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Preparation and Evaluation of 5-Fluorouracil loaded Nano-Structured lipid carrier by double emulsification techniques

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ABSTRACT

Skin cancer remains the second most common cancer causing death in majority of the population worldwide. Chemotherapeutical treatment generally includes treatment by administration of chemotherapeutical formulations mostly by intravenous route of administration which is painful, toxic, time consuming and costly for the patients. Chemotherapy also causes toxicity and cell death to other normal cells in the body apart from cancerous cells. The aim of the present investigation was to formulate a topical nano-particulate drug delivery system which causes lower exposure to normal body cells by higher efficacy of targeting the cancerous cells, producing lower toxicity rates and avoiding maximum possible side effects. Henceforth, an anti-neoplastic agent has been used in order to prepare Nano-structured Lipid Carriers (NLCs) which was further loaded into gel formulation for topical application. The nano-structured lipid carrier (NLCs) of 5-fluorouracil (5-FU) were prepared by using Compritol ATO 888 by double emulsification method. The lipids were selected based on the solubility studies and partition coefficient of 5-FU in lipids. The particle size of optimized formulation was found to be 246.2nm. The in vitro release studies of developed NLCs was carriers carried out at pH 7.4. Sodium carboxy methylcellulose, hydroxyl propyl methyl cellulose, and chitosan hydrogels loaded with NLCs were developed. The permeability behavior through dialysis membrane was performed for 24 hrs and Q 24 of optimized gel formulation was found to be $435.974\mu\text{g}/\text{cm}^2$. The steady-state flux (J_{ss}) was found to be $0.062102\text{ mg}/\text{cm}^2$, and permeability coefficient (K_p) was found to be $4.14013\text{ cm}/\text{hr}$ for optimized NLCs based gel formulation for ex-vivo skin permeability studies.

Keywords: Nano-structured lipid carrier (NLCs), Steady-state flux, Permeability coefficient

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INTRODUCTION

Skin cancers are cancers that arise from the skin. They are because of the advancement of irregular cells that can attack or spread to different parts of the body. There are three fundamental kinds of skin tumors: basal-cell skin cancer (BCC), squamous-cell skin growth (SCC) and melanoma. The initial two, alongside various less regular skin malignancies, are known as nonmelanoma skin growth (NMSC). Basal-cell malignancy develops gradually and can harm the tissue around it yet is probably not going to spread to far off zones or result in death. It frequently shows up as an effortless raised territory of skin, that might be gleaming with little vein running over it or may present as a raised zone with an ulcer. Squamous-cell skin malignancies will probably spread. Melanomas are the most forceful. Signs incorporate a mole that has changed in measure, shape, and shading, has sporadic edges, has in excess of one shading, is bothersome or bleeds.

Currently depending on the stage of cancer, skin cancer is been treated either by Chemotherapy, surgery, radiation therapy or by immunotherapy. Further, it is a well-known fact that the conventional dosage form when used for the treatment of skin cancer delivers the drug to both normal and cancerous tissues, thus leading to undesirable adverse effects. In order to overcome the drawbacks associated with available conventional chemotherapy, a system needs to be developed which is able to deliver the drug directly to the affected area of skin. Recently topical chemotherapy is attaining tremendous interest among scientific community for improving quality of life of patients.

Lipid Nanoparticles have been discovered to be one of the most efficient and bio-degradable drug delivery system for treating a number of diseases. There are generally two types of lipid based nano-particulate systems which includes Solid Lipid Nanoparticles (SLNs) and Nano Structured Lipid Carriers (NLCs). The advantage of NLCs over SLNs is the increased % Drug Loading and stability on-storage.

Drawbacks of SLNs:

- Solid lipid nanoparticle has poor drug loading capacity.
- Sometime expulsion of drug occurs because of polymeric transition during the storage.
- They have relatively high water content of dispersions, so that more chances of phase inversion.
- Low capacity to load hydrophilic drugs due to partitioning effects during the production process.

To overcome these effects formulation of nano-structured lipid carrier was necessary.

Advantages of NLCs over SLNs:

NLCs have been presented as the up and coming age of the SLNs to command the conceivable challenges of SLNs.

- Nano-structured lipid carrier improve the stability
- They also increase the capacity of loading of drug
- They prevent the expulsion of drug during the storage for long time.

For topical chemotherapy, it is necessary for the drug delivery system to delivering drugs precisely into target tissues in order to achieve the maximum benefit with minimum side effects.

In the current study, the NLCs loaded with 5-Fluorouracil with enhanced skin permeability was formulated, which has the capacity to deliver the drug in a relatively short time by capturing the drug within the shell region.

MATERIALS AND METHOD

Material:

5-fluorouracil obtained from Chemdyes Corporation, Ahmedabad, Labrafac lipophilic WL 1349 was obtained from gift sample from Gattefosse India Pvt Ltd., Span 80 and Propylene Glycol Monoacrylate obtained from Gattefosse India Pvt Ltd., Tween 80 and Poloxamer 188 obtained from BASF India Ltd., Mumbai, India., Hydroxy propyl methyl cellulose K 4 M was obtained from Chemdyes Corporation, Ahmedabad., Carboxymethylcellulose sodium was obtained from Central drug house Ltd, Chitosan was obtained from Himedia laboratories Ltd.

Table 1: List of equipment

Name of equipment	Company
High speed homogenizer	IKA T25 digital ULTRA TURRAX, Germany
Ultrasonicator probe	Sonics Vibra-cell 750, Newtown, Connecticut, USA
Franz multicell diffusion apparatus	ORCHID scientific, India
UV apparatus	Shimadzu UV-1800., Japan

Identification of 5-Fluorouracil:

Melting Point Determination of 5-Fluorouracil:

The Thiel's tube method for the determination of melting point in the presence of liquid paraffin was applied for determining the melting point of 5-Fluorouracil. The capillary was filled with drug crystals and placed in the melting point apparatus. The temperature at which the solid drug crystals started melting was marked as the melting point of the drug.

Table 2: Melting point report of 5-Fluorouracil

Standard melting point	Observed melting point
280-282 ⁰ C	282 ⁰ C

UV Determination of Drug

10 µg/ml solution of 5-Fluorouracil was prepared in distilled water and was analysed using Shimadzu-1800 double beam UV-Visible Spectrophotometer in the range of 200 nm to 400 nm.

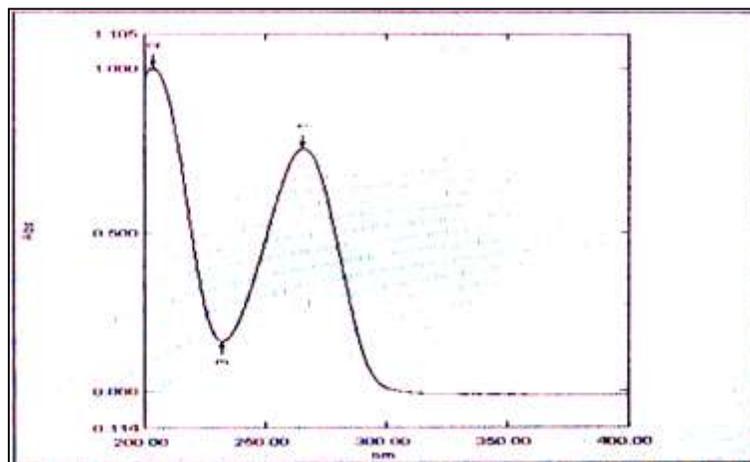


Figure 1: UV spectra of 5-Fluorouracil in 7.4 phosphate buffer

Establishment of calibration curve in 7.4 pH phosphate buffer saline

Procedure:

100mg drug A was dissolved in 7.4 phosphate buffer saline upto 100 ml to produce solution of 1000 µg/ml. After 10 ml of solution was taken and diluted with 7.4 phosphate buffer saline upto 100 ml to produce 100 µg/ml stock solution concentration.

Preparation of sample solution:

Sample solution was prepared from the stock solution of 100 µg/ml. For the preparation of 3 µg/ml solution 0.3 ml from the stock solution was taken and diluted upto 10ml with 7.4 phosphate buffer. In the same way 6 µg/ml, 9 µg/ml, 12 µg/ml, 15 µg/ml and 18 µg/ml solutions were prepared.

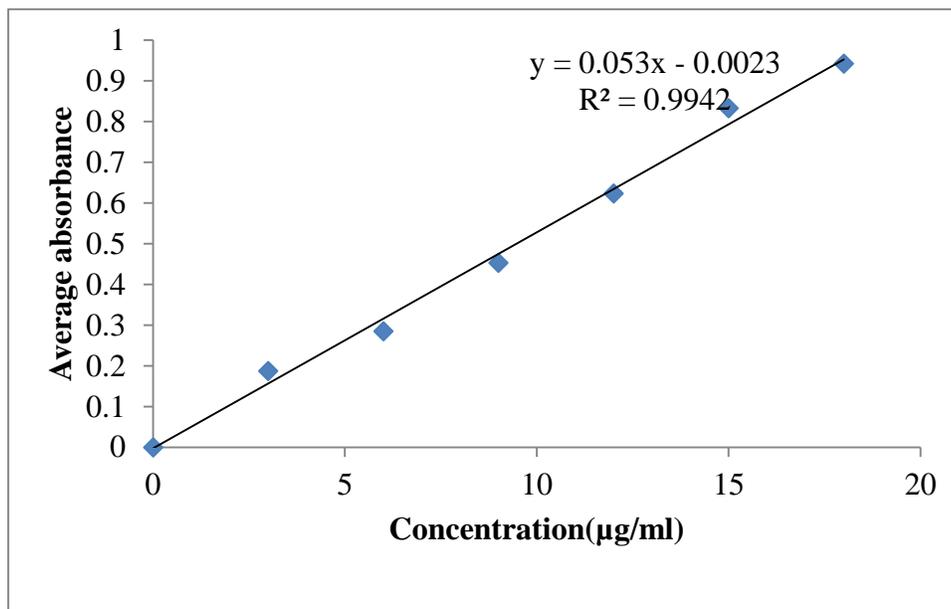


Figure 2: Calibration curve of 5-florouracil in phosphate buffer saline

Establishment of calibration curve in chloroform ethanol mixture

100mg drug A was dissolved in chloroform ethanol mixture (50:50) up to 100 ml to produce solution of 1000 µg/ml. 10 ml from this solution was taken and diluted with chloroform ethanol mixture up to 100 ml to produce 100 µg/ml stock solution concentration.

Preparation of sample solution:

Sample solution was prepared from the stock solution of 100 µg/ml. For the preparation of 2 µg/ml solution 0.2 ml from the stock solution was taken and diluted up to 10ml with mixture of chloroform and ethanol. In the same way 4 µg/ml, 6 µg/ml, 8 µg/ml and 10 µg/ml solutions were prepared.

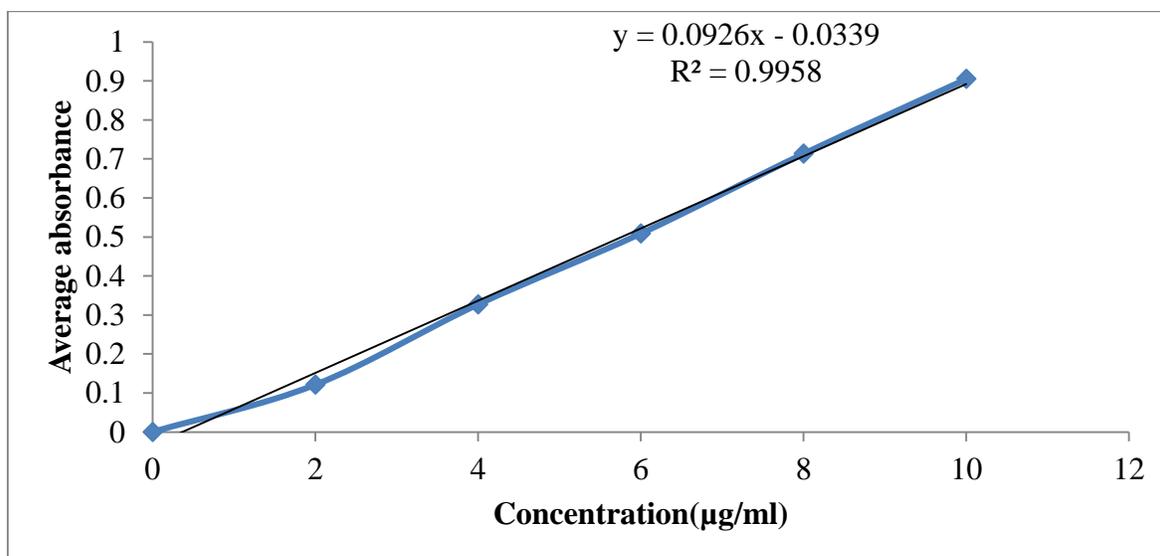


Figure 3: Calibration curve of 5-florouracil in chloroform ethanol mixture

Development of Nano-Structured Lipid Carrier System NLCs

Solubility studies for selection of lipids:

Different type of solid and liquid lipids were evaluated for determining the solubility of 5-Fluorouracil. One gram of solids/liquid lipids were accurately weighed and transferred to glass vials. The drug was added to the melted lipids (in increments of 2mg), and solid/liquid lipids-drug mixture was then heated at 10°C above the melting point of solid lipids, and agitated at 100 rpm using an incubator shaker bath for 24 h. Following dissolution of the initial amount of drug that was added to the lipid medium, the amount of the drug in the lipid was increased until drug crystals fails to dissolve in the molten lipid after shaking for 24 h.

Partitioning studies for selection of lipids:

One gram of lipids were melted with respect to their melting point. After that, 10 ml of double distilled water was added. Then, this mixture kept for vortexing for 15 minutes. After complete amalgamation of mixture 10 mg of 5-Fluorouracil was added to above emulsion. Both the phases were vortexed and then allowed to cool. The lipid was separated by centrifugation at 15,000 rpm. Then, filter the aqueous phase through syringe filter and UV of this aqueous phase was taken at 267nm.

Preparation of NLCs

5-Fluorouracil-NLCs were prepared using HSH and probe sonicator. In brief, Compritol ATO 888 and Labrafac WL 1349 and Span 80 or PGMC were heated (10°C above the melting point of solid lipid) until a uniform and clear lipid phase was observed. 5-Fluorouracil was dissolved in hot distilled water at 80°C. The drug solution then transferred to melted lipid phase with simultaneous stirring in high speed magnetic stirrer at 1500 RPM. The Aqueous surfactant solution was prepared separately using double distilled water and was heated to the same temperature as that of lipid phase. The hot aqueous surfactant solution was transferred to above pre-emulsion with simultaneous stirring for 2 minute. which was subsequently subjected to probe sonication for 2 min at 30% amplification. Later HSH was done at about 9000 RPM for 2 minutes.

Characterization of 5-fluorouracil loaded nanostructured lipid carrier

1. Particle size and polydispersity index of nanostructured lipid carrier
2. Differential scanning calorimetry
3. XRD
4. Scanning electron microscopy
5. In-Vitro Release Studies

Particle size and polydispersity index of nanostructured lipid carrier

Nano-structured lipid carrier were characterized for average particle size(Z-average) and polydispersivity index(PI) using Horiba Particle Size Analyzer. Particle size diameters were determined at 90th,50th and 10th percentile of particle undersized.

Differential scanning calorimetry

Possibility of any interaction between drug and excipients was assessed (DSC-60 shimadzu .The thermogram of the samples were obtained at a scanning rate of 10⁰C conducted over a range of 0-400⁰C under an inert atmosphere flushed with nitrogen at the rate of 20 ml/minute.

X-Ray Powder Diffraction(XRD)

X-ray powder diffraction (XRD) is a rapid analytical technique primarily used for phase identification of a crystalline material and can provide information on unit cell dimensions. The analyzed material is finely ground, homogenized, and average bulk composition is determined.

Scanning Electron Microscopy(SEM)

The SEM was performed in EVO 1800 Research ZEISS. The scanning electron microscope (SEM) uses a focused beam of high-energy electrons to generate a variety of signals at the surface of solid specimens. The signals that derive from electron-sample interactions reveal information about the sample including external morphology (texture), chemical composition, and crystalline structure and orientation of materials making up the sample.

In-Vitro Release Study of NLCs

In-vitro drug release study was carried out by taking 5ml of NLCs into dialysis membrane with molecular weight cut-off of 12kDa. The bag was further suspended in 50 ml of PBS pH 7.4 Buffers at 37°C in water bath at 100 rpm. Aliquots 2 ml were taken at the time points of 1h, 2h, 3h, 4h, 6h, 8h, 12h, 16h, 24h and 72h and replenished by equal amount of the buffer in order to maintain the sink condition and subjected to analysis for the presence of drug in the respective buffer at 267 nm by UV-Vis spectrophotometry.

Preparation of Gel Formulation and Characterization of the Formulation:

NaCMC (2% w/w), HPMC (2% w/w) and chitosan (2% w/w), as a prototype of negatively charged matrix, hydrophilic gel base and cationic polymer respectively were tried as gel bases for NLCs formulation.

Hydroxypropyl methylcellulose

Hydroxypropyl methylcellulose K4M was used for the preparation of gel. For the preparation of hydro gel 0.8g of hydroxypropyl methylcellulose was dissolved in 20 ml of double distilled water. After that 10% of glycerol was added to double distilled water and kept the resultant dispersion to magnetic stirrer at 300 rpm without application of heat. Process was continued till the HPMC was

fully dissolved. Required amount of NLCs dispersion was added to the polymeric solution to prepare hydrogel(2% w/w).

Sodium carboxyl methyl cellulose

Sodium carboxyl methyl cellulose was used for the preparation of gel. For the preparation of hydrogel 0.8 g of sodium hydroxypropyl methylcellulose was dissolved in 20 ml of double distilled water. After that 10% of glycerol was added to double distilled water and kept the resultant dispersion to magnetic stirrer at 300 rpm without application of heat. Process was continued till the sodium carboxyl methyl cellulose was fully dissolved. Required amount of NLCs dispersion was added to the polymeric solution to prepare hydrogel(2% w/w).

Chitosan

Chitosan was cationic polymer. Various acids were used for the dissolved chitosan polymer. In this, 0.8 g of chitosan was dispersed in 20 ml of double distilled water containing 10% glycerol and 1% acetic acid. After that, the resultant dispersion kept for 300 rpm with heating at 50⁰C for 30 minute. Required amount of NLCs dispersion was added to the polymeric solution to prepare hydrogel(2% w/w).

Characterization of Gel Formulation Containing 5-Fluorouracil:

Diffusion Study of Nano-Structured Lipid Carrier Containing Gel Formulation

The permeation study of 5-FU from NLCs was investigated using a modified Franz diffusion cell system: A dialysis membrane was placed on the upper donor chamber of the diffusion cell, separating this compartment from the receptor chamber. Accurately weighed quantities of varying NLCs formulations containing 5-FU were placed on the membrane and suspended in PBS. The receptor chamber was completely filled with the corresponding buffer PBS (20 mL, pH 7.4). The receptor was stirred at 100 rpm and aliquots of 1mL were extracted from the receptor chamber at predetermined time intervals. The aliquots were filtered and finally measured at 267 nm using a spectrophotometer.

Ex-vivo Skin Permeability Studies

Franz diffusion cell was used for the evaluation of Ex-vivo skin permeability testing. To performed this abdominal skin of rat was used. Surgical removal of subcutaneous tissue was done. After removal of subcutaneous tissue the dermis side of skin wiped with isopropyl alcohol. Isopropyl alcohol was used to remove the fats. Then skin was washed with double distilled water. The skin was then mounted between the donor and receptor compartment. The speed of rotor was 100 rpm.

Experimental Section:

Factors related to process and formulation widely affect the formulation owing to its efficacy and stability. Hence preliminary trials were conducted to evaluate the process parameters. The aim of the present work was to develop NLCs with optimal size and maximum entrapment efficacy.

Screening of Excipients:

Selection of Solid Lipids

For the selection of solid lipids two kind of studies were performed.

Solubility studies

The selection of solid lipid was based on maximum solubility of drug in it. A number of lipids were chosen for the study.

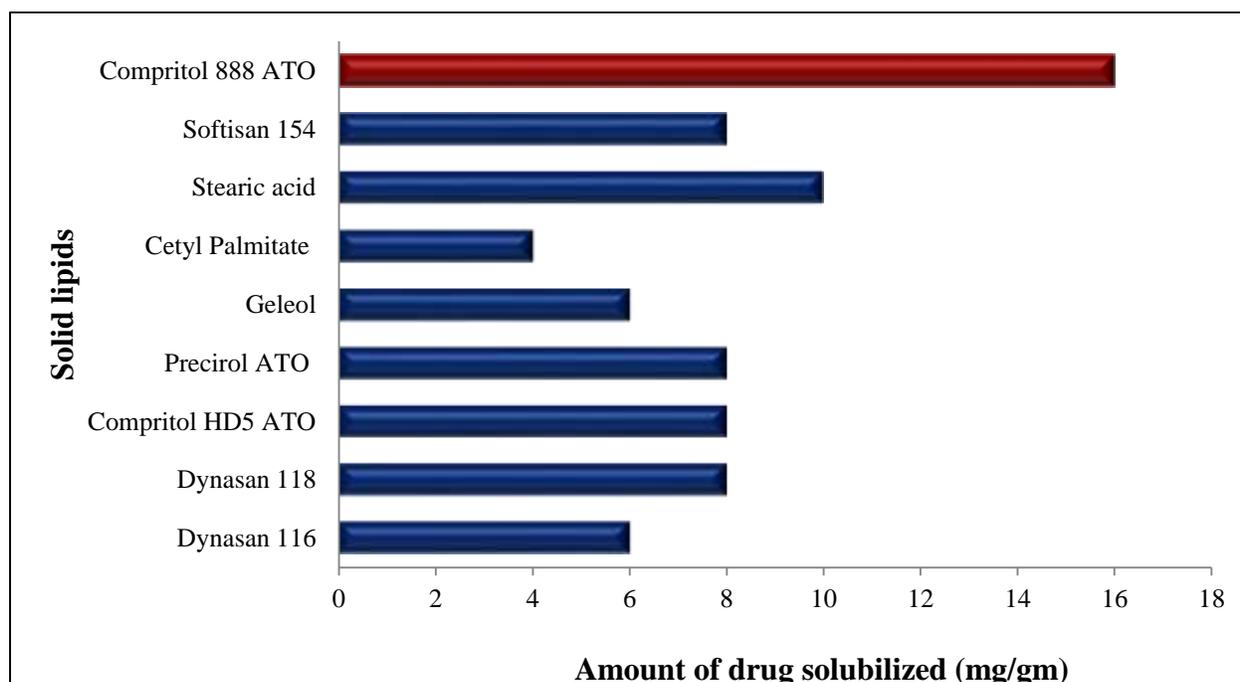


Figure 4: Solubility of drug in various lipids

Based on the solubility of drug it was observed that 5-Fluorouracil shows maximum solubility in Compritol 888 ATO(16 mg/gm).Followed by stearic acid, compritol HD5 ATO and Precirol ATO shown 10 mg/gm,8 mg/gm and 8 mg/gm respectively. Drug was maximum solubilized in Compritol ATO 888, therefore we use Compritol ATO 888 for further studies.

Partitioning studies:

Partitioning study was based on maximum partition in different solid lipids.

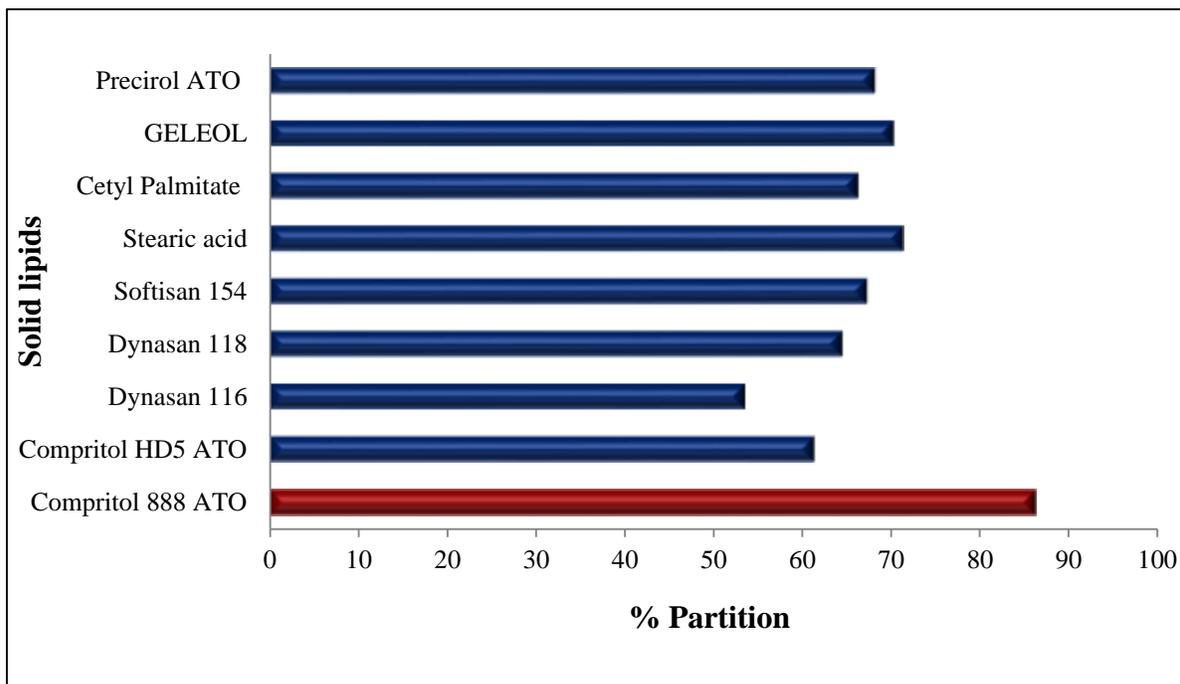


Figure 5: Partition studies of various lipids

It was observed that compritol ATO 888 shows maximum partition 86.32%. After that, Geleol, stearic acid shows 70.31% and 71.43% respectively. Therefore, we select Compritol ATO 888 for further studies.

Selection of Liquid Lipids

The selection of liquid lipid was based on maximum solubility of drug in it. A number of lipids were chosen for the study.

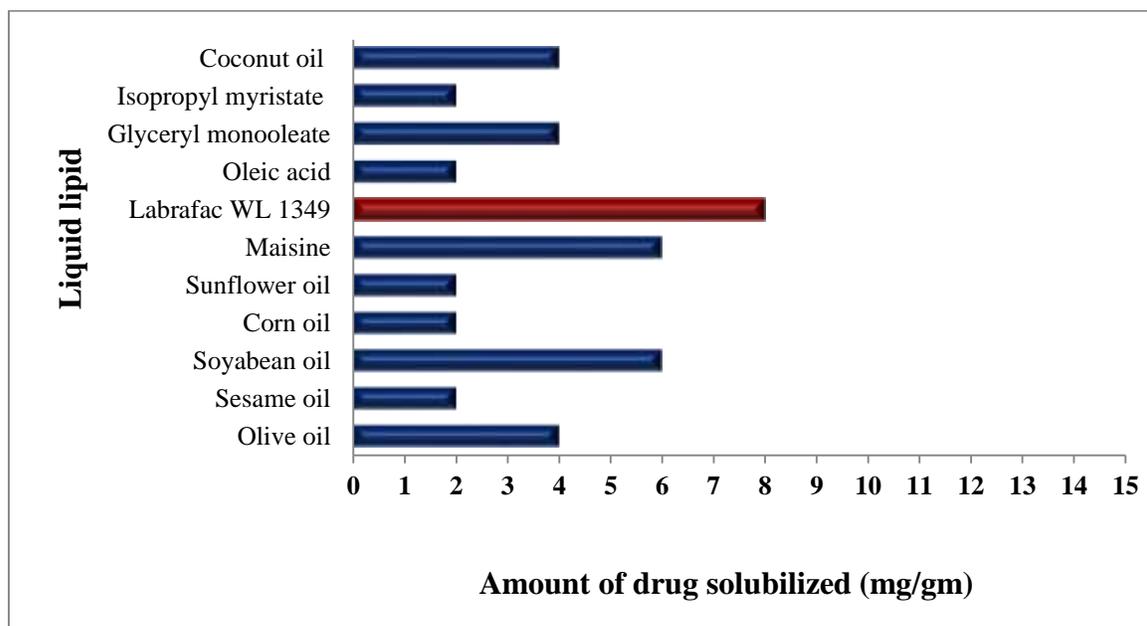


Figure 6: Solubility of liquid lipids

Solubility studies data clearly shows that Labrafac WL 1349 shows maximum solubility of 5-florouracil about 8mg/gm. In contrast to this, sesame oil, corn oil, sunflower oil and oleic acids shows minimum solubility 2 mg/gm. Labrafac WL 1349 was shown maximum solubility in compared to other lipids therefore Labrafac WL 1349 was used for further studies.

Selection of ratio of Solid lipids and Liquid lipids

Mixture of solid lipid and liquid lipid were prepared at different ratio:

Table 3: Different ratio of solid lipids: liquid lipids

Serial no.	Solid lipids: Liquid lipids	Observation
1	60:40	Stains
2	70:30	Stains
3	80:20	Stains
4	85:15	No stains
5	90:10	No Stains
6	95:5	No Stains

It was observed that mixture containing 85% solid lipid and 15% liquid lipid shown no stains. Therefore, we use this ratio for further studies.

Selection of Type of Surfactant

Various types of non-ionic surfactants were evaluated. Based upon the drug solubility profile surfactants were selected. To achieve this, various surfactant were screened.

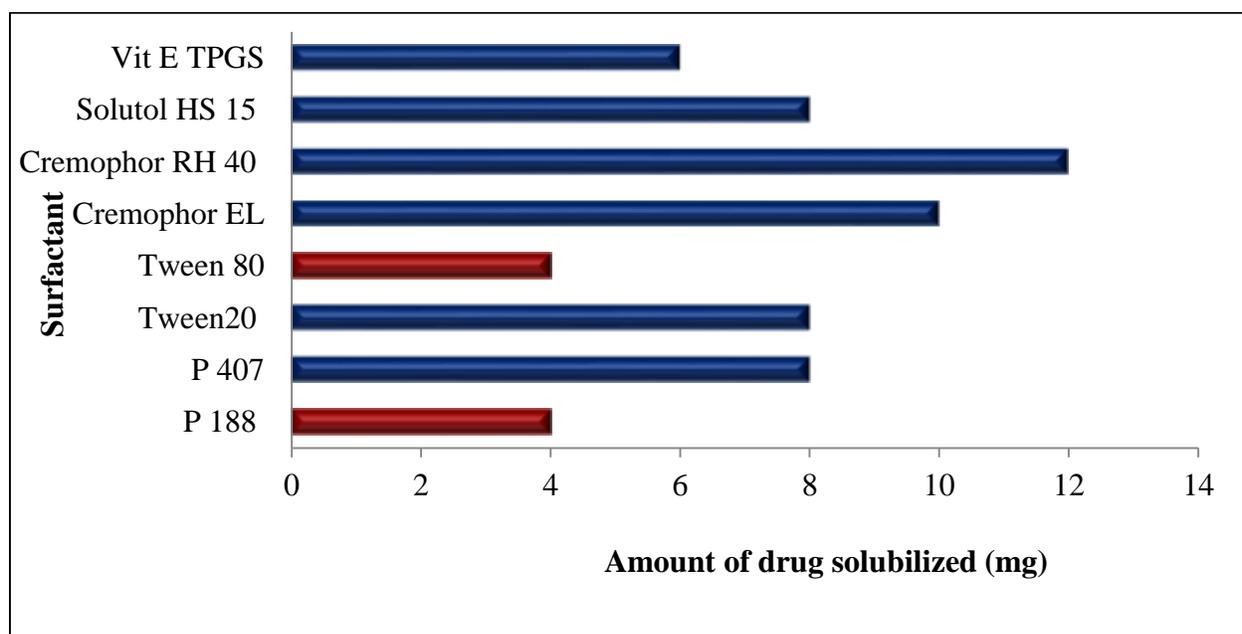


Figure 7: Solubility of drug in various surfactants

It was observed that Poloxamer 188 and Tween 80 shows solubility of drug 4mg/ml in both the case. In contrast to these, Cremophor RH 40 and Cremophor EL shows 12mg/ml and 10mg/ml

respectively. Poloxamer 188 and Tween 80 surfactants shows less solubility of drug , therefore we use this for further studies.

Preliminary trials:

Effect of Span80 and Tween 80 on preparation of NLCs

- Concentration of Tween 80 was kept at 3% and 6% respectively.
- Concentration of lipid and oil were kept constant 85% and 15% respectively.
- Concentration of Span 80 was vary from 1% to 5%.

Table 4: Effect of Span 80 and Tween 80 concentration on particle size

Batches	Span80 (%W/W)	Tween80 (%W/V)	D 90(nm)	Polydispersivity index	%EE
ST1	1	3	227	0.358	60.61
ST2	3	3	199.8	0.384	52.29
ST3	5	3	117.4	0.362	46.37
ST4	1	6	175.8	0.386	49.85
ST5	3	6	128.9	0.36	46.74
ST6	5	6	76.8	0.386	39.22

(Amount of Drug-10 mg, Speed of Homoginizer-9000RPM, Amplification-30%,2 min, Concentration of lipid-1 gm)

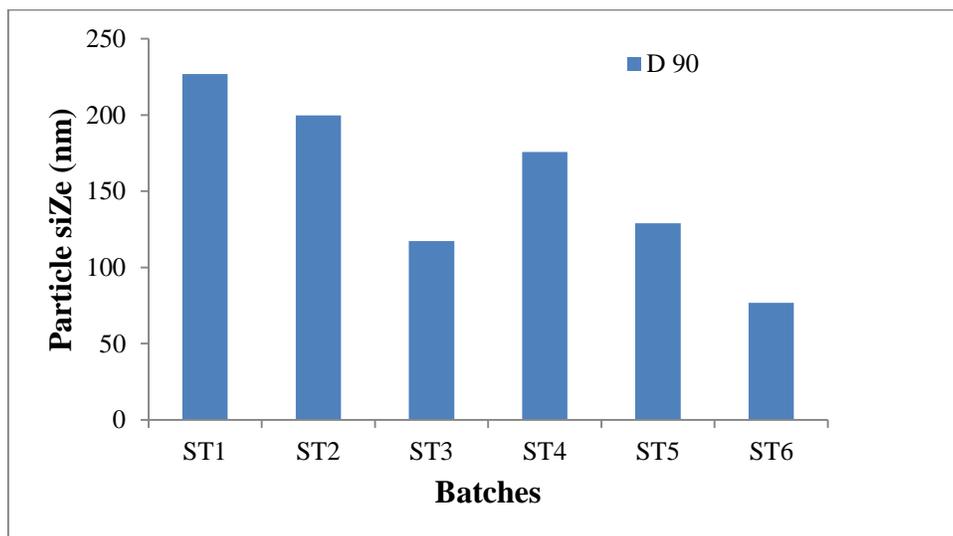


Figure 8: Effect of concentration of surfactants span 80 and tween 80 on Particle size

As the concentration of surfactant increases particle size value decreases, this could be due to breakdown of lipid droplets into smaller size at higher concentration of surfactant. Further, It was also observed that increase in concentration of surfactant leads to decrease in %EE. This could be due to increase in surface area of particles as a result of decrease in particle size (with increase in surfactant concentration), which leads to increased leaching of drug from the system during preparation of NLCs.

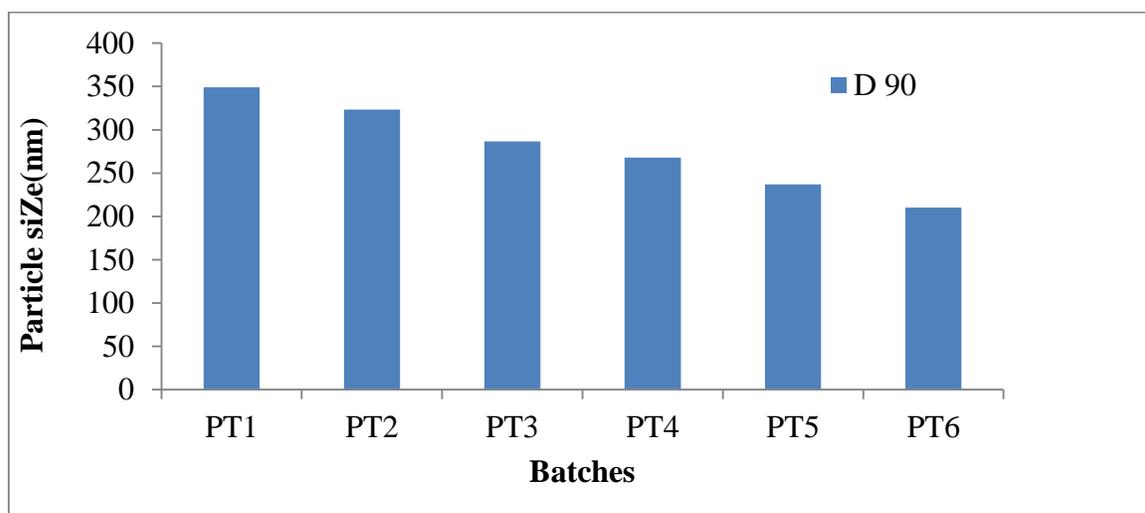
Effect of Propylene glycol Monocaprylate(PGMC) and Tween 80 on preparation of NLCs:

1. Concentration of Tween 80 was kept at 3% and 6% respectively.
2. Concentration of PGMC was vary from 1% to 5%.

Table 5: Effect of PGMC and Tween 80 concentration on particle size

Batches	PGMC (%)	Tween 80 (%)	D 90(nm)	Polydispersivity index	% EE
PT1	1	3	349.1	0.326	78.73
PT2	3	3	323.2	0.372	71.5
PT3	5	3	286.5	0.366	65.26
PT4	1	6	268	0.331	68.54
PT5	3	6	237	0.317	59.18
PT6	5	6	210	0.31	51.23

(Amount of Drug-10 mg, Speed of Homoginizer-9000RPM, Amplification-30%, 2 min, Concentration of lipid-1 gm)

**Figure 9: Effect of concentration of surfactant tween 80, PGMC and poloxamer 188 on particle size.**

As the concentration of surfactant increases particle size value decreases, this could be due to breakdown of lipid droplets into smaller size at higher concentration of surfactant. Further, It was also observed that increase in concentration of surfactant leads to decrease in %EE. This could be due to increase in surface area of particles as a result of decrease in particle size (with increase in surfactant concentration), which leads to increased leaching of drug from the system during preparation of NLCs.

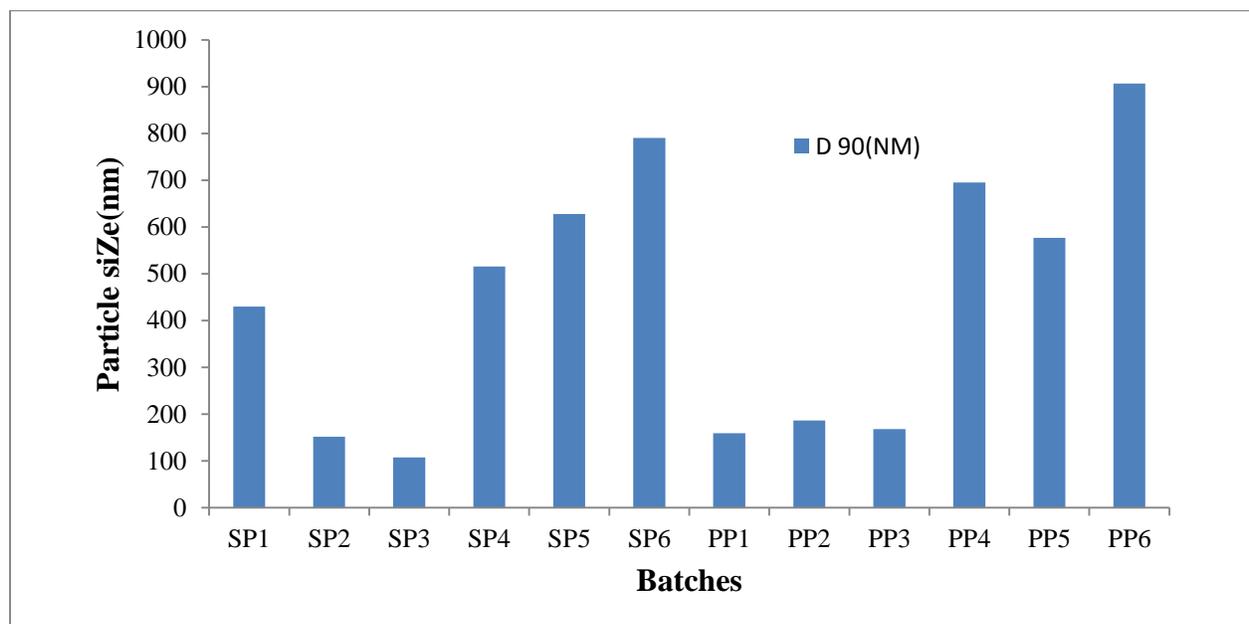
Effect of Span 80, PGMC and Poloxamer 188on preparation of NLCs:

- Concentration of Poloxamer 188 was kept at 3% and 6%.
- Concentration of span 80 and PGMC were varied from 1% to 5%.

Table 6: Effect of Span 80 and PGMC and Poloxamer 188(3% and 6%) on particle size

Batches	Span 80 (%)	PGMC (%)	Poloxamer188 (%)	D 90(nm)	Polydispersivity index
SP1	1	-	3	429.9	0.446
SP2	3	-	3	151.8	0.465
SP3	5	-	3	107.5	0.466
SP4	1	-	6	515.4	0.544
SP5	3	-	6	627.8	0.534
SP6	5	-	6	790	0.508
PP1	-	1	3	159	0.468
PP2	-	3	3	186.3	0.458
PP3	-	5	3	168.2	0.497
PP4	-	1	6	695.1	0.554
PP5	-	3	6	576.9	0.572
PP6	-	5	6	906.8	0.527

(Amount of Drug-10 mg, Speed of Homoginizer-9000RPM, Amplification-30%, 2 min, Concentration of lipid-1 gm)

**Figure 10: Effect of Span 80, PGMC and Poloxamer 188on particle size**

In this case, at 3% concentration of Poloxamer 188, the PI was above 0.400. At 6% concentration of Poloxamer 188, the PI was above 0.500. Apart from this gelation of batches was observed at 6% w/w concentration of Poloxamer 188.

Effect of amplification on preparation of NLCs

Table 7: Effect of amplification on Particle size and entrapment efficacy

Batches	Amplification	Time	D 90(nm)	Polydispersivity index	% EE
US 1	30	1 min	388.7	0.485	-

US 2	30	2 min	349.1	0.326	78.73
US 3	40	1 min	333.2	0.316	75.15
US 4	40	2 min	216.2	0.358	59.18

(Concentration of lipid-1 gm, Concentration of Tween 80-3%, Concentration of PGMC-1%, Amount of Drug-10 mg, Speed of Homoginizer-9000RPM, Concentration of lipid-1 gm)

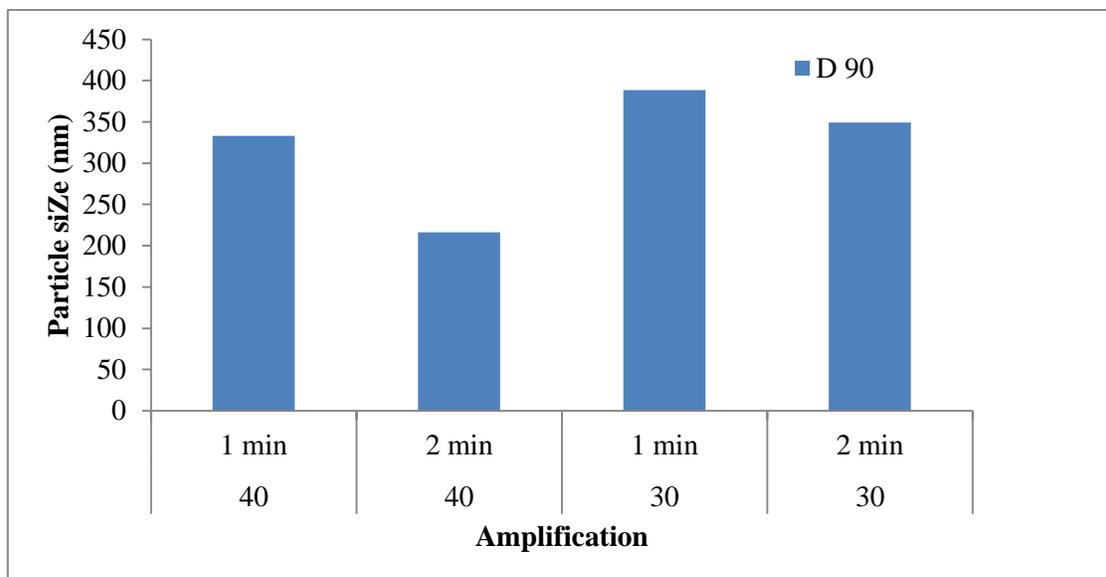


Figure 11: Effect of amplification on particle size and D 90

In batch US 1, 30% amplification for 1 min particle size was 388.7 was observed. But in this case PI value was found to be more than 0.485. This could be due to aggregation of particles because of time of amplification was less. In batch US2, 30% amplification for 2 min particle size was found to be 349.1 nm. As we increase the amplification particle size of the NLCs were decreased. In batch US3, 40% amplification for 1 min particle size 333.2nm was observed. After that, we increase the time to 2 min further reduction in particle size was observed.

Effect of High speed homogenizer on preparation of NLCs:

Table 8 : Effect of HSH on particle size and entrapment efficacy

Batches	RPM	D 90	Polydispersivity index	% EE
DS 1	9000	349.1	0.326	78.73
DS 2	10000	221.7	0.358	57.21
DS 3	11000	138.9	0.369	47.88

(Concentration of lipid-1 gm, Concentration of Tween 80-3%, Concentration of PGMC-1%, Amount of Drug-10 mg, Amplification-30% for 2 minute ,Concentration of lipid-1 gm)

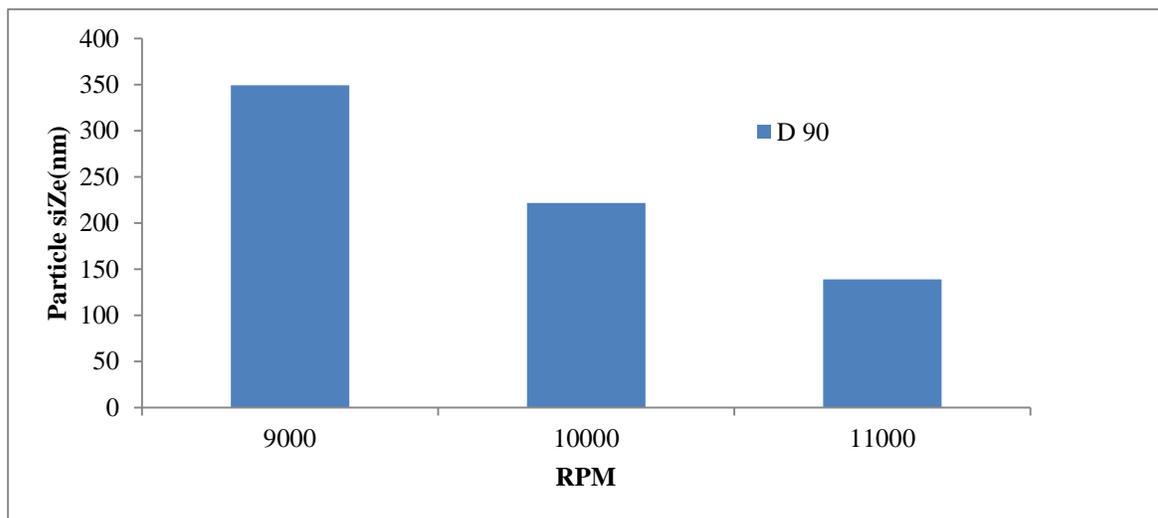


Figure 12: Effect of RPM on particle size and D 90

It was clearly seen that as the RPM increase from 9000 to 11000 there was significant reduction in particle size was observed. We set RPM of HSH to 9000, 10000 and 11000 the particle size observed were 349.1, 221.7 and 138.9 respectively. This effect was observed because of as we increase the RPM from 9000 to 11000 the liquid was pass through high shear between the rotor and stator and particle size was decrease due to generation of cavity. As we increase the speed, the particle size was found to be smaller.

Effect of drug concentration on preparation of NLCs

Concentration of drug varies from 10mg to 20 mg. Amount of PGMC and tween 80 were kept constant. 30% amplification was given for 2 minutes. HSH speed was kept constant 9000 RPM for 2 min.

Table 9: Effect of drug concentration on particle size and entrapment efficacy

Batches	Drug concentration	D 90(nm)	Polydispersivity index	% EE
DC 1	10 mg	349.1	0.326	78.73
DC 2	20 mg	248.4	0.301	83.59

(Concentration of lipid-1 gm, Concentration of Tween 80-3%, Concentration of PGMC-1% , Amplification-30% for 2 minute , speed of homogenizer-9000RPM,Concentration of lipid-1 gm)

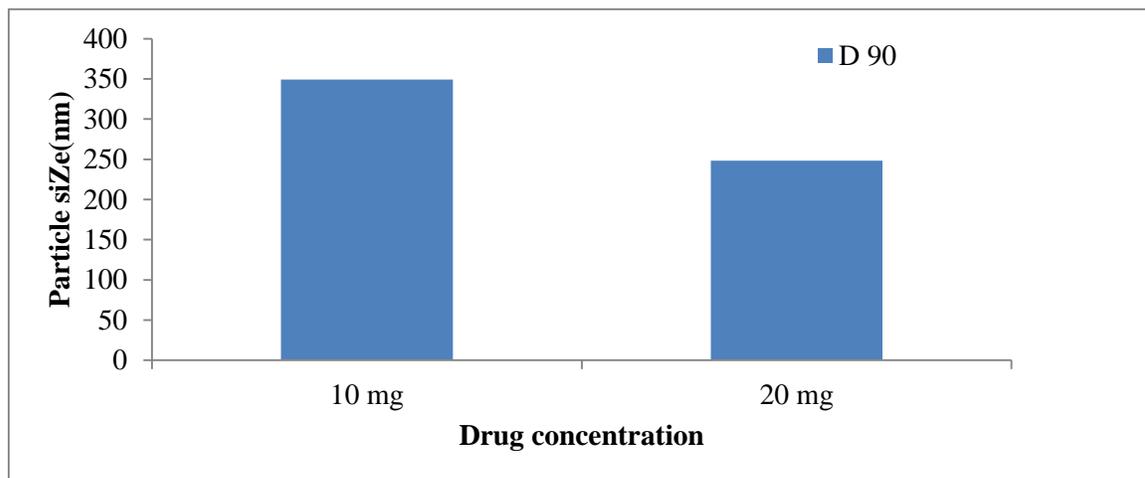


Figure 13: Effect of drug concentration on particle size and Z average

As the drug concentration increase from 10 mg to 20 mg the particle size were decreased to 349.1 to 248.4 nm respectively. This could be due to a decrease in viscosity of the system (W/O emulsion) upon increase in volume of aqueous phase added during preparation of W/O emulsion. In contrast, percentage entrapment efficacy of 20 mg of drug concentration was more than 10 mg concentration of drug. This could be due to a more drug was available for entrapment.

Design of experiments

From preliminary trial it was observed that concentration of drug effects particle size and %EE, PGMC and RPM effects particle size. Hence 2^3 factorial design was applied. A full factorial design is the one of the process in which we can determine the causes between the process and output of the process variable. We can measure the relationships between the dependent variable which can affect the independent variable.

In general full factorial designs, each factor can have a different number of levels, and the factors can be quantitative, qualitative or both. These are factorial designs where the number of levels for each factor is restricted to two. Restricting the levels to two and running a full factorial experiment reduces the number of treatments (compared to a general full factorial experiment) and allows for the investigation of all the factors and all their interactions. If all factors are quantitative, then the data from such experiments can be used for predictive purposes, provided a linear model is appropriate for modeling the response (since only two levels are used, curvature cannot be modeled). So, we sets input variables to gets optimize output results. From the preliminary trial was observed that speed of High speed homogenization ,concentration of PGMC and concentration of drug were effect the particle size and entrapment efficacy of hydrophilic drug.

On the basis of preliminary trials, the independent variable selected were concentration of surfactant, concentration of drug and speed of HSH. The dependent variables were Z-average ,PI,D

90 and % entrapment efficacy. Mathematical model for deriving equations indicating relationship between the independent and dependent variable are as follow:

$$Y=B_0+B_1X_1+B_2X_2+B_3X_3+B_{12}X_1X_2+B_{13}X_1X_3+B_{23}X_2X_3+B_{123}X_1X_2X_3+B_{11}X_1^2+B_{22}X_2^2+B_{33}X_3^2$$

Where, B_0 -Intercept

B_1, B_2, B_3 -Co-efficient of X_1 and X_2 variable

$B_{12}, B_{23}, B_{13}, B_{123}$ -Co-efficient of interactions

X_1, X_2 -Variables

Table 10: Design of experiment data with the coded value, actual value and response

Batches	Independent variables			X1: Drug (mg)	X2: PGMC (%)	X3: RPM	Dependent variables		
	X ₁ ^a	X ₂ ^b	X ₃ ^c				D 90(nm)(Y ₁)	PI	%EE (Y ₂)
F1	-1	-1	-1	15	0.5	8 K	291.6	0.345	80.2
F2	+1	-1	-1	25	0.5	8 K	246.2	0.358	88.31
F3	-1	+1	-1	15	2	8 K	201.9	0.263	71.64
F4	+1	+1	-1	25	2	8 K	188.9	0.314	75.64
F5	-1	-1	+1	15	0.5	10 K	171.7	0.317	72.56
F6	+1	-1	+1	25	0.5	10 K	152.2	0.221	68.78
F7	-1	+1	+1	15	2	10 K	130.4	0.319	64.76
F8	+1	+1	+1	25	2	10 K	113.5	0.349	61.44

In the design batch F2 it was clearly shows that Particle size was higher as compared to other design batches, so it was predicted that the entrapment efficacy was higher. It was clearly shown that design batch F2 shows more entrapment efficacy than others.

Variables	Low(-1)	High(+1)
X ₁ ^a	15	25
X ₂ ^b	0.5	2
X ₃ ^c	8K	10K

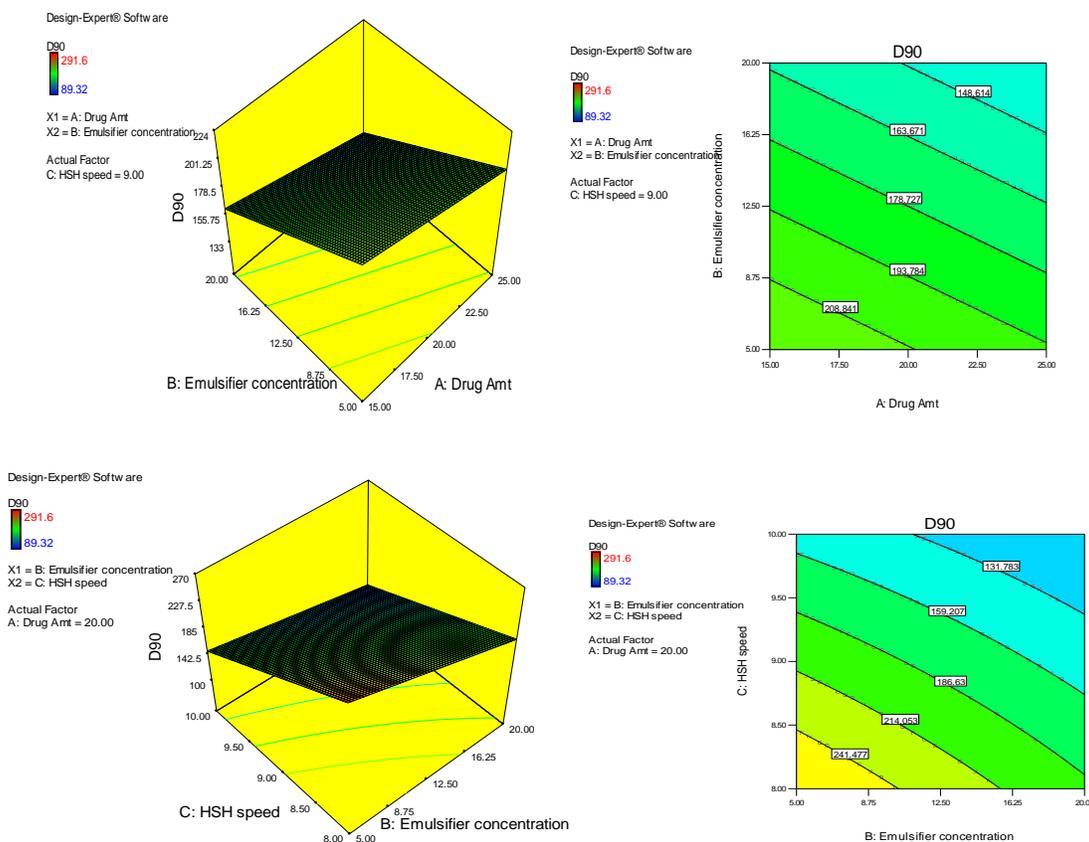
(^a Amount of Drug(mg),^b Concentration of PGMC(%),^c Speed of Homoginization(RPM))

D 90

Equation for D 90:

$$D\ 90 = +178.73 - 14.32X_1 - 30.85X_2 - 51.42 + 7.90X_2X_3$$

Effect of **emulsifier concentration** and **drug amount** and **RPM** on D 90:



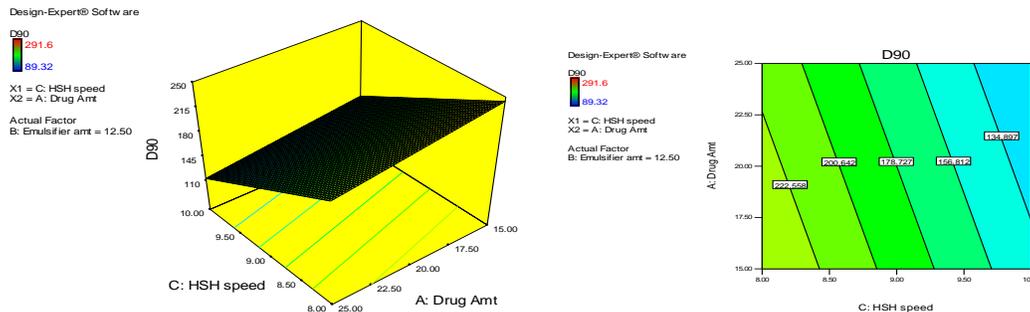


Figure 14: Response surface counter plot graph for D 90

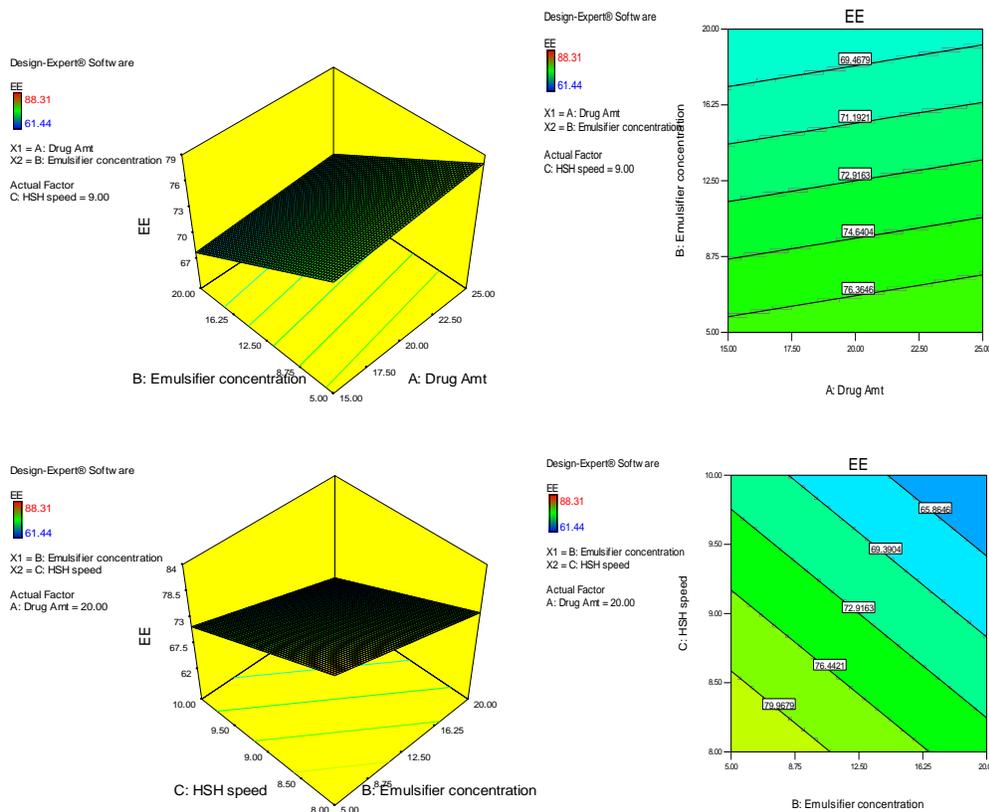
Surface counter plot graph for D 90 describes that as amount of drug was increased the particle size was decreased. On other hand, emulsifier concentration has inverse effect on D90. On the other hand, D 90 value was decrease as HSH speed was increase.

Percentage entrapment efficacy:

Equation for % entrapment efficacy:

$$\%EE = +72.94 + 0.63X1 - 4.55X2 - 6.03X3 - 2.40X1X3$$

Effects of **emulsifier amount** and **drug amount** and **HSH** on **% EE:**



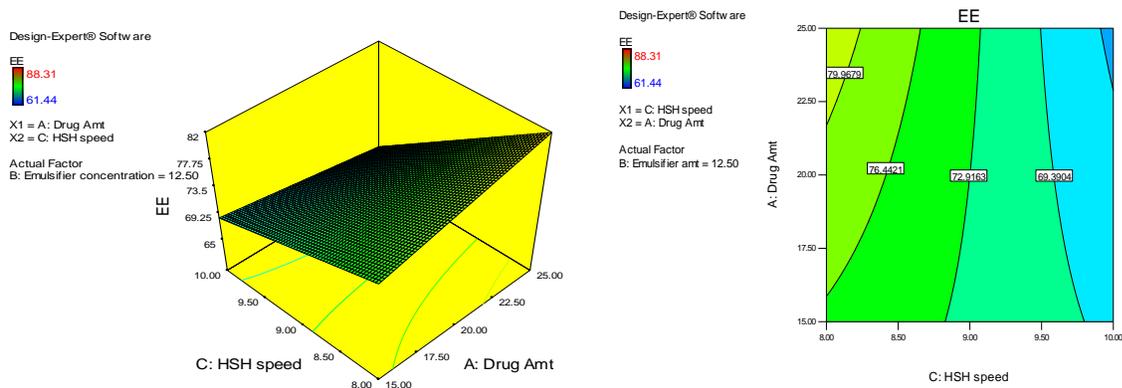


Figure 15: Response surface counter plot graph for % EE.

% EE increase with drug amount increase from 15mg to 25mg. On other hand, as emulsifier amount increase from 5mg to 20mg percentage entrapment efficacy decrease. As HSH speed increase, % EE decrease.

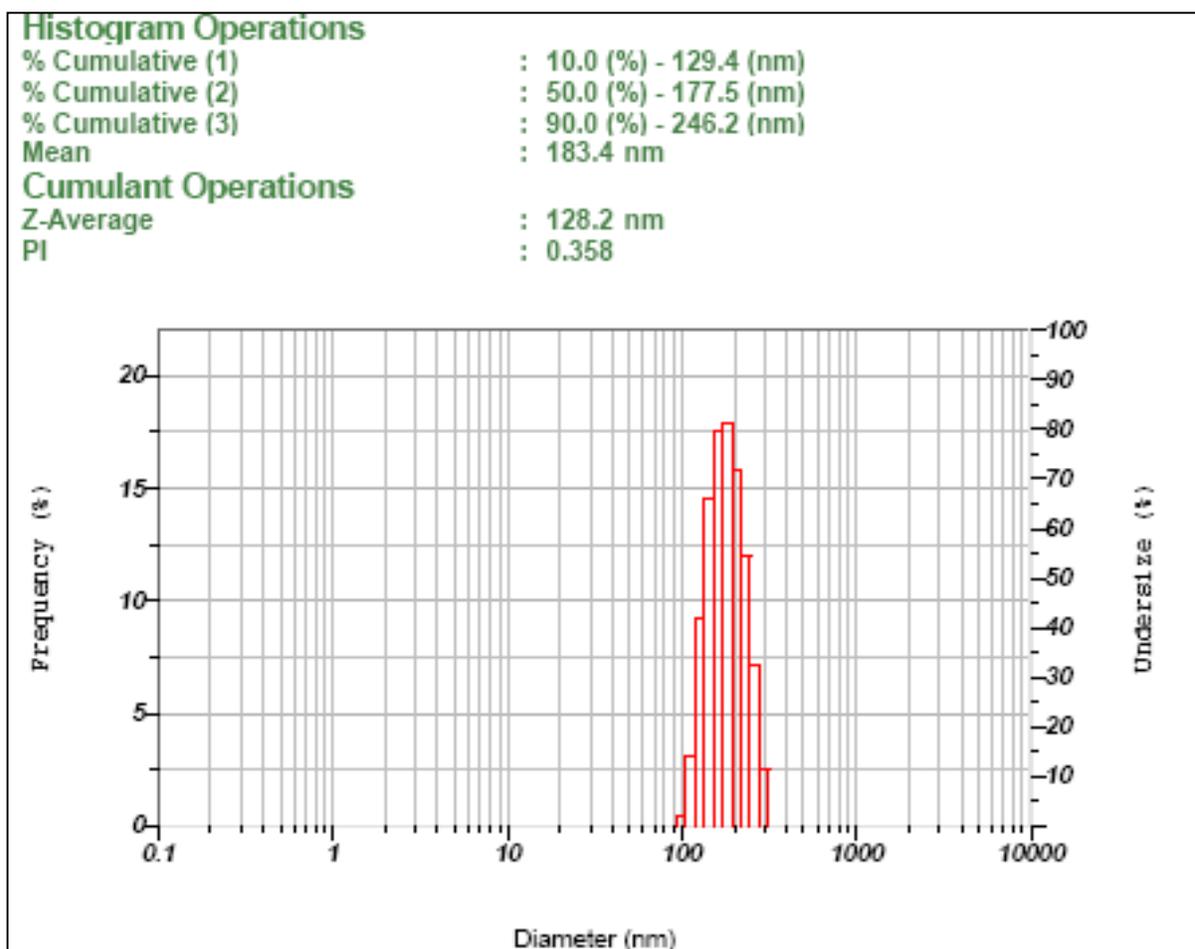


Figure 16: Particle size data for F2 batch

In vitro release of 5-Flourouracil from gels enriched with NLCs:

The amount of drug permeation studies were performed in multi cell diffusion apparatus. The dialysis bag was placed in upper donor compartment of Franz diffusion cell, Separating this compartment from the receptor compartment. The accurate quantity of 5-florouracil containing nanostructured lipid carrier was placed in donor compartment and receptor compartment filled with 7.4 PH phosphate buffer saline solutions. Stirring in receptor compartment kept constant at 100 rpm and samples were collected at pre-determined interval of time for up to 24 hrs. Samples were measured at 267 nm in UV visible spectrophotometer.

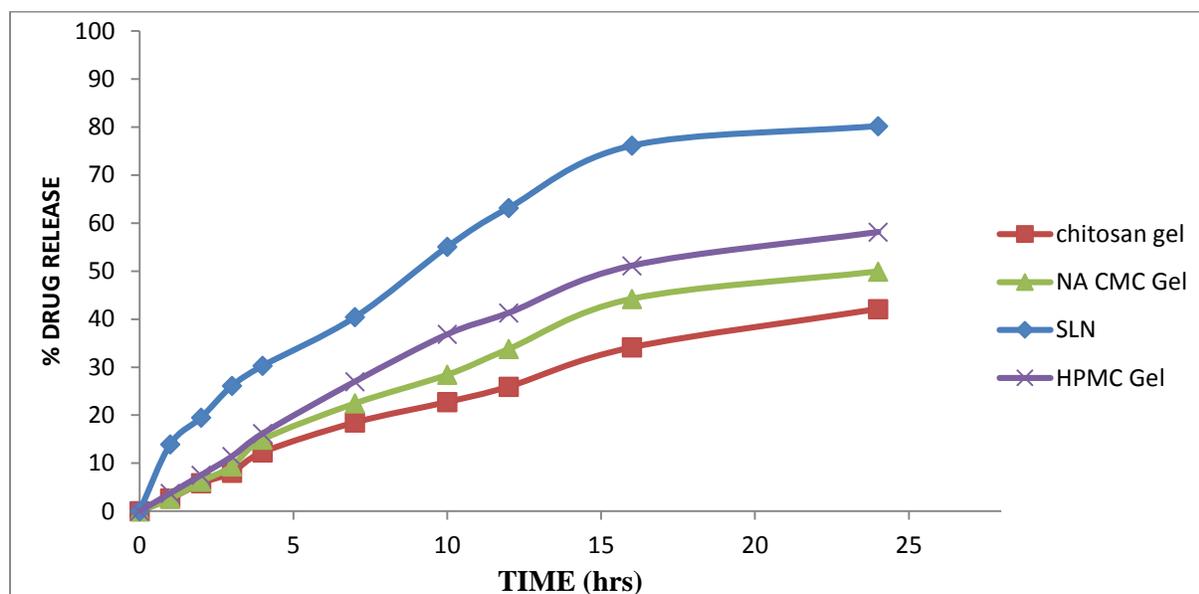


Figure 17: In vitro release of 5-florouracil from gels enriched with nanostructured lipid carrier dispersions.

It was observed that HPMC containing gel formulation shows highest diffusion through dialysis membrane. In contrast, chitosan containing gel formulation shown as less amount of drug release through dialysis membrane. It was observed that nanostructured lipid carrier containing formulation shows highest amount of release because of its hydrophobic nature.

Ex-vivo skin permeability studies:

Franz diffusion cell was used for the evaluation of Ex-vivo skin permeability testing. To perform this abdominal skin of rat was used. To perform this skin of rat with full thickness was excised from abdominal region. After removal of skin from the abdominal region hair was removed. Surgical removal of subcutaneous tissue was done. After removal of subcutaneous tissue the dermis side of skin wiped with isopropyl alcohol. Isopropyl alcohol was used to remove the fats. Then skin was washed with double distilled water. The skin then mounted between the donor and receptor compartment. The speed of rotor was 600 rpm.

Permeation data analysis:

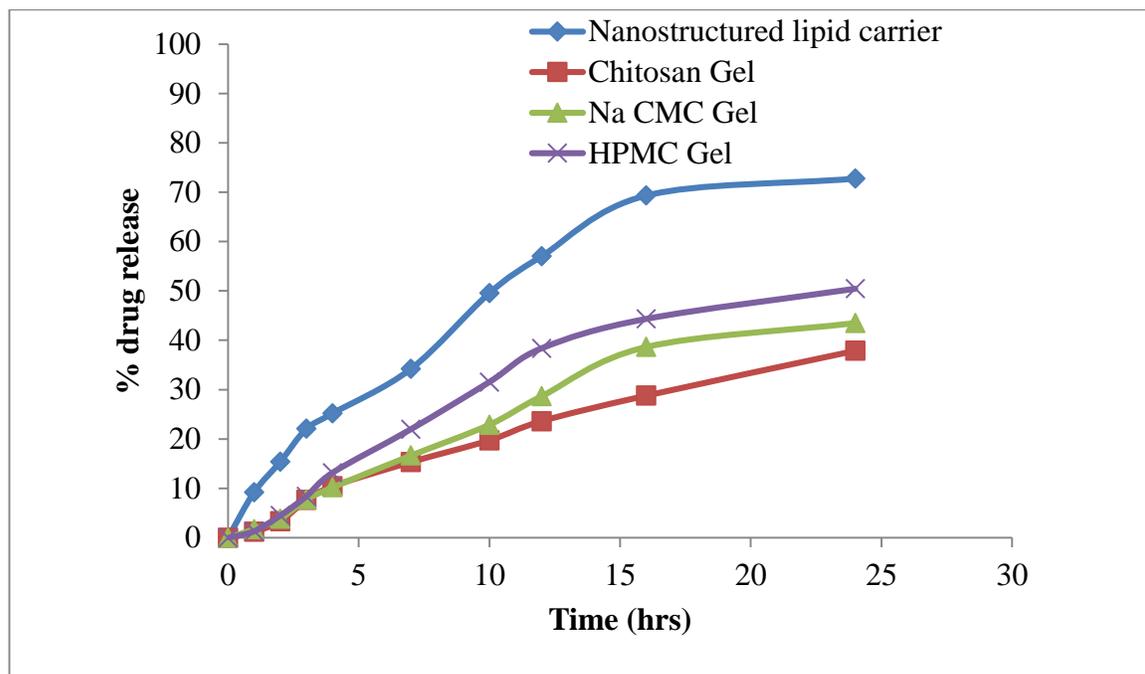


Figure 18: Ex-vivo skin permeability of 5-fluorouracil from gels enriched with nanostructured lipid carrier dispersions

Table 11: Permeability parameter of different formulation in in-vitro skin testing

Formulation	Q 24($\mu\text{g}/\text{cm}^2$)	Permeability coefficient($\text{Kp}(\text{cm}/\text{hr})$)
Nano structured lipid carrier	601.57	0.802093333
Hydroxy propyl methyl cellulose Gel	435.975	0.5813
Sodium carboxy methyl cellulose Gel	374.4	0.4992
Chitosan Gel	315.82	0.421093333

In in-vitro skin permeability studies it was observed that nanostructured lipid carrier passes through skin layer easily. After that, HPMC gel formulation passes through skin layer easily. In contrast, chitosan gel was less permeable through skin membrane.

Differential scanning calorimetry (DSC):

It is a technique that shows that the thermal behavior of a drug and other excipients occurring due to the formulation of NLCs. Thus to check and confirm the interactions between the drug, lipid and other excipients, DSC characterizes the stability of formulation significantly.

As observed in figure, the transition peak of pure 5-fluorouracil was observed at 287.51°C . The DSC analysis of lipid component (Compritol ATO 888 and Labrafac WL 1349) exhibited peak at 72.97°C . When a blank batch was analyzed transition peak at 71.77°C was observed. When drug loaded nanostructured lipid carrier was shown transition peak at 71.85°C and exothermic peak was shown at 324.18°C .

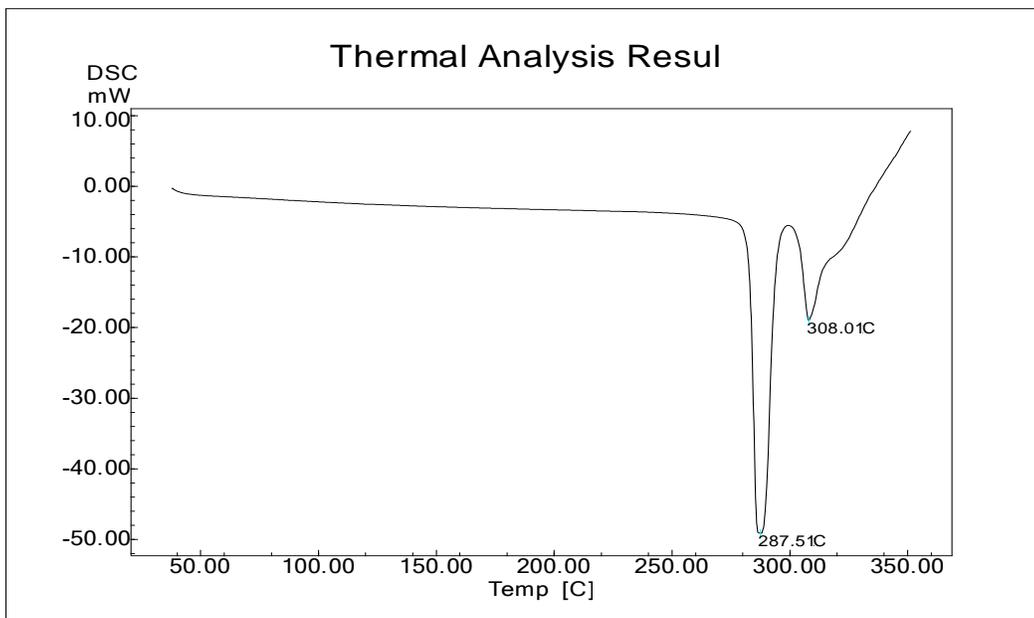


Figure 19: DSC analysis of pure drug shows peak at 287.51°C

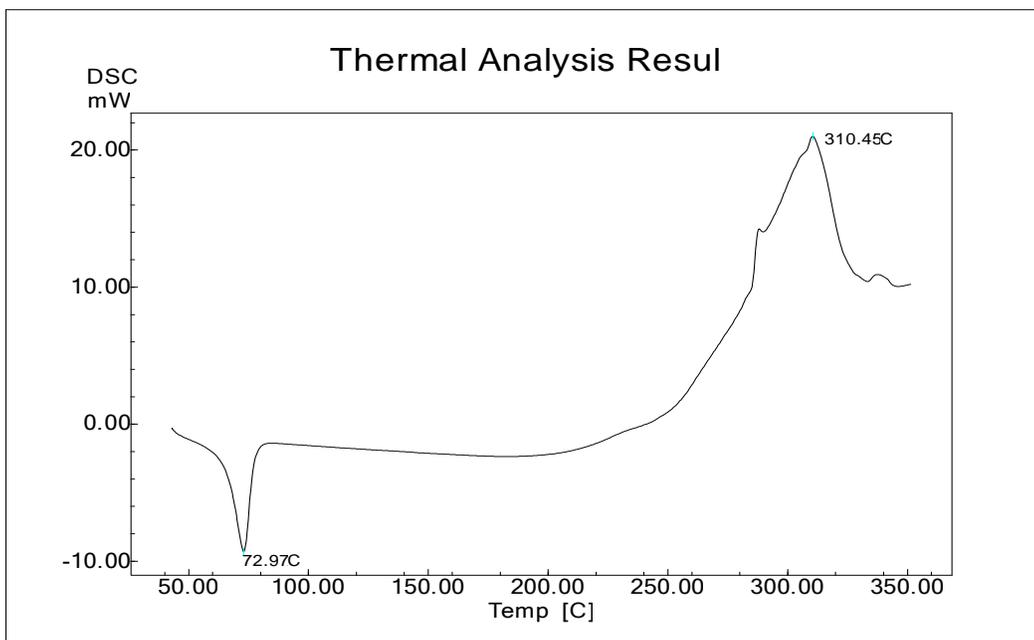


Figure 20: DSC analysis of lipid mixture

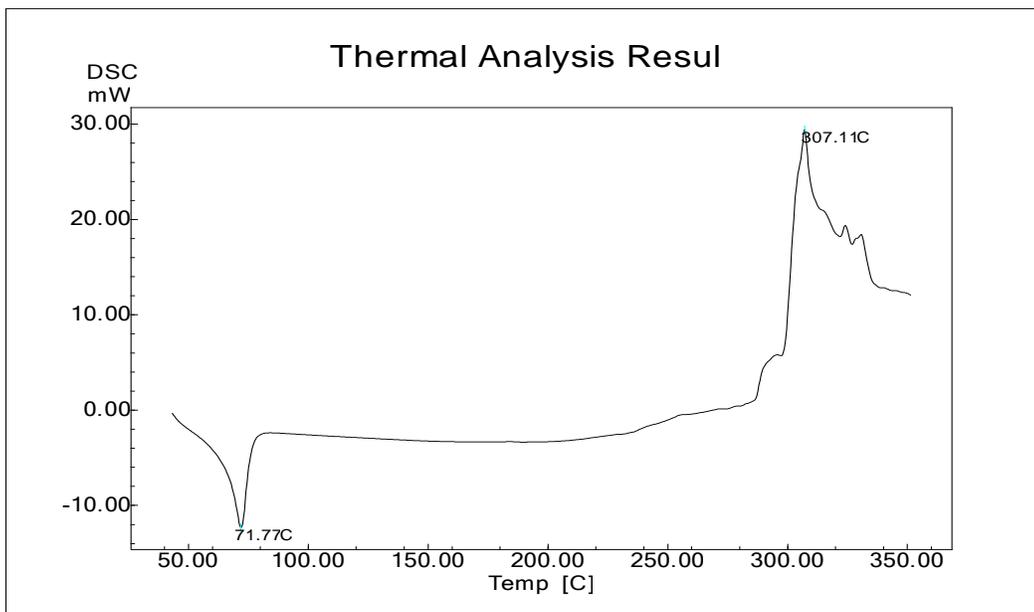


Figure 21: DSC analysis of blank mixture of nanostructured lipid carrier

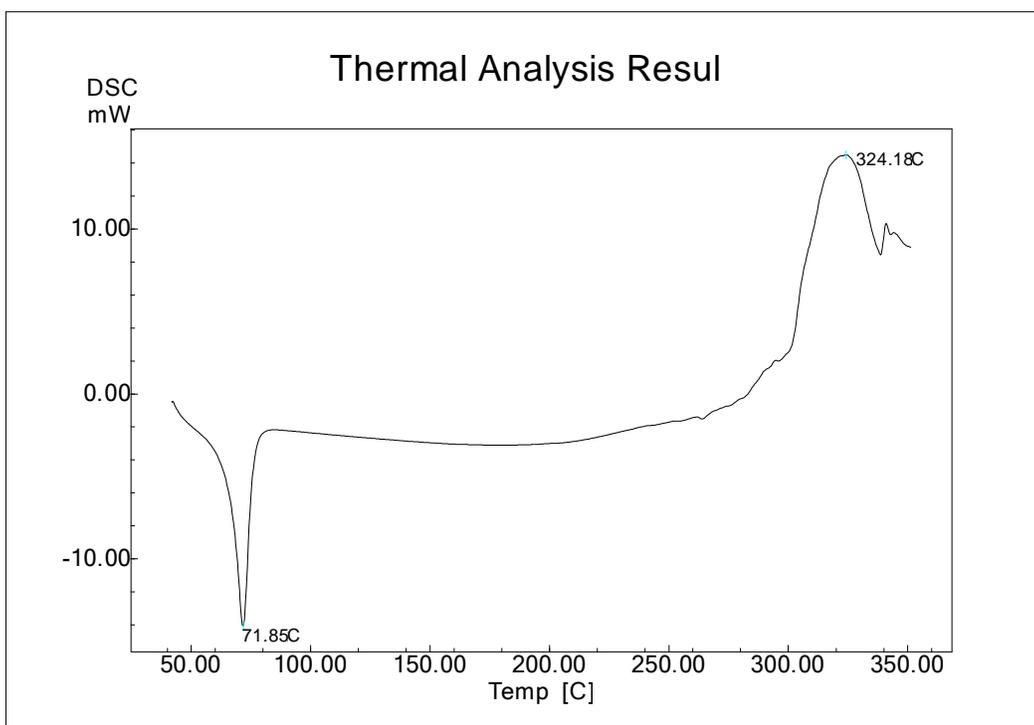


Figure 22: DSC analysis of drug loaded nanostructured lipid carrier

DSC analysis of pure drug shows endothermic peak at 287.51⁰C. Drug loaded NLC shows no peak at 287.51⁰C.

Scanning Electron Microscopy(SEM)

Shape and surface morphology of prepared NLCs were evaluated for scanning electron microscopy. The study revealed that most of the NLCs was spherical in shape and the surface of the particle showed a characteristic smoothness.

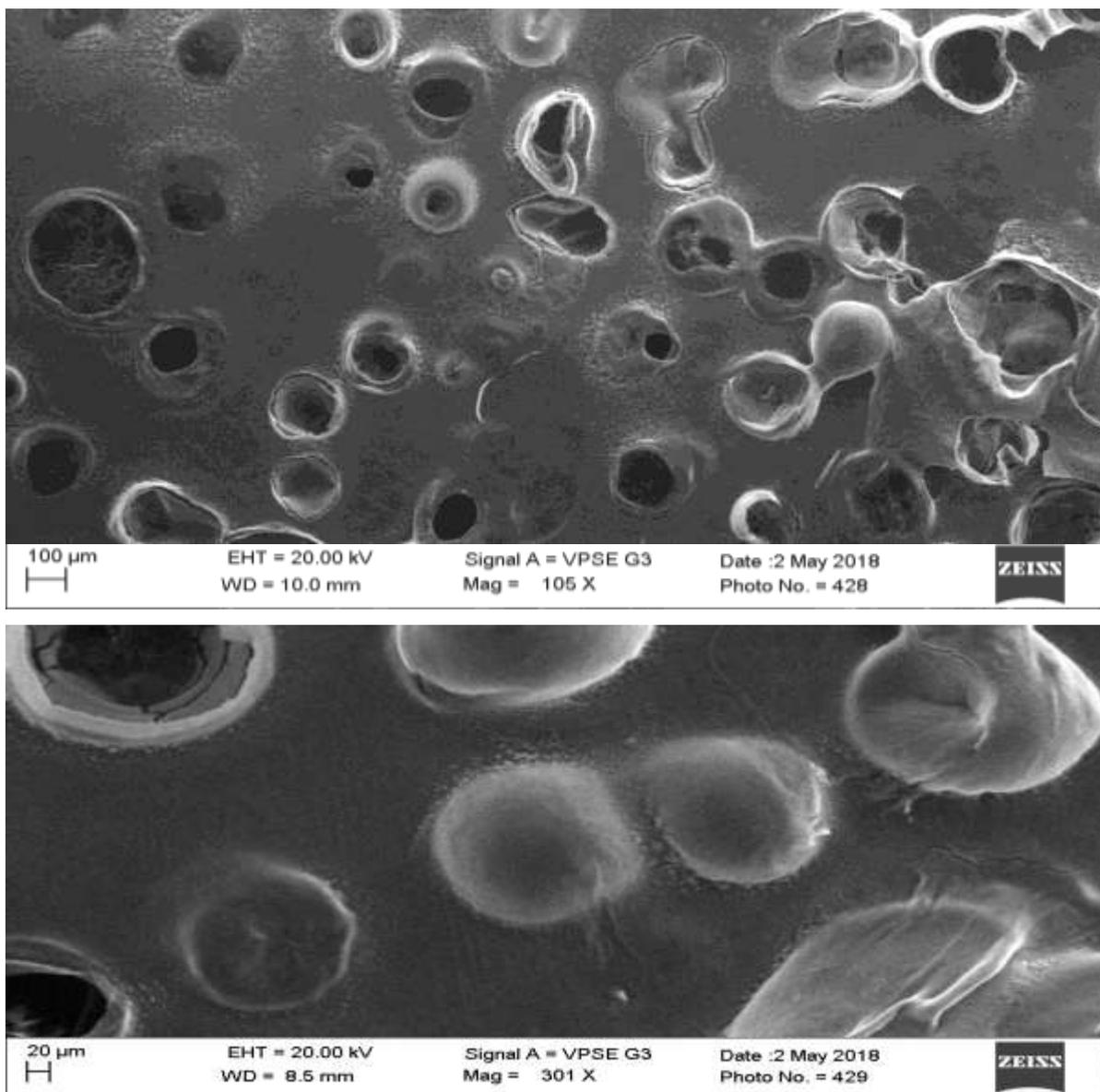


Figure 23: Scanning electron microscopy of NLCs.

X-ray diffraction(XRD)

XRD can identify specific crystalline compounds based on their crystal structure. In XRD, the monochromatic beam of X-ray is diffracted at angles determined by the spacing of the planes in the crystals and the type and arrangement of the atoms, which is recorded by a detector as a pattern. The intensity and position of the diffractions are unique to each type of crystalline material. XRD pattern can predict the manner of arrangement of lipid molecules, phase behaviour, and characterize and identify the structure of lipid and drug molecules. However, best results are

observed when NLCs dispersions are investigated directly as solvent removal may change the modification.

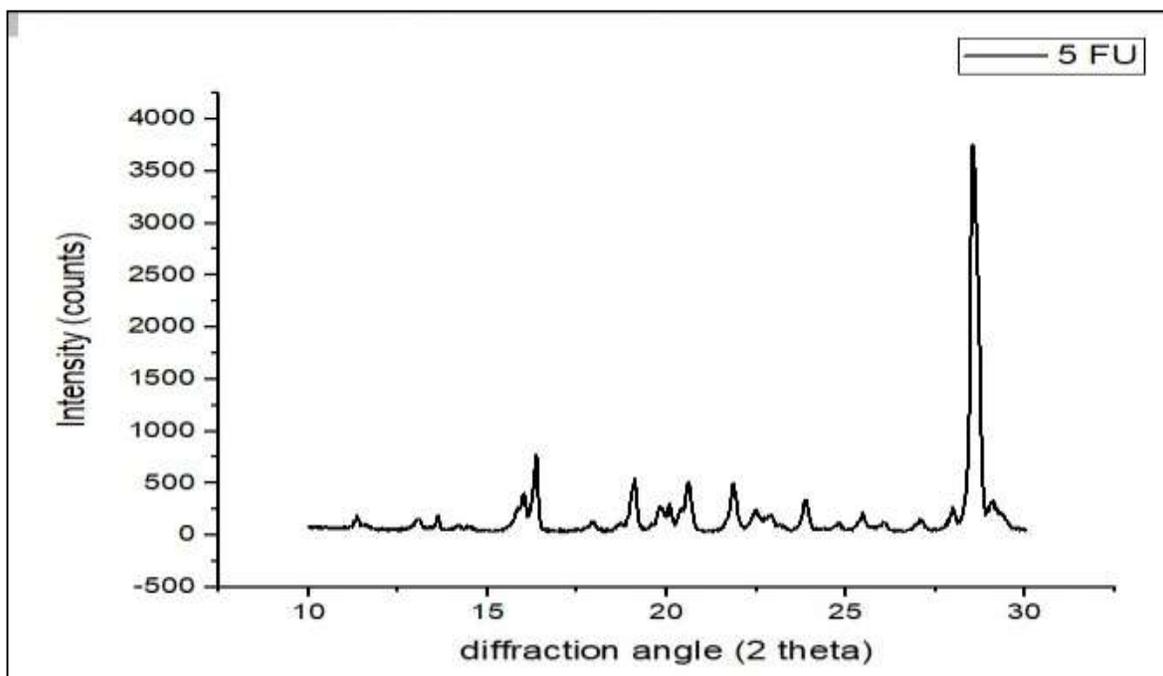


Figure 24: X-ray diffraction studies for 5-Fluorouracil

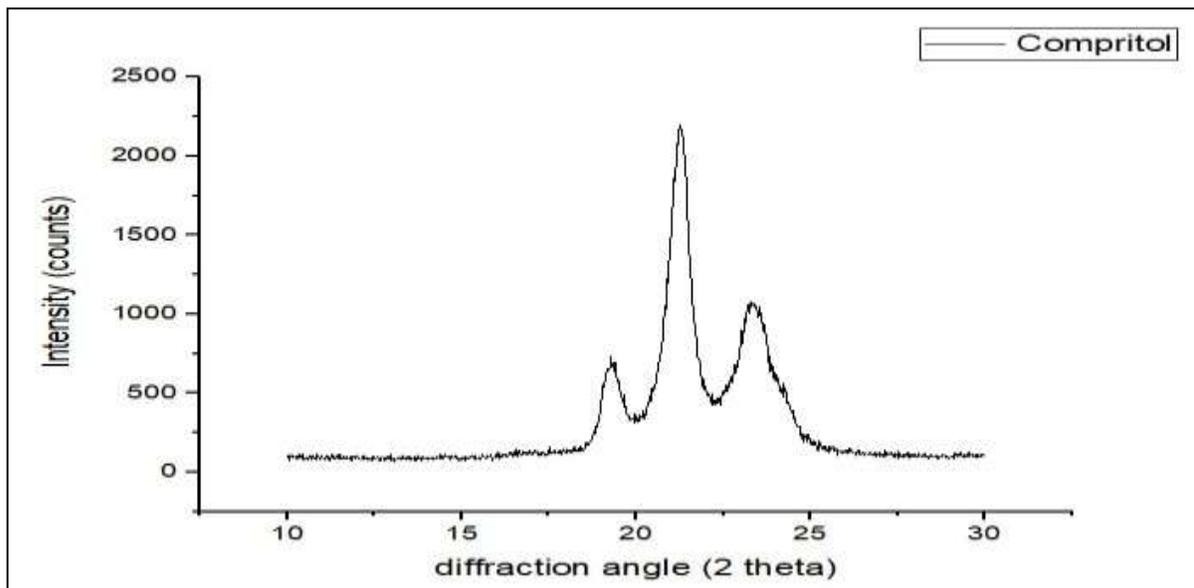


Figure 25: X-ray diffraction studies for Compritol ATP 888

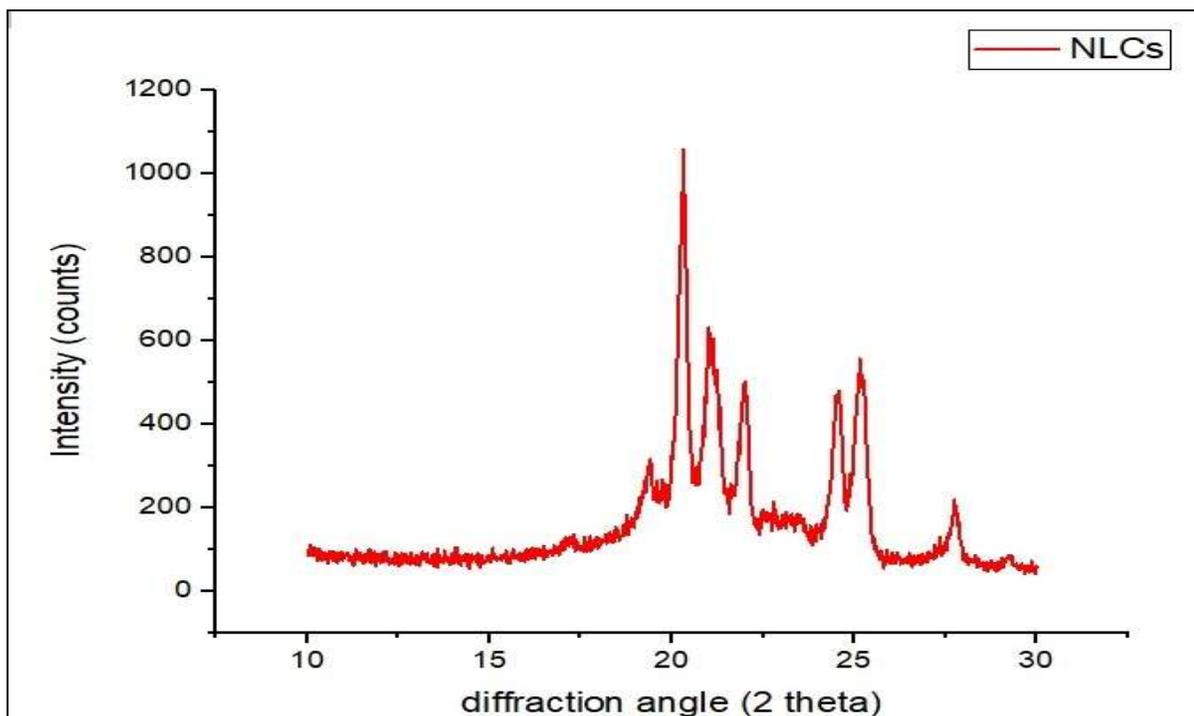


Figure 26: X-ray diffraction studies for NLCs

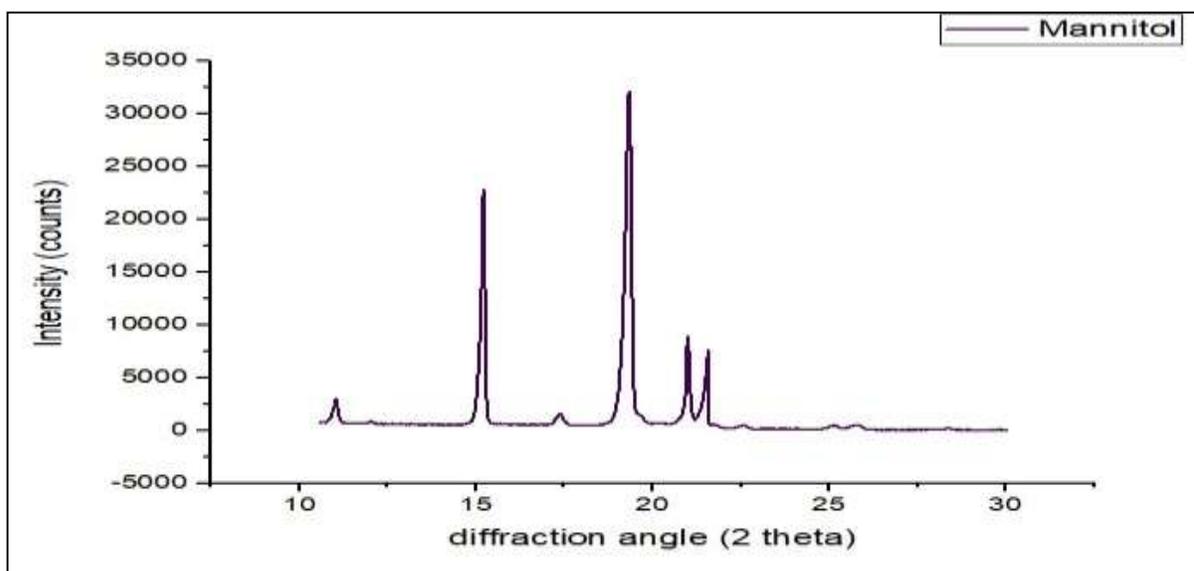


Figure 27: X-ray diffraction studies for mannitol

XRD experiment revealed that 5-Fluorouracil was uniformly and molecularly distributed in the NLCs. Which is an interesting result for distribution of a hydrophilic compound in lipophilic matrix. Hence, we interpret from the result that drug was fully entrapped in NLCs.

SUMMARY:

Skin cancer has been discovered as the second most common cancer causing death to the Individuals worldwide. Cancer basically comprises of uncontrolled growth of non functionalizing

cells which tend to occupy into the tissues and keeps growing. Further they feed on external nutrient sources present in the body fluid as well as take up the newly formed blood vessels for their growth creating several complications. Thus the unorganized cellular growth occurring in the skin of the body leading to several complications has been termed as skin cancer.

The conventional solid lipid nanoparticulate based gel formulations were available in market but the percentage drug loading or entrapment efficacy of this formulation was less .Apart from this, stability was the one of the biggest problem of this kind of formulation especially for hydrophilic drugs. So, that the toxicity was one of the major concerned for this formulation.

Nanoparticulate drug delivery systems has several advantages over the conventional dosage forms in terms of higher efficacy, novel routes of uptake and which can provide better results than the available dosage forms. In order of which Nano-structured lipid carries (NLCs) were prepared which can be orally or topically administered and are able to target the cancer affected cells by making them recognizable to receptor based cell mediated endocytosis and thereby causing minimal toxicity to the normal cells and henceforth show efficacious result in terms of treatment and lower toxicity. The NLCs were prepared using digital high speed homogenization technique. The purpose of using high speed homogenizer was due to its distinct quality of size reduction which uniformly reduces the size of the particles. A variety of lipids were chosen for the drug incorporation of which compritol ATO 888 was found to be capable of achieving the desired quality of product. The lipid was selected based on a number of studies that were conducted prior to the lipid selection. In order to achieve a desired quality of the product, preliminary trials were conducted to evaluate the effect of surfactant on the product, effect of speed of homogenization, amplification of ultrasonicator, concentration of co-surfactant, effect of drug concentration on the % entrapment efficiency and the drug loading capacity of the product. Based on the preliminary trials, the concentration of drug, concentration of co-surfactant and effect of RPM of HSH were evaluated of having an impact on the final quality of the product. In regards of which, these factors were evaluated using full-factorial Design of optimization and the parameters such as particle size, D90, PDI, % entrapment efficiency and % drug loading of the formulation were studied.

In –vitro diffusion and ex-vivo skin permeability studies were done for the evaluation of diffusion trough dialysis membrane and skin membrane were evaluated.

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