International Journal of Biological Macromolecules 85 (2016) 258–270

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Application of Box–Behnken design for preparation of levofloxacin-loaded stearic acid solid lipid nanoparticles for ocular delivery: Optimization, *in vitro* release, ocular tolerance, and antibacterial activity

Mirza Salman Baig [a], Abdul Ahad [b], Mohammed Aslam [a], Syed Sarim Imam [c], Mohd. Aqil [a,*], Asgar Ali [a]

[a] *Faculty of Pharmacy, Jamia Hamdard (Hamdard University), M.B. Road, New Delhi 110062, India*
[b] *Department of Pharmaceutics, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia*
[c] *Glocal School of Pharmacy, Glocal University, Saharanpur, 247121 Uttar Pradesh, India*

ARTICLE INFO

Article history:
Received 27 November 2015
Received in revised form
17 December 2015
Accepted 22 December 2015
Available online 29 December 2015

Keywords:
Box–Behnken design
Solid lipid nanoparticles
SLN
HET-CAM
Levofloxacin
Ocular drug delivery
Optimization
Antibacterial activity

ABSTRACT

The aim of the present study was to develop and optimize levofloxacin loaded solid lipid nanoparticles for the treatment of conjunctivitis. Box–Behnken experimental design was applied for optimization of solid lipid nanoparticles. The independent variables were stearic acid as lipid (X_1), Tween 80 as surfactant (X_2) and sodium deoxycholate as co-surfactant (X_3) while particle size (Y_1) and entrapment efficiency (Y_2) were the dependent variables. Further *in vitro* release and antibacterial activity *in vitro* were also performed. The optimized formulation of levofloxacin provides particle size of 237.82 nm and showed 78.71% entrapment efficiency and achieved flux 0.2493 μg/cm^2/h across excised goat cornea. *In vitro* release study showed prolonged drug release from the optimized formulation following Korsmeyer–Peppas model. Antimicrobial study revealed that the developed formulation possesses antibacterial activity against *Staphylococcus aureus*, and *Escherichia coli* equivalent to marketed eye drops. HET-CAM test demonstrated that optimized formulation was found to be non-irritant and safe for topical ophthalmic use. Our results concluded that solid lipid nanoparticles are an efficient carrier for ocular delivery of levofloxacin and other drugs.

1. Introduction

Topical drug delivery into eyes is the most popular and accessible route of administration for the treatment of various eye diseases including bacterial conjunctivitis. However, one of the major disadvantages of conventional ophthalmic drug delivery is the low drug bioavailability because of factors like lachrymation, nasolachrymal drainage and metabolic degradation [1,2]. Because of such factors, the residence time of common commercial eye drops for topical application is no more than 5 min. Moreover, the relative impermeability of cornea leads to only 1–5% of the applied drug penetrating into intraocular area [3,4]. It is being proposed that ocular therapy would be significantly improved if the precorneal residence time of drug could be increased. It is also generally agreed that for patient compliance the preferred topical ophthalmic drug delivery system would be in the form of an eye drop, without causing blurred vision and irritation. In the recent years, research efforts have been focused on developing systems that would improve precorneal residence time to obtain a required bioavailability [1,5,6]. Solid lipid nanoparticles (SLN), are a good alternative carrier to traditional systems such as solutions, suspensions and ointments [7,8]. The mean diameter of SLN is in the range of approximately 50 and 1000 nm [9]. One of the advantages of SLN is the fact that the lipid matrix is made of physiologically compatible lipids, from natural or synthetic sources, which minimizes the danger of acute or chronic toxicity. Due to its nonirritant and nontoxic properties, SLN are regarded as one of the most suitable carriers in ocular remedies. Sustained and controlled drug release properties of SLN can be beneficial for ophthalmic preparations. The proposed ability of SLN to

* Corresponding author at: Department of Pharmaceutics, Faculty of Pharmacy, Jamia Hamdard (Hamdard University), M.B. Road, New Delhi 110062, India.
E-mail addresses: aqilmalik@yahoo.com (Mohd. Aqil), abdulahad20@yahoo.com, aahad@ksu.edu.sa (A. Ahad).

http://dx.doi.org/10.1016/j.ijbiomac.2015.12.077
0141-8130/

increase pre-corneal retention time may help to improve bioavailability of ocular drugs when compared with their conventional ophthalmic solutions [10–12]. SLN combines all the advantages of fat emulsion, polymeric nanoparticle and liposome while overcoming their shortcomings such as drug leakage, hydrolysis and un-stability drug storage [13,14].

Acute conjunctivitis is a prevalent infection, which affects many people and imposes economic and social burdens. It is estimated that acute conjunctivitis affects 6 million people annually in the United States [15,16]. The cost of treating bacterial conjunctivitis alone was estimated to be $377 million to $857 million per year [17,18]. Bacterial conjunctivitis is the second most common cause and is responsible for the majority (50–75%) of cases in children; it is observed more frequently from December through April [19–21]. Generally a treatment with ocular antibiotics is recommended to eradicate the pathogen.

Fluoroquinolones eye drops have been the most often recommended treatment of bacterial conjunctivitis. Levofloxacin, a third generation fluoroquinolone antibiotic shows good activity against *Staphylococcus aureus* on cornea and conjunctiva. The dosage regimen includes one drop in every 1–2 h for 3 days and then in every 4–5 h [22]. Levofloxacin, being a hydrophobic drug is therefore a suitable candidate for the formulation of SLN to develop a prolonged ocular drug delivery system with the rationality of reducing the frequency of dosing. Many researchers reported that treatment with SLN system increases the drug bioavailability, reduces administration frequency and promotes drug targeting for various ocular ailments [1,3,8,10,23–25]. In the present investigation, we attempted to develop and optimize SLN loaded with levofloxacin for improved retention time and permeation through cornea using a three-factor three-level Box–Behnken design.

2. Materials and methods

2.1. Materials

Levofloxacin hemihydrate was a kind gift from Algen Healthcare Ltd., Delhi, India. Stearic acid was procured from Qualikems Fine chem. Pvt., Ltd., Delhi, India. Tween 80 and acetonitrile were purchased from S.D. Fine-chem Limited, Mumbai, India. Sodium deoxycholate from Titan Biotech Ltd., Delhi, India. Dichloromethane was procured from Rashtriya Chemicals Corporation, Chandigarh, India. Methanol (HPLC grade) from Real Chemsys Products Pvt., Ltd., Ghaziabad, India. All other solvents and materials used were of analytical grades.

2.2. Screening of solvents

The solubility of levofloxacin in various solvents (water, chloroform, glacial acetic acid, simulated tear fluid, dichloromethane) was determined by adding excess amounts of drug in 3 ml of solvent in small vials. The vials were tightly stoppered and were continuously stirred to reach equilibrium for 72 h at 25 °C in a biological shaker. After that, the mixtures were centrifuged at 5000 rpm for 30 min. The supernatant was separated, dissolved in methanol and solubility was quantified by UV Spectrophotometer at 288.5 nm [26].

2.3. Screening of lipids

The solubility determination of levofloxacin in various lipids (Stearic acid, Glyceryl monosterate, Gelucire 39/1, Gelucire 50/13, Gelucire 54/14, Compritol 888 ATO) was performed by adding levofloxacin in increments of 1 mg until it failed to dissolve further in the molten lipids (which were heated at 10 °C above their melting point). Vials were vortexed meanwhile to assist solubilization. End point for the process was appearance of turbid solution [27].

Table 1
Variables in Box-Behnken design for formulation development of levofloxacin loaded SLN.

Variables	Levels		
	−1	0	+1
Independent variables			
X_1 = Lipid (% w/w)	4	6	8
X_2 = Surfactant (% w/w)	2	3	4
X_3 = Co-surfactant (% w/w)	1	2	3
Dependent variables			
Y_1 = Particle size (nm)			
Y_2 = Entrapment efficiency (%)			

2.4. Statistical experimental design

Box–Behnken statistical design with 3-factors, 3- levels, and 17 runs was employed for the optimization study using Design-Expert software (Design-Expert 8.0.5.2, State-Ease Inc., Minneapolis, USA). Lipid (X_1), surfactant (X_2) and co-surfactant (X_3) were selected as independent variables and they were set at low, medium and high levels on the basis of the results of initial trials. Table 1 summarizes the coded values of different variables.

In accordance with the design, 17 SLN formulations were prepared and characterized for particle size (Y_1), and entrapment efficiency (Y_2) which were chosen as response parameters (Table 1). This design explains the main effects and interaction effects of the independent variables on the formulation characteristics. Objective function for the present study was selected as maximizing entrapment efficiency, while minimizing particle size. Box–Behnken design was specifically selected, because it requires fewer runs than a central composite design, in cases of three or four variables [28–30].

Analysis of variance (ANOVA) was used to establish the statistical validation of the polynomial equations generated by Design Expert software. All the responses observed were simultaneously fitted to linear (first order), second order, and quadratic models. Various feasibilities were conducted over the experimental domain to find the compositions of the optimized SLN formulation. 3D response surface plots were generated by the Design Expert software, whereby intensive grid search performed over the whole experimental region. Five optimum checkpoint formulations were selected to validate the chosen experimental domain. The resultant experimental values of the responses were quantitatively compared to that of the predicted values.

2.5. Method of preparation of levofloxacin loaded SLN

Various SLN formulations of levofloxacin for ocular delivery were prepared by employing Box–Behnken design applying solvent evaporation technique [31] using following composition: Lipid *i.e.* Stearic acid (4–8% w/w), Surfactant *i.e.* Tween 80 (2–4% w/w), and Co-surfactant *i.e.* Sodium deoxycholate (1–3% w/w). Briefly, levofloxacin was dissolved in sufficient quantity of dichloromethane and then stearic acid was gradually added to the drug-dichloromethane solution by *vortexing* at room temperature (25 °C). Separately, aqueous phase was prepared in distilled water by solubilizing Tween 80 (surfactant) and sodium deoxycholate (co-surfactant) and probe sonicated for 6 min with 28% amplitude 500 W power output. During sonication, organic phase was gradually added (3 ml/min) for emulsification using syringe and needle.

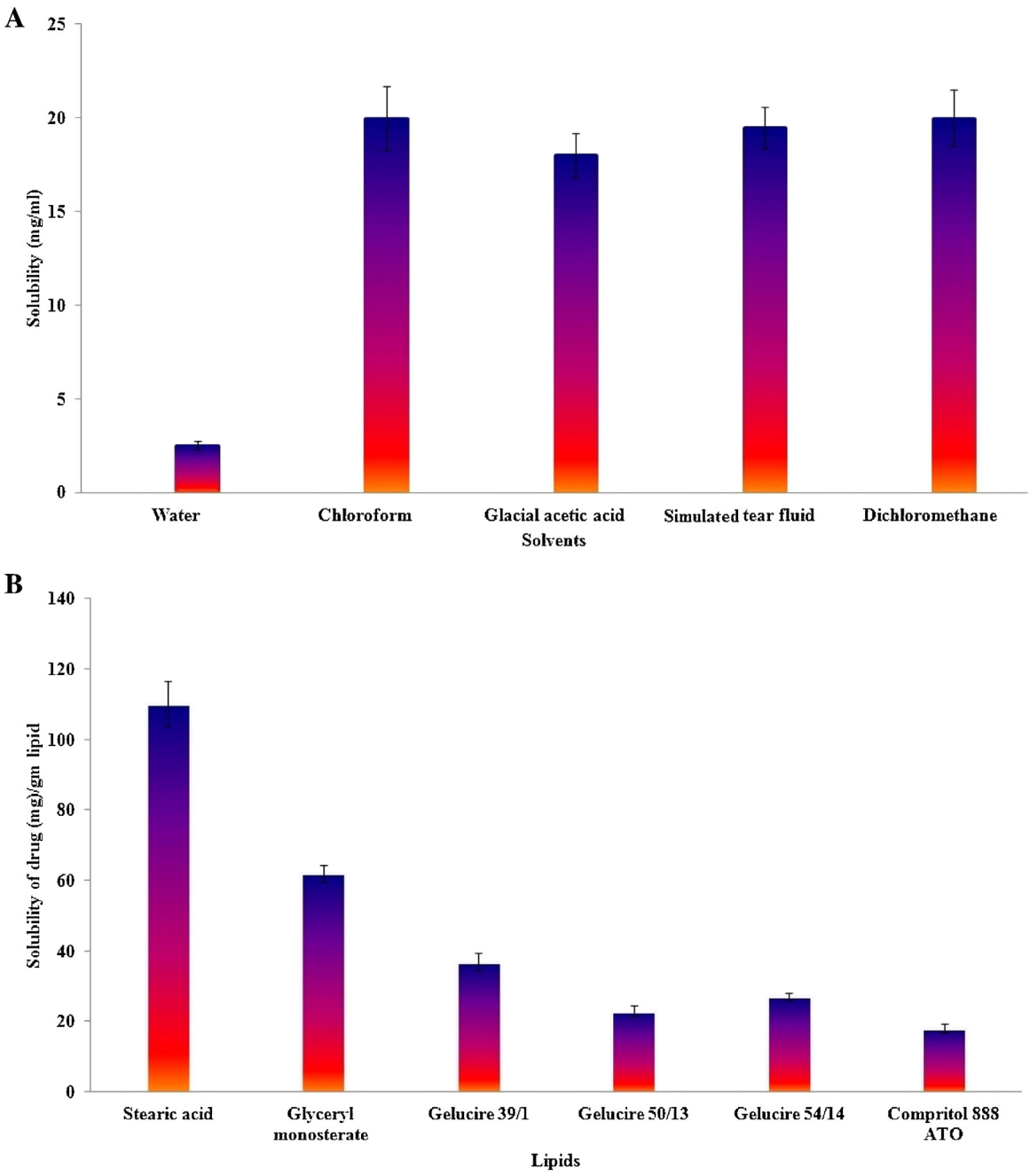

Fig. 1. Solubility of levofloxacin in various (A) solvents and (B) lipids.

2.6. Analysis of particle size and morphology of SLN

The particle size analysis of levofloxacin loaded SLN formulations was done by photon correlation spectroscopy (PCS), using a computerized inspection system (Zetasizer, HAS 3000; Malvern Instruments, Malvern, United Kingdom) at 25 ± 1 °C and at a scattering angle of 90°. Samples were appropriately diluted with double-distilled water previously filtered with 0.45 μm membrane filters before analysis.

Morphology of the prepared SLN was studied using transmission electron microscope. A drop of SLN was placed over the copper grid coated with carbon film.

Subsequently, drop of SLN stained with 2% phosphotungstic acid solution and dried at room temperature. SLN particles were visualized by using a Morgagni 268D transmission electron microscope (Fei Electron Optics), digital Micrograph and Soft Imaging Viewer software were used to perform the image capture and analysis.

2.7. Drug entrapment efficiency

For determination of entrapment efficiency the unentrapped drug in SLN formulations was separated by the use of the centrifugation method [32]. A known dilution of the SLN was prepared with double distilled water and was centrifuged at 14,000 rpm (Cooling Centrifuge, C24, REMI Instruments Ltd., Mumbai, India) for 40 min at 10 °C. The amount of drug entrapped into the SLN was the difference between the total amount used to prepare the SLN and the amount that was found in the supernatant. The amount of free drug in the supernatant was analyzed by UV-spectrophotometer

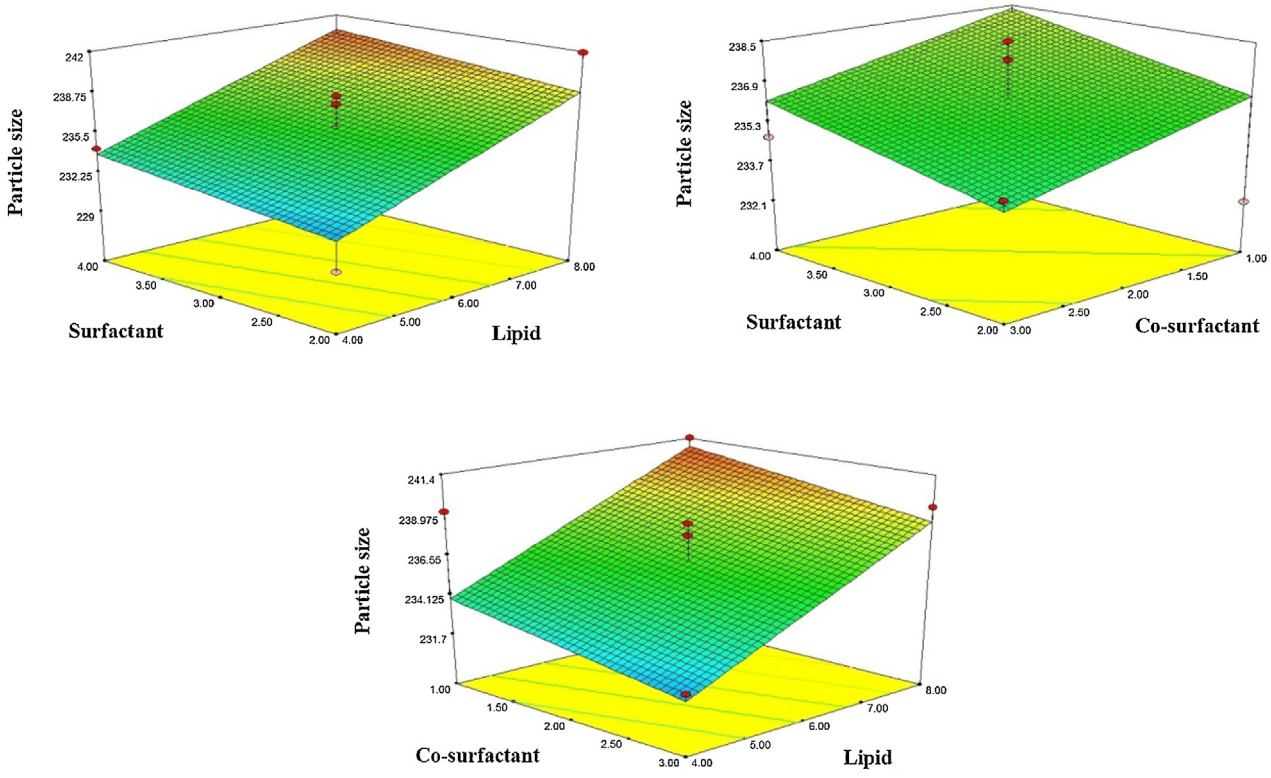

Fig. 2. Three-dimensional response surface plot showing effect of independent variables on SLN particle size.

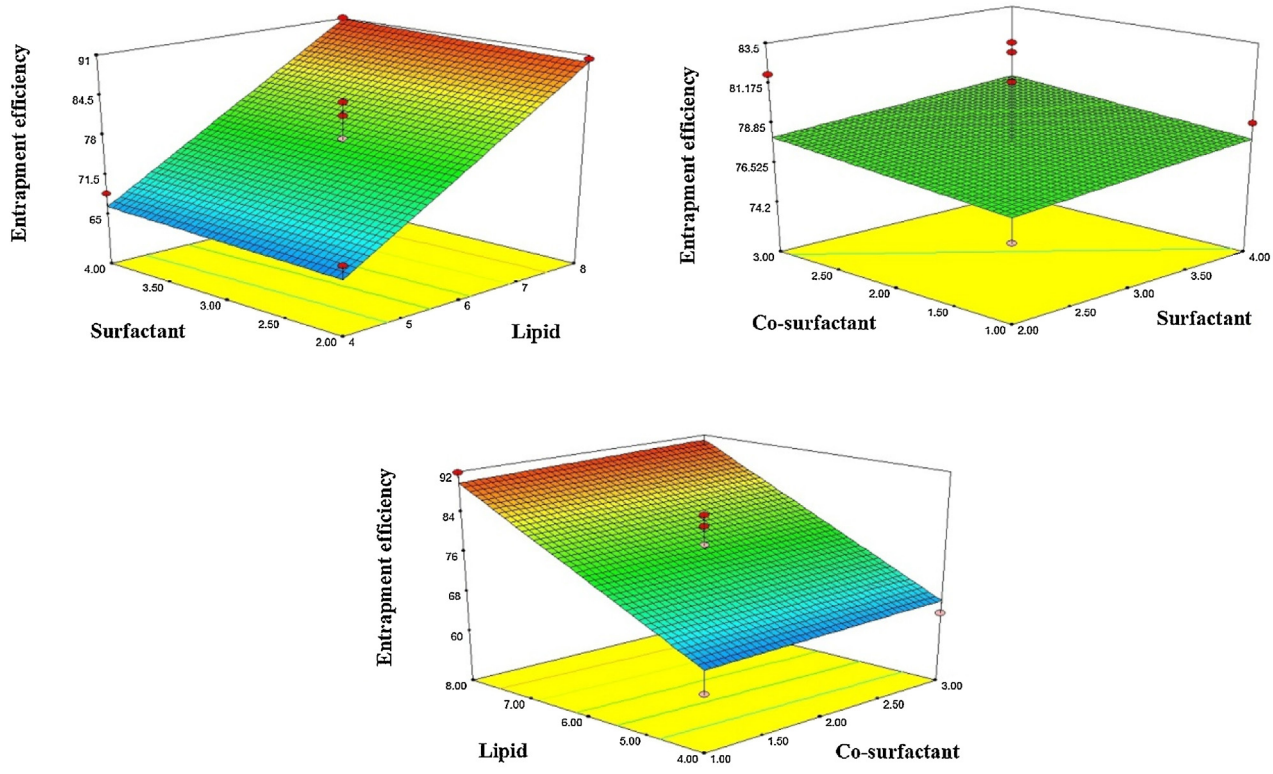

Fig. 3. Three-dimensional response surface plot showing effect of independent variables on SLN entrapment efficiency.

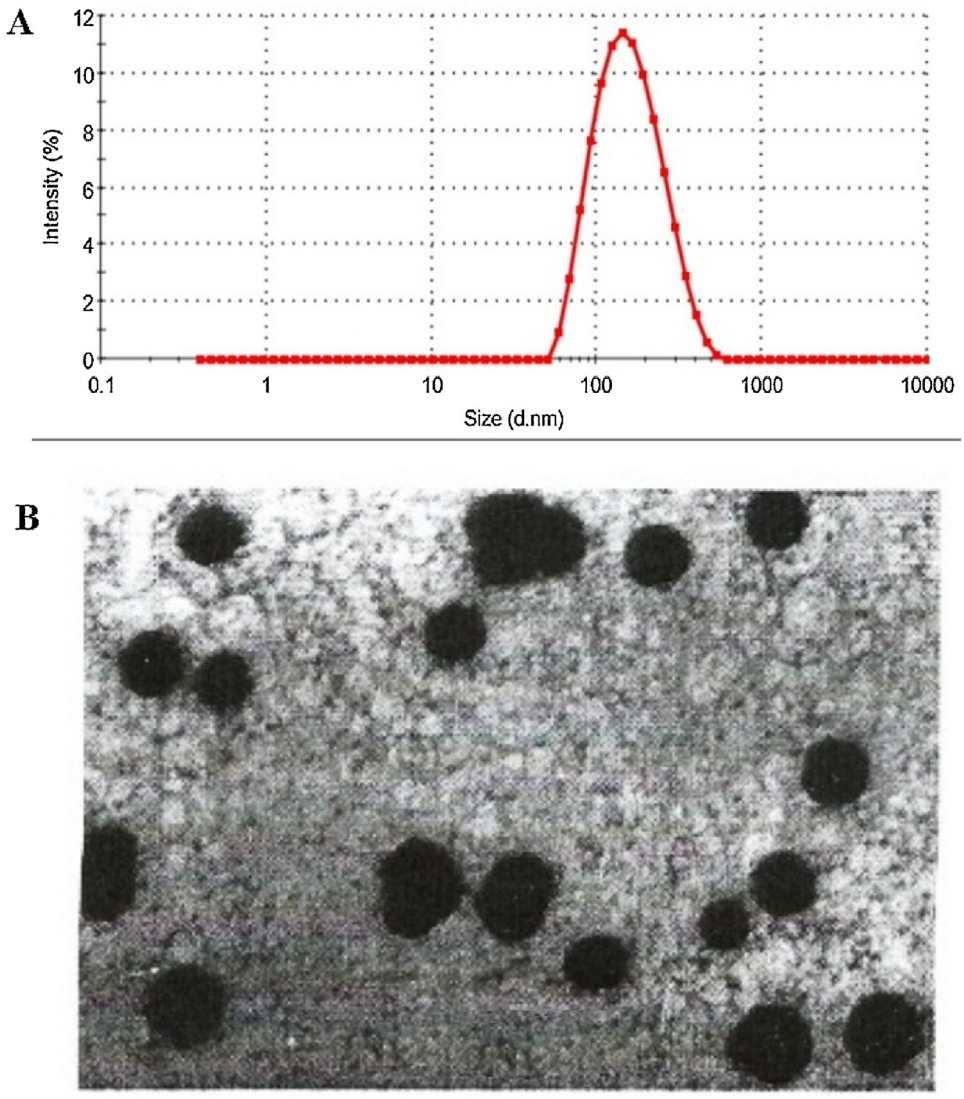

Fig. 4. (A) Graph showing particle size distribution and (B) Transmission electron micrograph of L-SLN-OPT.

at 288.5 nm [26]. The drug entrapment efficiency was calculated by the following equation.

Entrapment efficiency(Druginitial − DrugfreeDruginitial × 100

2.8. Differential scanning calorimetry

Differential scanning calorimetry (DSC) was performed to identify the crystal form of levofloxacin dispersed in the lipid matrix. Thermograms were recorded by means of DSC6 (Perkin Elmer, Uberlingen, Germany) for the identification of crystallinity. For calorimetric measurements, standard aluminum pans with accurately weighed 5 mg samples were taken and sealed in aluminum hermetic pans. Samples were heated at a scanning rate of 10 °C/min over a temperature range between 40–400 °C. An empty pan was used as reference. An inert atmosphere was maintained by purging with nitrogen (20/ml).

2.9. X-ray diffraction analysis

X-ray diffraction (XRD) was done to investigate the crystalline character of the formulated SLN. XRD pattern of physical mixture of levofloxacin and stearic acid, and freeze dried SLN were obtained using PANalytical, Netherlands, PW 3710 diffractometer. A voltage of 35 kV and a current of 30 mA for the generator were used with Cu as the tube anode material. The samples were exposed to a Cu–Kα radiation over a range of 2θ angles from 5° to 70°. At the rate of 0.25 second time per step.

2.10. In vitro drug release studies

To study the release kinetics, data obtained from *in vitro* release studies were fitted in various kinetic models. *In vitro* drug release studies were carried out using Franz diffusion cells using dialysis membrane. The dialysis membrane was treated with 0.35% w/v sodium sulfite solution in water at 80 °C for 1–2 min, then acidified with H_2SO_4 (0.2%, v/v) and then stored for 12 h in double distilled water. The SLN formulation (1 ml) in 0.9% NaCl solution and marketed eye drops solution was introduced into the donor compartment and the open ends of the apparatus sealed with parafilm to prevent evaporation. Simulated tear fluid (pH 7.4) was used as the receiver vehicle and was stirred with a magnetic bead throughout the experiment. Aliquots of 1 ml of the samples were withdrawn at predetermined time intervals and the withdrawn

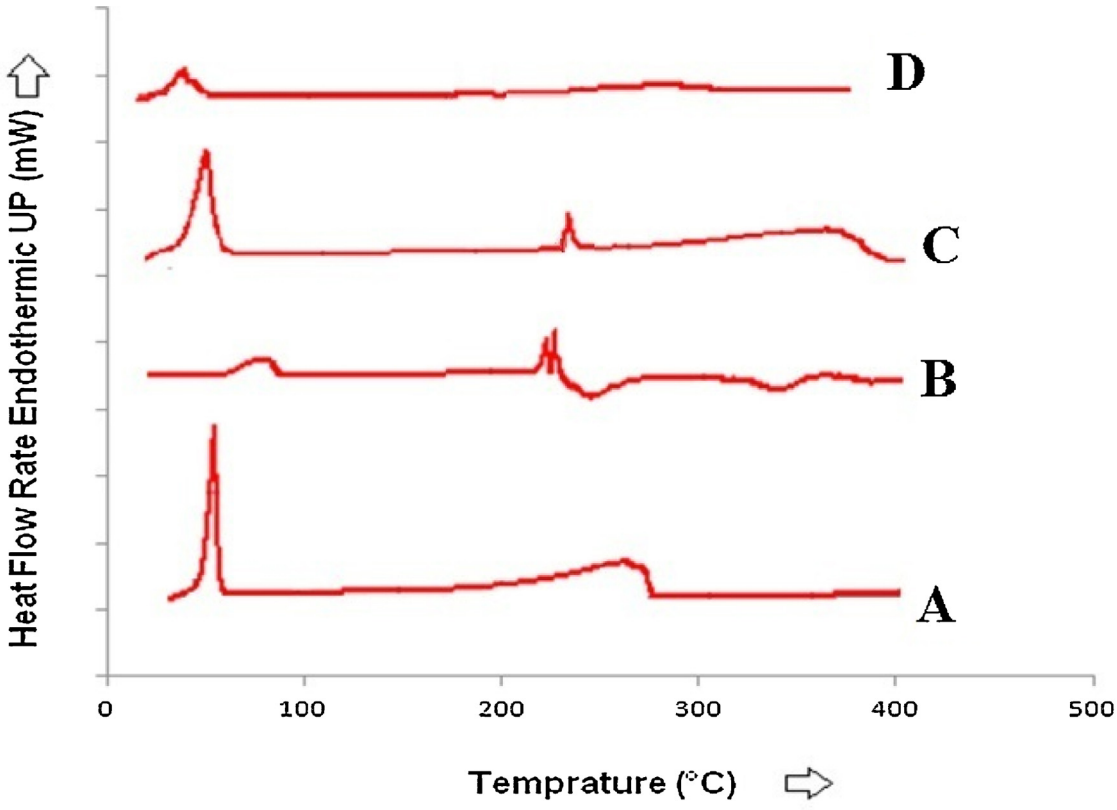

Fig. 5. DSC thermograms of (A) stearic acid (B) levofloxacin (C) stearic acid-levofloxacin physical mixture (D) lyophilized L-SLN-OPT.

Table 2
Scoring chart for HET-CAM test.

Effect	Scores	Inference
No visible hemorrhage	0	Nonirritant
Just visible membrane discoloration	1	Mild irritant
Structures are covered partially due to membrane discoloration or hemorrhage	2	Moderatelyirritant
Structures are covered totally due to membrane discoloration or hemorrhages	3	Severe irritant

HET-CAM, hen's egg chorioallantoic membrane.

samples analyzed for drug content by UV–vis spectrophotometer [26].

2.11. Ex vivo corneal permeation study

Freshly excised goat cornea was fixed between clamped donor and receptor compartments of Franz diffusion cell in such a way that its epithelial surface faced the donor compartment. SLN formulation was placed in donor compartment and it was covered with parafilm to prevent evaporation. Receptor compartment was filled with 10 ml of simulated tear fluid (pH 7.4) and was stirred with a magnetic bead during the experiment. Temperature of assembly was maintained at 37 °C. Corneal portion available for diffusion was 1 cm^2. The samples (1 ml) were withdrawn from the receptor compartment via sampling port at different time intervals *i.e.* 0, 0.25, 0.50, 0.67, 1, 2, 3 and 4 h and analyzed for drug content using UV spectrophotometer [26]. The receptor phase was immediately replenished with equal volume of fresh simulated tear fluid.

2.12. Antibacterial activity

In order to evaluate the antibacterial activity of levofloxacin-loaded SLN, the agar cup plate method was carried out against *S. aureus* and *Escherichia coli*. The method was based on inverse relation between minimum inhibitory concentration (MIC) and diameter of zone of inhibition. Zone of inhibition was found to be proportional to concentration of drug. A layer of nutrient agar (20 ml) was allowed to solidify in *Petri* plate then cultures of microbe in nutrient broth were transferred on solidified agar and culture was uniformly dispersed. SLN formulation and marketed eye drops were poured in cups. All these procedure was carried out aseptically; all *Petri* plates were kept at room temperature for 2 h to diffuse the drug into medium then incubated for 24 h at 37 °C. Diameter of zone of inhibition was noted at various time intervals [33,34].

2.13. Ocular tolerance test

For evaluating the ocular tolerability of the developed formulation, modified hen's egg chorioallantoic membrane (HET-CAM) test was carried out. The potential irritancy of compounds may be detected by observing adverse changes that occur in the CAM of the egg after exposure to test chemicals [35]. Briefly, fertilized hen's eggs were obtained from poultry farm. These selected eggs (50 and 60 g) were incubated in humidified incubator at a temperature of 37 ± 0.5 °C for 3 days. The eggs were rotated manually in a gentle manner after every 12 h. On the third day, 3 ml of egg albumin was removed from the pointed end of the egg by using sterile techniques. The hole was then immediately sealed by 70% alcohol sterilized parafilm with the help of a heated spatula. The eggs were kept away from the shell in the equatorial position for the development of CAM. The eggs were candled everyday and then on the fifth day, nonviable embryos were removed. On the tenth day CAM was exposed at the equator of the eggs through which formulations (100 μl) were instilled directly onto the CAM surface and left in contact for 5 min. The membrane is examined for vascular damage and the time taken for injury to occur is recorded. Saline

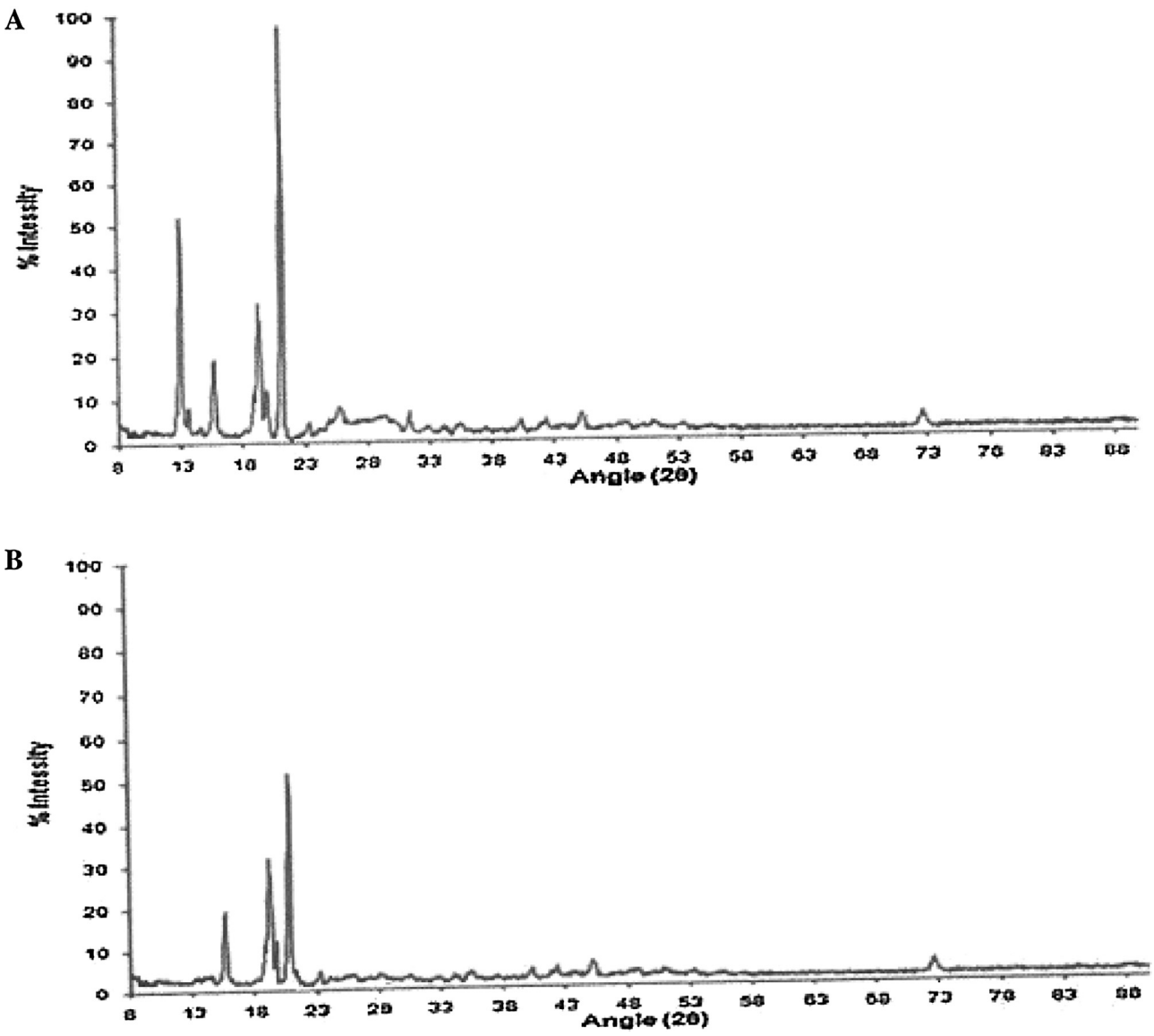

Fig. 6. XRD diffractograms of (A) stearic acid-levofloxacin physical mixture and (B) lyophilized L-SLN-OPT.

solution (0.9% NaCl w/v) was used as a control as it is reported to be practically nonirritant. The scores were recorded according to the scoring schemes as shown in Table 2.

3. Results and discussion

3.1. Screening of components

Solubility of levofloxacin in various solvents and lipids is given in Fig. 1. As per the results of solubility studies, levofloxacin showed maximum solubility in dichloromethane (20 mg/ml). Screening of lipid was performed to find out the lipid which could give maximum drug loading.

As shown in Fig. 1, stearic acid was the only lipid in which drug solubility was found to be 110 mg/g of lipid; hence stearic acid was selected for preparation of SLN. Also solubility of stearic acid with dichloromethane was determined; 350 mg of stearic acid found to be completely soluble in l ml of dichloromethane at room temperature. In addition levofloxacin also presented maximum solubility in dichloromethane hence stearic acid as lipid and dichloromethane as solvent were selected for the preparation of levofloxacin loaded SLN formulations.

3.2. Characterization of SLN

A total 17 runs were generated by using the Design Expert software (Design-Expert 8.0.5.2, State-Ease Inc., Minneapolis, USA) and the response parameters (particle size and entrapment efficiency) so observed are shown in Table 3.

All the responses observed for 17 formulations prepared were simultaneously fitted to first order, second order and quadratic models using Design Expert®. It is evident that all the three independent variables, namely the lipid (X_1), surfactant (X_2), and co-surfactant (X_3) have interactive effects on the two responses, e.g., Y_1; particle size (nm), Y_2; entrapment efficiency (%).

Three dimensional plots were prepared and are shown in Figs. 2 and 3, for responses Y_1 and Y_2, respectively. These plots are known to study the interaction effects of the factors on the responses as well as are useful in studying the effects of two factors on the response at one time. When the experimental values of responses for 17 runs were fitted to different models of

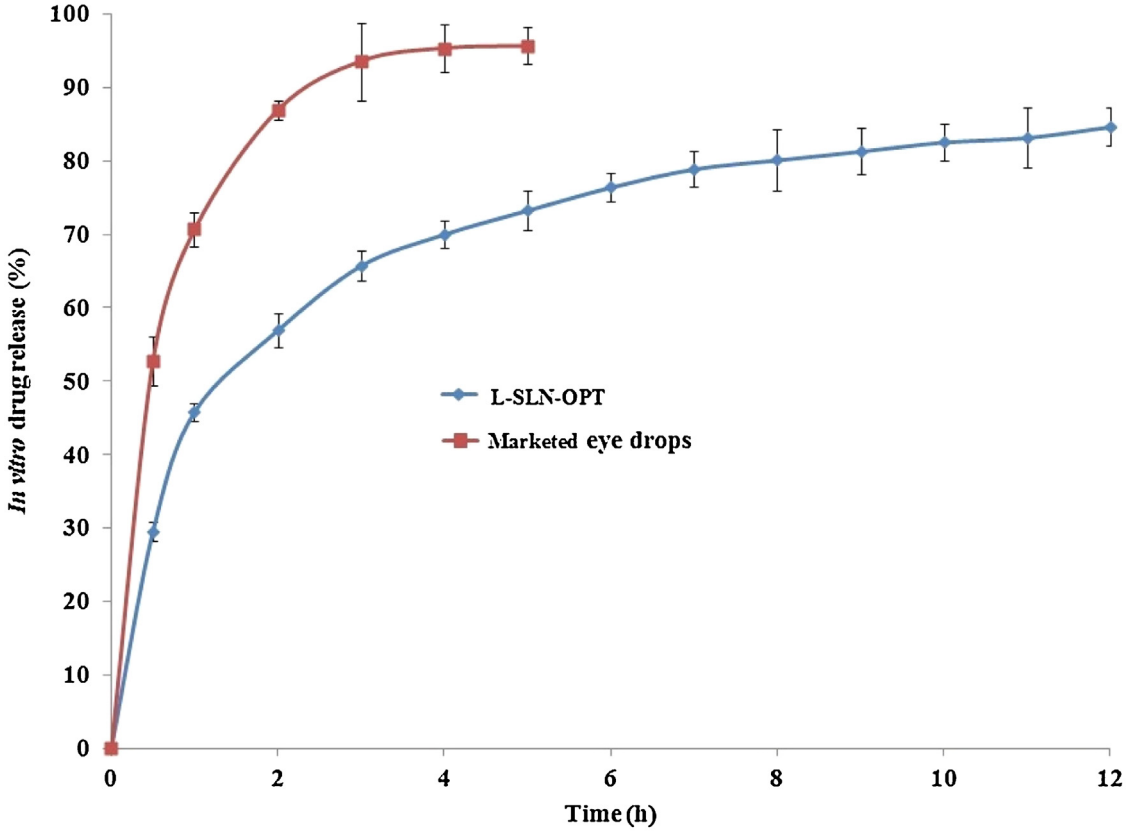

Fig. 7. *In vitro* drug release profile from L-SLN-OPT and marketed eye drops.

Table 3
Observed response in Box–Behnken design for levofloxacin loaded SLN.

Formulation code	Independent variables			Dependent variables			
				Observed value		Predicted value	
	X_1	X_2	X_3	Y_1	Y_2	Y_1	Y_2
F1	4.00	3.00	3.00	232.1	63.7	231.71	66.29
F2	6.00	3.00	2.00	235.8	77.5	236.28	78.04
F3	4.00	4.00	2.00	234.2	68.4	233.78	66.24
F4	4.00	3.00	1.00	239.2	60.6	233.91	65.19
F5	8.00	3.00	3.00	239.5	85.3	238.66	90.89
F6	8.00	2.00	2.00	241.9	90.3	238.78	89.84
F7	8.00	3.00	1.00	241.4	91.8	240.86	89.79
F8	6.00	4.00	3.00	234.7	80.6	236.16	79.09
F9	6.00	3.00	2.00	237.8	81.3	236.28	78.04
F10	6.00	3.00	2.00	238.5	83.5	236.28	78.04
F11	8.00	4.00	2.00	240.0	91.0	240.73	90.84
F12	6.00	4.00	1.00	237.0	78.9	238.36	77.99
F13	6.00	3.00	2.00	233.6	74.3	236.28	78.04
F14	6.00	2.00	1.00	232.1	75.6	236.41	76.99
F15	6.00	3.00	2.00	234.9	74.8	236.28	78.04
F16	4.00	2.00	2.00	229.5	67.3	231.83	65.24
F17	6.00	2.00	3.00	234.6	81.7	234.21	78.09

Where, X_1 = Lipid (% w/w), X_2 = Surfactant (% w/w), X_3 = Co-surfactant (% w/w), Y_1 = Particle size (nm), Y_2 = Entrapment efficiency (%).

Box–Behnken design, it was observed that the best-fitted model for all of the two dependent variables was the linear model.

3.2.1. Particle size

To derive a relation between the different factors and SLN particle size, different mathematical models like linear, 2FI, Quadratic and cubic were analyzed for test of fit using the Design Expert software. Box–Behnken design suggests linear model for SLN particle size analyses. As far as ANOVA report concerned for SLN particle size optimization, the Model *F*-value of 5.86 implies the model is significant. There is only a 0.93% chance that a "Model *F*-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. Fig. 2 is the three dimensional plot which shows the effect of independent variables on particle size of SLN (Y_1). The polynomial equation to fit the surface response has only X_1, X_2 and X_3 as a variable, as presented in Eq. (1). The other factors and their interactions were not significant.

$$\text{Particle size} = +236.28 + 3.48 \times X_1 + 0.98 \times X_2 - 1.10 \times X_3 \quad (1)$$

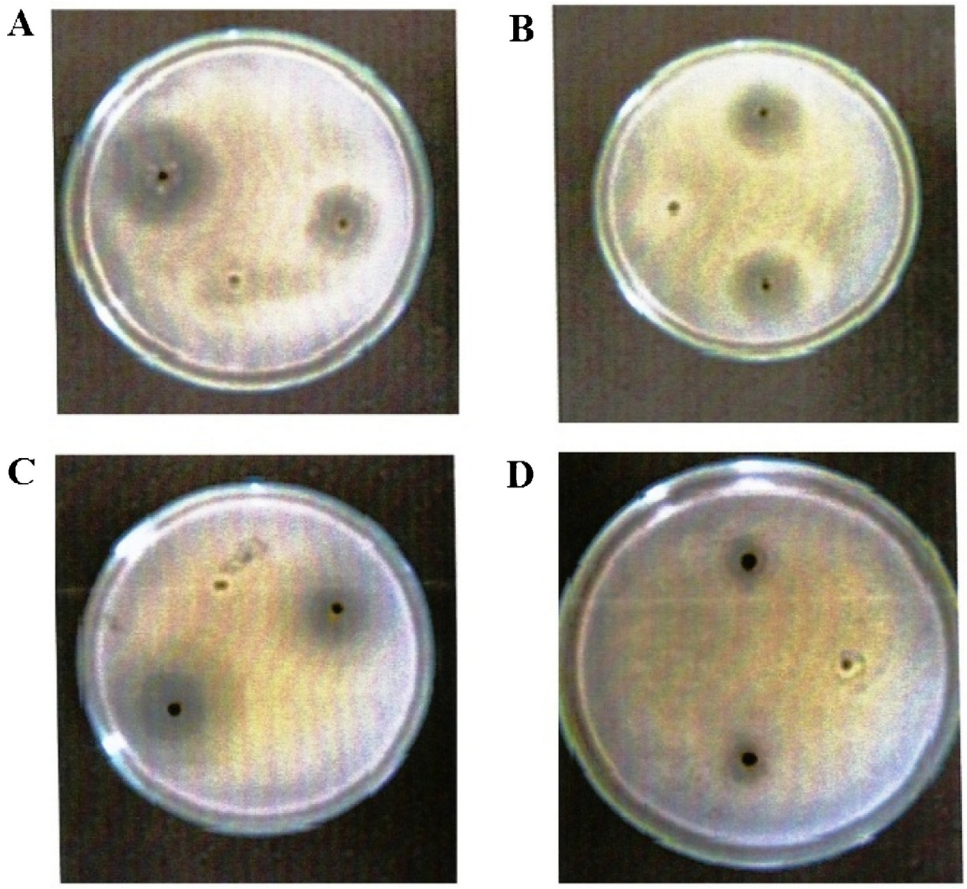

Fig. 8. Agar plates incubated for antibacterial efficacy of (A) marketed eye drops against *E. coli* (B) L-SLN-OPT against *E. coli* (C) marketed eye drops against *S. aureus* (D) L-SLN-OPT against *S. aureus*.

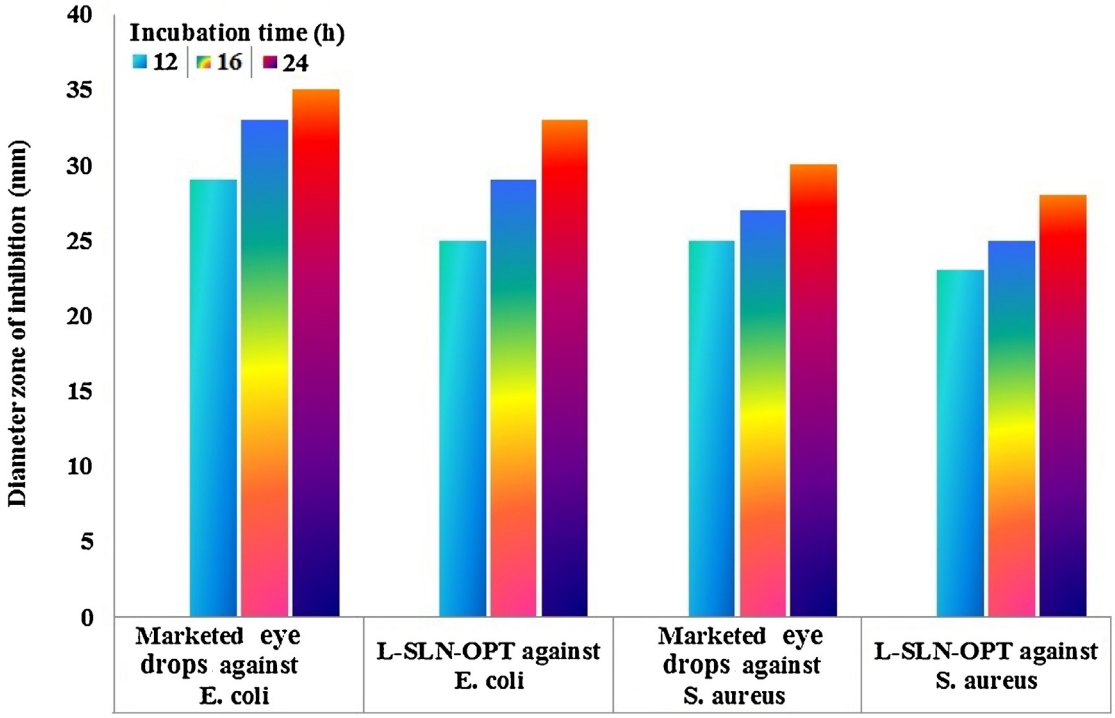

Fig. 9. Zone of inhibition produced by marketed eye drops and L-SLN-OPT formulation against *E. coli* and *S. aureus*.

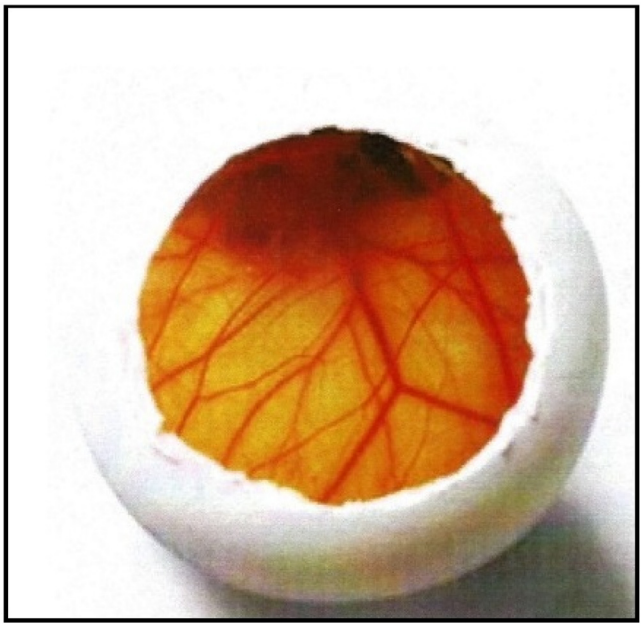

Fig. 10. Hens Egg Test Chorioallantoic Membrane (HET-CAM).

Considering all experiments in Table 3, an averaged particle size was 236 nm, with minimum and maximum of 229.5 nm and 241.9 nm, respectively. The percent of lipid has the significant effect on the particle size of the SLN formulation because it solubilizes the drug in formulation.

The 3D-graph (Fig. 2) showed that, on increasing the concentration of stearic acid (lipid) from 4% to 8%; the particle size of the SLN formulations were also increased (Table 3). Formulations F6, F7, F5, and F11, which contained the highest amount of lipid, comparatively showed the larger particle size than that of formulations F16, F4, F1, and F3, respectively, which containing the low amount of lipid. It is observed that, there was no prominent effect was seen on the particle size when the percent of surfactant in formulations was increased as seen in Table 3. The formulation F6 and F11 showed a decrease in particle size on increasing the percent of surfactant from 2% to 4%, although the decrease in particle size is not significant. While it is clear from the data (Table 3) that, there was a gradual decrease in the particle size from 239.2 nm of formulation F4 to 232.1 nm (F1) when the percent of co-surfactant in formulation was increased (Table 3). Similar results have observed for formulation F7 (241.4 nm) to 239.5 for formulation F5 where lipid and surfactant (% w/w) were kept constant.

3.2.2. Entrapment efficiency

A polynomial equation was fitted to the entrapment efficiency for levofloxacin is given in Eq. (2). It is evident that all the three independent variables, that is, concentration of lipid, surfactant, and co-surfactant, have positive effects on the response Y_2.

$$\text{Entrapment efficiency} = +78.04 + 12.30 \times X_1 + 0.50 \times X_2 + 0.55 \times X_3 \quad (2)$$

Considering all experiments in Table 3, averaged levofloxacin entrapment efficiency for the 17 experiments was found to be 78%, with minimum and maximum of 60.6% and 91.8%, respectively. The lipid concentration produced a significant effect on the levofloxacin entrapment efficiency. The 3D-Response graph (Fig. 3) showed that on increasing the lipid content in formulations; higher entrapment efficiency of levofloxacin was observed in SLN. This may be due to the increasing internal phase; more amounts of lipid were available for the dissolving of drug. Nature of the drug plays a significant role in the entrapment efficiency of drug because the drug is entrapped in lipid phase. Levofloxacin is a lipophilic drug, and its solubility is also higher in stearic acid, so that the entrapment efficiency noticeably was found to be higher.

It is observed that on increasing the lipid content which reduces the partition of drug in outer phase lead to increase in the entrapment efficiency of drug in SLN. As evidence; formulations F6, F7, F5, and F11, which contained the highest amount of lipid (8% w/w), comparatively showed the higher levofloxacin entrapment efficiency than that of formulations F16, F4, F1, and F3, respectively, which containing the low amount of lipid (4% w/w) (Table 3). Surfactant and co-surfactant ratio plays a major role in the solubility of drug in external phase and in our study, their concentration also has produced effect on the entrapment efficiency (Fig. 3) although the effects were not found prominent.

3.3. Optimization

On the basis of three-dimensional response surface graphs generated by the Design-Expert software, it can be said that the lipid, surfactant and co-surfactant concentrations produces a substantial effect on SLN particle size and entrapment efficiency. 3D-Graphs show that with increasing the percent of lipid in formulations; the particle size and entrapment efficiency of SLN increased. On the other side, second factor, surfactant and co-surfactant optimum concentration ratio was found to be responsible for optimum SLN particle size and improved levofloxacin entrapment efficiency. The response surface graphs represented that the chosen independent variables (stearic acid as lipid, Tween 80 as surfactant and sodium deoxycholate as co-surfactant) were presented good effect on formulations and found to be better parameters for the selection of optimized formulation.

The optimum formulation of levofloxacin-loaded SLN system was selected based on the criteria of attaining the maximum value of entrapment efficiency and minimizing the particle size by applying numerical point prediction optimization method of the Design Expert software®. The formulation composition with stearic acid (6.31% w/w), surfactant (3.0% w/w), and co-surfactant (1.0% w/w) was found to fulfill requisites of an optimized levofloxacin SLN formulation, the optimized formulation named as L-SLN-OPT.

Particle size distribution of L-SLN-OPT is presented in Fig. 4. The L-SLN-OPT has presented the particle size of 237.82 nm. The particle size was found to be within acceptable size range *i.e.*10 μm which is tolerable particle size for ophthalmic instillation [36]. L-SLN-OPT showed the entrapment efficiency of 78.71%. These experimental observed values of particle size (237.82 nm) and entrapment efficiency (78.71%) presented by the L-SLN-OPT found in agreement with the predicted value of particle size (237.92), and entrapment efficiency (79.39) respectively generated by design expert software, suggesting that the optimized formulation was trustworthy and rational.

3.4. Morphology of SLN

In order to investigate the morphology of the optimal L-SLN-OPT, transmission electron microscope was used. Transmission electron photomicrograph of the L-SLN-OPT is shown in Fig. 4. The transmission electron microscopy image show the drug enclosed in the lipid matrix. The transmission electron images of L-SLN-OPT show uniform size distribution of lipid nanoparticles having coarsely spherical shape, displaying sealed SLN structure. The uniformity in particle size distribution correlates with the small polydispersity index (0.251) obtained via PCS. The particle size after the transmission electron microscopy study was found to be in the

range of 220–245 nm, which is in agreement with the particle size range depicted by particle size analysis.

3.5. Differential scanning calorimetry

The DSC thermograms of pure levofloxacin, stearic acid, physical mixture (levofloxacin and stearic acid) as well as SLN formulation (L-SLN-OPT) are shown in Fig. 5. The thermal curve of levofloxacin showed endothermic peak at 230.5 °C. Physical mixture of stearic acid and drug showed endothermic peaks at 61.8 °C and 230 °C respectively (Fig. 5).

The melting endothermic peak of stearic acid in the prepared SLN was slightly shifted to a lower temperature (52 °C). The decrease in melting temperature of stearic acid in nanoparticles compared with stearic acid alone have been attributed to their small size (nanometer range), the dispersed state of the lipid, and the presence of surfactants. The thermogram of the lyophilized L-SLN-OPT did not show the melting peak for the levofloxacin. This indicated that levofloxacin was not in crystalline state but rather present in amorphous state and drug was completely entrapped within the nanoparticles. These findings are in accordance with previously reported literature [37,38].

3.6. X-ray diffraction analysis

To find out the physical state of drug, the physical mixture of levofloxacin and stearic acid was compared with that obtained by XRD diffractogram of lyophilized L-SLN-OPT (Fig. 6).

In physical mixture there are clear crystalline peak of drug whereas this peak was not observed in lyophilized L-SLN-OPT system which shows the drug undergo microcrystallization with in lipid matrix. Absence of drug peak reflections in the diffractogram of SLN, which may be assumed due to the dilution effect exerted by lipid matrix, indicated that the drug was entrapped within the lipid matrix. Moreover, in lyophilized L-SLN-OPT, it was observed that the lipid matrix are less crystalline, it may be expected that the amorphous portion would accommodate drug as there would be enough spaces where drug would be incorporated, compared with their physical mixture. Reduction in the intensities of reflections in the SLN indicated absence of any dynamic polymorphic transformation and progression to highly ordered lipid SLN due to polymorphic transition would not occur. As a result, incorporated drug would remain in the SLN and higher drug entrapment efficiency in SLN would be experienced in the above mentioned lipid matrix. This result was in agreement with DSC analysis, as no endotherm was observed in SLN system.

3.7. In vitro drug release studies

The *in vitro* release profile curves obtained by the dialysis membrane method from L-SLN-OPT, and marketed eye drops of levofloxacin which was used as reference for comparison were investigated over 12 h and comparative results are shown in Fig. 7.

Cumulative percentage drug release of L-SLN-OPT showed 84.67% in 12 h. *In vitro* release curve showed the initial burst release with the approximate 45% of drug release during the first two hours; after that release sustained from the L-SLN-OPT. Burst release occurred due to the presence of the free levofloxacin in the external phase and on the surface of the SLN. The lipophilic nature of the levofloxacin could be the reason for sustained release of the drug from internal lipid phase after initial burst release. It reported that, initial burst release rate was affected by the change of concentration of lipid and surfactant in external phase. When the lipid concentration increased, the initial burst release rate decreased; this may be due to the higher concentration of drug presence in the inner core [39,40]. Whereas surfactant concentration increases, the initial burst release rate increases due to the increased solubility of drug in external phase.

In order to propose the possible release pattern from L-SLN-OPT, the release data was evaluated to check the goodness of fit for zero-order release kinetic, First-order release kinetic, Higuchi's matrix [41] and Korsmeyer–Peppas model [42,43]. The goodness of fit was evaluated by R^2 (correlation coefficient) values. The model showing highest value of R^2 was considered as best model for release kinetic. The R^2 values for different models are presented in Table 4.

The correlation coefficients obtained after fitting the *in vitro* release data to the respective model equations indicate that best fit is obtained with Korsmeyer–Peppas model. The highest value of the correlation coefficient ($R^2 = 0.970$) was observed for Korsmeyer–Peppas model, followed by the first-order ($R^2 = 0.914$), Higuchi's ($R^2 = 0.905$) and zero order ($R^2 = 0.773$) models, as shown in Table 4. The same release kinetics for lipid nanoparticles formulations across dialysis membrane was described by previous researchers [11,44]

Table 4
Mathematical models used to study the release kinetics and their R^2 value obtained after fitting *in vitro* release data.

S. no	Model	Equation	R^2 value for marketed eye drops	R^2 value for L-SLN-OPT[a]
1	Zero order	$M_0 - M = kt$	0.6475	0.773
2	First order	$\ln m = kt$	0.9196	0.914
3	Higuchi's matrix	$M_0 - M = kt^{1/2}$	0.8732	0.905
4	Korsmeyer–Peppas	$\log (M_0 - M) = \log k + n \log t$	0.9337	0.970

Where, m_o and m is initial drug content at time t_o and drug content at time t, respectively.

[a] Optimized solid lipid nanoparticles formulation of levofloxacin.

Table 5
Scores recorded for HET-CAM ocular irritation test.

Group	Time (min)								
		5	10	15	30	60	120	240	360	480
Scores										
Normal saline solution (0.9% NaCl)	Egg-1	0	0	0	0	0	0	0	0	0
	Egg-2	0	0	0	0	0	0	0	0	0
	Egg-3	0	0	0	0	0	0	0	0	0
	Mean	0	0	0	0	0	0	0	0	0
L-SLN-OPT[a]	Egg-1	0	0	0	0	0	0	0	0	1
	Egg-2	0	0	0	0	0	0	0	0	0
	Egg-3	0	0	0	0	0	0	0	0	1
	Mean	0	0	0	0	0	0	0	0	**0.67**

[a] Optimized solid lipid nanoparticles formulation of levofloxacin.

3.8. *Ex vivo* corneal permeation study

Transcorneal permeation of L-SLN-OPT presented cumulative amount permeated through cornea of 69.54 μg/cm^2 in 4 h and produced a flux of 0.2493 μg/cm^2/h. This better permeation through L-SLN-OPT across the cornea could be attributed to the agglomeration of SLN as depot near the cornea from which the drug is slowly delivered to the precorneal area. Corneal hydration is generally used as an important parameter to evaluate damage to the corneal tissue. Generally, corneal hydration remained in the normal range of 75–80% [45,46], when the pH of the formulation was between 6.2 and 6.8. Therefore, the L-SLN-OPT could be considered safe and non-damaging to the eye as the pH of L-SLN-OPT was found to be 6.4, which indicates that L-SLN-OPT did not cause any damage to corneal tissue.

3.9. *Antibacterial activity*

The antimicrobial study was carried out by agar cup plate method as qualitative assessment to compare antibacterial efficacy of developed L-SLN-OPT with marketed eye drops (Fig. 8).

Marketed eye drops preparation shows a zone of inhibition of 35 mm for *E. coli* compared to zone of inhibition of 33 mm obtained in L-SLN-OPT in 24 h. While L-SLN-OPT shows a zone of inhibition of 28 mm for *S. aureus* compared to zone of inhibition of 30 mm obtained in marketed eye drops in 24 h (Fig. 9).

According to the results, L-SLN-OPT possess antimicrobial activity against *S. aureus*, and *E. coli*. As it can be seen, there is no observable difference between the zone of inhibition of free levofloxacin and SLN-loaded levofloxacin. This means that, loading of levofloxacin on SLN does not lead to an enhancement in the antimicrobial function of levofloxacin. In other words, SLN has no antimicrobial activity itself. Finally it concluded that the developed L-SLN-OPT are equivalent to marketed eye drops in antibacterial action.

3.10. *Ocular tolerance test*

Ocular irritation of the developed formulation was checked by HET-CAM test (Fig. 10).

It is based on the direct application of formulation onto the CAM. The CAM of the chick embryo is a complete tissue including veins, arteries, and capillaries and it responds to injury with a complete inflammatory process, a process similar to that induced in the conjunctival tissue of the rabbit eyes [47].

HET-CAM is a rapid, sensitive and inexpensive method for assessing the irritation potential of ophthalmic formulation [48]. Test was performed using normal saline solution as control for irritancy test, scores was allocated to different levels of irritation based on observation as indicated in (Table 2). Scores was recorded as shown in Table 5, an average score of 0.67 was observed until 8 h (480 min). Study revealed that optimized formulation was found to be non-irritant and safe in given dose range, for topical ophthalmic use.

4. Conclusion

Levofloxacin loaded SLN using stearic acid as lipid showed potential outcome which were optimized using 3-factor, 3-level Box–Behnken design. The application of Box–Behnken design to study the preparation process for SLN showed to be a very important tool, allowing establishing the relationships among the factors and quality attributes. Our results demonstrate that using stearic acid as lipid, Tween 80 and sodium deoxycholate as surfactant and co-surfactant respectively increases levofloxacin entrapment, and produces SLN with the desired size, size distribution, and morphological properties. The levofloxacin-SLN obtained *in vitro* release experiments exhibited a biphasic release pattern with burst release at the initial phase followed by sustained release. SLN had a comparative antibacterial activity against *S. aureus* and *E. coli in vitro* with respect to marketed eye drops. However, *in vivo* studies for levofloxacin-SLN should be performed to determine its ophthalmic delivery efficacy.

Conflict of interest

All authors have approved the final manuscript and the authors declare that they have no conflicts of interest to disclose.

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