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Preparation and Characterization of Nano Structured Lipid Carriers for Ocular Bacterial Infection

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The problem of bacterial conjunctivitis has dramatically increased in recent years due increased pollution and modern lifestyle. The present study was focused to fabricate Sparfloxacin loaded nanostructured lipid carriers (Spar-NLCs) for ophthalmic application to improve ocular penetration of drug and give sustained release of drug to reduce dosing frequency and toxic effect of drug associated with ocular membrane. A regular two-level factorial design was used to optimize the formulation parameters that are significantly affecting the formulation attributes. Spar-NLCs with particle size 171.1 ± 11 nm, zeta potential -49 ± 6.47 mV, entrapment efficiency 89.5 ± 5% and spherical in shape was obtained. Besides this, FTIR spectroscopy, differential scanning calorimetry, and transmission electron microscopy results suggest that the drug is successfully incorporated in NLC and has excellent compatibility with the excipients. In vitro release study follows Korsmeyer peppas model and suggests that 81.35 ± 6.2% release of drug from Spar-NLCs in 12 hours. The result of ex-vivo permeation study demonstrated 349.75 ± 7.3 μg/cm^2 of permeation of drug, 44.482 μg cm^{-2} hr $^{-1}$ of flux, and 0.1482 cm hr^{-1} of permeability coefficient which is 1.7 folds higher than pure drug suspension. The antimicrobial activity of Spar-NLCs was better than the pure drug suspension and equivalent to the marketed formulation. Spar-NLC formulation did not showed any ocular damage, swelling, and redness in in -vivo Draize test. The ocular

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tolerance test (HET-CAM test) also suggests that the Spar-NLC formulation and its excipients were nonirritant to the ocular tissues. The formulation was found to be stable over the three month of stability study. Therefore, this work strongly suggest that Spar-NLCs has higher penetration and extended release of drug which can be effectively used in prevention of bacterial conjunctivitis.

Keywords: Nano lipid carries; ocular; ex-vivo permeation; sparfloxacin; bacterial conjunctivitis.

1. INTRODUCTION

Eye is one of the vital organs of our body. The eye is made up of several component i.e., cornea, iris, pupil, lens, retina, macula, optic nerve. The average size of eye is about 1inch. It serves very important function- the sense of light.

Eyes consist of three layers. Outer layer consists of sclera and cornea; middle layer consists of iris, choroid and ciliary body; inner layer consists of photoreceptors and neurons which consist of retina. There are three chambers in eye. First one is anterior chamber which lies between cornea and iris; second is posterior chamber which lies between iris and lens; third one is vitreous chamber which is present between retina and lens. Anterior chamber and posterior chamber contain aqueous humor which is produced by ciliary body. It helps in maintaining intraocular pressure. The vitreous chamber is filled with thick gel like vitreous humor. The conjunctiva is mucous membrane present in the eye. It contains three parts: bulbar conjunctiva, palpebral conjunctiva and fornix or the conjunctival sac [1].

Allergic diseases are dramatically increased in last decades; and eye is not exception for that. Different type of allergies affects 15-20% of world population. Ocular allergy is more common these days because of environmental changes, pollution, and changed lifestyle. In one study researcher tested 5000 allergic children out of which 32% of children had ocular diseases [2]. Various reports shows that the frequency of allergic diseases have been increased in past 40 years and continue to growing [3]. The duration of ocular allergies ranges from some days to some weeks and if not treated in time it can cause complication. There are several causes of ocular allergies like air pollution, genetics, exposure to different cosmetics etc. Conjunctivitis is common allergic disease found in society. It causes in all age groups and in all socioeconomic classes [4]. Acute conjunctivitis is self-limiting disease which cure by taking simple precautions and medicines. Conjunctivitis is always mistaken for red eye. Red eye is inflammatory response to the various sight threatening diseases. Therefor it important to proper diagnosis of conjunctivitis. The symptoms of conjunctivitis include redness in eye, watery discharge, presence of pain, itching, blurred vision, and eyelid characteristic [5]. Non-infectious conjunctivitis is caused by allergens and irritants. Infectious conjunctivitis caused by bacteria, viruses, fungus, chlamydial and parasites. There are four types of allergic conjunctivitis i.e. seasonal allergic conjunctivitis (SAC), perennial allergic conjunctivitis (PAC), vernal keratoconjunctivitis (VKC) and atopic keratoconjunctivitis (AKC). There is other type of conjunctivitis also found like contact lens related keratoconjunctivitis, toxin related conjunctivitis, medication-induced keratoconjunctivitis. Conjunctivitis further divided into acute conjunctivitis and chronic conjunctivitis. Acute conjunctivitis generally resolves in 3 to 4 weeks and chronic conjunctivitis takes more than 4 weeks [6].

Fluoroquinolones have great activity against Gram-negative and Gram-positive bacteria. Sparfloxacin provide improved efficacy in ocular infection than conventional fluroquinolones. Sparfloxacin is newer fourth generation fluoroquinolone which has more in vitro activity than ciprofloxacin in gram positive bacteria and mycobacteria. The activity of sparfloxacin is reduced with acidic pH and in the presence of cations. It exerts it action by inhibiting topoisomerase II (DNA gyrase) and topoisomerase IV enzymes, which are essential in bacterial DNA replication, transcription, and recombination. Sparfloxacin is useful in the treatment of community acquired pneumonia, urinary tract infection, lower respiratory tract infection etc [7].

Solid lipid nanoparticles (SLNs) are one of the promising drug delivery system for the ocular disorders. It contains lipidic core which is stabilized by surfactants. It can dissolve both lipophilic and hydrophilic drug moieties [8]. Nanostructured lipid carriers (NLCs) are second generation lipid nanoparticles which contain liquid lipid along with solid lipid in the core [9]. It

overcomes the problem of drug expulsion during storage and provide high drug loading capacity than SLNs. NLCs have amorphous lipid core therefore it provides more flexibility for drugs. NLCs have better structural integrity, higher drug loading capacity, better stability during storage, controlled drug release and good biocompatibility than conventional dosage forms [10]. Solid lipids used in preparation of SLNs and NLCs are glyceryl behenate, stearic acid, glyceryl monostearate, triglycerides, esters of fatty acids and fatty alcohols etc. Liquid lipids used in preparation of NLCs are medium chain triglycerides, oleic acid, paraffin oil, corn oil etc. To stabilize lipid nanoparticles surfactants plays major role. Commonly used surfactants are Tween 80, Tween 20, Cremophore EL, Span 80, Pluronic F127 etc [11].

2. MATERIALS AND METHODS

2.1 Materials

Sparfloxacin was kindly gifted by Rakshit Pharmaceuticals Ltd. The solid lipids glyceryl monostearate obtained from CDH laboratory. Stearic acid was acquired from Loba Chemie Pvt. Ltd. Compritol 888 ATO was gifted by Gattefosse India Pvt Ltd. Liquid lipids oleic acid, isopropyl myristate, and paraffin oil were purchased from S. D. Fine Chem Ltd. Corn oil was acquired from Amrut industries, Thane. Surfactants Tween 80 and Tween 20 were procured from Loba Chemie Pvt Ltd. Cremophore EL and Kolliphore RH 40 were gifted by BASF. All other chemical and solvents used were analytical grade.

2.2 Methods

2.2.1 Lipid and surfactant screening

Compritol 888 ATO (Glyceryl dibehenate), stearic acid, glyceryl monostearate, precirol ATO 5 were reported suitable to use in ocular drug delivery system [12]. These four lipids were screened for their ability to dissolve maximum amount of sparfloxacin. Oleic acid, paraffin oil, corn oil and isopropyl myristate were selected for solubility study. These liquid lipids were suitable for use in ocular formation and reported to be used in preparation of NLCs [13]. Drug was added in excess amount in liquid lipid. The mixture then cyclomixed and shaken for 72hr in water bath shaker. Then these Liquid lipids centrifuged and analyzed by UV spectrophotometry [14,15]. Surfactant requires in formulation to stabilize aqueous and lipid phase. Tween 80, Tween 20, Cremophore EL and Kolliphor RH40 were screened for solubility study [16]. Surfactant were screened by same process as liquid lipids.

2.3 Preparation of Spar-NLC

There are various methods of preparation of nanostructured Lipid Carriers (NLCs). Melt Emulsification sonication was chosen for the present investigation as it is easy and convenient at the given lab settings. In this method two phases were prepared i.e., aqueous phase and lipid phase. Glyceryl monostearate (GMS) and oleic acid were taken in one beaker and heated 5-10^{0}c above the melting point of the glyceryl monostearate (GMS). Then sparfloxacin was added and dissolved in the lipid phase. Aqueous phase contains water and surfactant were taken into another beaker. Aqueous phase was also heated to the same temperature as the lipid phase. The lipid phase was continuously stirred on magnetic stirrer. Then aqueous phase was poured dropwise in the lipid phase with constant stirring, after complete addition of aqueous phase the emulsion vigorously stirred for 10 mins. Hot emulsion is then probe sonicated for size reduction. This obtained hot nano-emulsion was immediately added in cold water for the solidification of NLCs. This formulation was then stored in well caped glass vials [8].

2.4 Experimental Design and Statistical Analysis

The formula for NLC preparation was optimized by three factor-two level factorial design in trial version of design expert software. Solid lipid to liquid lipid ratio (A), surfactant concentration (B) and sonication time (C) were selected as three independent variables. Low level and high level for each factor were listed in table. Total 9 batches of formulation were designed including one center point. Further analysis was performed by putting values of responses got after the analysis of the batches. There were four responses recorded for each trial. The responses were particle size (Y1), polydispersity index (Y2), zeta potential (Y3), and entrapment efficiency (Y4).

2.5 Particle Size, Pdi and Zeta Potential

Particle size of NLCs were analysed by zeta-sizer Nano ZS. The zeta-sizer works on principle of Dynamic light scattering (DLS). The NLC formulations were diluted to 10 times and 1-1.5 mL of sample was placed in disposable particle size cuvette. This cuvette then placed in

instrument and scanned for particle size. To analyse zeta potential, a clear zeta potential cuvette was used. The sample was diluted with distilled water 10 times and 1 mL of diluted sample was placed in zeta potential cuvette. The cuvette was then placed in slot designed for it in the instrument. The instrument uses a Helium Neon Laser set at an angle of 90° at a temperature of 25°C.

2.6 Entrapment Efficiency

The entrapment efficiency of sparfloxacin NLCs were calculated by measuring the amount of drug present in supernatant. Firstly, formulation was diluted 2 times and then 1 mL of sample filled in the ultracentrifuge tubes. The ultracentrifuge tubes placed in the instrument and subjected to ultracentrifugation at 80000 rpm for 1hr. The instrument used was Optima Max XP ultracentrifuge (Beckman Coulter, U.S.A). Then the formulation was separated in two phases. The NLCs cake were formed at the surface of formulation and clear solution formed at bottom. The clear solution formed at bottom was then removed by injection and stored in another tube. This supernatant diluted with water suitably and analysed by UV-Spectroscopy at wavelength of 290 nm using distilled water as blank. The drug entrapment efficiency (E) and drug loading (L) of formulation were calculated by following equations [17].

2.7 TEM Analysis

The morphology of sparfloxacin loaded NLCs were observed by transmission electron microscopy (Tecnai T20, FEI Company^{TM}, USA). The sample preparation was done before analysis. A drop of sample was diluted 50 folds with double distilled water. Then this sample was placed on 400 mesh copper grids coated with carbon film. Then the sample was negatively stained by 1% phosphotungstic acid. After analysis images of sample were obtained at various magnification [18].

2.8 DSC Analysis

The thermal characteristics of sparfloxacin were analysed using differential scanning calorimetry (DSC). The DSC thermogram of pure sparfloxacin was obtained using DSC (DSC STAR E system, Mettler Toledo, Switzerland), equipped with intercooler 2P cooling accessory. About 10mg of sparfloxacin loaded NLCs was weighed and filled in DSC pan and sealed properly. Then this pan was placed in DSC instrument along with reference pan and heated from 30^0c to 300^0c. The heating rate of pan was maintained at 10^0c/min. The nitrogen gas is purged at the rate of 20mL/min during experiment to maintain inert environment and endotherm was recorded.

2.9 *In-vitro* Release Study of Spar-NLC

The in-vitro release study of sparfloxacin loaded NLC dispersion was carried out using dialysis membrane. The dialysis membrane was hydrated in simulated tear fluid (STF). The release study was carried out in 100 mL beaker and release medium was used as STF. 1 mL of formulation was added in dialysis membrane and both ends of membrane sealed by threads. Then it was placed in beaker filled with STF with continuous stirring at 100 rpm. The aliquots were withdrawn at specific time point. After removal of aliquot same volume of fresh STF was added in release medium. The sample then analysed by UV-Spectroscopy at wavelength of 290 nm using STF as a blank.

2.10 Ex-Vivo Permeation Study of Spar-NLC

Freshly excised goat eyeballs were procured from local butcher shop. The eyeballs were stored at 4^0C in normal saline solution (0.9% NaCl solution). The cornea was dissected using forceps and scissor. The cornea was carefully removed with 5-6 mm of surrounding sclera. This excised cornea was kept in simulated tear fluid with air purged in beaker by air pump to avoid tissue damage [19]. To study permeability study franz diffusion apparatus was used in study. The franz diffusion cell contain donor compartment which has formulation, an acceptor compartment which has release medium. The donor compartment is surrounded by water jacket which maintain proper temperature of release medium for study. The volume of acceptor compartment is 7.8 mL which is suitable for ocular permeability study. The acceptor compartment is filled with 7.8 mL simulated tear fluid as a release medium. The acceptor compartment has side extension which has provision to take aliquots. The goat cornea mounted in between donor compartment and acceptor compartment by using clamps. Then 0.5 mL of sparfloxacin loaded NLC dispersion was added in donor compartment. The release medium was continuously stirred at 25 rpm and maintained at 34^0C using water jacket. Aliquot of

1 mL was withdrawn at definite time interval up to 8 hours and equal quantity of fresh simulated tear fluid was added to system to maintain sink condition. The aliquots then analysed by UV spectroscopy at wavelength of 290nm using STF as blank. After analysis, the percent release of drug, permeability of drug was calculated. The drug permeation parameters such as steady state flux (Jss) and permeability coefficient (Paap) was calculated using formula. Steady state flux is the amount of drug that passes the semipermeable membrane.

2.11 *In-Vivo* Ocular Irritation Study (Draize Test)

The sparfloxacin loaded NLCs were tested for the any potential irritancy to the eye. Sparfloxacin loaded NLCs were evaluated using modified draize test. In this study six albino rabbits (2-3 kg) were used. These rabbits were placed in animal house. The health of all rabbits maintained, and proper diet and water were given to all rabbits daily. Then 100 µl of test formulation was instilled in the conjunctival sac of left eye of the rabbit. The right eye was instilled with 0.9% NaCl solution which serves as control for this test. The test formulation was instilled every day for the 7 days. Both eyes of rabbits were checked for the swelling, redness, and any corneal damage at 1, 2, 4, 8, 24, 48 and 72 hr after instillation of test formulation [20-22].

2.12 Antimicrobial Study

The ability of formulation to inhibit the growth and colonisation of bacteria's is assessed by antimicrobial testing. The agar cup method was used to test antibacterial activity of formulation. *Staphylococcus aureus* was used as test organism. To compare the results, pure drug suspension and marketed ciprofloxacin eye drop used along with test formulation. Firstly, the petri plates and all glassware required for study were autoclaved. Enough nutrient agar was made and seeded with *S. aureus.* Then this seeded nutrient agar was poured into petri plates and allow to solidify. Then cups were made on each agar plate with cork borer. Then 200 µl of test formulation, drug suspension and marketed ciprofloxacin eye drop were poured in cup of different plates. This complete procedure was carried out in aseptic condition. These petri plates 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 37^0 C for 24 hours [12,23]. The petri plates were examined, and zone of inhibition was calculated using zone reader. The zone of inhibition of test formulation was compared with plain drug suspension and marketed ciprofloxacin eye drop.

2.13 Ocular Tolerance Test (HET-CAM Test)

Ocular tolerability of sparfloxacin loaded NLCs were tested by modified Hen's egg test-Chorioallantoic membrane (HET-CAM) test. Briefly the fertile 10 days incubated hen eggs were procured from Central Poultry Development Organization, Mumbai. The test was done in triplicate on each formulation. In the HET-CAM test 0.9% NaCl used as negative control and 1% NaOH solution used as positive control. The eggs were placed in tray in such way that the narrower end facing upward. The eggs were kept steady to stabilize membrane. Then the eggs were cleaned properly. Then window of 2*2 cm was made on egg with the help of sterile syringe and forceps. The white membrane was removed carefully without injuring the CAM. Then 0.2 mL of 0.9% NaCl, 1% NaOH solution and test formulation were dropped on CAM membrane of each egg. The observation CAM membrane for any haemorrhage, vessel damage, and coagulation were done for 5 min. Negative control should not show any damage to the membrane while positive control should show irritant to the membrane. The time taken to develop irritancy was noted. Then the formulation treated eggs were compared with positive and negative control to conclude the toxicity of formulation [24,25].

2.14 Stability Study

The stability of sparfloxacin loaded NLCs were checked to assess the long-term usability of formulation by ICH guidelines Q1A. The stability study of formulation gives us idea about potential excipient reaction, long term drug stability, possible drug expulsion from formulation. Stability study also assess the any possibility of suspension breaking during storage of formulation. It also assesses the stability of formulation at different environment and storage condition. Briefly, 5 mL of sparfloxacin loaded NLCs was poured in glass vial and sealed properly. Then these vials were stored at room temperature (25 ± 2^0C/60% ± 5% RH), at accelerated condition (40 ± 2°C/75% ± 5% RH) and in refrigerator (5 ± 3^0C) for the duration of 3 months. The formulation was investigated every

month for change in particle size, zeta potential, and entrapment efficiency [26].

3. RESULTS AND DISCUSSION

3.1 Lipid and Surfactant Screening

Glyceryl monostearate, stearic acid, precirol ATO 5, and compritol 888 ATO these are suitable lipids for ocular drug delivery. Glyceryl monostearate is glycerol ester of stearic acid. It has self-emulsifying property. It is used in emulsifier in food industry, solid lipid for preparation of lipid nanoparticles. Sparfloxacin is highly lipophilic drug. Glyceryl monostearate is glycerol ester of stearic acid with 21 carbons. It has long lipophilic hydrocarbon chain which is responsible for solubilization of lipophilic drug such as Sparfloxacin. The glyceryl moiety present on GMS provides slight hydrophilicity to the compound. The sparfloxacin also contain carboxylic group. The hydrogen bond between carboxylic acid group and hydroxy group on glyceryl monostearate enhances solubility of drug in the lipid [27]. The solubility study shows that sparfloxacin has least solubility in stearic acid. Amongst the four lipids precirol ATO 5 and compritol 888 ATO has 1.28±0.27 mg/g and 2.63±0.34 mg/g of solubility of drug. Solid lipid screening study reveals that the glyceryl monostearate can solubilize more drug than other with solubility 4.26±0.26 mg/g.

When liquid lipids present in the core of lipid nanoparticles it creates imperfection in crystal structure of lipid core in the cooling process. The amorphous core of nanostructured lipid carriers prevents drug expulsion from the core, and it provides more drug loading capacity to the lipid system. Therefore, assessment of solubility of drug in the liquid lipid is important concert for the further study. Oleic acid, isopropyl myristate, paraffin oil and corn oil were assessed for their ability to solubilize the model drug. Out of the four liquid lipids, oleic acid shows the maximum ability to dissolve the sparfloxacin i.e., 0.597±0.06 mg/g. The solubility data for all liquid lipid summarized in table. The solubility of sparfloxacin in oleic acid attributed to is lipophilic nature. Oleic acid has 18-carbon long hydrocarbon chain. The hydrocarbon chain also contains unsaturation at 9^{th} position which enhances its lipophilic nature [28]. Therefore, oleic acid is selected as liquid lipid for the formulation development.

Various surfactants were screened for the solubility study. Tween 80, Tween 20, Cremophore EL and Kolliphore RH40 were studied for their ability to dissolve the drug. Chemically Tween 20 and Tween 80 are polyoxyethelene sorbitan monolaurate and polyoxyethylene sorbitan monoleate, respectively. Out of four surfactants Tween 80 has the least ability to solubilize drug. The surfactant with more solubility of drug may hamper the drug loading and responsible for the drug expulsion. Therefore, the surfactant with less solubility of drug in it were selected for the further study. The solubility of sparfloxacin in Tween 80 is 0.230±0.06 mg/g.

3.2 Optimization of Spar-NLC

Factorial design was selected for the optimization of formulation excipients and process parameters. Three factors i.e., Liquid lipid concentration, surfactant concentration and sonication time were selected. Total 9 batches were prepared by using a factorial design of 3 factors at 2 levels to obtain optimization of independent variables to prepare Sparfloxacin loaded NLCs with desired results. By observing the results obtained by factorial design the best suitable model illustrated to see the impact of various variables on formulation parameters. Based on the model suggestion, a polynomial equation is derived to correlate the various formulation factors to the responses obtained by ANOVA test. To get the optimum value of factors to get desirable responses, statistical relationship between factors and responses were generated using trial version of design expert statistical software. The equation for each response was calculated by least square regression method using design expert statistical software.

3.3 Effect of Variables on Particle Size and PDI

The surfactant concentration and sonication show negative effect on particle size. As the surfactant concentration increases from 1% in F2 to 3% F3, particle size decreases from 182.6nm to 158.5 nm, respectively. This explains that increase in surfactant concentration leads to form smaller NLCs. Same as the sonication time increases form 4 min in F2 to 8 min in F4, particle size decreases from 182.6 to 168.5 nm. Therefore, it concluded that sonication breaks the NLCs particles into smaller ones. The software shows liquid lipid concentration (A) does not have significant effect on particle size. Liquid lipid concentration (A) exhibits negative effect and surfactant concentration (B) exhibit

positive effect on polydispersity index (PDI). As liquid lipid concentration increases from 30% in F2 to 50% in F1, PDI decreases from 0.343 to 0.244. As surfactant concentration increases from 1% in F2 to 3% in F3, the PDI also increases from 0.343 to 0.412.

3.4 Effect of Variables on Zeta Potential

The zeta potential of all formulations was measured using Malvern zeta sizer. The zeta potential of all 9 batches were ranged from -52 mV to -43.1 mV. The analysis done by software indicated direct relationship of surfactant concentration (B) and liquid lipid concentration (A) with zeta potential (Y3). The surfactant concentration and liquid lipid concentration (A) show positive effect on zeta potential (Y3). As the surfactant concentration increases from 1% in F2 to 3% F3, zeta potential (Y3) increases from -56 mV to -49 mV, respectively. This explains that increase in surfactant concentration leads to increase in surface potential on NLCs. Same as the liquid lipid concentration (A) increases form 30% in F2 to 50% in F8, zeta potential increases from -56 mV to -48.3 mV. This explains that increase in liquid lipid concentration leads to increase in surface potential on NLCs. The software shows sonication does not have significant effect on zeta potential.

3.5 Effect of Variables on Entrapment Efficiency

The entrapment efficiency of all formulations was measured using formula. The entrapment efficiency of all 9 batches were ranged from 82% to 95.7%. The analysis done by software indicated direct relationship of surfactant concentration (B) and liquid lipid (A) with entrapment efficiency (Y4). The liquid lipid concentration (A) shows positive effect on entrapment efficiency. As the liquid lipid concentration increases from 30% in F3 to 50% F9, entrapment efficiency increases from 83.6% to 88.92%, respectively. This explains that increase in liquid lipid concentration leads to more drug loading in NLCs. As surfactant concentration increases form 1% in F2 to 3% in F6, entrapment efficiency decreases from 91.93% to 82.5 %. The software shows sonication (C) does not have significant effect on entrapment efficiency.

3.6 Estimation of Quantitative Effect of the Variables on NLCs

To estimate quantitative effect of factors on the responses, student t-test was performed. In Table 5 factor effects in factorial design and *p*-values of all responses resulted from ANOVA test was represented. If any factors affect the response, the value of significantly differ from zero and the *p*-value less than 0.05. A positive value represents synergistic effect of variable on the response and negative value represents antagonist effect of factor on response. The statistical data shows that liquid lipid (A) has significant synergistic effect on zeta potential (Y3) and entrapment efficiency (Y4) with *p*-values of 0.0074 and 0.0007, respectively. Surfactant concentration (B) has significant antagonist effect on particle size (Y1) and entrapment efficiency (Y4) with *p*-values of 0.0002 and <0.0001, respectively. While on zeta potential (Y3) it has positive effect with *p*-value of 0.0133. The third independent variable i.e., sonication time has antagonist effect on particle size and the *p*-value is 0.0058.

3.7 DSC Analysis

The thermal analysis of sparfloxacin loaded NLCs were studied by differential scanning calorimetry (DSC). The DSC data give us idea about the melting point, crystallinity, and degradation of sample. The DSC of pure sparfloxacin was obtained from literature. The DSC thermogram of sparfloxacin shows sharp endothermic peak at 262^0C which indicates the melting point of sparfloxacin, and narrow peak indicates its crystallinity. DSC thermogram of glyceryl monostearate obtained from literature [25]. The thermogram shows melting endotherm of glyceryl monostearate starts at 50.62^0C, peak at 55.76^0 C, and ends at 57.10^0C. The DSC thermogram of developed formulation displayed in Fig. 5. It shows onset of melting endotherm at 97.85^0C, peak at 104.83^0C, and end set at 112.27^0C. Thermogram of sparfloxacin loaded NLCs does not show any peak sparfloxacin peak at 262^0C. The thermogram also shows higher melting point than the glyceryl monostearate. This is due to dissolved state of sparfloxacin in lipid core of NLCs and amorphous nature of lipid core. The absent of sparfloxacin peak in formulation thermogram and shifting melting point to higher temperature indicate successful dissolution and encapsulation of drug in NLCs. DSC thermogram of formulation is broader than the pure glyceryl monostearate which is

amorphous in nature. This is due to homogeneous mixture of solid lipid and liquid lipid in the NLCs which forms amorphous lipid core [12].

3.8 TEM Analysis

The shape and surface morphology of sparfloxacin 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 Fig. 6. The images suggested the particle size of sparfloxacin 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 greyish coloration of NLCs may be due to the encapsulation of drug in NLCs core [29].

3.9 *In vitro* Release Study of 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 sparfloxacin 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 sparfloxacin loaded NLCs was obtained by plotting % cumulative release verses time.

The graph of In-vitro release study of pure drug suspension and drug loaded NLCs were displayed in Fig. 7. Sparfloxacin is BCS class II drug which has low solubility and high permeability. The main problem in absorption of sparfloxacin is dissolution of drug in release medium. The pure drug suspen**s**ion dissolution study shows that the only 32.18± 3.45**%** of total drug is dissolved in medium in 12 hrs. This may be due to the saturation of medium [30]. The sparfloxacin loaded NLCs suspension releases about 81.35± 3.67% of drug in 12 hrs. This is due to the smaller size of NLCs. In pure drug suspension the sparfloxacin is in the crystalline state, therefore it takes more time to dissolve the drug. As in NLCs the sparfloxacin is in dissolved in lipid core and the lipid core of NLCs is amorphous in nature, therefore the dissolution of drug from NLCs is better than the drug suspension.

Kinetic models for in-vitro release study of sparfloxacin 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 values of all models are tabulated in table. The R^2 value of Korsmeyer peppas model for Sparfloxacin loaded NLCs suspension is 0.9966 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 sparfloxacin loaded NLCs. The dissolution of drug is dependent on diffusion of drug through lipid matrix and swelling of lipid matrix.

3.10 *Ex-vivo* Permeation Study

The results of ex-vivo release study was showed in Fig. 8. The ex-vivo release study shows that 54± 2.67% of drug release from the sparfloxacin loaded NLCs suspension in 8 hours. The pure drug suspension showed only 31.22± 2.74% of drug release in 8 hours. The main problem in drug permeation in ex-vivo study is epithelial barrier. The structure of epithelium of ocular tissue contains lipid bilayer. sparfloxacin loaded NLCs contain drug enriched lipid core. The lipids present in NLCs provides better permeation of drug through the epithelium of ocular membrane. Therefore, it enhances permeation of drug through membrane than normal drug suspension. The smaller size of NLCs and amorphous nature of drug in lipid core also enhances the drug release and permeation through membrane [31].

Sparfloxacin loaded NLCs has better penetration than the drug suspension. This is due to the lipidic core of NLCs which help in penetration of drug through the ocular membrane. The permeation parameters of sparfloxacin loaded NLCs such as steady state flux (Jss) and permeability coefficient (Paap) are significantly better when compared to pure drug suspension. The values of steady state flux and permeability coefficient of pure drug suspension are 26.079 µg cm^{-2} hr^{-1} and 0.08693 cm hr^{-1} respectively. For sparfloxacin loaded NLCs the values of steady state flux and permeability coefficient are

44.482 µg cm^{-2} hr^{-1} and 0.1482 cm hr^{-1} respectively. The enhancement ratio of sparfloxacin loaded NLCs over pure drug suspension is 1.7048.

3.11 *In-vivo* Ocular Irritancy Test (Draize Test)

The Draize test evaluate the degree of irritation of excipients in formulation to the ocular membrane. The ocular irritancy score was divided in four grades. For non-irritating formulation, the score was 0-3, for slightly irritating substance 4-8, for moderately irritating substance 9-12 and the substances which were severely irritating the score was 13-16. The test formulation and control did not show much difference on ocular membrane Fig. 9. The eye which was treated with test formulation did not show any harm to cornea, iris, and conjunctiva. No watery drainage, redness in the eye or swelling were observed in both control and test eye. This test represents good tolerability of ocular tissue for the test formulation and harmless for ocular tissue.

3.12 Antimicrobial Study

The activity of Sparfloxacin loaded NLCs was compared with pure drug suspension and marketed ciprofloxacin eye drop. The shows that sparfloxacin possess the antimicrobial activity against the *Staphylococcus aureus*. The zone of inhibition was measured by zone reader. The zone of inhibition of pure drug suspension was found to be 21 mm and the zone of inhibition of sparfloxacin loaded NLCs was found to be 33 mm. Marketed formulation of ciprofloxacin showed 32 mm of zone of inhibition. From the results it is observed that sparfloxacin loaded NLCs has better zone of inhibition than the pure drug suspension. This result attributed to better penetration of drug, sustained release, and enhanced uptake of drug through sparfloxacin loaded NLCs. Sparfloxacin loaded NLCs and marketed ciprofloxacin eye drop does not show measure difference in antimicrobial activity. This conclude that the developed formulation has equivalent activity as other marketed formulation.

Table 1. Excipients used in screening

Solid lipids	Liquid lipids	Surfactants
Compritol 888 ATO (Glyceryl dibehenate)	Oleic acid	Tween 80 (Polysorbate 80)
Stearic acid	Isopropyl myristate	Tween 20 (Polysorbate 20)
Glyceryl Monostearate (GMS)	Paraffin oil	Cremophore EL (Propane-1,2,3-triol)
Precirol ATO 5 (Glyceryl palmitostearate)	Corn oil	Kolliphore RH 40 (Polyoxyl castor oil)

Table 2. Independent factors for optimization of formulation

Factor	Low level (-1)	High level (+1)
Liquid lipid conc. (%)	30	50
Surfactant conc. (%)	1	3
Sonication time (min)	4	8

Table 3. Regression equations of fitted models of sparfloxacin loaded NLCs

Responses	Equations
Mean particle size (Y1)	+214.47500 – 15.11250 * surfactant – 3.61875 * sonication
Polydispersity index (Y2)	+0.383250 – 0.002975 * liquid lipid + 0.034125 * surfactant
Zeta potential (Y3)	-61.0500 + 0.171667 * liquid lipid + 2.22500 * surfactant
Entrapment efficiency (Y4)	+91.52250 + 0.143583 * liquid lipid – 4.09625 * surfactant

Table 4. Experimental design with actual values of factors with responses obtained for critical quality attributes from experimental batches

Formulation no.	A Liquid lipid (%)	B: Surfactant (%)	C: Time of Sonication (min)	Y1: Size (nm)	Y2: PDI	Y3: Zeta (mV)	Y4: EE (%)
1	50	1	8	171	0.244	-49	95.72
2	30	1	4	182.6	0.343	-56	91.93
3	30	3	4	158.5	0.412	-49	83.6
4	30	1	8	168.5	0.32	-51.1	92.52
5	50	3	8	145	0.361	-43.1	87.76
6	30	3	8	136.7	0.374	-49.7	82.5
7	40	2	6	165	0.398	-52	92.88
8	50	1	4	188.5	0.227	-48.3	95.38
9	50	3	4	149.5	0.26	-44.8	88.92

Table 5. ANOVA summary of models for sparfloxacin loaded NLCs

Response	*p*-value for model	R-squared	Adjusted R-squared	Adequate- precision (S/N ratio)
Particle size	0.0004	0.9578	0.9409	15.069
Polydispersity index	0.0179	0.7997	0.7196	6.7122
Zeta potential	0.0063	0.8682	0.8155	8.5857
Entrapment efficiency	<0.0001	0.9803	0.9724	22.6043

Table 6. Optimized spar-NLC formulation

Factor	Optimal values	Responses	Observed values
Liquid lipid concentration	45%	Particle size	170.1
Surfactant concentration	2%	Zeta potential	-47.5
Sonication	6 min	Entrapment efficiency	89.5

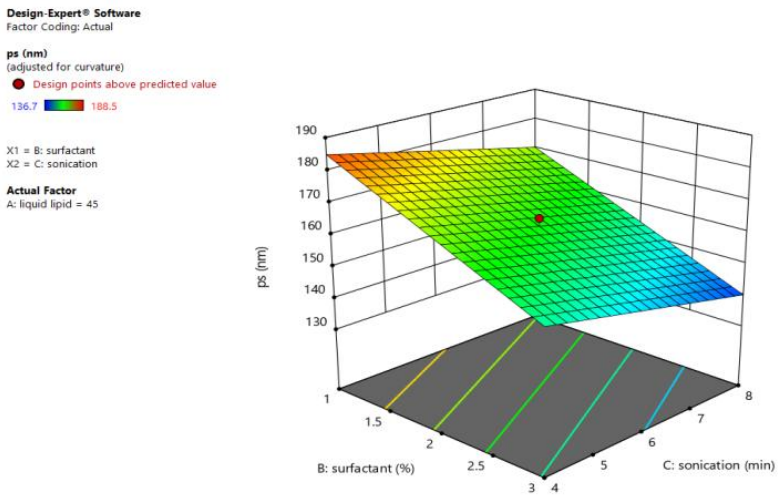


Fig. 1. Response surface 3D plot illustrating effect of surfactant concentration (B) and sonication (C) on particle size (Y1)

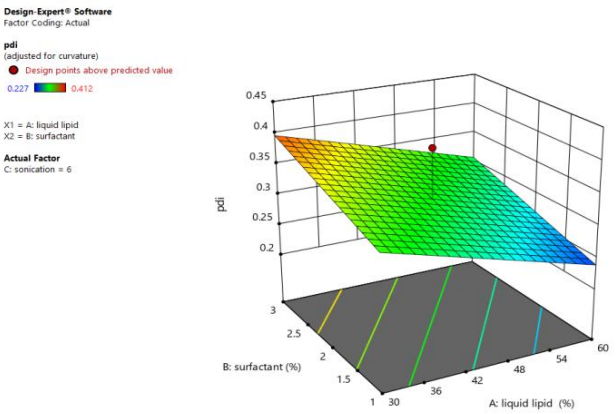


Fig. 2. Response surface 3D plot showing the effect of surfactant concentration (B) and liquid lipid concentration (A) on polydispersity index (Y2)

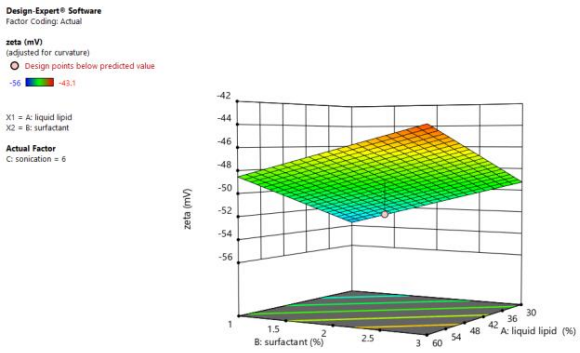


Fig. 3. Response surface 3D plot showing the effect of liquid lipid concentration (A) and surfactant concentration (B) on zeta potential (Y3)

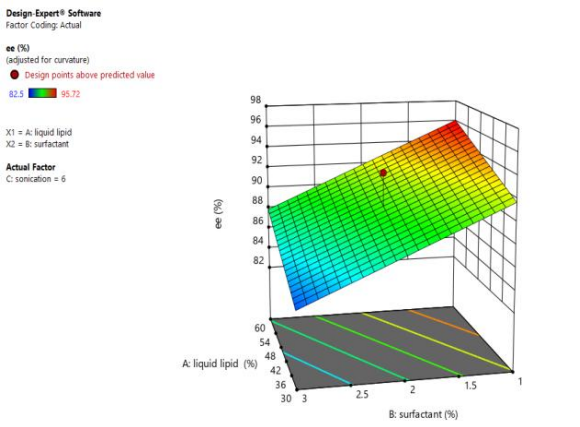


Fig. 4. Response surface 3D plot illustrating the effect of liquid lipid concentration (A) and surfactant concentration (B) on entrapment efficiency (Y4)

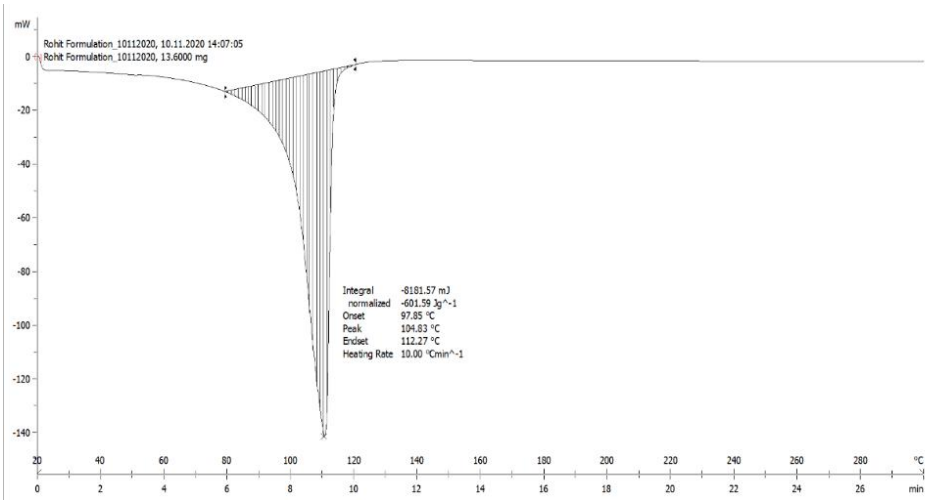


Fig. 5. DSC thermogram of sparfloxacin loaded NLCs

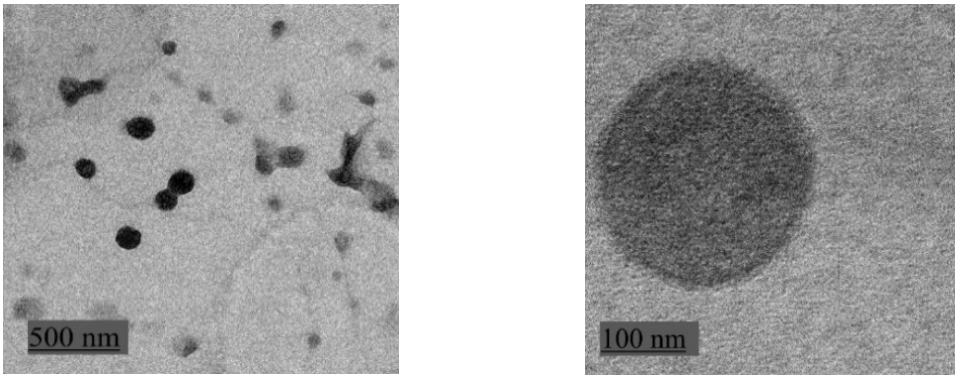


Fig. 6. Transmission electron microscopy (TEM) analysis of sparfloxacin loaded NLCs

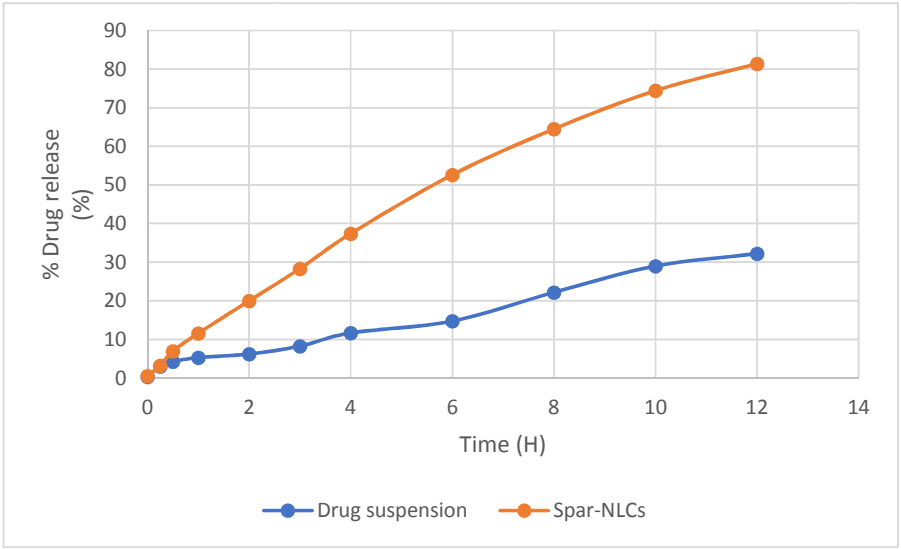


Fig. 7. *In-vitro* release profile of pure drug suspension and Spar-NLCs suspension

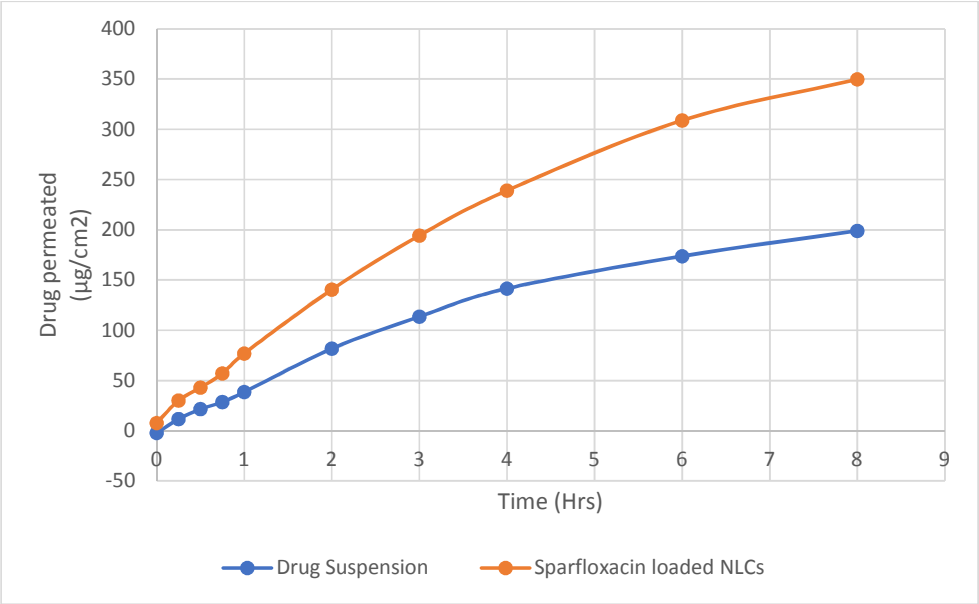
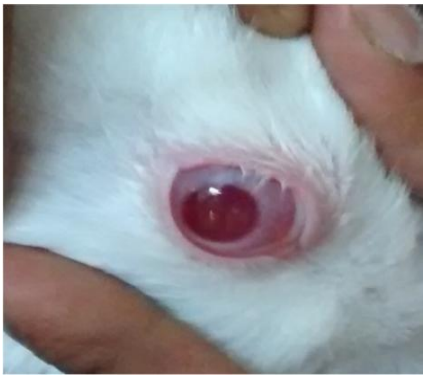
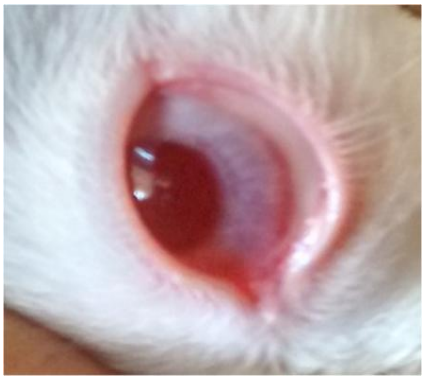


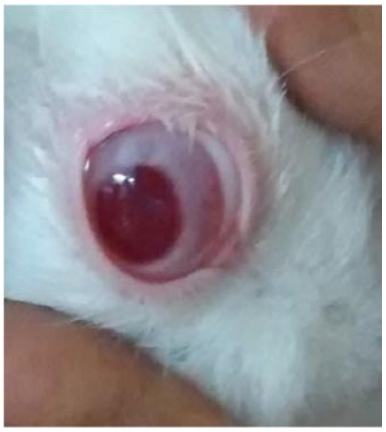
Fig. 8. Permeation of pure drug suspension and sparfloxacin loaded NLCs



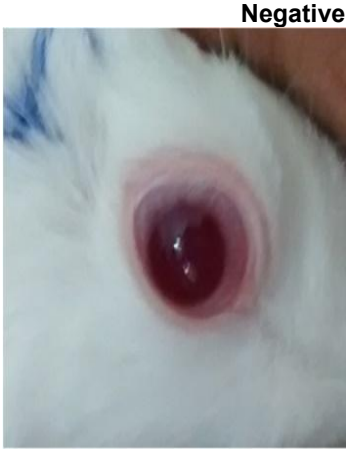
Positive control



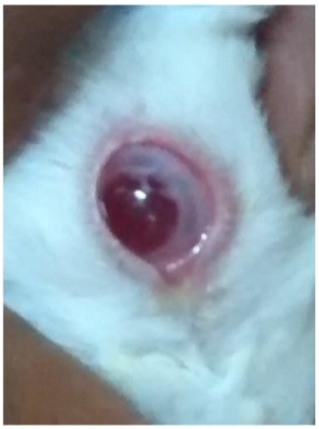
Negative Control



Test (After 1 Hr)



Test (After 24 Hrs)



Test (After 7 Days)

Fig. 9. Images of ocular irritation study

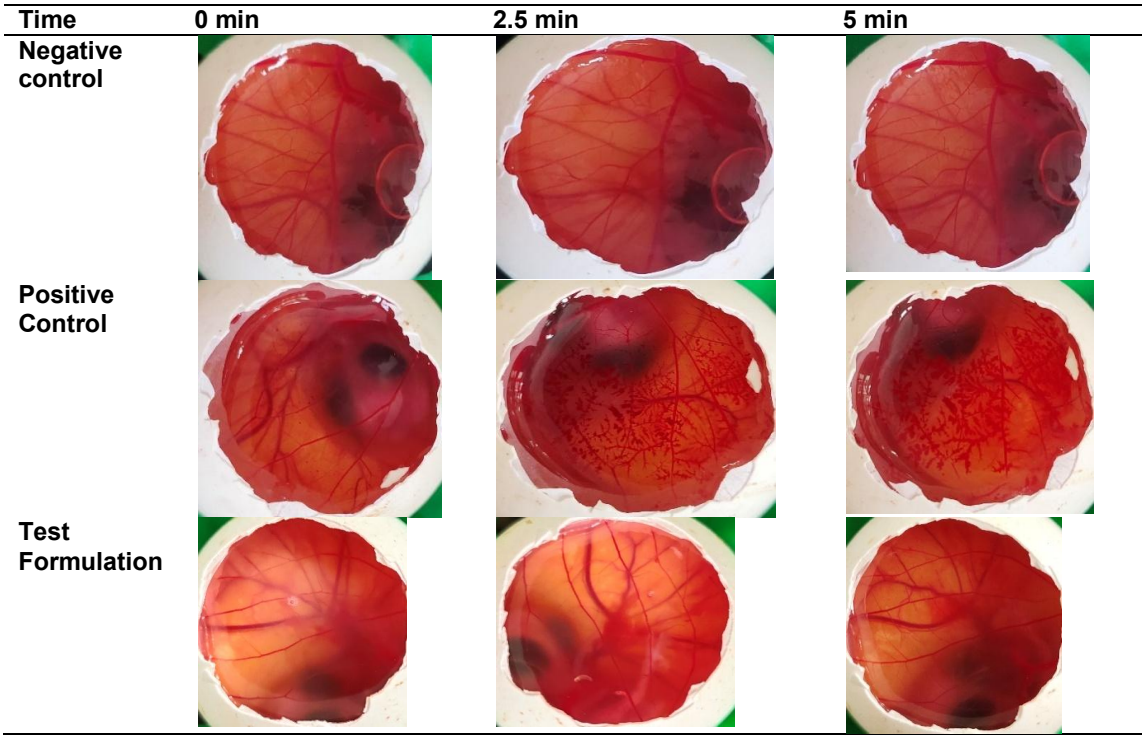


Fig. 10. Ocular tolerance test (HET-CAM test) of sparfloxacin loaded NLCs

3.13 Ocular Tolerance Test (HET-CAM Test)

HET-CAM test is inexpensive, qualitative, and rapid test for ocular tolerance which does not have any legal and ethical obligation[23]. The negative control i.e., 0.9% NaCl does not show any hemorrhage, blood clotting or vessel damage. The images displayed in Fig. 10 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 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. 0.2 mL of sparfloxacin 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 sparfloxacin loaded NLCs suspension is nonirritant to the Hens Egg Chorioallantoic Membrane and safe to use for the ocular drug delivery.

3.14 Stability Study

The results show that increase in particle size at 5 ± 3^0C from 170.1 nm to 189.7 nm over the period of 3 months. The entrapment efficiency decreases from 89% to 76.2%. The change in particle size and entrapment efficiency at room temperature is more than that of refrigerator temperature. The particle size increases from 170.1 nm to 196.3 nm over the period of 3 months. The decrease in entrapment efficiency at room temperature from 89% to 70.7% over the period of 3 months. The particle size increases at accelerated condition was from 170.1 nm to 210.5 nm over the period of 3 months. The decrease in entrapment efficiency at room temperature from 89% to 64.5% over the period of 3 months. The increase in particle size during storage condition is due to the formation of more stable modification of lipids in formulation and forming larger more stable particles. The decrease in entrapment efficiency of formulation is due to the leakage of drug from lipid core to the medium [26]. Leakage of drug from

formulation is slower when the formulation is stored in refrigerator.

4. CONCLUSION

Within the limit of experimental design, the study achieved following outcomes. Successful development and optimization of sparfloxacin loaded nanostructured lipid carriers for ocular delivery of drug. Characterization of sparfloxacin loaded nanostructured lipid carriers concluded desired formulation attributes. In-vitro and ex-vivo study suggested developed formulation has good efficacy, sustained release, and better penetration through ocular membrane. Developed formulation displayed better antimicrobial activity and non-irritant to ocular membrane. Hence the sparfloxacin loaded nanostructured lipid carriers can be proved to be effective alternative for currently available therapeutic approaches to conjunctivitis.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The institutional animal ethical committee guidelines were severely followed to carry out experiments (CCP/IAEC/Feb 2021/19).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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