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LIPID BASED NANOCARRIERS OF TAZAROTENE FOR THE TREATMENT OF PSORIASIS: OPTIMIZATION AND IN VITRO STUDIES

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ABSTRACTS

The present investigation dealt with the formulation development, optimization, in vitro and in vivo study of tazarotene loaded nanocarriers for the treatment of psoriasis. The lipid based nanocarriers i.e. SLN and NLCs were prepared by melt emulsification-sonication. Process variables were optimized by one variance at time (OVAT) approach. Experimental design -Box Behnken design and Artificial Neural Network were used for optimization of various formulation parameters of Tazarotene loaded NLCs and SLNs. Various physicochemical parameters such as particle size, zeta potential, assay and % entrapment efficiency were measured. Further drug loaded SLNs and NLCs were loaded in Carbopol 980 gel. The gel was characterized for pH, viscosity, drug content uniformity, homogeneity, stability study and occlusion test. Drug loaded NLCs, SLNs with or without gel base was evaluated for *in-vitro* drug diffusion study, cellular uptake study, MTTassay, and cytotoxicity study using A431 skin cancer epithelial cell line. The particle size range of the optimized formulation was215.2 nm ± 4.5 nm with zeta potential -37.8 mV ± 3.4 mV, and entrapment efficiency was 57±3% for SLNswhile in case of NLCs, the particle size was around 235.22 nm ± 6.8 nm with zeta potential of -39.8 mV ± 3.7 mV, andentrapment efficiency of 60-65% for NLC. The

cell viabilitystudy also indicated that formulation was non-toxic to cells. The cellular uptake study was done using confocal microscopy which indicated that the drug loaded SLNs and NLCs were taken up by the cells in peripheral region.

Abbreviations: NLC - nano lipid carrier; SLN – solid lipid nanoparticle, MTT - [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]

KEYWORDS: Tazarotene, NLC, SLN, Psoriasis, Melt emulsification, Artificial Neural Network, Topical drug delivery system

1.1 INTRODUCTION

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

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

liposomes, ethosomes, microemulsions and lipid nanocapules are studied as a topical drug delivery systems.[5, 6]

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

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

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

1.2 MATERIALS AND METHODS

1.2.1 List of materials: Tazarotene was gift sample from sun pharmaceutical industries ltd, Vadodara, India. Chremophor El, Cutina GMS, Galol GMS, Precitrol ATO 5, Compritol 88, Gelucire 50/13 pellets, strearic acid, Dynasan 112, Dynasan 114, Imvitor 948, Miglyol 829, Imwiotr 491, Arlamol, Acconon 6 were procured from Gattefose, France. Polxamer F 127 was provided by BASF,Mumbai as a gift sample. Tween 80,triethanolamine, olive oil and

PVA were procured from LOBA Chemie,India . Carbopol 98 NF was provided by Lubrizol as a gift sample.

1.2.3 METHODS

1.2.3.1 Estimation of Tazarotene using UV spectrometer at 351nm

For the estimation of Tazorotene, UV-Vis analytical method was developed in methanol : chloroform (8:2) and phosphate buffer pH 6.5. A double beam UV/Visible spectrophotometer shimadzu (japan) model UV – 1800 connected to a computer loaded with software UV probe ver. 2.10 with automatic wavelength accuracy of 0.1 nm and matched quartz cell of 1 cm path length was used for all measurements was used. Standard stock solution was prepared by dissolving tazarotene in methanol: chloroform: 8:2 and phosphate buffer pH 6.5. Suitable aliquots of the stock solution were pipetted out into 10 mL volumetric flask and the volume was made up to 10 mL and was mixed by vortex mixture to give final concentrations ranging from 1-5 µg/mL. λ_{max} was determined by UV spectroscopic scanning (200-400 nm) of 100 µg/mL solution against reagent blank using UV-Visible spectrophotometer (model UV – 1800, Shimadzu, Japan). Absorbance of the solution ranging from 1-5 µg/mL was measured at λ_{max}. Validation of analytical method was carried out as per ICH guideline.

1.2.3.2 Screening of liquid lipid and solid lipid: The screening of liquid liquid or solid lipid was based on the solubility of drug to give a visible clear solution under normal light when seen with the naked eye. 50 mg drug and varying quantities of solid lipids were heated above the melting point of the lipid in a temperature controlled water batch (Remi, Mumbai, India) in 15 ml glass vials. After melting the lipid in vials, the solubility of drug observed under normal light. Solubility of drug in liquid also carried out by same method with 24 hr stirring.[13] The results were presented in Table 6.

1.2.3.3 Partitioning behavior of drug in various lipid : Partition behavior of drug in various lipids was carried out.[14] 50 mg drug was dispersed in a mixture of melted lipid and hot distilled water. The mixture was shaken for 24 hrs in a hot water bath at 65°±5° C. After cooling, the aqueous phase was separated by ultracentifugation (Remi, Mumbai, India) and the drug content was analyzed. Partition coefficient was calculated by equation (1), and results were shown in Table 7.

$$PC = \frac{(A_I - A_W)}{A_W} \quad \text{.....Equation (1)}$$

Where, A_I is initial amount of drug added i.e. 50 mg

A_W is the amount of drug present in the aqueous phase.

1.2.3.4 Preparation of NLC and SLN: The NLCs were prepared by melt emulsification – sonication method.[15] Using the same method, SLN was also prepared by replacing the liquid lipid part with solid lipid to compare with NLC for the topical application. The solid lipid (Cutina GMS), and liquid lipid (Cremophor EL) were selected based on drug solubility study from various lipids.Lipid and drug were taken in a beaker and heated up to 60° C. In another beaker, hydrophilic surfactant dissolved in dist. water was heated to same temperature as of lipid melt. The lipid melt was added to surfactant solution and was stirred by Ultra turrax T25 basic at 19000 rpm for 5 min with continued heating. The prepared emulsion was sonicated using probe sonicator for 5 min. The emulsion was allowed to cool naturally and the NLCs were evaluated.

Factors identification and optimization: FishBone diagram was generated to understand which variables affect, or the stable formulation development of Tazarotene NLCs as shown in Fig 1.[16] During formulation optimization batch size (10ml) and temperature of processing (60° C) were kept constant. From the Fish Bone diagram, the variables were identified. Those variables which affected less to the final quality product of tazarotene NLCs were kept constant based on preliminary batches formulation reseults and literature survey. Those variables which had medium risk were optimized by one variable at a time (OVAT) approach.

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

2015[@]. Levenberg-Marquardt algorithm was used for training. The feed forward back propagation neural network was used.

Table 1: Coded and actual levels of the variables used in Box-Behnken Desingn.

Factors (Variables)	Code of variables	Actual levels of the variables		
		-1	0	1
Solid lipid: Liquid lipid ratio	X1	7:3	8:2	9:1
Lipid: Drug ratio	X2	9:1	11:1	13:1
Surfactant Concentration (w/w)	X3	1%	1.5 %	2%

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

1.2.3.6Preparation of SLNs or NLCs loaded gel: The NLCs or SLNs dispersion was incorporated in the carbopol 980 NF. 0.8%w/w carbopol gel was prepared by pH change method. Triethanolamine (0.23%w/v) was used as the alkylating agent. The Carbopol 980 NF was allowed to swell for 3 hr. The gel was neutralized by dilute solution of triethanolamine. The NLC or SLN dispersion was added to the gel and mixed properly by using high speed stirrer at 500 rpm for 2 min. The gel was evaluated for parameters like appearance, viscosity and Ph.[17]

1.2.3.7 Characterization of Nanostructured lipid carriers

i. **Physicochemical characterization**: The NLC dispersions were characterized for their physicochemical properties such as colour, odour and stability after centrifugation. Centrifugation was performed at 200 g for 20 min using centrifuge

ii. **Particle size and Zeta potential**: The particles size and zeta potential analysis of nanoparticles was performed Malvern Zetasizer Nano ZS (Malvern, UK). Each sample was suitably diluted with filtered distilled water (10 times) to avoid multi scattering phenomena and placed in a small disposable size cell. The analysis was carried out at 25° C.

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

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

$$\%DE = \frac{Drugentrapped}{Totaldrugadded} * 100$$ .....Equation (2)

v. **Occlusion test** : The occlusion test was performed as following.[18] 50 ml of dis water was taken in 125 ml beaker. All beakers were covered with filter paper. NLC/SLN dispersion, NLC/SLN gels or plain gel was applied uniformly on the filter paper. One beaker was covered with filter paper but without sample served as a reference. The Area of the filter paper was 23.74 cm^2. The Initial weight of all beakers was recorded and the samples were weighed after 6, 24 and 48 h, giving the water loss due to evaporation at each time (water flux through the filter paper). The Occlusion factor was calculated according to the following equation

F = ((A-B)/A) x 100 .....Equation (3)

Where A is the water loss without sample (Control) and B is the Water loss with sample. (An occlusion factor of Zero means no Occlusive effect compared with the reference, and 100 is the maximum occlusion factor.)

1.2.3.8 Characterization of Gel[19]

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

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

iii. Homogeneity: Gel was tested for homogeneity by visual inspection after the gel was set in the container. It was tested for appearance and presence of any aggregates. For determination of the homogeneity three samples of 1 gm gel were taken from the different

sites from the gel container. The gels were extracted with chloroform: methanol mixture (3:7). The mixture was diluted sufficiently and analyzed by UV spectrophotomerter.

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

1.2.4 In Vitro drug diffusion study: It was performed using modified Franz diffusion cell to evaluate the drug release profile form the formulation. Artificial Dialysis Membrane 70 (Himedia, Mumbai India) having pore size 2.4 nm and molecular weight cut-off of 12000 Dalton was used.[20] The formulation equivalent to 10 mg drug was applied to the donor compartment and the receptor Compartment was filled with Phosphate buffer pH 6.8 (100 ml). The area of the diffusion is 3.79 cm^2. During the experiments, the solution in the receptor side was maintained at 37°C and stirred at 100 rpm with Teflon-coated magnetic stirring bars. At fixed time intervals, 5 ml aliquot was withdrawn and analyzed by UV spectroscopy after suitable dilution. To Find out flux of the drug from the membrane quantity of the drug released is divided by area of drug release (Co./A). The plot of Co/A vs time was plotted. The slope of the plot indicates the flux.

1.2.5 Stability study : The stability of the developed SLNs & NLCs loaded gel was conducted for 6 months as per International Conference on Harmonization ICH Q1A (R2) guidelines.[21] The optimized formulation was selected for the stability study. Briefly, sample were stored in the sealed amber colored glass vials at 25° C± 2° C, 60% RH ± 5% RH. After 1, 2, 3 and 6 months, the samples were characterized with respected to physical appearance, pH, viscosity and drug content.

1.3 RESULT AND DISCUSSION

1.3.1 Estimation of drug using UV spectrophotometer

For evaluating the accuracy of the method, different concentrations were prepared and was analyzed for the drug content.

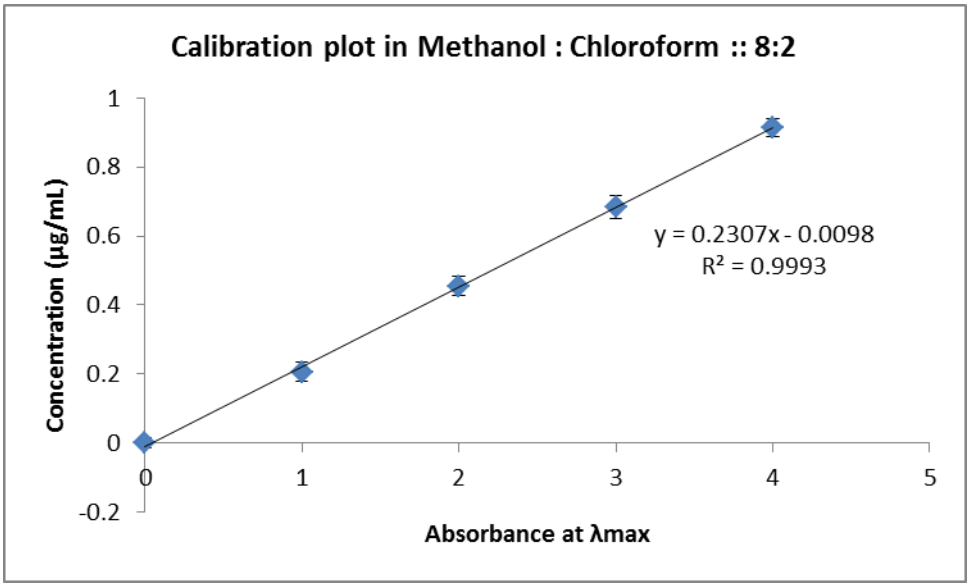


Figure 1: Tazarotene calibration plot in Methanol: Chloroform: 8: 2.

Table 2: Accuracy for the estimation of Tazarotene in methanol: chloroform: 8: 2.

Amount of Pure drug added (%)	Theoretical Concentration (µg/mL)	Amount Found (µg/mL)	Percent Recovery (%)
80	5.4	5.29 ± 0.108	97.96
100	6	6.02 ± 0.068	100.39
120	6.6	6.46 ± 0.134	97.88

The result of accuracy within range of 97.96 % to 100.39% for Methanol: Chloroform :: 8:2 ensures accuracy of the method. Recovery greater than 95% with low standard deviation justifies the accuracy of the developed method.

Table 3: Precision study for the estimation of Tazarotene in methanol: chloroform:: 8:2.

Concentration of drug (µg/mL)	Intra-day precision		Inter-day precision	
	Absorbance (Mean±S.D.)	% RSD	Absorbance (Mean±S.D.)	% RSD
2	0.46±0.008	1.97	0.44±0.008	1.94
4	0.91±0.016	1.83	0.914±0.016	1.85
6	1.41±0.026	1.85	1.41±0.022	1.86

The DL and QL were found to 1.5394µg/mL and 4.6650µg/mL receptively for this developed method. The results of DL indicates that upto 1.5394 µg/mL concentration of tazarotene can be detected but may not quantized in methanol: chloroform: 8:2. The results of QL of 4.6650 µg/mL indicates this much concentration of tazarotene can be quantitatively determined with suitable precision and accuracy.

VALIDATION: For evaluating the accuracy of the method, different concentrations were prepared and was analyzed for the drug content.

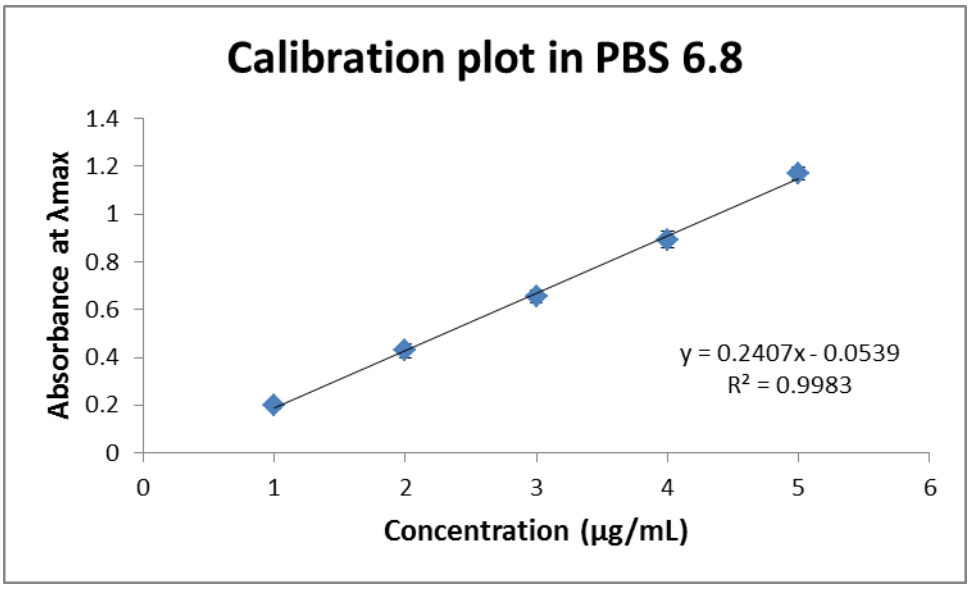


Figure 2: Tazorotene calibration plot in phosphate buffer saline pH 6.8.

The result of accuracy within range of 98.17% to 98.64% for PBS 6.8 ensures accuracy of the method. Recovery greater than 95% with low standard deviation justifies the accuracy of the developed method.

Table 4: Accuracy for the estimation of Tazarotene in PBS 6.8.

Amount of Pure drug added (%)	Theoretical Concentration (µg/mL)	Amount Found (µg/mL)	Percent Recovery (%)
80	5.4	5.32 ± 0.082	98.52
100	6	5.89± 0.093	98.17
120	6.6	6.51 ± 0.065	98.64

Table 5: Precision study for the estimation of Tazarotene in PBS 6.8.

Concentration (µg/mL)	Intra-day precision		Inter-day precision	
	Absorbance (Mean±S.D.)	% RSD	Absorbance (Mean±S.D.)	% RSD
2	0.42±0.009	2.08	0.43±0.003	0.71
4	0.89±0.011	1.25	0.91±0.016	1.71
6	1.30±0.013	1.00	1.31±0.015	1.18

The DL and QL were found to 1.7232µg/mL and 5.2845µg/mL receptively for this developed method. The results of DL indicates that upto 1.7232µg/mL concentration of tazarotene can

be detected but not quantized in PBS 6.8. The results of QL of 5.2845 µg/mL indicates this much concentration of tazarotene can be quantitatively determined with suitable precision and accuracy.

1.3.2 Preformulation

1.3.2.1Screening of Lipid: Various solid lipids and liquid lipid were tried to find in which lipid there is highest solubility of drug. Result of solubility study were shown in Table 6. In various solid lipid, Cutina GMS showed better solubilization capacity for Tazarotene molecule. Other than solubilization, Cutina GMS exhibited good flow property, non-toxicity, approved regulatory status, cost, etc. also favors its choice as solid lipid. Hence, Cutina GMS lipid was selected for the preparation of SLNs, NLCs. The solubility of tazarotene was found to be more in liquid lipid than solid lipid. Cremophor EL showed highest solubility of drug compare to other liquid lipid. Having better solubilization capacity, Cremophor EL may provided better penetration of drug molecule through the skin because of its good permeability.[22] Hence Cutina GMS VPH and Cremphor EL were selected for further study from 7:3 to 8:2 ratio.

Table 6: Solubility of Tazarotene in different lipids.

Solubility study in solid lipid		**Solubility study in Liquid lipid**	
Name of Solid Lipid	Amount of solid lipid required to dissolve 50 mg of drug	Name of Liquid Lipid	Solubility of drug in Liquid lipid (mg/mL)
Galol GMS (Gattefosse)	436.5 ± 2.3 mg	Olive oil	13.16 ± 0.74 mg/mL
Cutina GMS VPH	**422.3 ± 3.53 mg**	Imwitor 948	16.67 ± 0.45 mg/mL
Precirol ATO 5	793 ± 2.24 mg	Imwiotr 491	9.6 ± 0.65 mg/mL
Compritol 88	560 ± 3.74 mg	Miglyol 829	5.82 ±0.34 mg/mL
Gelucire 50/13 pellets	498.2 ± 3.32 mg	Miglyol 849	5.53 ±0.64 mg/mL
Stearic acid	590.2 ± 3.23 mg	**Cremophor EL**	**45.45 ±0.34 mg/mL**
Dynasan 112	691.2 ± 4.43 mg	Arlamol	25 ±0.47 mg/mL
Dynasan 114	630.4 ± 2.26 mg	Acconon cc 6	32.54 ± 0.83 mg/mL

1.3.2.2Partition co-efficient: Partition of drug in different lipid waspresented in Table 7. Tazarotene showed highest partition co-efficient for Cutina GMS lipid. So Cutina GMS was selected as lipid for further optimization.

Table 7: Partition Study.

Lipid	Partition Coefficient
Galol GMS (Gattefosse)	2.289
Cutina GMS VPH	3.202
Precirol ATO 5	1.485
Compritol 88	2.086
Gelucire 50/13 pellets	2.125
Stearic acid	1.838
Dynasan 112	1.514
Dynasan 114	1.634

1.3.2.3 Preliminary Screening trials for selection of amount of lipid.

Keeping 1% PVA solution, the trials were taken for selection of Cutina GMS VPH to drug from 5:1 to 13:1. From the results (Table 8), it was concluded that when lipid amount was very less i.e. 5:1, the NLC were not formed making it necessary to increase the amount of lipid used. The formation of NLCs started at the ratio of 8:1. Non-formation of NLCs at lower lipid amount might be because of formation of lipid along with drug is encapsulated inside the micellar structure of surfactant used.[23] By increasing the concentration of lipid; the random disordered structure of solid lipid was formed due to presence of liquid lipid which solubilize the drug inside.[24] Increasing the ratio from 8:1 to 13:1, there was increase in size of NLCs and %EE as well. At 13:1 ratio, entrapment is 84.23±5.53%, but the size is 289±38 nm which was on higher side.[25] So further trials were taken to increase %EE, because it affected size of NLCs. Hence, Cutina GMS VPH: Drug ratio (13:1) were selected for further trials.

Table 8: Preliminary Screening trials for selection of amount of lipid.

Cutina GMS VPH: Drug ratio	Size (nm)	PDI	%EE
5:1	Not formed	-	-
6:1	Not formed	-	-
7:1	Not formed	-	-
8:1	160 ± 29	0.121 ± 0.007	40.35 ± 4.63
9:1	172 ± 38	0.213 ± 0.012	56. 56 ± 3.87
10:1	215 ± 35	0.172 ± 0.009	62. 78 ± 4.35
11:1	225 ± 24	0.111 ± 0.013	73.42 ± 5.32
12:1	250 ± 40	± 0.017	79.52 ± 4.98
13:1	**289 ± 38**	**± 0.012**	**84.23 ± 5.53**

1.3.2.4 Preliminary Screening trials for selection of Selection of surfactant

Poloxamer F 127, Tween 80 and PVA were taken at 1% concentration. The lower amount may not be sufficient to stabilize the NLC dispersion and higher amount causes the frothing problem, so 1% was selected from the literature review.[25] Based on the size, zeta potential and storage stability at 4° C for 2 days, surfactant was finalized as shown in Table 9. Zeta Potential of -46.5 mV indicated the stability of formulation as compared to Poloxamer F 127 and Tween 80 with PVA.[25] Even the storage stability of NLCs displayed the same. Even though the size factor is being affected when compared with Tween 80, but storage stability is important for final NLCs formulation till it reaches to the patient's hand.[26] In tween 80 formulations there was gel formation at the wall indicating instability due to separation of the two phases of NLCs during storage at 4 °C. Hence PVA was selected for further study.

Table 9: Screening of surfactant.

Surfactant (1%)	Morphological Parameters	Particle Size (nm)	Poly Dispersive Index	Zeta Potential (mV)	Physical appearance during storage
Poloxamer F 127	White dispersion	293 ± 15	0.115 ± 0.010	-21.1 ± 3.8	Aggregated Particles
Tween 80	Slight yellowish dispersion	275 ± 22	0.120 ± 0.017	-14.5 ± 2.3	Gel formation at wall
PVA	Off white dispersion	280 ± 19	0.139 ± 0.013	-46.5 ± 5.3	No change in Dispersion

1.3.3 Optimization by Box Behnken Design

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

Table 10: Results of Box-Behnken design for optimization.

Sr No	Solid : Liquid Lipid	Lipid : Drug	% Surfactant	Size ± SD (nm)	Zeta Potential ± SD (mV)	%EE ± SD
2	1	-1	0	224±09.41	-40.12±2.96	56.11±5.12
6	1	0	-1	250±11.24	-31.25±4.26	63.29±6.43
14	0	0	0	225±05.49	-40.10±4.28	65.20±2.34
8	1	0	1	219±08.54	-48.12±4.62	62.23±6.27
15	0	0	0	225±05.49	-40.10±4.28	65.20±2.34
11	0	1	-1	285±09.86	-30.03±2.75	70.23±2.65
16	0	0	0	225±05.49	-40.10±4.28	65.20±2.34
7	-1	0	1	260±08.75	-50.11±2.65	65.13±7.53
3	-1	1	0	266±09.43	-39.13±3.15	70.68±3.15
13	0	0	0	225±05.49	-40.10±4.28	65.20±2.34
5	-1	0	-1	295±05.48	-22.53±5.29	69.43±5.37
12	0	1	1	243±04.74	-45.52±4.32	62.12±4.96
17	0	0	0	225±05.49	-40.10±4.28	65.20±2.34
9	0	-1	-1	273±08.65	-28.14±3.16	58.21±6.53
10	0	-1	1	260±11.74	-50.02±2.71	55.07±4.98
1	-1	-1	0	273±09.25	-40.14±4.53	60.25±5.26
4	1	1	0	225±12.93	-44.42±5.23	65.12±8.47

For all the dependent variables, summary is shown in Table 11. The selected design of experiment is Box-Behnken design which is a type of surface response design. The resulted 17 runs were randomized in order to remove any bias error of the model. The design model for the three factors was quadratic model which indicates complex relationship between input and the output variables.

Table 11: Design summary.

Response	Units	Minimum	Maximum	Mean	Std. Dev.	Model
Size, R1	Nm	219	295	246.941	25.0511	Quadratic
Zeta Potential, R2	mV	-50.11	-22.53	-39.4135	7.64596	Quadratic
Entrapment, R3	%	55.07	70.68	63.7571	4.46096	Quadratic

Fraction of design space (FDS) graph was generated at process sigma, s = 1 and α risk level of 0.05 as shown in Fig. 3. It displayed the area/volume of the design space having a mean std error less than or equal to a specified value. The ratio of the area/volume to total area/volume is the fraction of design space. This figure indicated that 37% of design space has a relative std error of less than 0.5 from the graph. It suggested that only 37% design space is precise enough to predict the response.

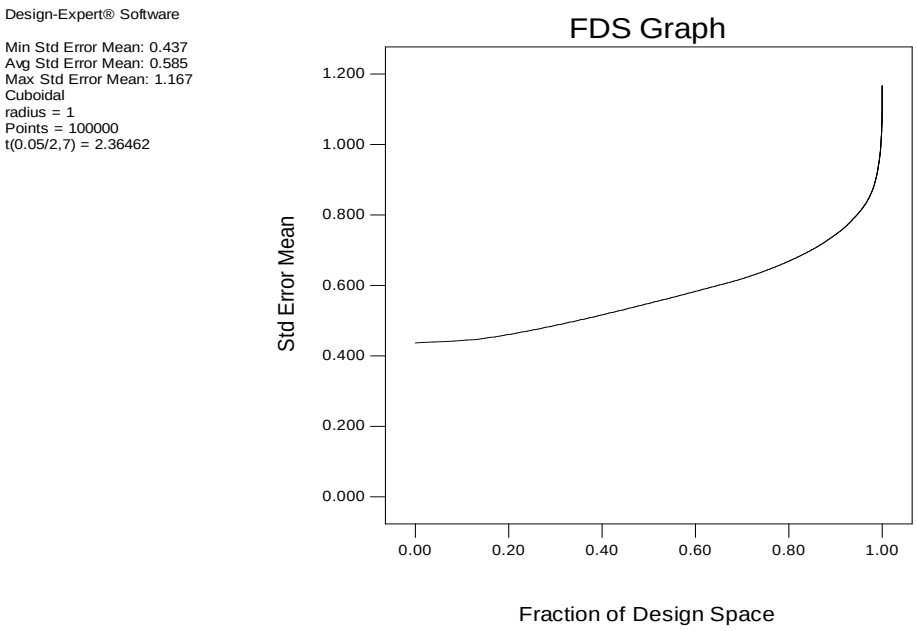


Figure 3: FDS graph for design model.

Statistical analysis for size (Y1): Based on the R2 value for model selection as shown in Table 12, quadratic model was selected for the response Y1.

Table 12: Model selection for Y1 response (Size).

Source	Adjusted R^2	Predicted R^2	PRESS value	
Linear	0.4700	0.3298	6729.33	
2FI	0.3477	-0.1335	11381.53	
Quadratic	0.9861	0.9725	276.00	Suggested
Cubic	1.0000		-	Aliased

Due to lack of orthogonality, even though adjusted R^2 value is high for cubic model, it was aliased. The PRESS (predicted residual error sum of squares) statistics also indicate that the selected model, quadratic is perfect. To identify the significant parameters and their interaction, analysis of variance was performed for each parameter. The ANOVA for particle size was given in Table 13.The value of ANOVA showed that the effects of factors were significant and hence the model was significant for particle size. From the p value (<0.0001), it was confirmed that the model is significant for the selected response. Form the F value, it was observed that the independent variable X_1 (Solid: Liquid Lipid) was affecting highest to the dependent variable Y1 (size). Whereas Lipid: drug (variable X_2) was the leas affecting variable to response Y1. It holds true for the selected input range of the variables for creating the design space. Out of the design space the effect of variables varies drastically. To avoid

the variation which affects the final NLCs size, based on the preliminary screening the range of input variables was selected.

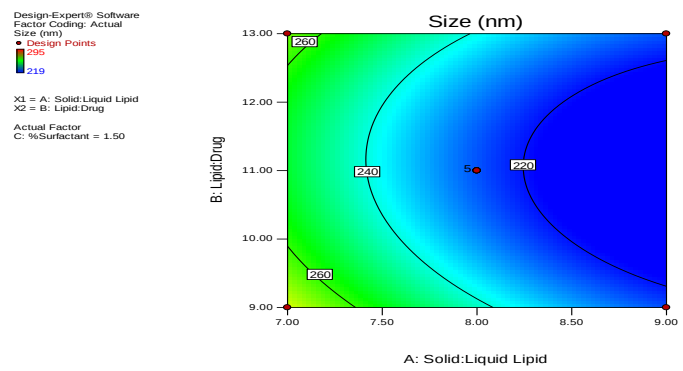
Table 13: ANOVA for response surface quadratic model for the Y1 (Size) Response

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F
Model	10023.69	9	1113.74	451.95	< 0.0001
X_1	3872.00	1	3872.00	1571.25	< 0.0001
X_2	15.13	1	15.13	6.14	0.0424
X_3	1830.13	1	1830.13	742.66	< 0.0001
X_1X_2	16.00	1	16.00	6.49	0.0382
X_1X_3	4.00	1	4.00	1.62	0.2433
X_2X_3	210.25	1	210.25	85.32	< 0.0001
X_1^2	171.12	1	171.12	69.44	< 0.0001
X_2^2	1027.96	1	1027.96	417.14	< 0.0001
X_3^2	2553.22	1	2553.22	1036.09	< 0.0001
Residual	17.25	7	2.46		
Lack of Fit	17.25	3	5.75		
Pure Error	0.000	4	0.000		
Cor Total	10040.94	16			

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

$$\text{Size } (Y_1) = +1548.59 - 138.00\ X_1 - 83.75\ X_2 - 262.00\ X_3 + 1.00\ X_1X_2 + 2.00\ X_1X_3 - 7.25\ X_2X_3 + 6.38\ X_1^2 + 3.90\ X_2^2 + 98.50\ X_3^2 \quad \text{.....Equation (3)}$$

The equation(3) indicated the complexity between the input and output variables.From the quadratic equation or its co-efficient one cannot draw a conclusion whether the effect of any independent variable is a positive or negative on the response. The interrelation between the dependent and independent variables is shown in 2D contour (Fig 4) plot and 3D response surface curves (Fig 5).



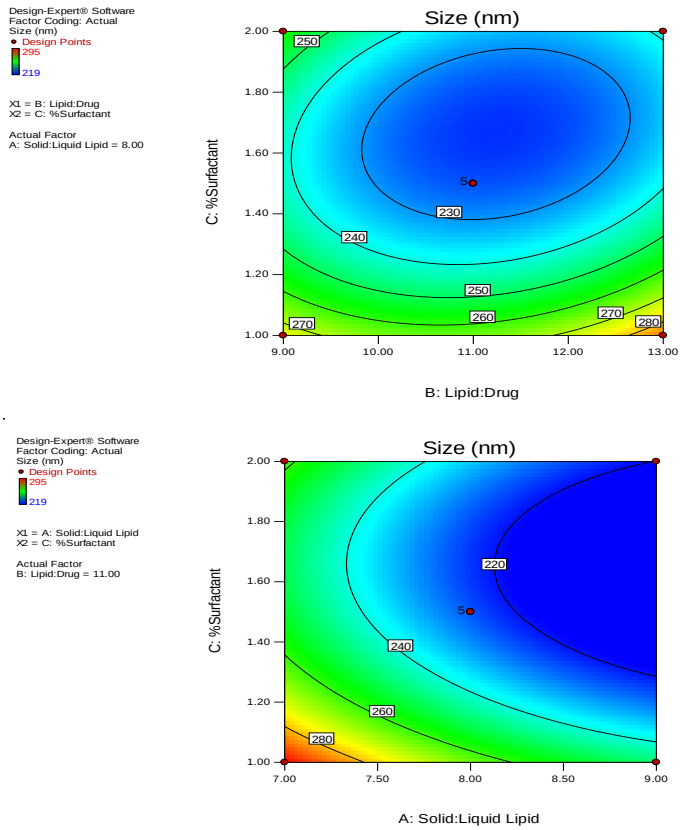
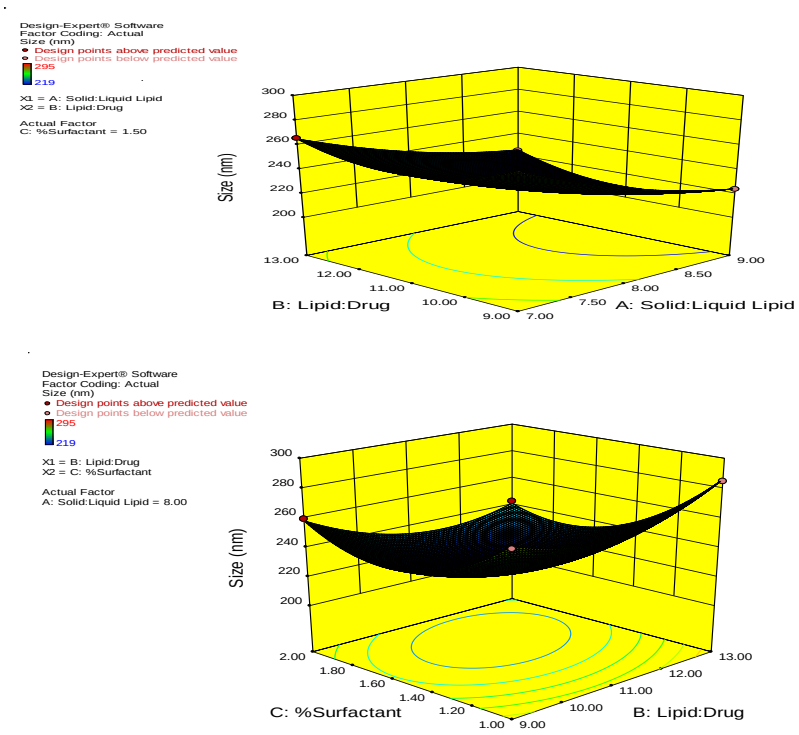
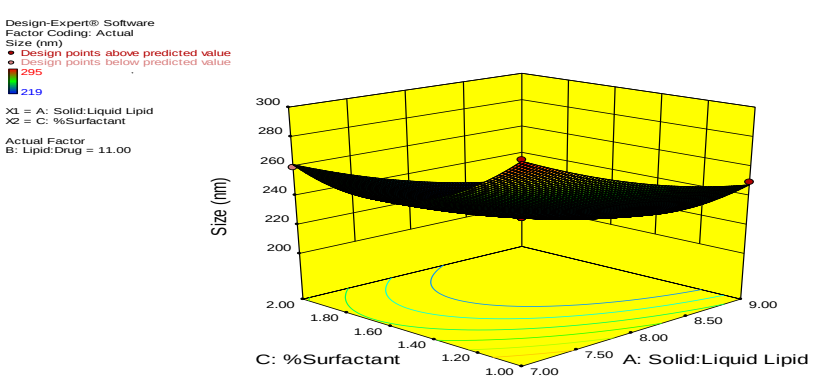


Figure 4: ANOVA for response surface quadratic model for the Y1 (Size) Response(2D contour plot).





Figur 5: ANOVA for response surface quadratic model for the Y1 (Size) Response (3D response surface curves).

From this 2D (fig. 2) and 3D (Fig 3) graphs for response Y1 – size, the effect of interrelation between the independent variables can be seen. The effect of variable Solid:Liquid Lipid (X_1) and Lipid:Drug Ratio (X_2) on size (Y1) can be interpreted from the contour lines as well as its 3D replica. From both the graphs, it is shown that the individual components may have statistically significant effect on Y1 but the interaction of X1 and X2 is not much as the colour coding for Y1 is varying between green to blue which is lower middle range for colour coding. But when the surfactant (X3) is changed either with Solid:Liquid Lipid (X1) or Lipid:Drug Ratio (X2), there is large variation in size as seen from the colour coding of contours for the selected input range. The contours are varying from the lower range of size (Y1) 219 nm – blue colour to highest size (Y1) – 295 nm. Thus we can conclude that the effect of X1 is highest for the response Y1 as seen from the ANOVA table and from the interaction terms highest effect is of X2X3.

Statistical analysis for zeta potential (Y2): Based on the R2 value for model selection as shown in table 10, quadratic model was selected for the response Y2. Due to lack of orthogonality, even though adjusted R^2 value is high for cubic model, it was aliased. The PRESS (predicted residual error sum of squares) statistics indicate that linear model is perfect for the response Y_2. These values of R^2 and PRESS values for statistical significance of model selection are contradictory in nature. So, the data were interpreted using both the models. After data treatment using both the models, it was found that using linear model the complex interrelationship between interaction terms of input variables cannot be explained. So quadratic model was chosen to understand the overall effect of independent variables on dependent variables, which shows effect of individual variables, its interaction with other variables and the square of the variable. (e.g. X_1, X_1X_2 and $X1^2$)

Table 14: Model selection for Y2 response (Zeta Potential).

Source	Adjusted R^2	Predicted R^2	PRESS value	
Linear	0.8941	0.8223	166.23	Suggested
2FI	0.9409	0.8202	168.15	
Quadratic	0.9734	0.8138	174.14	Suggested
Cubic	1.0000		-	Aliased

ANOVA Analysis: To identify the significant parameters and their interaction, analysis of variance was performed for each parameter. The ANOVA for particle size is given in table 11. The value of ANOVA shows that the effects of factors were significant and hence the model is significant for zeta potential. From the p value (<0.0001) also, it is confirmed that the model is significant for the selected response. Form the F value, it is observed that the independent variable X3 (% Surfactant) is affecting highest to the dependent variable Y2 (Zeta Potential). Whereas X1 and X2 are the least affecting variables to response Y2. This was also seen from the preliminary trials. Change in surfactant such as Tween 80, Poloxamer F 127 and PVA showed drastic change in zeta potential. This preliminary trial showed that even though the Lipid: Drug ratio and Solid : Liquid Lipid were fixed variables the change in zeta was due to surfactant only. After selection of PVA as a formulation ingredient for further optimization of its concentration to be used, the ANOVA also showed that the major effect on zeta potential was due to PVA (%surfactant, X3) only.

Table 15: ANOVA for response surface quadratic model for the Y2 (Zeta Potential) Response.

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F
Model	924.49	9	102.72	66.07	< 0.0001
A-Solid:Liquid Lipid	18.00	1	18.00	11.58	0.0114
B-Lipid:Drug	0.058	1	0.058	0.037	0.8526
C-%Surfactant	836.81	1	836.81	538.22	< 0.0001
AB	7.05	1	7.05	4.53	0.0707
AC	28.68	1	28.68	18.44	0.0036
BC	10.21	1	10.21	6.57	0.0374
A2	0.19	1	0.19	0.12	0.7354
B2	1.72	1	1.72	1.10	0.3281
C2	22.49	1	22.49	14.47	0.0067
Residual	10.88	7	1.55		
Lack of Fit	10.88	3	3.63		
Pure Error	0.000	4	0.000		
Cor Total	935.37	16			

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

Zeta Potential = $23.06 + 1.19\ X_1 + 6.38\ X_2 - 108.60\ X_3 - 0.66\ X_1X_2 + 5.35\ X_1X_3 + 1.60\ X_2X_3 - 0.21\ X_1^2 - 0.16\ X_2^2 + 9.25\ X_3^2$ ……Equation (4)

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

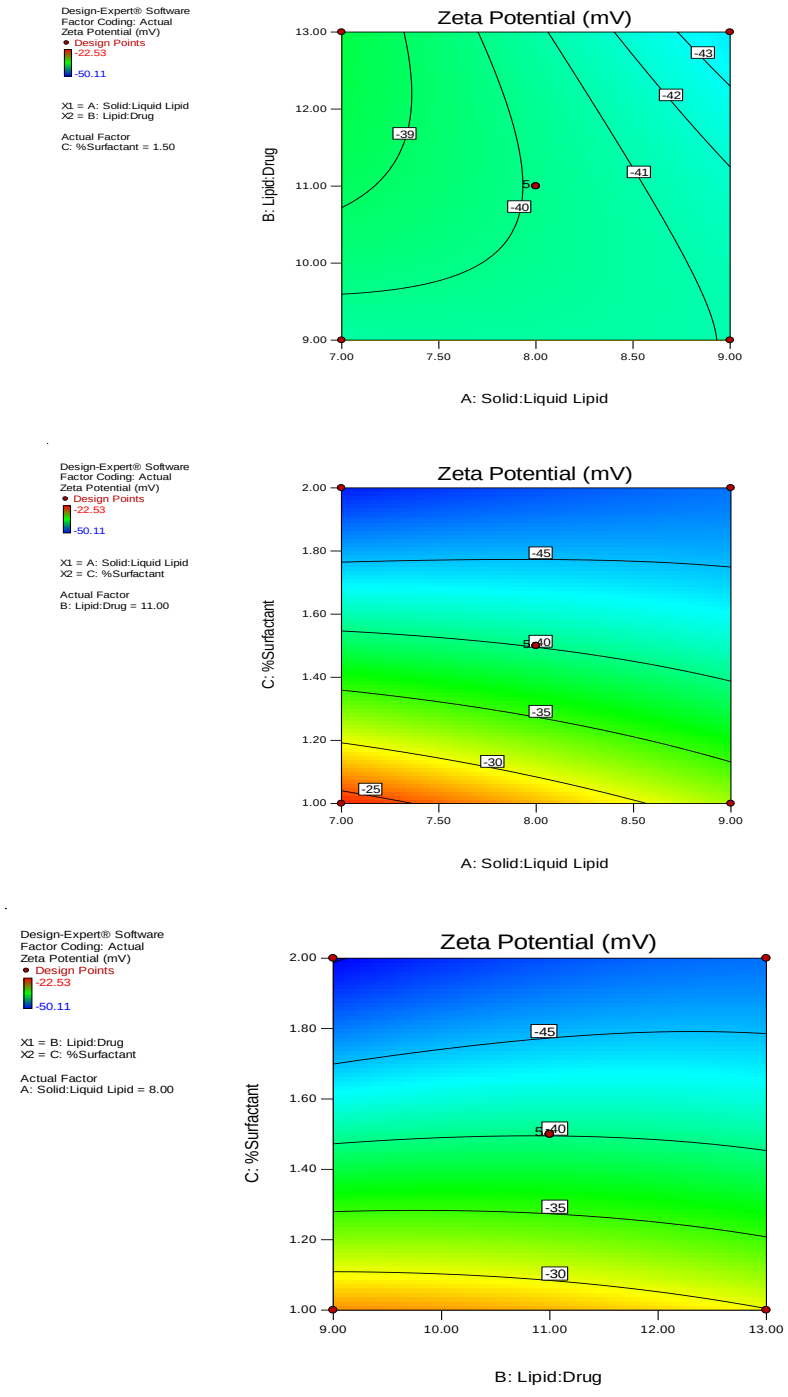


Figure 6: 2D contour plot for Zeta Potential.

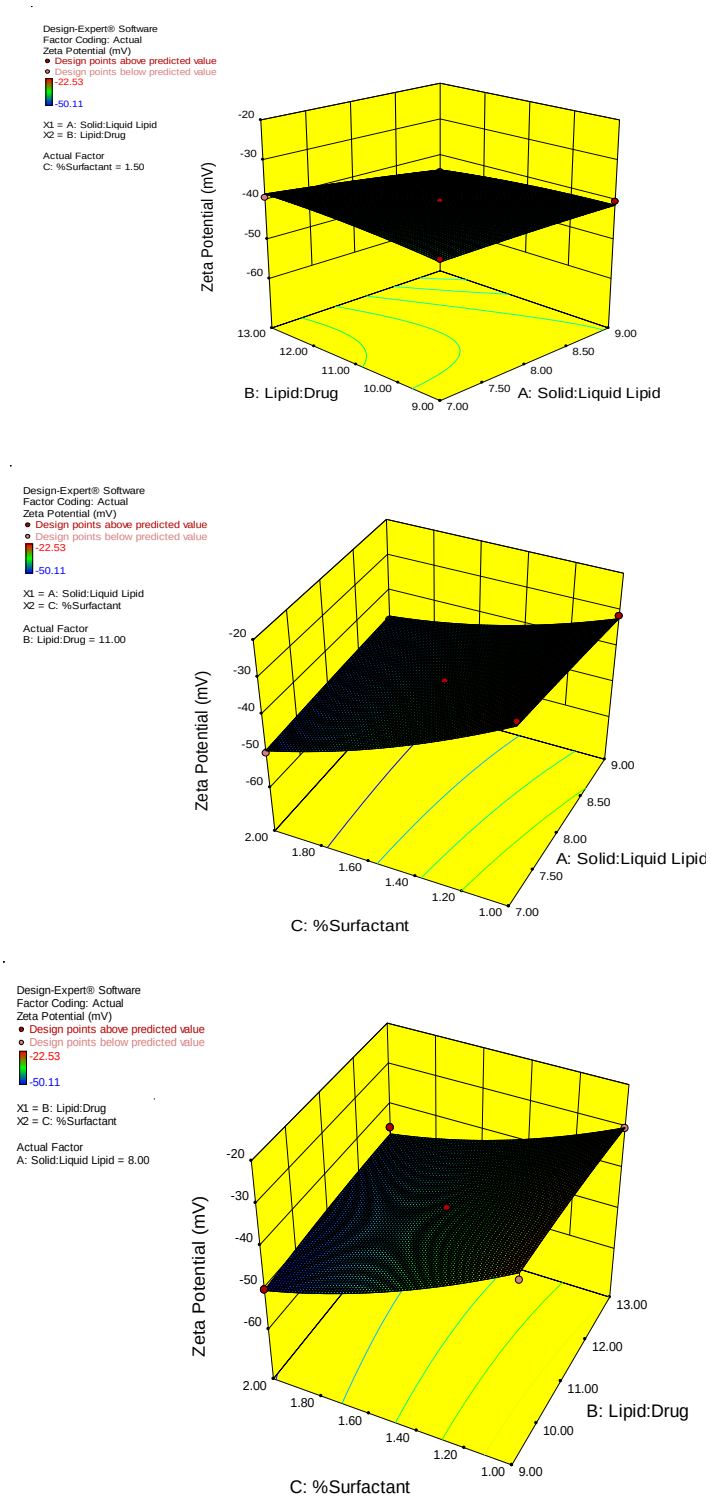


Figure 7: 3D response surface curves for Zeta Potential.

From this 2D (Fig 4) and 3D (fig 5) graphs for response Y2 – zeta potential (Fig. 6 & Fig. 7), the effect of interrelation between the independent variables can be seen.

The effect of variable Soilid:Liquid Lipid (X_1) and Lipid:Drug Ratio (X_2) on zeta potential (Y2) can be interpreted from the contour lines as well as its 3D replica. From both the graphs,

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

Statistical analysis for %EE (Y3): Based on the R2 value for model selection as shown in table 16, quadratic model was selected for the response Y3.

Table 16: Model selection for Y2 response (Zeta Potential).

Source	Adjusted R^2	Predicted R^2	PRESS value	
Linear	0.7888	0.6723	104.34	
2FI	0.7722	0.3995	191.20	
Quadratic	0.9684	0.7785	70.53	Suggested
Cubic	1.0000		-	Aliased

Due to lack of orthogonality, even though adjusted R^2 value is high for cubic model, it was aliased. The PRESS (predicted residual error sum of squares) statistics also indicate that the selected model, quadratic is perfect. To identify the significant parameters and their interaction, analysis of variance was performed for each parameter. The ANOVA for particle size is given in Table 17. The value of ANOVA shows that the effects of factors were significant and hence the model is significant for particle size. From the p value (<0.0001) also, it is confirmed that the model is significant for the selected response. Form the F value, it is observed that the independent variable X2 (Lipid: Drug ratio) is affecting highest to the dependent variable Y3 (%EE). Whereas X3 is the least affecting variables to response Y3. To optimize the amount of lipid (X2) and internal lipid ratio (X1) for %EE, the mathematical numerical optimization based on desirability was further carried out.

Table 17: ANOVA for response surface quadratic model for the Y2 (Zeta Potential) Response.

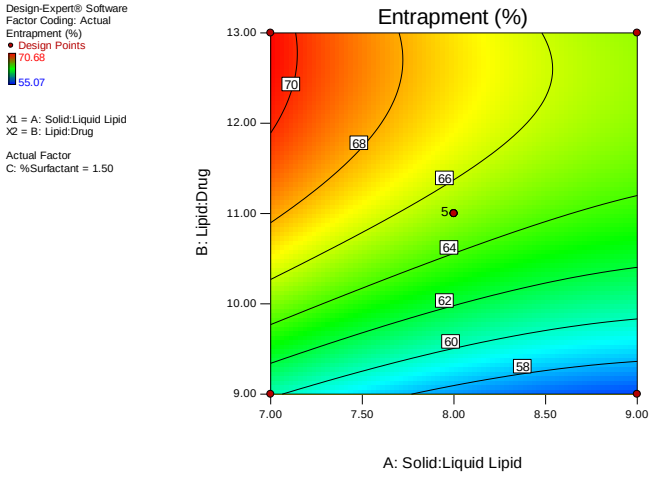
Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F
Model	314.00	9	34.89	55.40	< 0.0001
X_1	43.90	1	43.90	69.71	< 0.0001
X_2	185.38	1	185.38	294.38	< 0.0001
X_3	34.49	1	34.49	54.76	0.0001
X_1X_2	0.50	1	0.50	0.80	0.4007
X_1X_3	2.62	1	2.62	4.17	0.0805
X_2X_3	6.18	1	6.18	9.81	0.0166
X_1	2.22	1	2.22	3.53	0.1025
X_2	35.08	1	35.08	55.70	0.0001
X_3	3.46	1	3.46	5.49	0.0516
Residual	4.41	7	0.63		
Lack of Fit	4.41	3	1.47		
Pure Error	0.000	4	0.000		
Cor Total	318.40	16			

Mathematical model for %EE

In terms of actual components, the inter-relation between independent and dependent variables was derived.

$$\%EE = -1.97 - 14.44\ X_1 + 21.56\ X_2 + 7.43\ X_3 - 0.18\ X_1X_2 + 1.62\ X_1X_3 - 1.24\ X_2X_3 + 0.73\ X_1^2 - 0.72\ X_2^2 - 3.63\ X_3^2 \quad \text{.....Equation (5)}$$

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



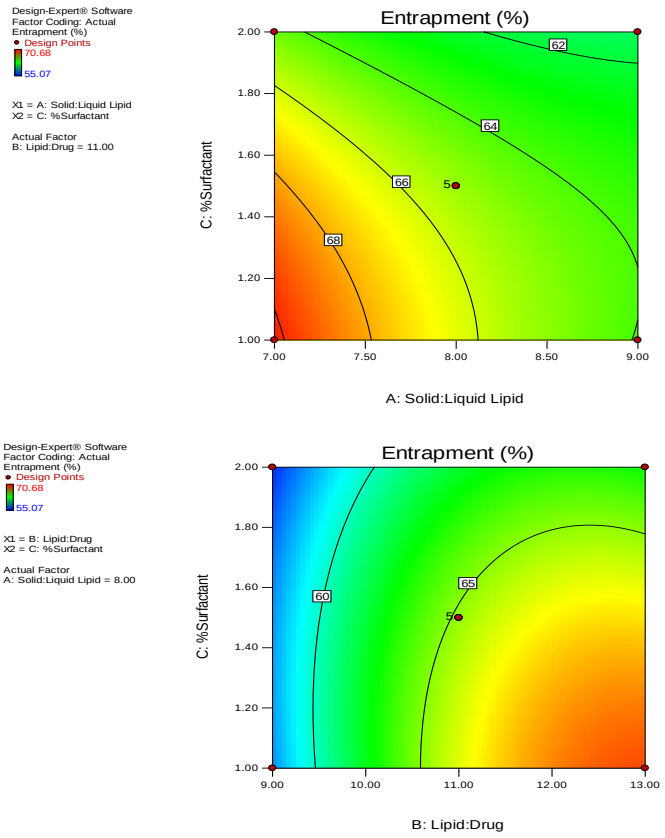
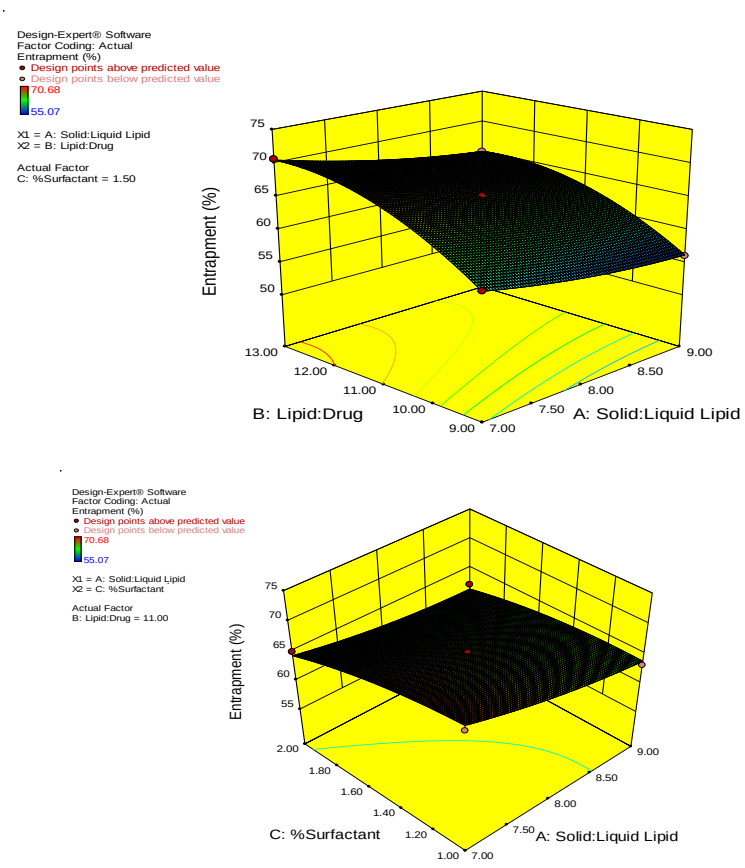


Figure 8: 2D contour plot for %EE.



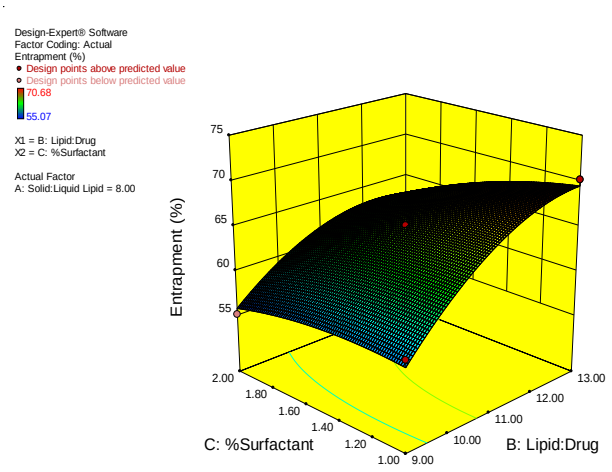


Figure 9: 3D response surface curves for %EE.

From this 2D (Fig. 8) and 3D graphs (Fig. 9) for response Y3 – %EE, the effect of interrelation between the independent variables can be seen. The effect of variable Soilid: Liquid Lipid (X_1) and Lipid:Drug Ratio (X_2) on %EE (Y3) can be interpreted from the contour lines as well as its 3D replica. From both the graphs, it is shown that the interaction of X1X2 and X2X3 is having more effect than X1X3. When the X2 is changed either with Solid:Liquid Lipid (X1) or Surfactant (X3), there is large variation in size as seen from the colour coding of contours for the selected input range. The contours are varying from the lower range of zeat to higher range and it is shoeing significant change.Thus, we can conclude that the effect of X2 is highest for the response Y3 as seen from the ANOVA table and even for the interaction terms.

1.3.4 Optimization using Desirability function

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

Table 18: Constraints applied on the variables.

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
X1: Solid:Liquid Lipid	Minimize	7	9	1	1	3
X2: Lipid:Drug	Minimize	9	13	1	1	3
X3: %Surfactant	Minimize	1	2	1	1	3
Y1: Size	Minimize	219	295	1	1	3
Y2: Zeta Potential	Minimize	-50.11	-22.53	1	1	3
Y3: Entrapment	Maximize	55.07	70.68	1	1	3

More is the liquid lipid, the structure of NLC will be more fluidic in nature leading to increase permeation and increased partition through the biological barrier. But as observed from the contour plots for size, higher is the solid lipid amount the lesser is the size. This is due to when more liquid lipid is added, it leads to distortedness in the final structure of NLCs leading to variation in Brownian motion of the NLCs in dispersing media. This leads to increase in size along with large values of PDI. But for its biological desired action lesser size with high permeation is desired. So, X1 was kept as minimize so as to incorporate less amount of solid lipid and Y1 was also kept minimize so as to get lesser size. The Factor X2 was kept as minimum, which will reduce the amount of lipid used for formulation. The X3 was kept low, because surfactant causes health hazard generally. Even though the range selected herein as per the GRAS limit for human consumption, but keeping patient health as priority, the X3 was kept minimal. Response Y1 (size) was kept minimum, as lower size is important for the increase surface area leading to increase contact to skin so the formulation can permeate. Response Y2 (zeta) was also kept minimum because it's on negative side. Lesser it is, more stable is the formulation. The higher %EE (Y3) is obviously desired so as to incorporate as much drug as possible. Based on the solution for the selected constraints the formulations were prepared (table 19).

Table 1: Solution selected for the applied constraints.

	Solid: Liquid Lipid X1	Lipid: Drug Ratio X2	%Surfactant X3	Size Y1	Zeta Potential Y2	Entrapment Y3	Desirability
Suggested	7.109	10.451	1.483	231.23	-38.825	60.32	0.912
Prepared	7.109	10.451	1.483	235.22±6.8	-40.53±3.7	62.72±6.7	-

*n=3

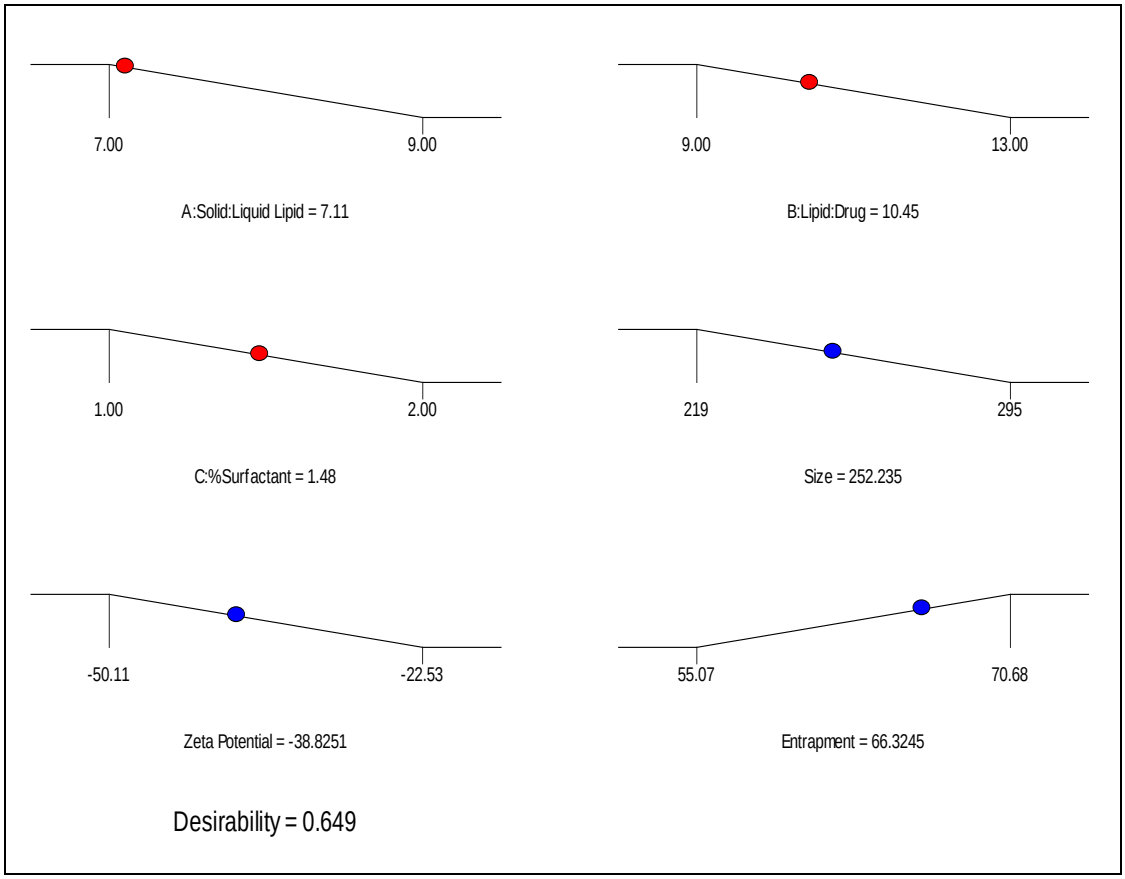


Figure 1: Shows the desirability graph for the applied constraints on the applied variables.

The prepared formulation as shown in Table 19. & fig 10, showed size of 235.22±6.8, zeta of -40.53±3.7 and %EE of 62.72±6.7.

1.3.5 Optimization by Artificial Neural Network[27, 28]

The major factor to be optimized for better penetration of drug from NLC through skin is its size. This lipophilic cargo of NLCs' partition into skin would be enhanced based on its size and size distribution. Lesser the size having monosized distribution will increase its partition. Added advantage of more surface area will lead to increased contact between NLCs and skin. This enhancement in contact due to optimized size leads to increased effectiveness of the tazarotene in NLCs than drug itself in gel. Based on the fact that size is the most important dependent variable needed to be optimized, ANN was applied for this optimization. The same data used in BBD were used as input variables in ANN. ANN feed forward back propagation framework was fitted to data of BBD as shown in fig. 11. The data set was divided into training (70%), validation (15%) and testing data (15%). The training of the network by Levenberg-Marquardt topology minimized the error sum of square for the training dataset to

give high R value. During training, the weights and biases of the network were adjusted iteratively to minimize the network performance function. Learning function was gradient descent with momentum (learngdm), because the momentum allows the network to ignore small features in the error surface. The number of training cycles were on the basis of MSE of the validation dataset. Fig. 12 shows the architecture of ANN.

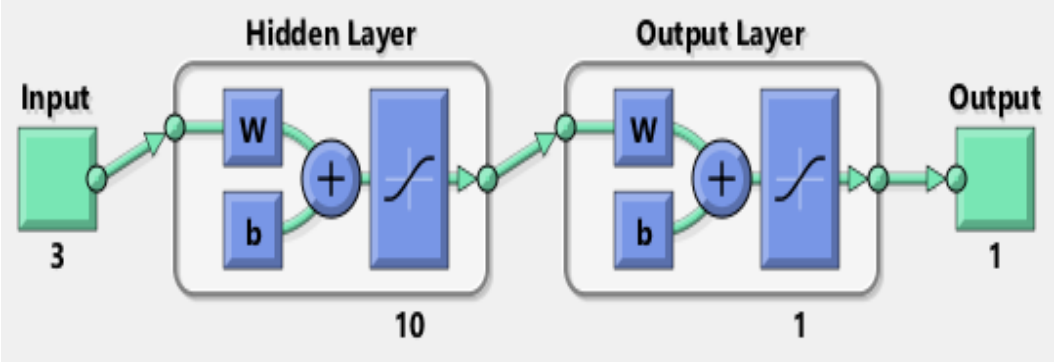


Figure 2: MLP feedforward back propagation network.

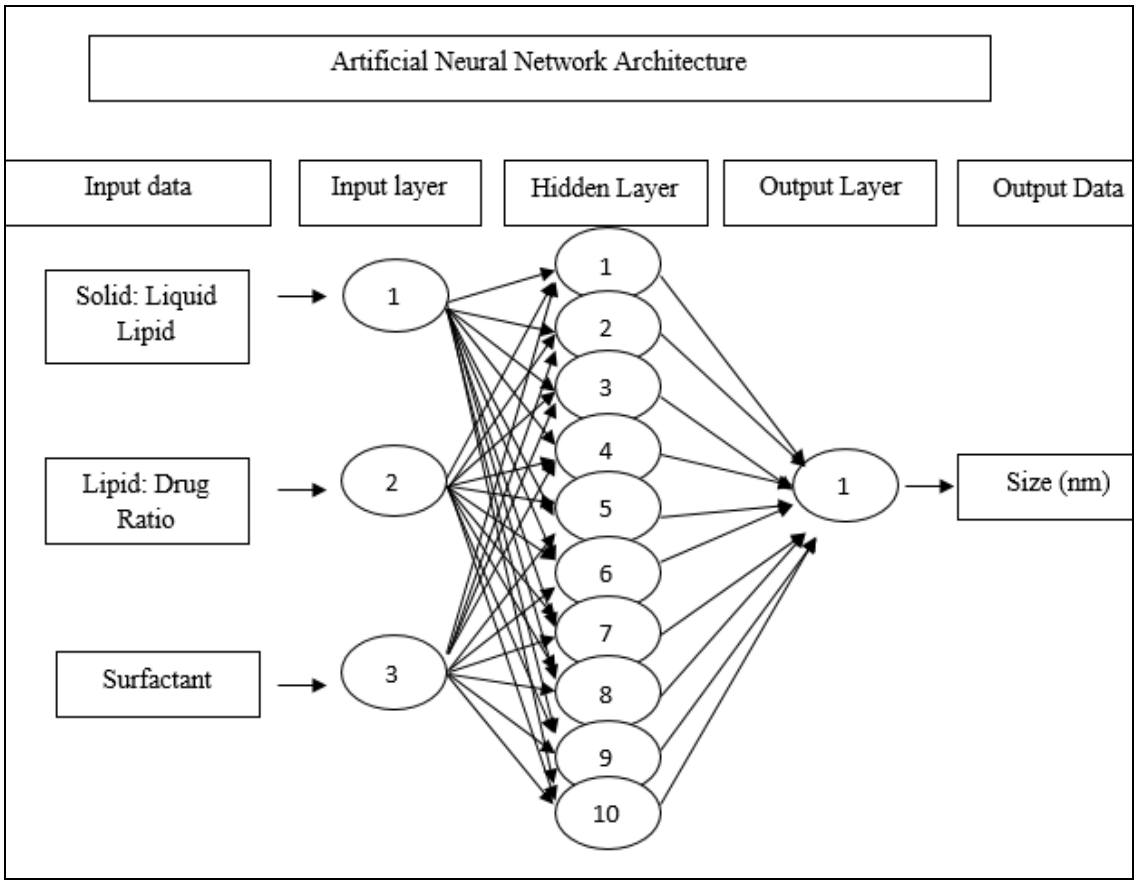


Figure 3: Architecture of Artificial neural network for optimization of Particle size.

Regression

Regression of the neural network dataset showed high R value. Overall value of the regression coefficient was found to be 0.99008 (Fig. 13) which was higher in comparison to regression coefficient value of 0.9861 of the quadratic model of BBD. This indicated that ANN model was trained to give better results in comparison to the BBD. Fig. 13 shows R value for the data set used herein for the developed network. As seen from the training R value (0.99999), the developed network is perfectly trained. This can be confirmed from the validation R value (0.98700). The test dataset showed R of 0.99299. The overall R value of the trained network for prediction of size of NLCs was found to be 0.99008. For this developed network simulation was carried out using the suggested dataset of BBD.

Simulation of the ANN network

For the simulation of the generated network, the new dataset same as the one suggested by the BBD was used as input for ANN. The amount of Solid: Liquid Lipid, Lipid: Drug Ratio and Surfactant were 7.109, 10.451 and 1.483 respectively. The output generated for the simulation dataset was 240.4318 nm. The batch prepared using same composition gave the actual size of 235.22±6.8 nm.

Comparison of BBD and ANN

The R value for the BBD based quadratic model was 0.9861 whereas for ANN it was 0.99008. The actual size was found to be 135.22 ± 6.8 nm. This indicates better predictability power of the ANN over BBD. The error for ANN was less as compared to BBD, indicating better accuracy in prediction of the size.

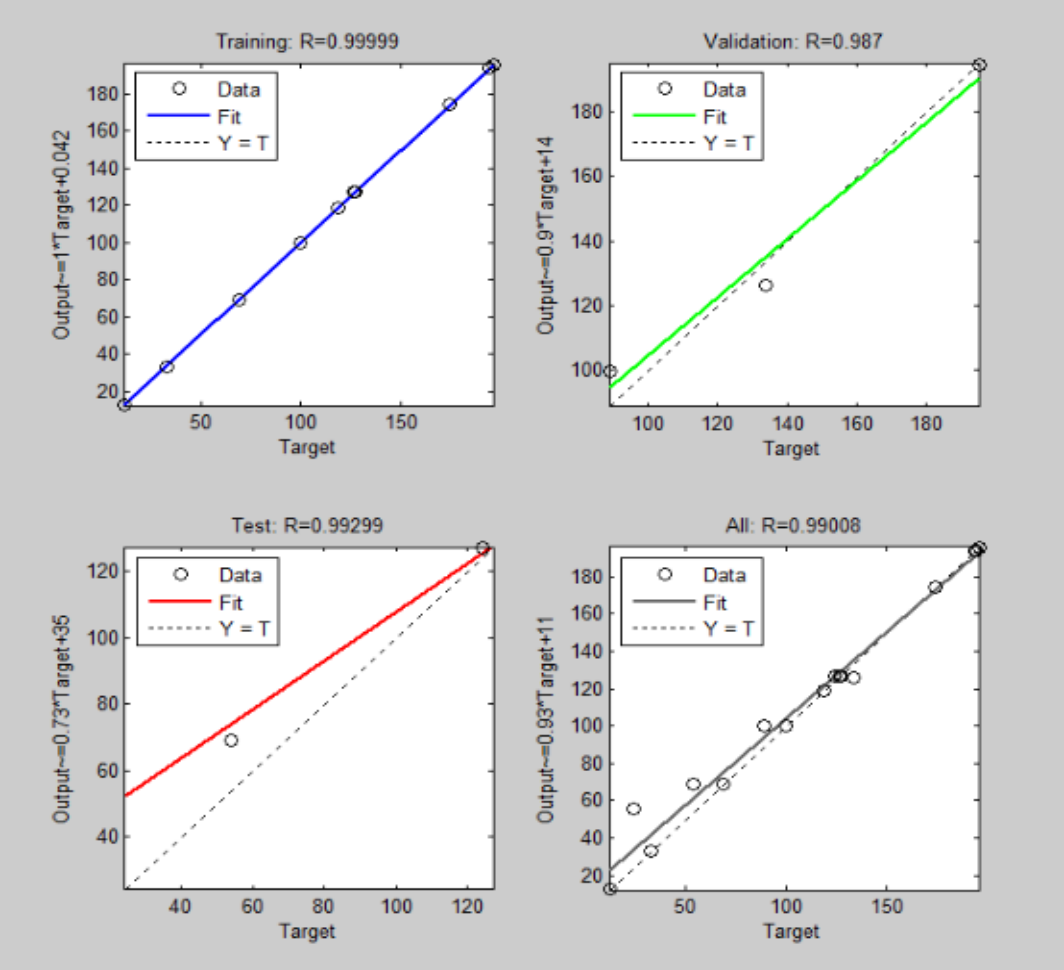


Figure 4: Regression of dataset showing R value.

Process variables optimization

Optimization of ultra turrax homogenization parameter: As the Ultraturrex plays the important role in size reduction of NLCs, the stirring speed of the homogenization are the important.[29] The effect of RPM (11000, 13000 and 19000 rpm) and time (3, 5, 7 and 10 min) is shown in the table 17. As seen from the Table 20, as the ultraturrax speed has been increased the size of NLCs decreases, but there is no much difference in %EE of drug. The homogenization speed affects size only. As there is no significant difference when rpm were increased from 13000 to 19000, the speed was fixed to 13000 rpm. At constant rpm of 13000, the time was varied for homogenization. There is no much change in size after increasing the time from 5 to 7 min. So, based on this OVAT approach for optimization of ultraturrax speed and time, 13000 rpm and 5 min were selected.

Table 2 : Optimization of homogenization rpm and time of rotation.

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

Optimization of sonication parameter : Sonication parameters such as amplitude and time of sonication were optimized at constant input variables as obtained from BBD. Different amplitude 10%, 30%, 50% and 70% were tried for 1, 3, 5 and 7 min. The cycle for sonication was kept constant at 0.5. The Table 21 shows optimization of sonication by OVAT approach. At constant input variables, at 30% amplitude, the time required for desired size is 5 min sonication as shown in Table 18. Increasing amplitude to 50% gives size reduction but comparatively it is not showing significant difference from 30%. Increasing it to 70% leads to decreased size at the cost of %EE. So, 30% amplitude was optimized.[27, 30] The sonication time was found to be 5 min as optimized as it gives less size with higher %EE.

Table 3: Optimization of Sonication parameter.

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

Incorporation of NLCs/SLN in gel: As the NLC and SLN dispersion has to be incorporated in the topical formulation its physicochemical properties are important to consider here. And also the developed gel was characterized for release pattern. From the Table 22, it is observed that the NLC/SLN formulation was of white colour and did not have any characteristic odor. These characteristics favor the use of NLC/SLN dispersion in topical formulation. The formulation is stable on centrifugation which shows that there were no aggregated particles present in the formulation. So, it can be concluded that the topical formulation incorporating this NLC formulation will be non-gritty.[31, 32]

Table 4: Physicochemical property of the NLCs/SLNS.

NLC DISPERSION	PROPERTY
Colour	White dispersion
Odour	Odourless
Stability on centrifugation (200 g for 20 minutes)	Stable on centrifugation
SLN DISPERSION	PROPERTY
Colour	White dispersion
Odour	Odourless
Stability on centrifugation (200 g for 20 minutes)	Stable on centrifugation

Particle Size Analysis : Result of particle size analysis of SLN and NLCs as measured by the zeta sizer is shown in Table 23. From the Fig. 14 and 15, it is observed that size of NLCs is comparatively more than SLNs. The liquid lipid present in NLCs may have contributed to the larger size but the size is in nano scale. Here both SLNs and NLCs are in nano range which will provide better occlusion and interaction with skin for increased penetration. The PDI in the range of 0.2 – 0.3 indicated that the samples were monodispersed in nature.[33]

Table 5: Particle Size and Zeta Potential of SLN and NLCs as measured by the Zeta Sizer.

Mean Particle size of SLN			Mean Particle size of NLCs		
Z-average (nm)	PDI	Zeta potential (mV)	Z-average (nm)	PDI	Zeta potential (mV)
215.2±4.5	0.272±0.08	-37.8±3.4	235.22±6.8	0.242±0.09	-39.8±3.7

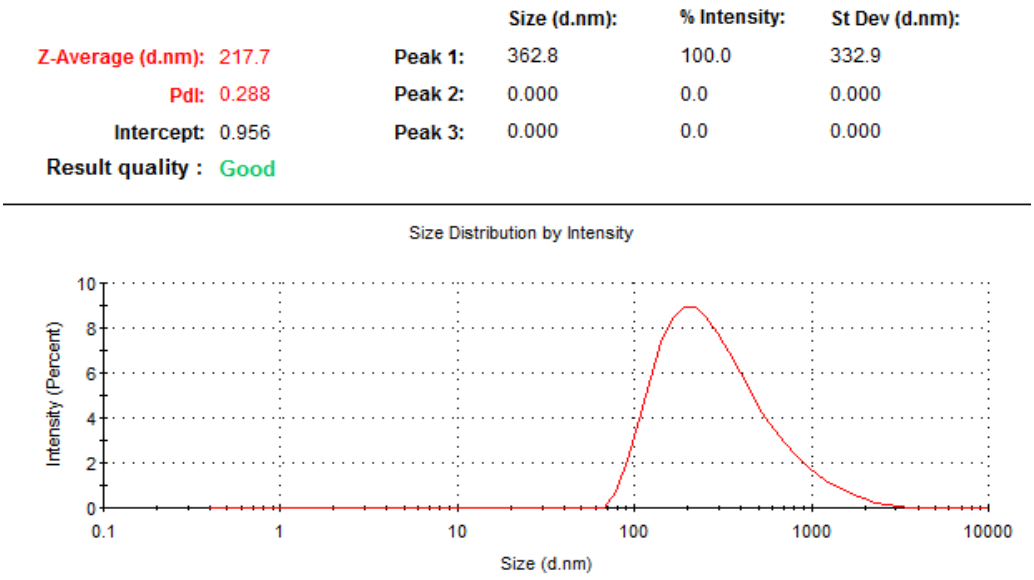


Figure 5: Particle Size and PDI of SLNs.

		Size (d.nm):	% Intensity:	St Dev (d.nm):
Z-Average (d.nm): 237.9	Peak 1:	220.2	100.0	54.94
PdI: 0.246	Peak 2:	0.000	0.0	0.000
Intercept: 0.872	Peak 3:	0.000	0.0	0.000
Result quality : Good				

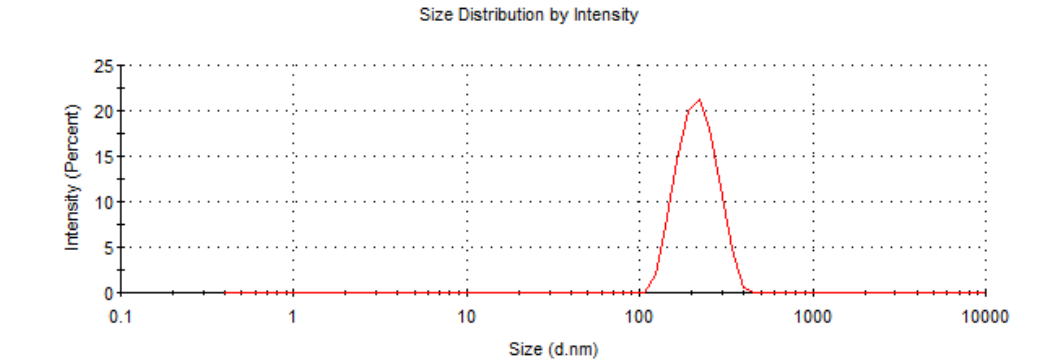


Figure 6: Particle Size and PDI of NLCs.

Zeta Potential : Zeta potential is a key factor to evaluate the stability of colloidal dispersion. In general, zeta values near to ±30 mV indicate that the SLNs give good stability in dispersion due to less chance of cohesion force between particles. As shown in Fig 16 and 17, zeta potential of optimized batch for SLNs and NLCs were -37.8 ± 3.2 mV and -39.8 ± 2.4 mV respectively, nearer to ±30mV. This indicates that the dispersions of NLC and SLN will remain deflocculated and would be physically stable.[32]

		Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -37.8	Peak 1:	-37.8	100.0	5.63
Zeta Deviation (mV): 5.63	Peak 2:	0.00	0.0	0.00
Conductivity (mS/cm): 0.0116	Peak 3:	0.00	0.0	0.00
Result quality : See result quality report				

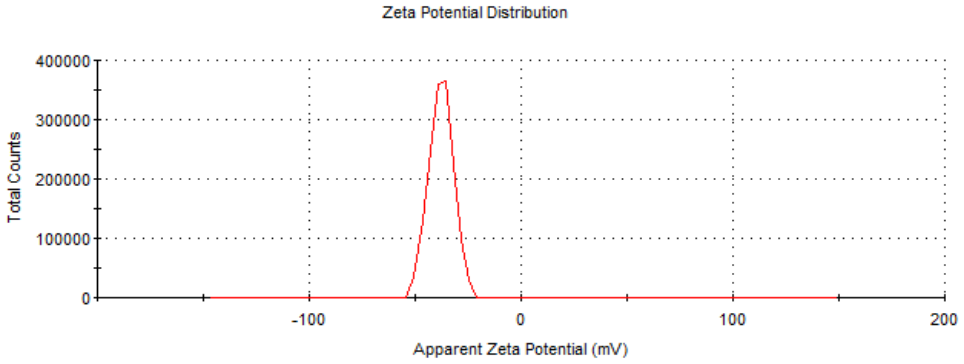


Figure 7 : Zeta Potential of SLNs.

		Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -39.8	Peak 1:	-39.8	100.0	5.47
Zeta Deviation (mV): 5.47	Peak 2:	0.00	0.0	0.00
Conductivity (mS/cm): 0.0155	Peak 3:	0.00	0.0	0.00

Result quality : See result quality report

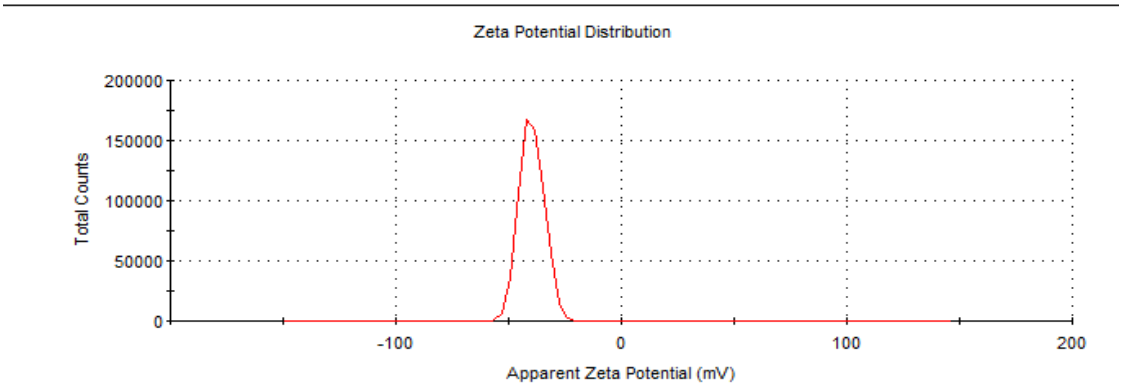


Figure 8: Zeta Potential of NLCs

Assay and % Drug Entrapment

Assay for either the SLNs or NLCs was found to be in the range of 97 to 98%. The loss may be due to processing steps involving transfer of the samples from one to another beaker or stirring.

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

Occlusion Test

The occlusion effect is one of the major effects responsible for penetration enhancement by lipid based nanoparticles.[34] The occlusion will increase the hydration of the skin and due to hydration, the penetration enhancement is achieved.[18] NLC have recently been investigated as carriers for enhanced skin delivery of sunscreens, vitamins A and E, triptolide and glucocorticoids.[35] It is thought their enhanced skin penetration is primarily due to an increase in skin hydration caused by the occlusive film formed on the skin surface by the NLC/SLN. A 31% increase in skin hydration has been reported following 4 weeks application of lipid nanoparticles enriched cream.

The Occlusion Factor for NLC Dispersion SLN Dispersion, Gel incorporating plain Drug and Gel incorporating NLC/SLN dispersion is shown in Table 24. An occlusion factor of zero

means no occlusive effect compared with the control, and 100 is the maximum occlusion factor.[18]

Table 6: Occlusion Study of SLNs/NLCs and incorporated Gel.

Time	Occlusion Factor				
	SLN Dispersion	NLC Dispersion	Gel Incorporating Plain Drug	Gel incorporating SLN dispersion	Gel incorporating NLC Dispersion
6	15±0.13	17±0.76	5±0.93	22±0.63	24±0.31
24	22±0.66	26±0.95	9±0.14	29±0.51	30±0.24
32	27±0.94	28±0.85	11.5±0.54	32±0.53	33±0.79
48	31±0.38	33±0.86	14±0.35	39±0.64	41±0.85

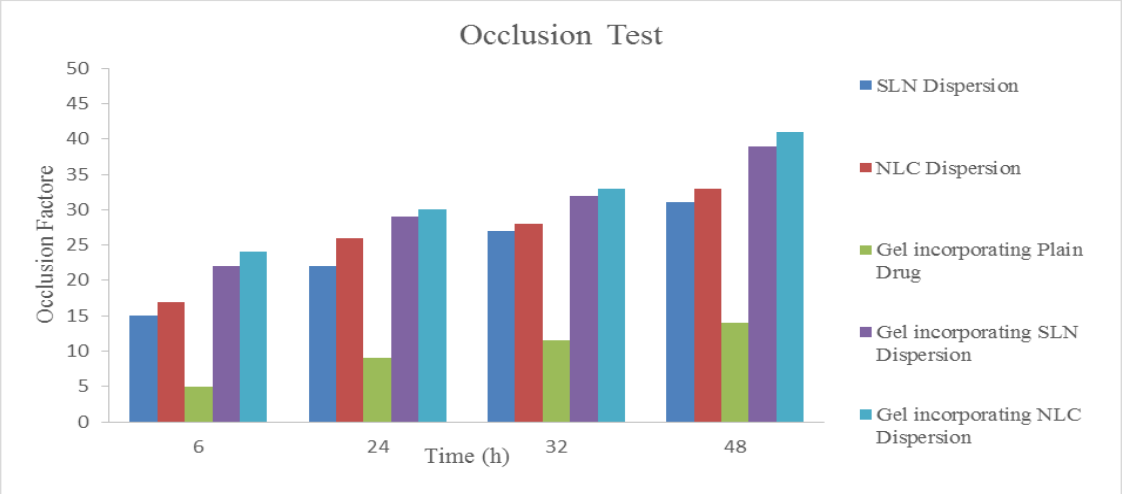


Figure 9: Occlusion Test.

As we can observe from results (Table 24 & Fig. 18) that the Gel incorporating NLC dispersion showed highest occlusion factor. The gel incorporating plain Drug showed significantly less occlusion factor therefore with comparison to Gel incorporating NLC/SLN dispersion, it showed less hydration and less penetration enhancement.

Evaluation of Gel

The pH of the gel containing either SLN or NLC formulation was found to be in the range of 6.3 to 6.8 which is much related to skin pH. So, the applied gel would not cause any irritation or discomfort to patient. Initially the gel formed with Carbopol showed acidic pH around 4 – 4.5. This is not suitable for application to skin. The consistency of that swelled dispersion was less rigid having less viscosity. After neutralization with triethanolamine the gel become rigid and pH of the gel is near to neutral. The hydration of Carbopol molecule when dispersed in water leads to partial uncoiling of every molecule. When this acidic Carbopol molecules

were neutralized with basic TEA, it converted to salt from its acidic property. This leads to thickening of the Carbopol and provided the desired pH for skin application. The assay of the drug in gel was found to be in the range of 96 – 98%, which is in acceptable limit. To draw a conclusion on homogeneity of the gel sample, appearance and assay was checked. For assay three samples taken from different area of container were compared. The sample was homogenous in nature and non-gritty. The assay for all samples were in the acceptable range. The viscosity of the NLC Gels and SLN Gels was found 29,235 cp and 29,789 cps at 25° C respectively. This viscosity favors the extrusion of the gel from the collapsible tube container as well as easy handling by patient during use.

1.3.7 In – vitro Drug Release

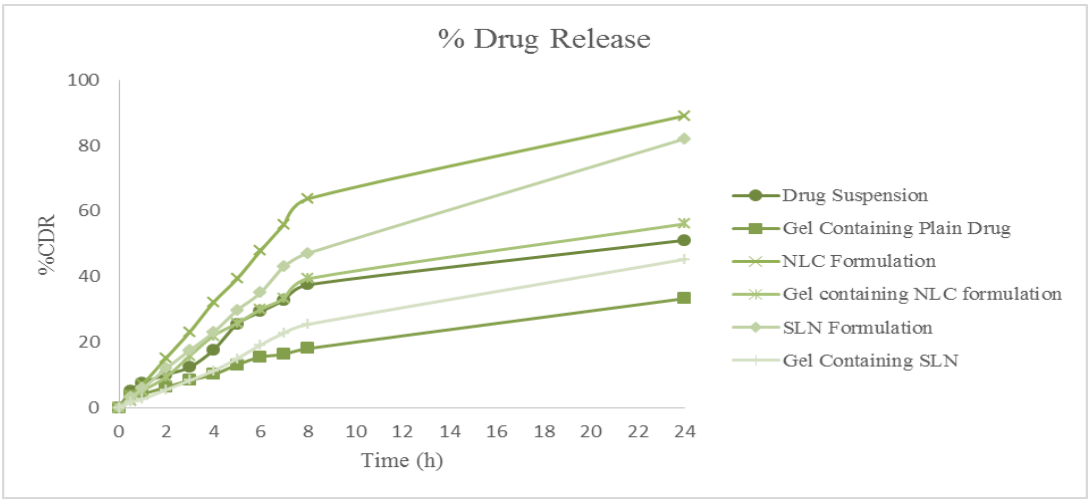


Figure 10 : In-vitro Drug Release.

Table 7: Comparative In-vitro drug release study.

Time	Drug Suspension	Gel Containing Plain Drug	NLC Formulation	Gel containing NLC formulation	SLN Formulation	Gel Containing SLN
0	0	0	0	0	0	0
0.5	5.2±1.4	3.5±1.5	3.7±2.2	2.2±1.5	3.2±2.5	1.7±2.4
1	7.6±2.7	4.2±2.1	7.1±2.7	5.1±2.8	5.9±3.1	2.6±1.5
2	10.1±2.1	6.3±3.9	15.2±1.7	9.4±3.1	11.9±4.2	5.5±1.4
3	12.4±3.6	8.4±2.8	23.1±2.9	15.8±3.8	17.5±2.8	8.3±2.5
4	17.6±1.3	10.1±2.6	32.1±3.4	21.9±2.6	22.9±3.1	11.2±3.1
5	25.5±3.6	13.1±3.1	39.3±1.5	25.7±3.7	29.7±2.6	14.9±4.2
6	29.3±2.6	15.5±4.2	47.9±2.5	29.9±4.2	35.3±1.4	19.1±1.8
7	32.9±1.7	16.3±3.7	55.9±3.9	33.3±1.9	43.1±3.1	22.8±2.4
8	37.5±3.5	18.0±4.2	63.7±2.8	39.3±2.6	47.2±3.2	25.4±3.1
24	51.1±1.3	33.3±5.3	89.1±3.9	56.1±2.1	82.7±1.6	45.3±2.6

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

Table 8: Model Fitting for drug release.

Model	R^2 Value					
	Drug Suspension	**Gel Containing Plain Drug**	**NLC Formulation**	**Gel containing NLC formulation**	**SLN Formulation**	**Gel Containing SLN**
Zero	0.5709	0.7800	0.6576	0.6493	0.8354	0.8699
First	0.8420	0.8815	**0.9851**	0.9033	**0.9943**	0.9575
Higuchi	0.9281	0.9631	0.9202	0.9343	0.9198	0.8958
Korsemeyer-Peppas	**0.9322**	**0.9928**	0.9342	**0.9457**	0.9743	**0.9700**
Hixon-Crowell	0.7708	0.8512	0.9808	0.8420	0.9863	0.9353
Baker-Lonsdale	0.9204	0.9510	0.8851	0.9205	0.8740	0.8742

For model fitting for drug release, the gel containing either drug/SLN/NLC followed korsemeyer-peppas model which is shown in Table 26.[36] Based on the n value for Korsemeyer Peppas model it was found to be anomalous non fickian drug release for all the gels. The n values were 0.621, 0.571 and 0.771 for drug containing gel, NLC containing el and SLN containing gel respectively. To Find out flux of the drug from the membrane quantity of the drug released is divided by area of drug release (Q/A).[36] The plot of Q/A vs. time was plotted. The slop of the plot indicates the flux. Here Flux is 0.812 and 0.662 from gel containing SLN and NLC respectively.

1.3.8 Stability study

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

Table 9: Effect of storage time and conditions on Physicochemical parameters of NLCs and SLNs loaded gel.

Sampling time: NLCs loaded gel	Storage condition LT stability study (25° C± 2° C, 60% RH ± 5% RH)			
	Appearance	**pH**	**Viscosity**	**Assay**
Initial	Smooth, off white, odourless	6.4 ±0.3	29,235 cps	98% ± 1.2%
After 1 Month	Smooth, off white, odourless	6.3 ±0.1	28,652 cps	97.5% ± 1.2%
After 2 Month	Smooth, off white, odourless	6.5± 0.2	31,974 cps	98% ± 1.2%
After 3 Month	Smooth, off white, odourless	6.3±0.2	27,168 cps	97% ± 1.2%
After 6 Month	Smooth, off white, odourless	6.6 ±0.2	28.634 5 cps	98% ± 1.2%
SLNs loaded gel	Appearance	pH	Viscosity	97% ± 1.4%
Initial	Smooth, off white, odourless	6.5 ±0.1	28,789 cps	98% ± 0.8%
After 1 Month	Smooth, off white, odourless	6.4±0.2	31,435 cps	97.5% ± 1.5%
After 2 Month	Smooth, off white, odourless	6.5±0.3	29,219 cps	98.1% ± 0.5%
After 3 Month	Smooth, off white, odourless	6.4±0.1	28,391 cps	97.6% ± 1.1%
After 6 Month	Smooth, off white, odourless	6.5±0.2	31,614 cps	96.9% ± 0.6%

CONCLUSION

From the experimental data it can be concluded that the developed formulation of tarozotine is effective in treatment of psoriasis. The lipid nanocarriers incorporated in the gel show retention at the site of application due to enhanced interaction with the skin with sustained release at the site of application with negligible toxicity which is desirable characteristic of the formulation. The stability of the developed formulation is also proven by stability studies which is prequisute for any new formulation for commercial use. The future aspect of the

developed formulation should be should be commercial scale up once the safety and efficacy is confirmed from clinical trials..

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