12	1.9892±0.27
300	78.34±0.78	12.340±1.67	2.1923±0.11
360	86.21±1.08	13.560±0.67	2.4145±0.86
420	94.13±0.98	15.340±0.11	2.7872±0.21
480	97.46 ±0.34	17.240±0.89	3.1293±0.43
540	-	18.870±0.09	3.5834±0.08
600	-	21.250±0.17	3.9234±0.21
660	-	22.030±0.97	4.4378±0.11
720	-	22.450±0.06	4.9212±0.28

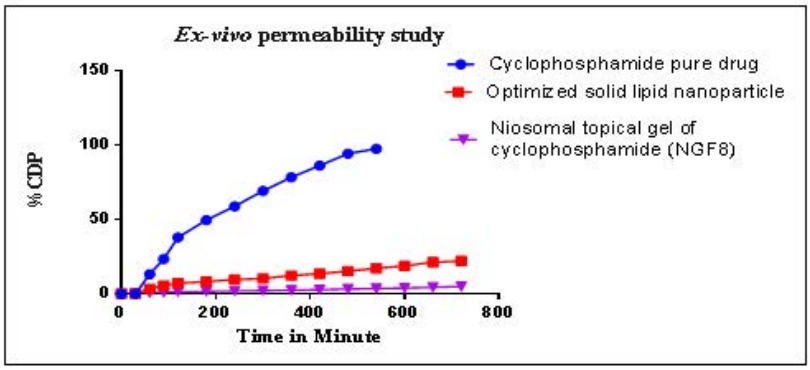


Figure 13: *Ex-vivo* permeability study profile of pure drug, optimized nanoparticle, F8 formulation and NGF8

Skin deposition study results and discussion:

The % drug deposition profile showing Niosomal topical gel containing cyclophosphamide (NGF8), reserved highest skin deposition as compared with the pure drug. The plain Cyclophosphamide showed less accumulation. As per mandatory requirement of ideal formulation, maximum deposition of drug in the squamous epithelium skin and minimum penetration throughout skin is able to release the drug for a prolonged period of time after topical administration. Hence niosome based topical gel of cyclophosphamide (NGF8) was found to be the best candidate for the topical administration.

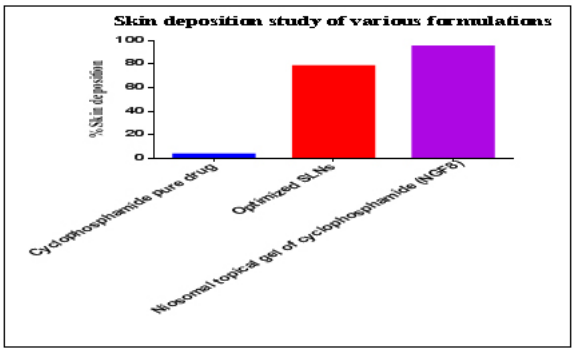


Figure 14: Skin deposition study of pure drug, optimized solid lipid nanoparticle, F8, and NGF8 Stability study:

As per ICH (R2) guideline optimized batch of niosomal dispersion and niosomal topical gel formulation (NGF8) were tested in general room temperature and refrigerator conditions. Samples were tested at different time intervals i.e. 15 days, 1 month, 3 months and 6 months.

Table 23: Product outcomes in general room temperature

Storage condition (30°C±2°C & 65%RH±5%RH)											
Optimized batch of topical niosomal gel (NGF8)						Optimized niosomal dispersion (N33)					
Evaluation parameters	Sampling time					Evolution parameters	Sampling time				
	Initial	15days	1month	3month	6month		Initial	15days	1month	3month	6month
pH	5.28	5.12	5.03	4.78	-NA-	Drug entrapment efficacy (%)	72.12	70.98	68.15	66.17	-NA-
Viscosity (CPS)	393	405	420	456	-NA-	Zeta potential (mV)	-40.16	-39.19	-38.13	-36.18	-NA-
Gel strength (%)	3.78	3.58	3.45	3.15	-NA-	Mean particle size (mm)	1.67	1.78	1.92	2.01	-NA-
Extrudability (gm/ cm2)	24.03	23.67	20.35	18.98	-NA-	-					-NA-
Drug content (%)	98.56	97.75	96.15	94.77	-NA-	-					-NA-
Spreadability(gm. cm/sec)	36.91	35.71	33.12	31.08	-NA-	-					-NA-
Bio-adhesive strength (gf)	86	86	87	87	-NA-	-					-NA-
In vitro gelation study	+++	+++	+++	+++	-NA-	-		+++	+++	+++	-NA-

Table 24: One-way ANOVA results for NGF8 batch during general stability study

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	53487	3	17829	F (1.000, 6.002) = 1.137	P = 0.3273
Individual (between rows)	6.970e+007	6	1.162e+007	F (6, 18) = 741.0	P < 0.0001
Residual (random)	282199	18	15678		
Total	7.003e+007	27			

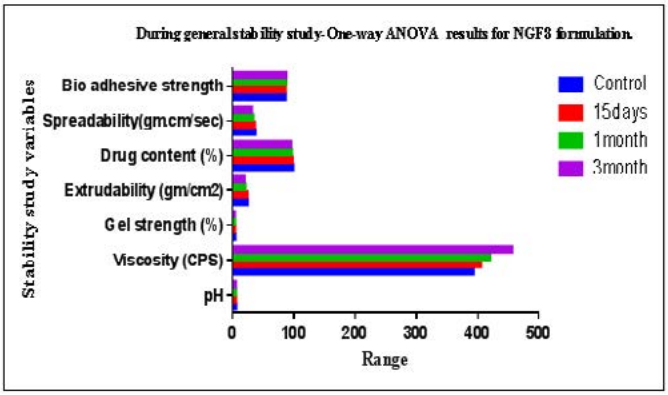


Figure 15: One way ANOVA results for NGF8 during general stability study

Table 25: One-way ANOVA results for N33 batch during general stability study

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	0.8868	3	0.2956	F (1.006, 2.013) = 0.05955	P = 0.8312
Individual (between rows)	23724	2	11862	F (2, 6) = 2389	P < 0.0001
Residual (random)	29.79	6	4.964		
Total	23754	11			

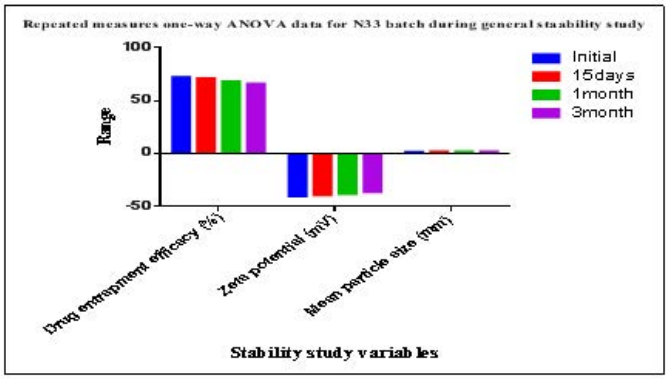


Figure 16: One way ANOVA results for N33 during general stability study

Table 26: Product stored in refrigerator at 5°C±3°C

Storage condition (5oC±3oc)											
Optimized batch of topical niosomal gel (NGF8)											
Evaluation parameters	Sampling time					Evolution parameters	Sampling time				
	Initial	15days	1month	3month	6month		Initial	15days	1month	3month	6month
pH	5.28	5.24	5.16	4.35	-NA-	Drug entrapment efficacy (%)	72.12	72.45	72.35	72.55	-NA-
Viscosity (CPS)	393	398	400	405	-NA-	Zeta potential (mV)	-40.16	-40.13	-40.09	-39.98	-NA-
Gel strength (%)	3.78	3.72	3.74	3.76	-NA-	Mean particle size (mm)	1.67	1.68	1.69	1.72	-NA-
Extrudability (gm/cm2)	24.03	23.85	23.67	23.41	-NA-	-					-NA-
Drug content (%)	98.56	98.25	96.45	97.77	-NA-	-					-NA-
Spreadability(gm.cm/sec)	36.91	36.84	36.12	36.01	-NA-	-					-NA-
In vitro gelation study	+++	+++	+++	+++	-NA-	-		+++	+++	+++	-NA-

Table 27: One-way ANOVA results for NGF8 batch during refrigerator stability study at 5°C±3°C

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	42.93	3	14.31	F (1.065, 6.389) = 0.4463	P = 0.5396
Individual (between rows)	5.917e+007	6	9.861e+006	F (6, 18) = 307571	P < 0.0001
Residual (random)	577.1	18	32.06		
Total	5.917e+007	27			

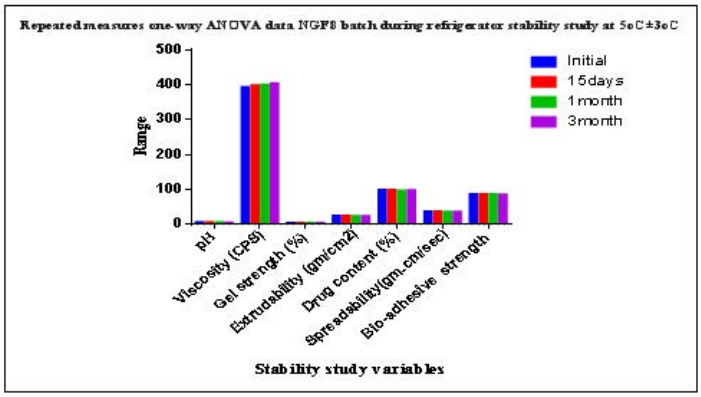


Figure 17: One way ANOVA results for NGF8 batch during refrigerator stability study at 5°C±3°C

Table 28: One-way ANOVA results for N33 batch during refrigerator stability study at 5°C±3°C

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	0.07309	3	0.02436	F (1.124, 2.247) = 3.009	P = 0.2151
Individual (between rows)	25850	2	12925	F (2, 6) = 1.596e+006	P < 0.0001
Residual (random)	0.04858	6	0.008097		
Total	25850	11			

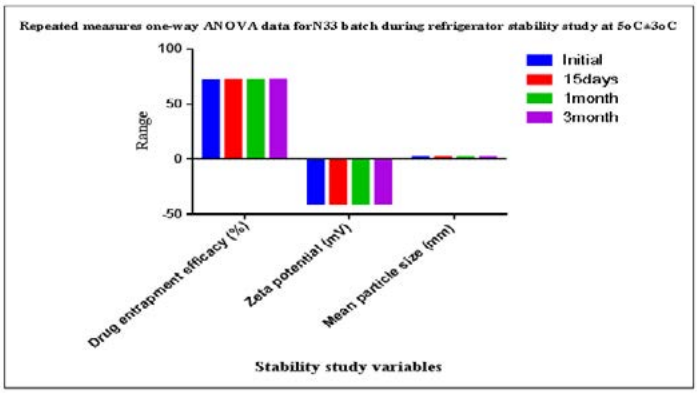


Figure 18: One way ANOVA results for N33 batch during refrigerator stability study at 5°C±3°C

Table 29: *In-Vitro* dissolution study of stability batches during general stability condition:

Dissolution profile of NGF8 during stability study					Optimized niosomal dispersion (N33)				
Time in hours	**Initial**	**15days**	**3month**	**6month**	**Time in hours**	**Initial**	**15 days**	**3 month**	**6month**
0	0	0	0	0	**0**	0.0	0.0	0.0	0
0.5	0.78	1.09	1.23	1.65	**0.5**	1.12	1.44	1.67	2.18
1	1.87	1.90	2.05	2.67	**1**	7.178	7.89	8.12	10.26
2	3.28	3.89	3.63	4.04	**2**	14.89	15.69	10.26	13.62
3	5.72	6.28	6.78	7.26	**3**	21.95	22.34	25.19	29.39
4	7.52	8.18	8.56	9.27	**4**	27.13	30.18	33.18	36.29
5	9.28	10.38	11.28	14.87	**5**	36.99	39.19	42.18	44.73
6	13.75	14.81	15.18	17.25	**6**	39.93	42.19	46.98	49.20
7	16.61	17.86	18.26	20.14	**7**	43.37	45.83	48.11	53.19
8	19.91	21.72	22.12	25.11	**8**	47.03	49.19	50.19	54.08
9	27.73	29.19	32.18	34.18	**9**	53.76	55.34	57.29	61.37
10	36.64	34.98	36.73	39.21	**10**	56.99	58.18	60.19	64.28
11	42.82	41.28	43.19	47.19	**11**	61.12	64.29	68.91	73.29
12	49.47	48.91	52.19	57.25	**12**	67.18	72.89	74.16	79.62
13	56.28	58.21	59.28	65.83	**13**	73.26	76.29	79.19	83.59
14	63.69	64.19	67.18	73.25	**14**	79.25	83.14	85.22	87.39
15	71.27	73.19	75.14	83.16	**15**	85.02	86.29	88.19	93.28
16	79.01	81.28	84.22	89.36	**16**	90.91	91.29	94.32	98.26
17	85.83	86.28	88.28	94.28	**17**	94.93	97.62	99.41	106.28
18	89.27	90.34	94.18	99.37	**18**	101.12	101.53	104.29	-
19	94.56	95.19	103.18	106.36	**19**	106.13	-	-	-
20	99.08	101.26	110.34	-	**20**	-	-	-	-

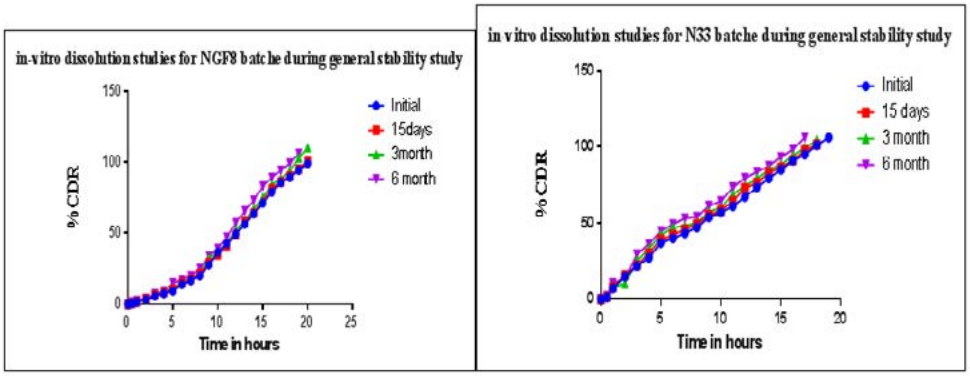


Figure 19: *In-Vitro* dissolution study of stability batches during general stability condition

Table 30: *In-Vitro* dissolution study of stability batches during refrigerator stability condition (5 °C±3 °C):

Dissolution profile of NGF8					Optimized niosomal dispersion (N33)				
Time in hours	**Initial**	**15days**	**3month**	**6month**	**Time in hours**	**Initial**	**15 days**	**3 month**	**6month**
0	0	0	0	0	**0**	0	0	0	0z
0.5	0.78	0.83	0.98	1.25	**0.5**	1.12	1.16	1.20	1.78
1	1.87	1.90	1.97	2.78	**1**	7.17	7.21	7.56	8.27
2	3.28	3.32	3.40	4.78	**2**	14.89	15.09	15.96	17.36
3	5.72	5.98	6.12	7.23	**3**	21.95	22.34	23.01	26.38
4	7.52	7.59	8.11	9.39	**4**	27.13	29.91	30.11	34.77
5	9.28	9.90	10.28	11.28	**5**	36.99	37.09	39.03	43.47
6	13.75	13.81	14.78	16.39	**6**	39.93	41.11	43.01	46.21
7	16.61	16.86	17.11	19.49	**7**	43.37	44.83	46.05	48.39
8	19.91	20.72	21.12	24.56	**8**	47.03	47.89	49.02	54.11
9	27.73	28.19	30.18	35.38	**9**	53.76	54.14	54.21	58.24
10	36.64	37.18	38.11	40.27	**10**	56.99	57.18	58.19	63.28
11	42.82	42.45	43.91	46.38	**11**	61.12	62.29	63.91	67.38
12	49.47	50.91	54.19	59.37	**12**	67.18	68.19	69.06	76.23
13	56.28	57.21	60.28	65.22	**13**	73.26	73.30	73.90	79.28
14	63.69	63.90	69.01	74.47	**14**	79.25	81.08	82.12	86.37
15	71.27	72.01	76.01	80.15	**15**	85.02	86.29	87.19	90.26
16	79.01	82.19	87.12	90.27	**16**	90.91	91.29	91.99	97.37
17	85.83	87.92	89.98	94.39	**17**	94.93	95.98	96.41	103.56
18	89.27	90.81	97.88	102.67	**18**	101.12	104.53	105.29	-
19	94.56	97.19	106.18	-	**19**	106.13	-	-	-
20	99.08	101.26	108.34	-	**20**	-	-	-	-

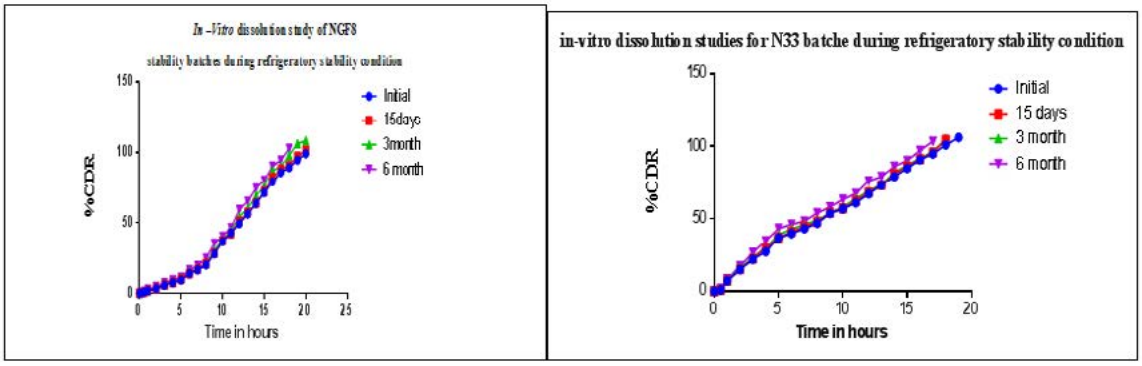


Figure 20: *In-Vitro* dissolution study of stability batches during refrigerator stability condition

Conclusion

Experimental studies reviled that maximum bioavailability and retention within the skin is possible using niosomal gel approach. Experimental studies also reviled that the optimized batch (NGF8) possess good gelling strength, moderate viscosity, optimum extrudability, good skin retention within the skin, optimum bio-adhesion and good gelling properties. Other studies like skin irritation studies on rat epidemics confirmed the absence of edema and erythema after seven days uses of the optimized formulation. The in vitro drug release studies shows, extended drug release up to a 20th hour, due to the various coating over drug and polymeric cross-linkage. Three-month stability studies indicating that the prepared formulation retains its maximum gelling property in refrigerator stability condition (5°C±3°C). More 3-month stability studies are a warrant to understand the physic-chemical behaviors of the optimized batch of the niosome based gel formulation. Hence it can be concluded that the prepared niosome based topical gel would have a good effect on skin cancer treatment, as especially in melanoma treatment [11-15].

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