MS.ID-IJPBS-0351
International Journal of Pharmacy and Biological Sciences-IJPBS™ (2022) 12 (4): 23-40
Online ISSN: 2230-7605, Print ISSN: 2321-3272
Research Article | Pharmaceutical Sciences | OA Journal | MCI Approved | Index Copernicus

Optimization and Physicochemical Characterization of a Gatifloxacin Loaded NLC For Ocular Antibacterial Application

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Received: 02 Jul 2022/ Accepted: 9 Aug 2022 / Published online: 1 Oct 2022
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Abstract
The study was designed to fabricate the Gatifloxacin nanostructured lipid carriers (GFX-NLCs) for opthalmic application to treat bacterial conjunctivitis. Initially, two level full factorial design was used to optimize the various factors significantly affecting the final formulation attributes. GFX-NLCs with particle size 85.66±4.65 nm polydispersity index 0.367± 0.008., zeta potential -28.5 mV, entrapment efficiency 91.45% ± 0.028 %, and spherical shape was achieved. The optimized GFX-NLCs demonstrated the Korsemeyer peppas model release kinetics with highest regression coefficient. Besides this, FTIR, differential scanning calorimetry results suggested that GFX had excellent compatibility with excipients. Furthermore, the results of ex-vivo permeation study demonstrated 0.82-fold enhancement ratio compared with marketed formulation. In addition, GFX-NLCs did not show any sign of ocular irritancy to the corneal tissue as confirmed by HET-CAM test. Therefore, the findings suggest that GFX-NLCs can be better alternative strategy to prevent and treat the bacterial conjunctivitis.

Keywords
Nanostructured lipid carriers, Gatifloxacin, Bacterial conjunctivitis, single emulsification and solvent evaporation, HET-CAM.

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INTRODUCTION:
Conjunctivitis is a generic term for inflammation of the conjunctiva of the eye caused due to various agents including bacteria, viruses, fungi, allergens or chemicals. Acute bacterial conjunctivitis is one type of conjunctivitis in which mucus membrane that covers the white part of the eye and inner eyelids are inflamed due to bacterial infection. In the developed world, acute red eyes account for 1–4% of all general practitioner consultations, and are most frequently diagnosed as acute bacterial conjunctivitis.[1] Dilation of conjunctival blood vessels secondary to viral or bacterial infection, chemical exposures, or allergies results in the redness seen on the examination. While viral and allergic conjunctivitis occurs more frequently, bacterial conjunctivitis is responsible for increased morbidity and provides a more challenging clinical scenario for physicians. Generally, its treatment regimen includes application of ocular antibiotics that will ultimately kill the causative agents that is pathogen. There are many other conditions like disruption of the natural epithelial barrier, insufficient tear production, trauma, and immunosuppressed status is also responsible bacterial conjunctivitis. [2]

Fluoroquinolone antibiotics are one of the most commonly used topical ophthalmic antibiotics used in the treatment of acute bacterial conjunctivitis. They, have a broad antimicrobial spectrum, are bactericidal in action, well tolerated, and have been proved less prone to bacterial resistance.[3]

Gatifloxacin(GFX), a broad-spectrum antimicrobial fluoroquinolone, is active against Gram-positive and Gram-negative bacteria and frequently used in infections like conjunctivitis, keratitis, keratoconjunctivitis and endophthalmitis. Bacterial conjunctivitis is usually caused by *Staphylococcus aureus, Streptococcus pneumoniae, Haemophilus species, Moraxella catarrhalis, Pseudomonas aeruginosa.* GFX has a molecular weight of 402.42 Da with a log P of 2.65.[4] Although fourth-generation fluoroquinolones show superior ocular penetration (compared with older generations), GFX has the tendency to recrystallize at neutral pH due to its poor water-solubility as shown by its log p values. It exerts it action by inhibiting topoisomerase II (DNA gyrase) and topoisomerase IV enzymes, which are essential in bacterial DNA replication, transcription, and recombination. GFX provide improved efficacy in ocular infection than conventional fluroquinolones. GFX is newer fourth generation fluoroquinolone which has more in vitro activity than ciprofloxacin and ofloxacin, second generation fluroquinolone in gram positive bacteria. [5]

Nanoparticles have improved drug dissolution due to their size and have been the area of research for decade now. Lipid based nanoparticles serves as an efficient formulation system for poorly water-soluble drugs. They demonstrate improved performance characteristics like good particle size, better drug loading, physical and chemical stability. Some of the promising and well reported lipid based nanosystems are liposomes, niosomes, Solid lipid nanoparticles (SLNs), Nanostructured lipid carriers (NLCs), nano-emulsions and polymer lipid hybrid nanoparticles (PLNs). Lipid nanoparticles consist of a solid lipophilic matrix in which active molecules can be incorporated. The particle size is mainly between 150-300 nm, smaller sizes, e.g. <100nm or larger size of up to 1000 nm can be obtained as a well for special need [6]. The lipid nanoparticles can be derived from oil-in-water nanoemulsions, where the liquid lipid of the oil droplets is replaced by a solid lipid, i.e., solid at body temperature. Due to this, lipid nanoparticles remain solid after administration to the body. This means, identical to polymeric nanoparticles, they provide a matrix for sustained release. At the same time the matrix can protect chemically labile actives against degradation.

Two generations of lipid nanoparticles are distinguished, the first generation are the solid lipid nanoparticles (SLN), the second generation are the nanostructured lipid carriers (NLC). SLN are made from solid lipid only, the NLC from a blend of solid and liquid lipids, still the blend being solid at body temperature. The addition of the oil compound distorts the formation of perfect lipid crystals, thus creating more imperfections with uptake capacity for drug or active. This so called “nanostructuring” increases the drug loading capacity.[7] When a lipid forms a highly crystalline particle, this leads to drug expulsion. SLN forms a matrix which has no imperfections, thus the space to host drug molecules, like the mortar between the bricks, is very limited. The amount of imperfections is much higher in NLC, because the addition of oil prevents the perfect recrystallisation of the solid lipid. Hence over time no changes in modification occur in NLC and thus no drug expulsion is obtained. Therefore, in general NLC possesses a higher physical stability than SLN. The delivery of drugs against infectious diseases in lipid nanoparticle aims to enhance precorneal retention by increasing concentration in the biophase,compared to conventional eye drops. Moreover, lipid nanoparticles should provide sustained drug release, so that the released drug can exert a suitable biocidal activity. These precise nanostructures are important for enhancing formulation durability along with increasing drug loading and bioavailability.[8]

The aim of the present study is to develop and characterize Gatifloxacin loaded NLCs

MATERIALS AND METHODS:

Materials:

GFX was received as a gift sample from Aarti drug limited, Mumbai, India. Glyceryl Monostearate was purchased from CDH laboratory, Bombay. Oleic acid was purchased from S. D. Fine Chem Ltd. Tween 80, Sodium chloride, Sodium bicarbonate, Sodium hydroxide, Calcium chloride dihydrate was purchased from Loba Chemicals Pvt. Ltd. Methanol AR was purchased from S.D. Fine chem Ltd. Chloroform AR was purchased from Loba chemical Pvt Ltd. Poloxamer 188 was obtained as gift sample from BASF, Mumbai, India.

Preparation of GFX loaded NLCs.

Single emulsification-solvent evaporation method was selected as method for preparation of GFX nanostructured Lipid Carrier (NLC). Excipients were selected by screening them based on solubility diligently. In this method, organic phase consists of solid lipid, liquid lipid, co-surfactant, drug, and organic solvent. The aqueous phase consists of water

and surfactant. Organic phase was heated in water bath at 50°C -60°C and added dropwise to the lipid phase with constant stirring for 40-50 mins. This coarse emulsion then probe-sonicated for size reduction to form NLC. This was further filtered with 0.22µm membrane filter to ensure sterility.[9–11]

Optimization by Full factorial Design:

The formula for NLC preparation was optimized by three factor-two level factorial design using Design Expert software® (Version 13.0.5.0 Stat Ease, Minneapolis, MN). Liquid lipid: Total lipid(A), Surfactant concentration(B) and total lipid concentration(C) were selected as three factors. All variables, including surfactant concentration, total lipid, and liquid lipid to total lipid ratio, were investigated at two levels: low (-1) and high (+1) with Centre point (0). The factorial design 24 runs with 8 Centre points and 4 blocks. Formulation was optimized for Particle size (Y1), PDI (Y2), entrapment efficiency (Y3) and zeta potential (Y4). The constraints applied were minimum particle size and PDI with maximum entrapment efficiency as shown in **Table I**. The factorial design designed 24 runs with 8 Centre points and 4 blocks.

Table I: Trial batches for optimization

Run number	Block number	Independent variable		
		Liquid lipid: total lipid (A)	Surfactant concentration (B)	Total lipid concentration (C)
1	Block 1	0.3	0.75	1.5
2		0.3	0.75	1.5
3		0.4	0.5	2
4		0.4	1	1
5		0.2	1	2
6		0.2	0.5	1
7	Block 2	0.3	0.75	1.5
8		0.4	1	2
9		0.4	0.5	1
10		0.2	1	1
11		0.2	0.5	2
12		0.3	0.75	1.5
13	Block 3	0.4	0.5	2
14		0.2	0.5	1
15		0.4	1	1
16		0.2	1	2
17		0.3	0.75	1.5
18		0.3	0.75	1.5
19	Block 4	0.2	0.5	2
20		0.4	1	2
21		0.3	0.75	1.5
22		0.2	1	1
23		0.3	0.75	1.5
24		0.4	0.5	1

Table II Protocol of In vitro release study with parameters

PARAMETERS:	
Apparatus	Franz Diffusion Cell
Permeation membrane	Freshly excised goat cornea
Stirring rate	100 rpm
Samples	GFX loaded NLC equivalent to 3mg of GFX and Marketed preparation (Gatiquin eye drops 0.3%w/v)
Release media	Simulated Tear Fluid (STF, pH-7.4)
Volume of release media	100ml
Temperature	32-34℃
Volume of aliquot	1 ml
Time points(hour)	0.25, 0.30, 0.75, 1, 2, 3, 4, 6, 8
Method of analysis	UV spectroscopic method (V -1900, Shimadzu, Japan)
Wavelength	285.5 nm

Table III- Experimental design with actual values of factors with responses obtained for critical quality attributes from experimental batches.

Run	lipid concentration: total lipid (A)	surfactant concentration (B)	amount of lipid (C)	particle size (Y1)	PDI (Y2)	Entrapment efficiency (Y3)	Zeta potential (Y4)
		%	%			%	mV
1	1	-1	1	115.3	0.431	95.5033	-24.6
2	0	0	0	102.6	0.286	91.4875	-28
3	0	0	0	99.7	0.279	91.5859	-27.4
4	-1	1	1	94	0.315	82.6618	-26.5
5	-1	-1	-1	102.6	0.531	61.6618	-32.6
6	1	1	-1	48.5	0.286	91.2059	-21
7	0	0	0	97.8	0.273	92.3553	-26.6
8	-1	1	-1	93.6	0.351	70.0568	-26
9	1	-1	-1	81.9	0.482	83.0242	-25.7
10	1	1	1	110.3	0.332	90.8157	-20.4
11	-1	-1	1	140.5	0.525	65.9997	-29
12	0	0	0	100.5	0.28	93.4491	-27.6
13	0	0	0	101.6	0.271	92.8411	-28.8
14	-1	1	1	90.7	0.301	83.6853	-26.3
15	1	-1	1	121.5	0.413	96.1185	-24.4
16	0	0	0	105.2	0.218	91.1236	-28.6
17	-1	-1	-1	101.2	0.512	62.0568	-32
18	1	1	-1	44.2	0.303	91.6123	-21.5
19	0	0	0	100.6	0.234	92.8962	-28.5
20	0	0	0	98.6	0.297	93.0242	-28.5
21	1	-1	-1	89.7	0.489	84.4491	-25.3
22	-1	-1	1	138.4	0.524	62.5272	-29.4
23	-1	1	-1	101.5	0.301	72.1185	-26
24	1	1	1	111.1	0.298	90.7888	-20.8

Table IV-Equations of responses

Responses	Equation
Particle size	99.65 + -8.75 * A + -12.325 B + 16.1625 * C + 0.5375 * AB + 8.075 * AC + -1.375 * BC + 10.7625 * ABC
PDI	0.399625 + -0.020375 * A + -0.08875 * B + 0.01425 * AB
Entrapment efficiency	80.2679 + 10.1719 * A + 3.85028 * B + 3.24468 * C + -3.18433 * AB + -0.377831 * AC + -0.374919 * BC + -3.38121 * ABC
Zeta potential	-25.7188 + 2.75625 * A + 2.15625 * B

Where, A=lipid concentration: total lipid; B=surfactant concentration; C= amount of lipid; AB=lipid concentration: total lipid * surfactant concentration; AC= lipid concentration:total lipid * amount of lipid
BC=surfactant concentration * amount of lipid; ABC=lipid concentration:total lipid * surfactant concentration * amount of lipid

Table V-Steady state flux and permeation coefficient of GFX-NLCs and Marketed formulation.

	GFX loaded NLCs	Marketed formulation
Steady state flux (µg cm^{-2} hr^{-1})	0.196855	0.243033
Permeation coefficient (cm hr^{-1})	0.065618	0.081011
Enhancement Ratio	0.82%	-

Physicochemical Characterization of GFX-NLCs:

Particle size and Polydispersity index:

The average particle size and polydispersity index (PDI) were performed with dynamic light scattering method using Malvern Zetasize (Nano ZS, Malvern Instruments, U.K.). The particle size and PDI Values were obtained by averaging of 10 measurements at an angle of 173° at 25 °C using disposable cells. Prior to the measurement, all samples were diluted with water to have a suitable scattering intensity.[12]

Zeta potential analysis:

Zeta potential was measured using disposable plain folded capillary zeta cells (Malvern Zetasizer Nano ZS). The zeta potential was calculated from the electrophoretic mobility using the Helmholtz Smoluchowski equation under an electrical field of 40 V/cm. The processing was done by the software included within the system. The measurements were done at 25 °C. [13]

Determination of the encapsulation efficiency:

Entrapment efficiency was determined by ultracentrifuge filtration method. The nano formulations were centrifuged at 80,000 RPM corresponding to 3,47,000×g for 60 min at 4 °C in an ultracentrifuge (Optima TM MAX-XP, Beckmann Coulter, USA). (13) The drug content in supernatant was analyzed at λ_{max}=293.5 nm by using validated UV spectroscopy method. It is calculated as:

$$\text{Entrapment efficiency} = \frac{(\text{Amount of drug loaded} - \text{Amount of drug in supernantant})}{(\text{Amount of drug loaded})} \times 100$$

Determination of pH:

pH of the formulation is a significant parameter for ophthalmic preparation as the formulation can cause irritation and cytotoxicity if not in desired pH. Hence Digital pH meter (Universal Enterprises, Mumbai, India) was used to determine the pH of the formulation.

Transmission electron microscopy (TEM):

High-resolution transmission electron microscope (Jeol, Jem 2100 plus) was used to examine the morphology of GFX-loaded NLCs. Prior to analysis, the sample was prepared. NLC was diluted in double-distilled water, and a drop of the NLC solution was placed on the carbon-coated grid for one minute. The grid was then dried at room temperature and placed on a drop of 1% phosphotungstic acid before being analyzed by TEM [14]

Differential Scanning Calorimetry (DSC):

DSC is the thermo-analytical technique in which the difference in the amount of heat required to increase the temperature of a sample and reference is measured as a function of temperature. It depicts the thermophysical properties of the drug thereby indicating the change in properties of the drug during formulation development. The DSC was obtained using DSC 1 STARe system (Mettler, Toledo, Switzerland).[15] About 10mg of sample was weighed and filled in a DSC pan and sealed properly. Then this pan was placed in a DSC instrument along with a reference pan and heated from 30 ºC to 300 ºC. The nitrogen gas is purged at the rate of 80 mL/min during the experiment to maintain an inert environment and an endotherm was recorded

***In vitro* drug release studies**:

In vitro drug release of GFX-NLC was performed in pH 7.4 simulated tear fluid using dialysis bag diffusion method. Overnight soaked dialysis membrane (cut-off molecular weight 12,000- 14,000 Da) was filled with 1 mL of GFX-NLC and immersed in 100 mL dissolution medium stirring with 100 rpm on magnetic stirrer and maintained at 32 -34°C. Meanwhile, 1 mL of sample pipette out at the defined interval up to 8 h and replaced with equal volume of fresh simulated tear fluid. Finally, the samples were analyzed in UV spectrophotometer at 285.5 nm to determine the concentration.

Ex-vivo corneal permeation studies:

The permeation study was performed by using Franz diffusion cell using freshly excised goat cornea for permeation of drug from GFX loaded NLC and marketed preparation. A freshly excised goat eye was received from the local slaughterhouse. As the viability of the cornea is crucial, it was stored in cold STF (pH 7.4). The cornea was then dissected carefully along with attached scleral tissue, and this acted as the permeability barrier. It was then equilibrated with STF (pH 7.4) for 2-3 hours. The study was conducted using Franz diffusion cell where the upper chamber served as a donor compartment in which samples (GFX Loaded NLCs, marketed formulation) under investigation was placed. The lower chamber served as receiver compartment that contained STF. Excised goat cornea was fixed between clamped donor and receptor compartments ensuring that the epithelial side of the cornea faced the donor compartment. [16] The aliquot was taken at predefined time points for 8 hr. The protocol and parameters were followed as given in Table II below:

Antimicrobial Activity

The agar diffusion well method was used to test antibacterial activity of formulation. *Staphylococcus aureus ATCC 6538* and *Pseudomonas aeruginosa ATCC 9027* were used as test organisms. To compare the results, marketed Gatifloxacin eye drops were

used along with test formulation. The well was made on each agar plate with sterile cork borer of 8mm diameter. Then test formulation and marketed Gatifloxacin eye drop were poured into wells of different plates. This complete procedure was carried out in aseptic condition. These petri plates are kept at room temperature for 2 hours to diffuse the formulation through the agar medium. Then these plates inverted and transferred into incubator and incubated at respective temperature and time period i.e., 30℃ for 18-24 hours.[17] The petri plates were examined, and zone of inhibition was calculated using zone reader. The zone of inhibition of test formulation was compared with marketed Gatifloxacin eye drop. These tests were done in duplicate.

Ocular irritancy test (Hen's egg test-chorioallantoic membrane):

For irritancy studies, an alternative method to Draize eye is used i.e., HET-CAM (Hen's Egg Test or Hulner Embryogen) Test. GFX loaded NLCs were tested by HET-CAM test. The eggs were procured from the Joshi poultry, Mumbai. The test was done in triplicate on each formulation. About 10 days after fertilization Hen's eggs were rotated in an incubator for 9 days after which time any defective eggs were discarded by observing them via candlelight testing method. The shell around the air cell is removed with the help of forceps and sharp needles and the inner membranes are extracted to reveal the chorioallantoic membrane. In this test 0.9% NaCl was used as negative control and 1% NaOH solution was used as positive control. Test chemicals i.e., positive control, negative control and test formulation are added to the membrane and left in contact for 5 min. The membrane was examined for vascular damage and the time taken for injury to occur is recorded. Irritancy is scored according to the severity and speed at which damage occurs. The potential ocular irritancy of a test substance is measured by its ability to induce toxicity in the chorioallantoic membrane of a chicken. The effects were measured by the onset of hemorrhage, coagulation, and vessel lysis. [18,19]

Stability:

According to the ICH guideline Q1A(R2), "The purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors such as temperature, humidity, and light, and to establish a retest period for the drug substance or a shelf life for the drug product and recommended storage conditions". This study was in accordance with the ICH guideline Q1A(R2) Stability testing of new drug substance and products. During this study, optimized Gatifloxacin loaded NLCs was sealed in transparent glass vials with rubber closure to evaluate the long-term stability of formulation. The formulation was evaluated at predefined time points for various parameters like Appearance, particle size, pH, zetapotential, entrapment efficiency, drug content. About 5ml of the GFX-NLCs formulation was sealed and stored in a transparent glass vials with silicone rubber closure and stored for 3 months at 2-8℃ and 25±4℃.[19]

RESULT AND DISCUSSION:

Optimisation of Gatifloxacin loaded nanostructured lipid carriers:

The trail runs were prepared according to the design designed by the software and following are the responses observed as mentioned in Table III.

Amongst all models, 3F1 model was best fitted models for all two independent variables. An optimized run was selected from the given solution for constraints which was then formulated and characterized

Particle size:

The particle size for optimization batches were in the range of 44.2-140.5 nm. The analysis done by software indicated direct relationship of amount of lipid (C), amount of lipid and liquid lipid: total lipid (AC), lipid concentration: total lipid * surfactant concentration * amount of lipid (ABC) with Particle size (Y1). The surfactant concentration(B) and liquid lipid: total lipid ratio(A) show negative effect on particle size. As the surfactant concentration increases from 0.5% to 1% in the particle size shows significant increase. The ANOVA for selected factorial model for particle size and PDI was analyzed. According to the analysis for particle size, the p-value for model terms A, B, C, AC, ABC was less than 0.0001 and p-value below 0.05 indicates the model terms are significant. Hence the mentioned model terms are significant. The Lack of fit F-value is 2.75, non-significant which is desirable. The R2 was found to be 0.9878. The standard deviation was 3.01 and mean was 99.65. **FIGURE 1**, **FIGURE 2 and FIGURE 3** below shows the pareto chart, contour plot and 3D surface graph for particle size respectively.

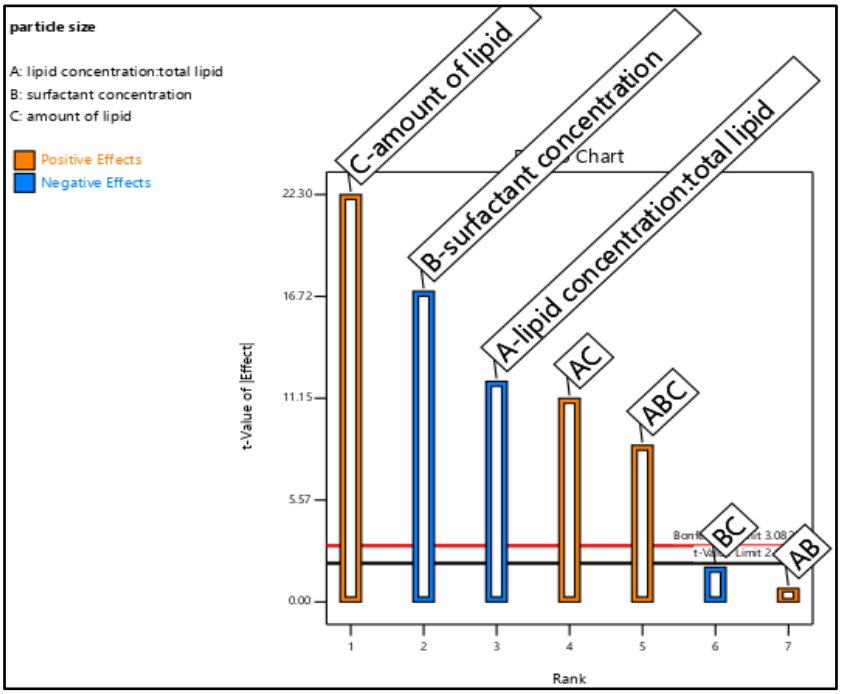

FIGURE 1-Pareto chart for particle size

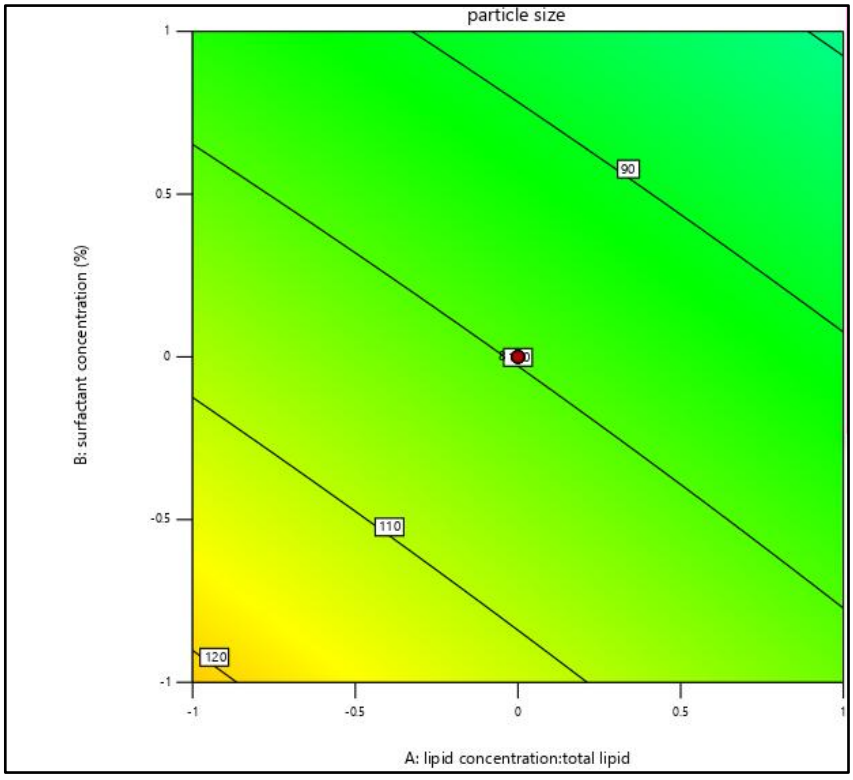

FIGURE 2-Contour plot for particle size

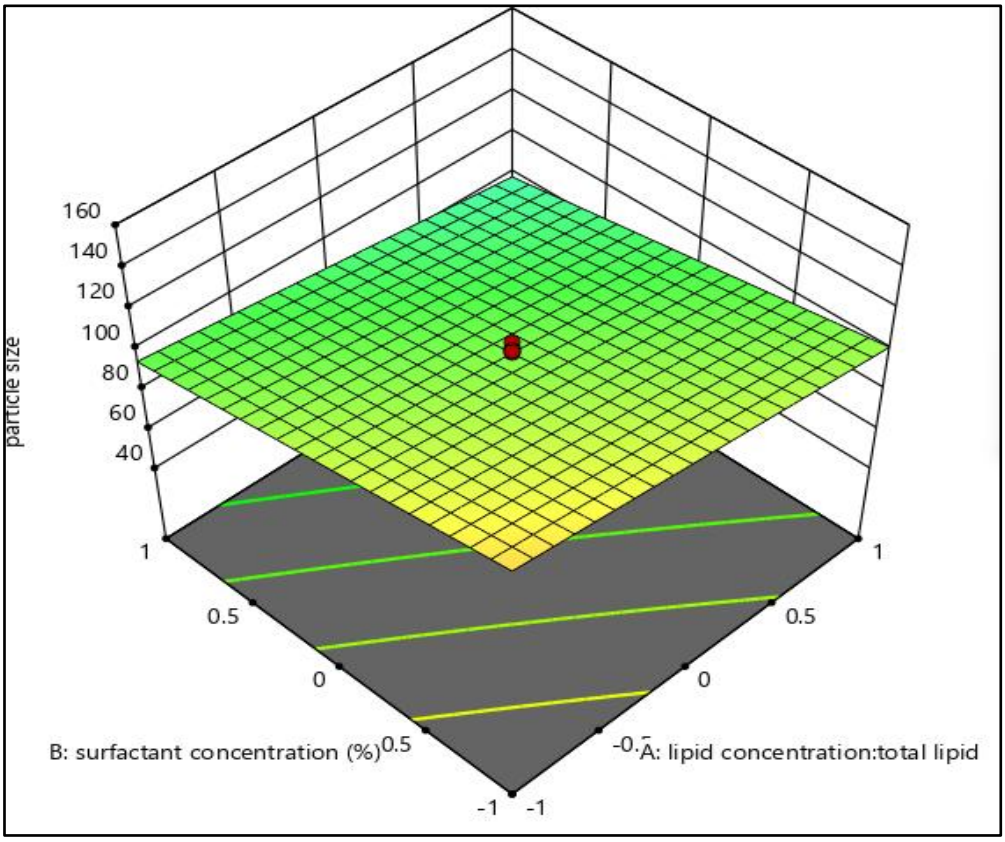

FIGURE 3-3D surface response graph for particle size

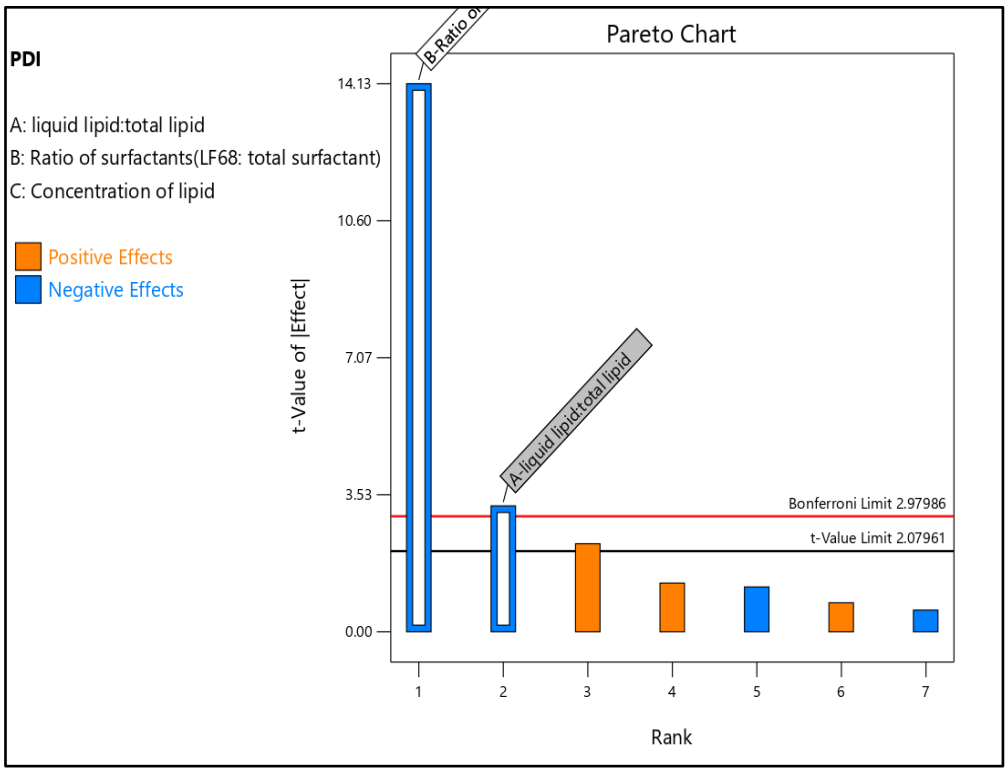

FIGURE 4-Pareto chart for PDI

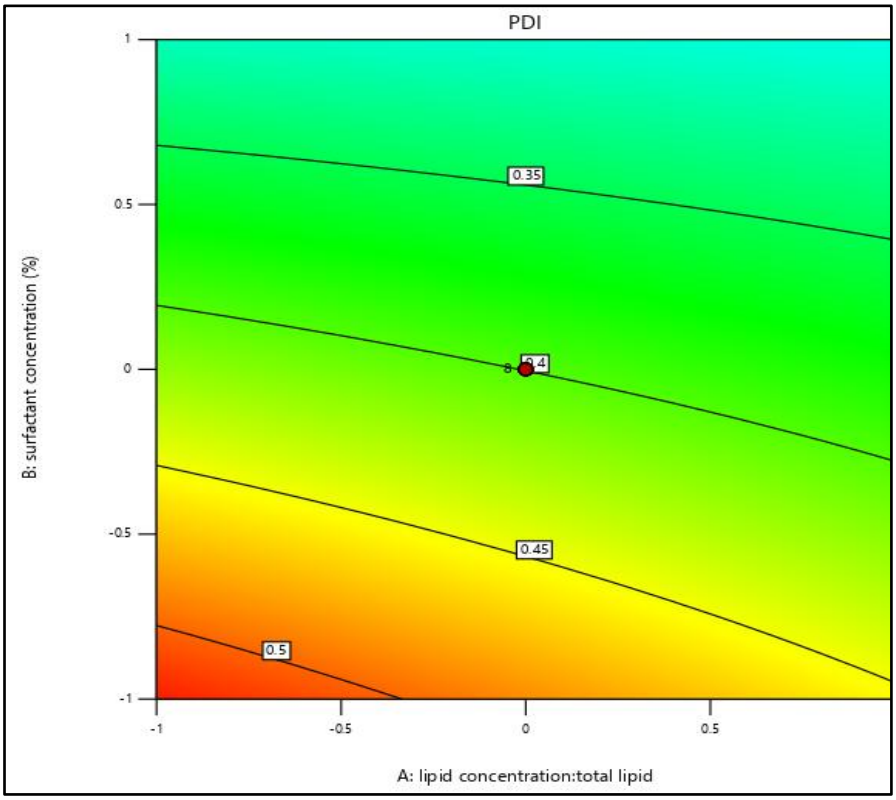

FIGURE 5-Contour plots for PDI

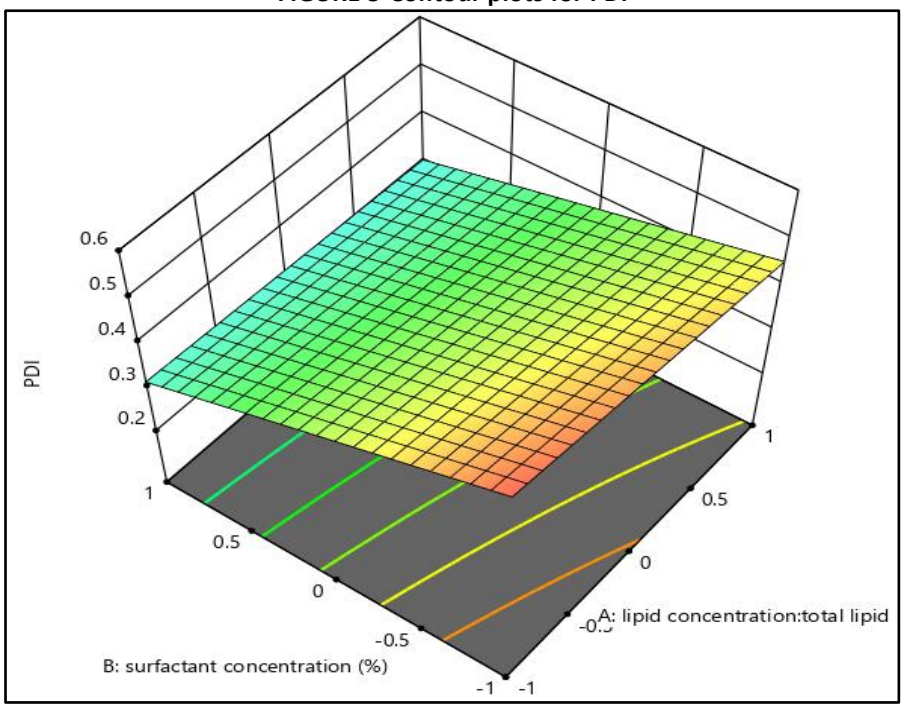

FIGURE6- 3D surface response graph for PDI

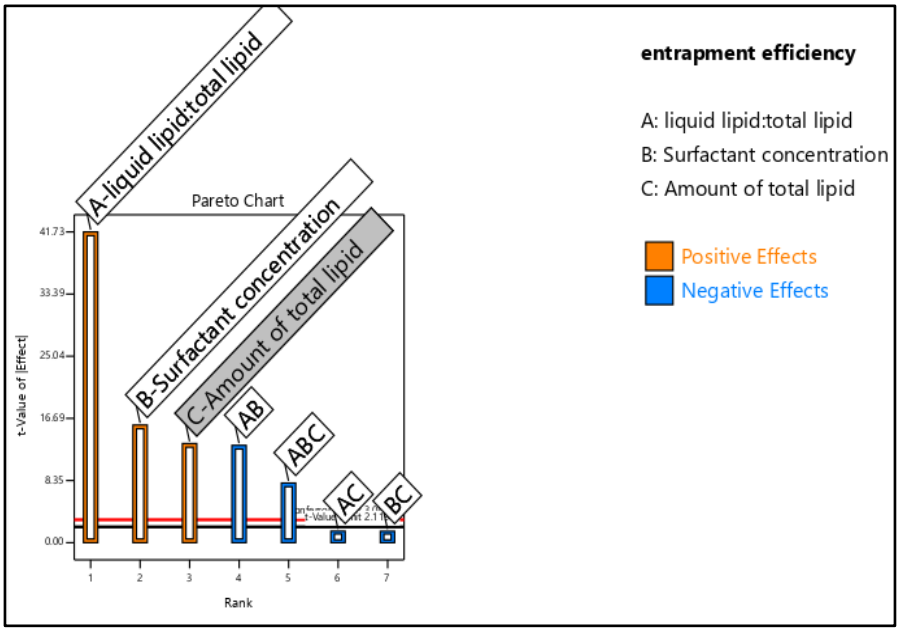

FIGURE 7- Pareto chart for entrapment efficiency

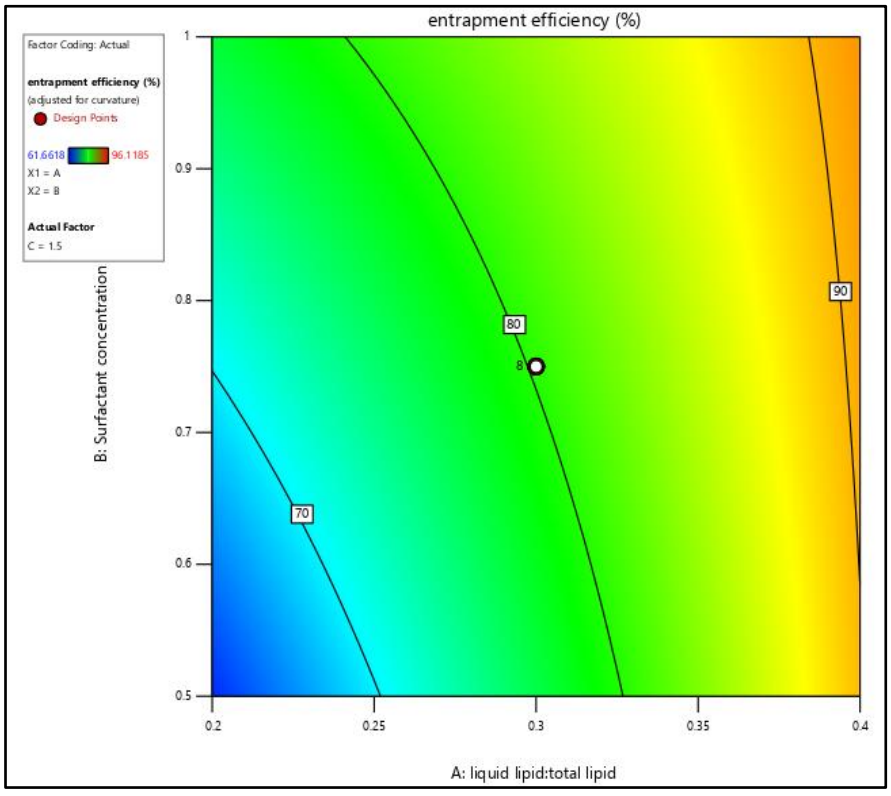

FIGURE 8- Countour plot for entrapment efficiency

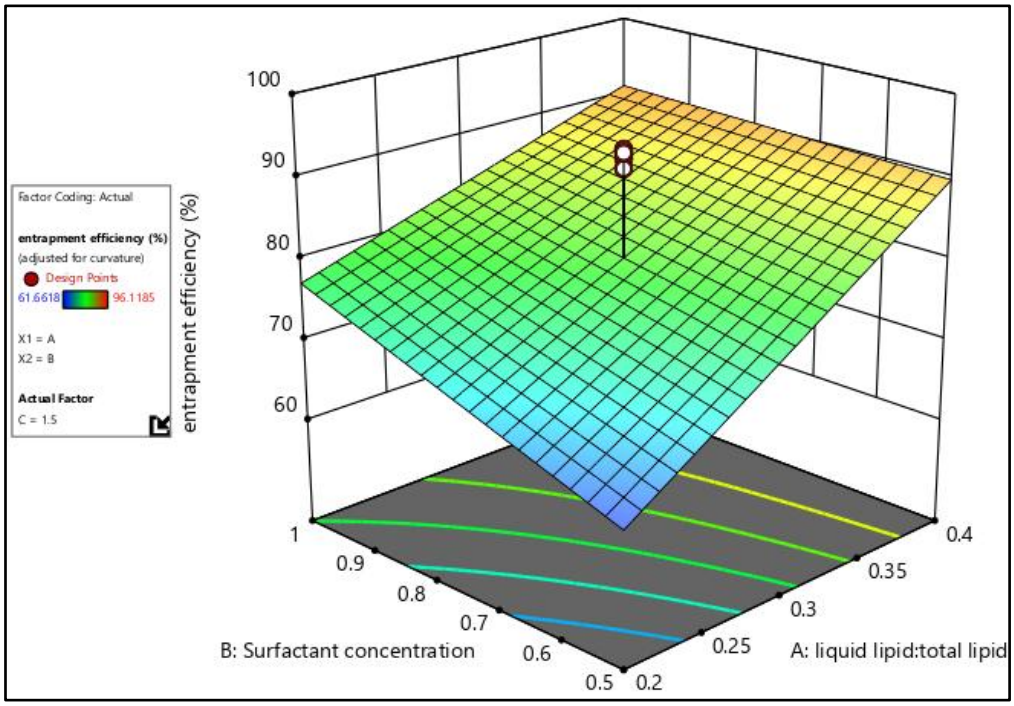

FIGURE 9- **3D surface response graph for Entrapment Efficiency**

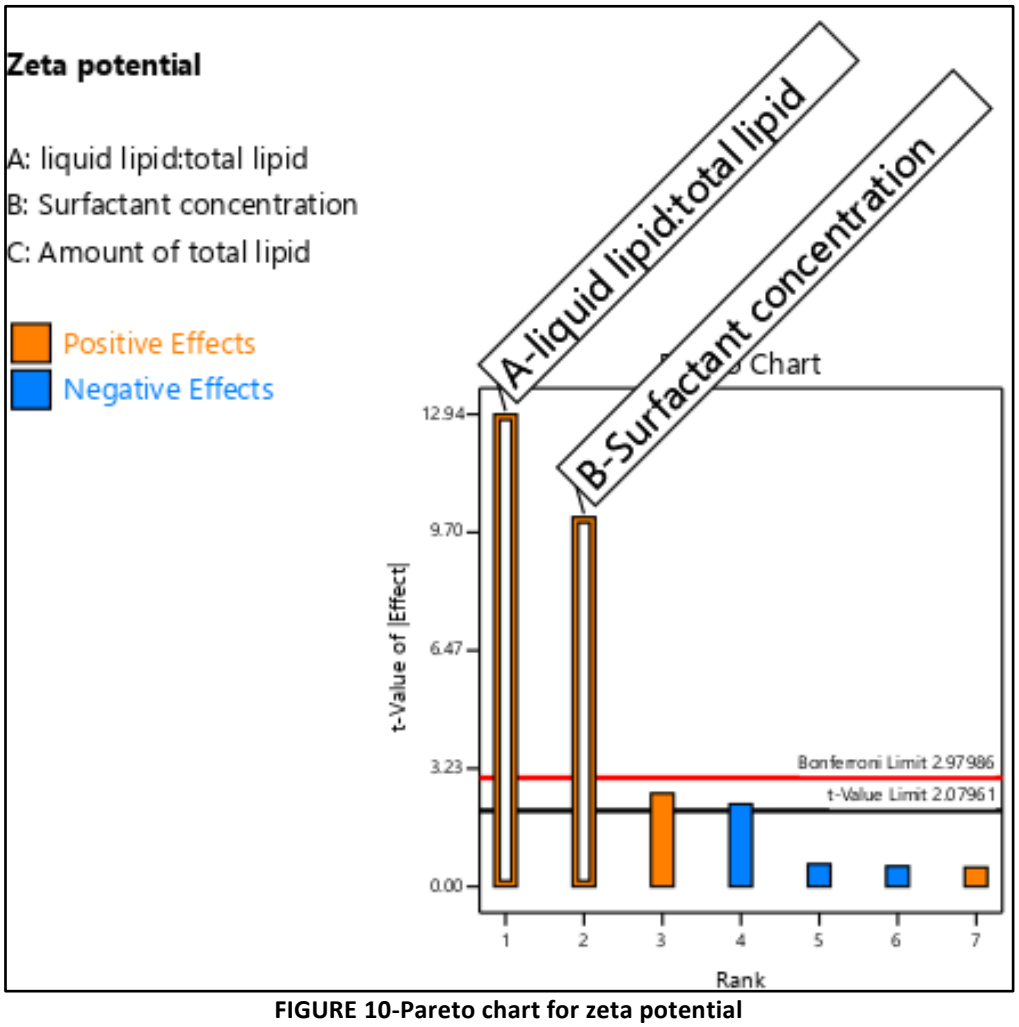

FIGURE 10-Pareto chart for zeta potential

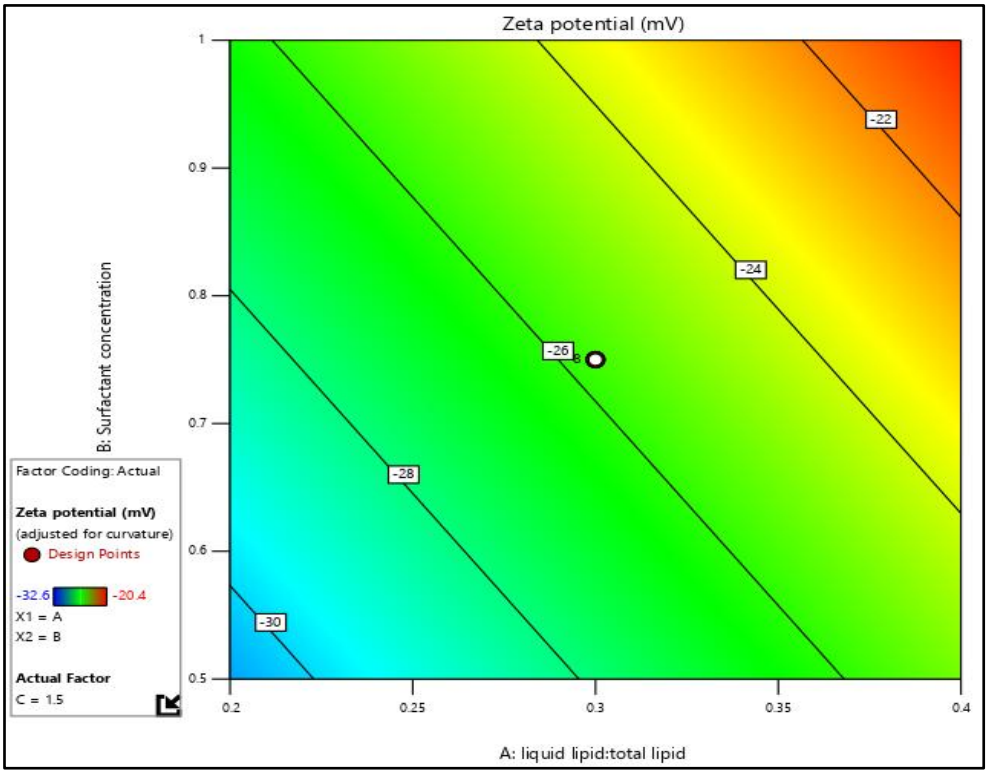

FIGURE 11-Contour plot for Zeta potenntial

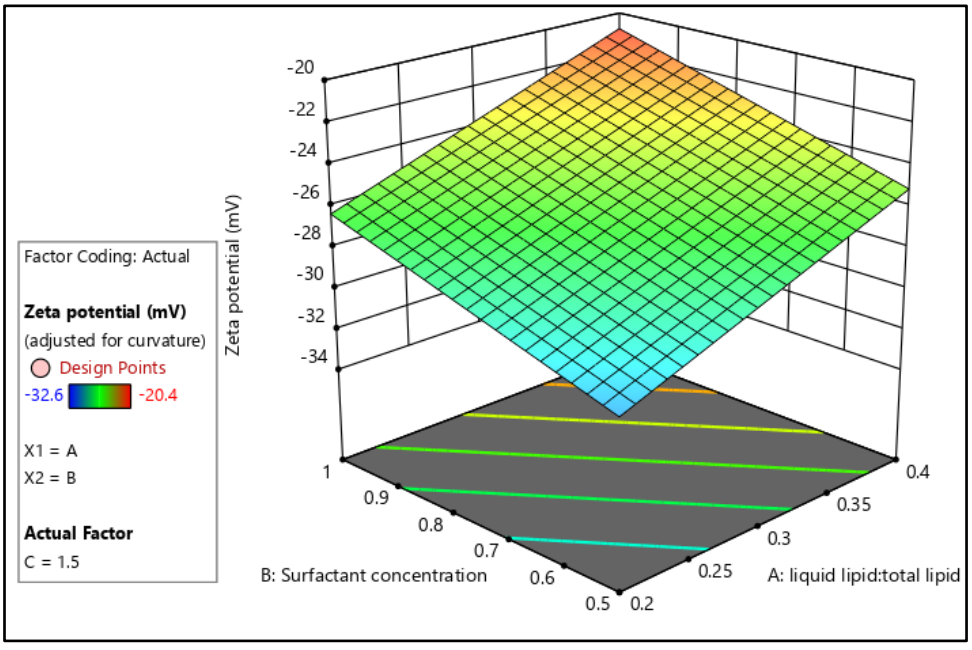

FIGURE 12-3D surface response graph for Zeta potential.

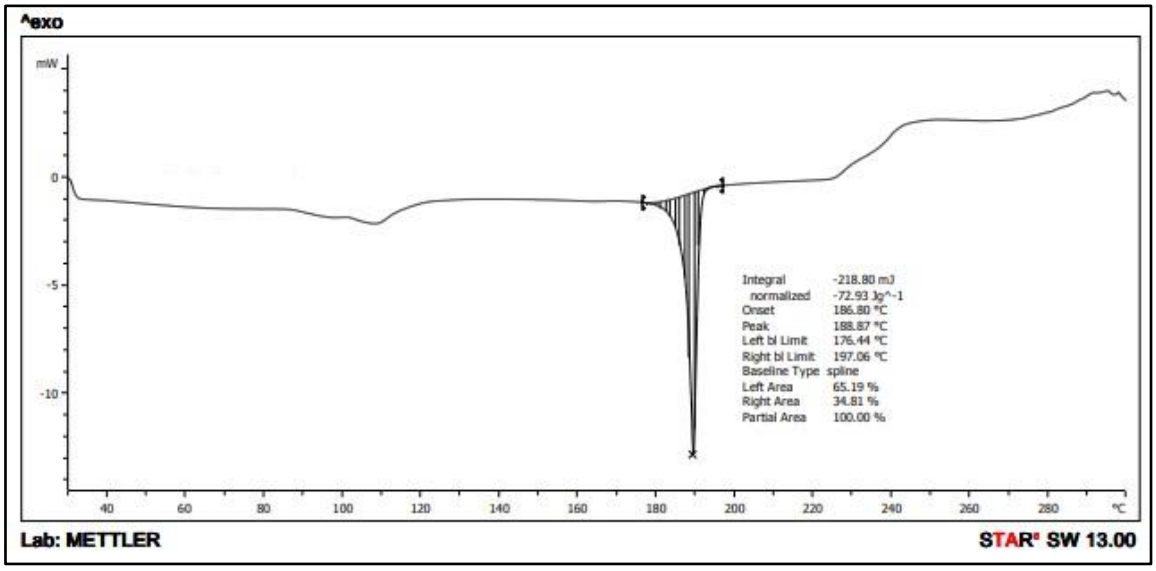

FIGURE 13-DSC thermogram of pure drug

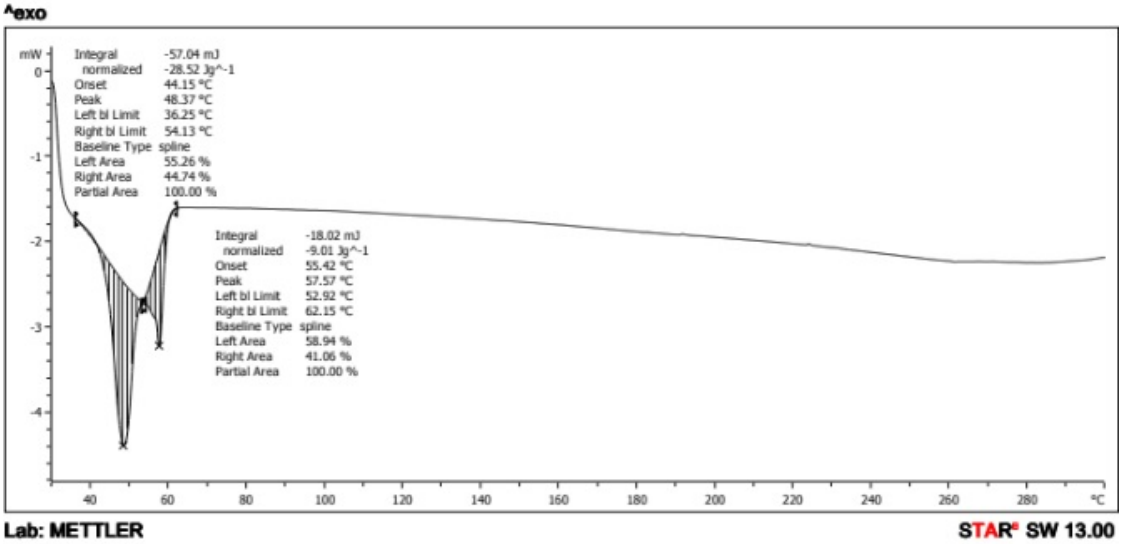

FIGURE 14-DSC of GFX loaded NLCs.

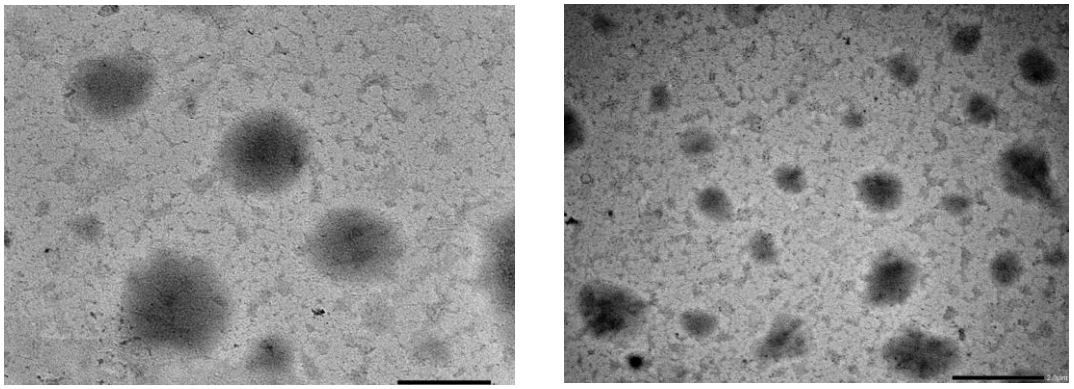

FIGURE 15- **Transmission Electron Microscopy (TEM) analysis of GFX loaded NLCs.**

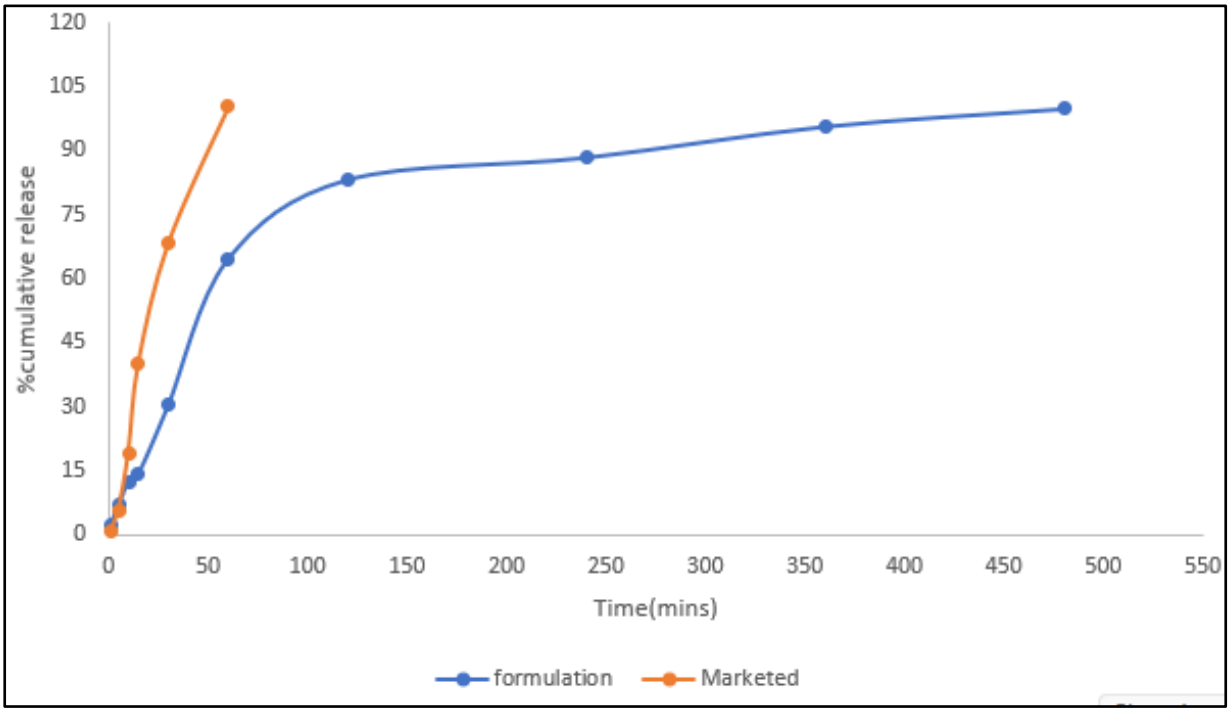

FIGURE 16- In vitro release profile of GFX-NLCs and Marketed formulation

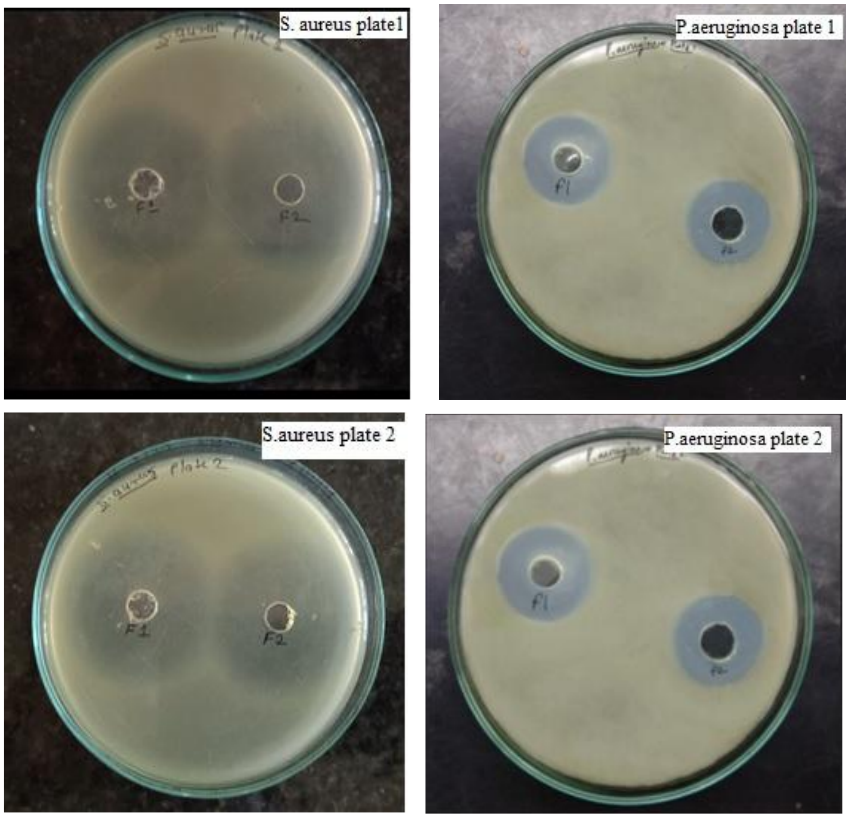

FIGURE 17-Antimicrobial study of GFX loaded NLC and Marketed formulation against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

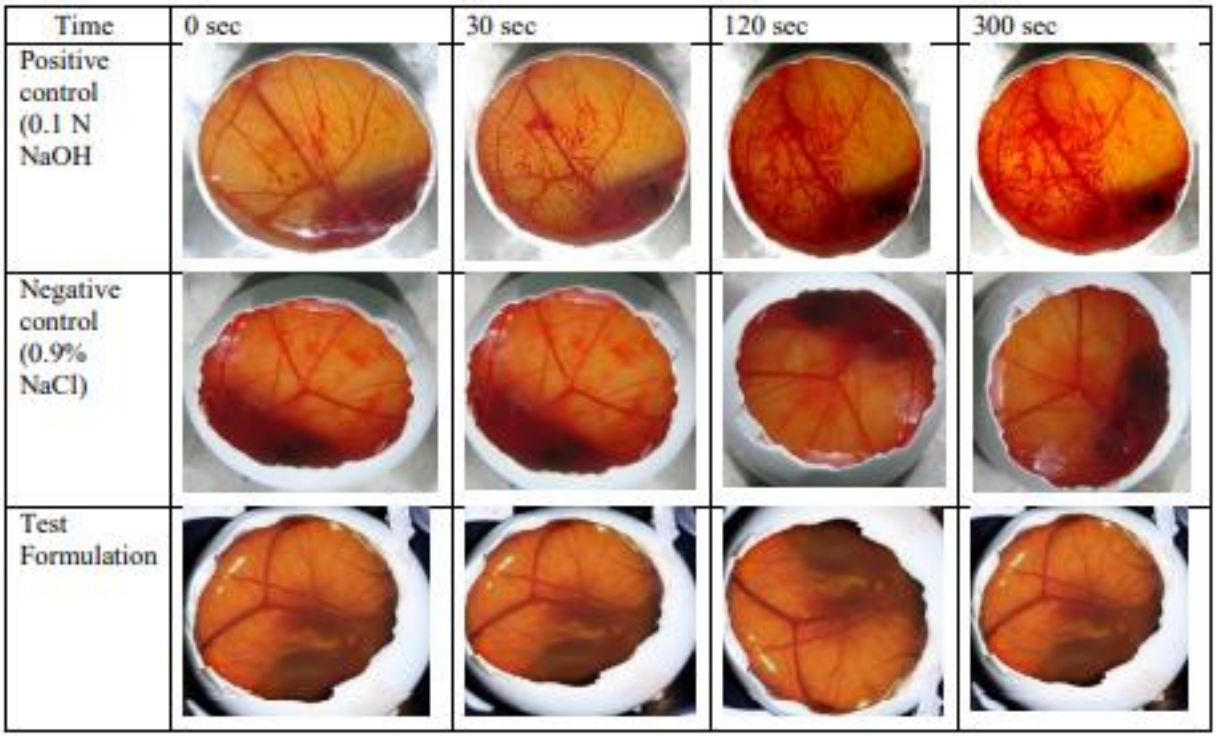

FIGURE 18 Vascular responses on the CAM after 300 sec of sample application

PDI:

The polydispersity index of the formulation signifies the uniformity of the particle size distribution of the nanoparticles. The PDI ranges from 0.225 -0.410. According to the analysis done by the software there is inverse relation of PDI with liquid lipid: total lipid concentration(A) and Surfactant concentration(B) whereas a combine positive effect of Liquid lipid concentration and surfactant concentration can be observed. According to the analysis for PDI, the p-value for model terms A, B, AB was less than 0.0001, indicating the model terms are significant. Hence the mentioned model terms are significant. The Lack of fit F-value is 0.45, non-significant which is desirable. The R2 was found to be 0.9438. The standard deviation was 0.0225 and mean was 0.355. **FIGURE 4**, **FIGURE 5 and FIGURE 6** below shows the pareto chart, contour plot and 3D surface graph for PDI respectively.

Entrapment efficiency:

The Entrapment efficiency of all formulations were measured using indirect method. Formula mentioned earlier was used to find out the entrapment efficiency. It ranged from 61.66% to 96.11%. According to the analysis done by the software it is observed that Liquid lipid: total lipid ratio, Surfactant concentration and total amount of lipid shows positive effect on the entrapment efficiency. The increase in EE with increase in liquid fraction is apparently due to high solubility of GFX in lipids and more voids produced in NLCs and the presence of sufficient surfactant prevent the drug expulsion and ultimately forms stable nanoparticles. According to the analysis for EE, the p-value for model terms A, B, C, AB, ABC was less than 0.0001 indicating the model terms are significant. Hence the mentioned model terms are significant. The Lack of fit F-value is 2.23, non-significant which is desirable. The R2 was found to be 0.9950. The standard deviation was 0.974 and mean was 84.29% **FIGURE 7**, **FIGURE 8 and FIGURE 9** below shows the pareto chart, contour plot and 3D surface graph for EE respectively.

Zeta potential:

Zeta potential of all the formulation was in the range of -32.6 mV to -20.4 mV. The analysis done by the software indicated that there is direct relation between the liquid lipid: total lipid ratio(A) and Surfactant concentration (B) on the zeta potential (Y4). As the liquid lipid concentration increased from 0.2 to 0.4 the Zeta potential also increased. The same effect is observed with the surfactant concentration, that is as the surfactant concentration increased from 0.5% to 1% zeta potential increased too. The software shows that there is no significant effect of amount of total lipid on zeta potential. According to the analysis for Zeta potential, the p-value for model terms A, B was less than 0.0001 indicating the model terms are significant. Hence the mentioned model terms are significant. The Lack of fit F-value is 5.12, non-significant which is desirable. The R2 was found to be 0.9438. The standard deviation was 0.8521 and

mean was -26.48 mV **FIGURE 10**, **FIGURE 11 and FIGURE 12** below shows the pareto chart, contour plot and 3D surface graph for Zeta potential respectively.

An equation was proposed by the software for the particle size, PDI, entrapment efficiency and zeta potential by the software which are tabulated in **Table IV.** The equation in terms of actual factors can be used to make predictions about the response for given levels of each factor.

From the above study an optimised batch was obtained it was prepared by the Single emulsification-solvent evaporation.

DSC Aanalysis:

The thermal analysis of Gatifloxacin loaded NLCs were studied by differential scanning calorimetry (DSC). The DSC data gives us idea about the melting point, crystallinity, and degradation of sample. The DSC thermogram (FIGURE 13) of Gatifloxacin shows sharp endothermic peak at 188.60^{o}C which indicates the melting point of gatifloxacin, and narrow peak indicates its crystallinity. The DSC thermogram of developed formulation (FIGURE 14) displayed shows two peaks, the onset of one peak of melting endotherm at 55.42℃, peaks at 57.57℃and ends at 52.92℃. The other peak onset of melting endotherm at 44.15 ^{o}C, peak at 48.37^{o}C, and end set at 36.25^{o}C. Thermogram of gatifloxacin loaded NLCs does not show any peak Gatifloxacin peak at 188.60^{o}C. There is shift in the melting point indicating the successful dissolution and encapsulation of drug in NLCs.

TEM analysis:

The shape and surface morphology of Gatifloxacin loaded NLCs were studied by Transmission Electron Microscopy (TEM) analysis. The TEM analysis of formulation shows that the NLCs are spherical in shape and has well-defined edges. The images suggested the particle size of Gatifloxacin loaded lipid nanoparticles were in agreement with the particle size that is obtained by Zeta sizer. Slight variation in size observed due to the polydispersity of particles in the formulation. The images show black colored spherical NLCs. The black coloration of NLCs may be due to the encapsulation of drug in NLCs core.

***In vitro* release study of Gatifloxacin loaded NLCs:**

In vitro release study is the measure of release of active pharmaceutical reagent from the formulation. It is important evaluation technique in formulation development and quality control study. In vitro release study of pure drug suspension and Gatifloxacin loaded NLCs were carried out using dialysis bag method. The dialysis bag was soaked in simulated tear fluid overnight. The study was done in triplicate and percent cumulative release of drug was calculated using formula. The release profile of pure drug suspension and Gatifloxacin loaded NLCs was obtained by plotting % cumulative release verses time The graphical representation is shown in FIGURE 16.

Drug release through GFX loaded NLCs was found to be 99.848±0.82% over the period of 8hrs. For the marketed preparation, the release was found to be 100.42±0.46% in 30 mins. It is observed that the drug release through NLCs is for longer period of time as compared to marketed formulation.

Kinetic models for in-vitro release study of Gatifloxacin loaded NLCs: The mechanism of drug release from formulation is obtained through kinetic release data. Kinetic models illustrate the factors involving in dissolution of drug from the formulation. The regression coefficient (R^2) of various kinetic models was obtained by plotting the data in graphs. The goodness of fit of any model is decided by how close the value of regression coefficient to the 1. The R^2 value of Korsmeyer peppas model for gatifloxacin loaded NLCs suspension is 0.9327 and it is closest to 1 compared to other three models. This indicate that Korsmeyer peppas model graph has best linearity than other models. This indicates anomalous non-Fickian diffusion from gatifloxacin loaded NLCs. The dissolution of drug is dependent on diffusion of drug through lipid matrix and swelling of lipid matrix.

Ex- vivo permeation:

Drug permeation from GFX loaded NLCs was found to be 53.564 ± 0.48 % over the period of 8 hrs. Drug permeation from marketed formulation (Gatiquin eye drops 0.3%w/v) was found to be 67.140 ± 0.049 % over the period of 8 hrs.The main problem in drug permeation in ex-vivo study is epithelial barrier. The structure of epithelium of ocular tissue contains lipid bilayer.

The steady state flux and permeation coefficient are given in the Table V.

Antimicrobial study:

The activity of Gatifloxacin loaded NLCs was compared with marketed Gatifloxacin eye drop. The shows that Gatifloxacin possess the antimicrobial activity against the Staphylococcus aureus and Pseudomonas aeruginosa. The zone of inhibition was measured by zone reader. The zone of inhibition for S. aureus of the marketed formulation was found to be 26 mm and the zone of inhibition of Gatifloxacin loaded NLCs was found to be 26.35 mm. It shows that the formulation is as active as the marketed formulation. This conclude that the developed formulation has equivalent activity as other marketed formulation. For P. aeruginosa, the zone of

inhibition for of the marketed formulation was found to be 14.19 mm and the zone of inhibition of Gatifloxacin loaded NLCs was found to be 15.46 mm. The FIGURE 17 shows the zone of inhibition in each plate.

Ocular Irritancy study:

HET-CAM test is inexpensive, qualitative, and rapid test for ocular tolerance which does not have any legal and ethical obligation. The negative control i.e., 0.9% NaCl does not show any hemorrhage, blood clotting or vessel damage. The images displayed in fig conclude that the negative control solution does not show any irritancy in 5 min test period. 0.1N NaOH solution used as positive control in the test and supposed to be irritant to the CAM membrane. After instillation of 0.5 mL of 0.1 N NaOH solution on CAM membrane, it started hemorrhage in blood vessels of CAM membrane. The images in FIGURE 18 show significant amount of blood vessel rupture and hemorrhage on CAM membrane at the end of 5 min test. This conclude that the 0.1 N NaOH solution has irritancy property to the chorioallantoic membrane. Third set of eggs were used to test the formulation. 0.3 mL of gatiloxacin loaded NLCs suspension was instilled on the CAM membrane. The CAM membrane does not show any hemorrhage in 5 min test period. This demonstrate that the Gatifloxacin loaded NLCs suspension is nonirritant to the Hens Egg Chorioallantoic Membrane and safe to use for the ocular drug delivery.

Stability study:

Stability study of GFX loaded NLCs was carried out for three months at the conditions mentioned in the experimental section. It was evaluated for various parameters. represents **s**tability of the Gatifloxacin loaded NLCs. The results observed are depicted below. The results suggest that at 5℃ there is increase in particle size from 85.66 nm to 144.4 nm over the period of 3 months whereas the entrapment efficiency decreases from 91.48% to 58.44 %. The particle size at 25±2℃ increased from 85.663 nm to 91.86 nm whereas the decrease in entrapment efficiency was from 91.48 % to 79.53 % over a period of 3 months. It is observed that the leakage of drug from the NLCs is slower when stored at 25±2℃. The decrease in entrapment efficiency is observed may be due to leakage of drug from the lipid to the outer medium.

CONCLUSION:

Successful formulation of non-toxic potential ophthalmic NLC system was carried out with a narrow size distribution, high GFX entrapment efficacy. The chosen components were Glyceryl monostearate, Oleic acid, Tween 80 and Lutrol F68. We propose the use of low levels of surfactant concentration and lipid concentration because then the critical stability parameters (Zeta potential, Particle size, PDI) and drug entrapment efficiency are suitable while emulsifier concentration can remain at low levels. The result of the study suggests that this formulation can be a better alternative to other fluroquinolones which have developed resistance over a period of time.

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