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Research Article

An antibiotic efficacy comparative study between market-prescribed and solid-lipid fluoroquinolone ophthalmic nanoformulation against conjunctivitis

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Abstract: Besifloxacin hydrochloride (BSF) drug substance (DS), is effective against gram-positive and gram-negative microorganisms. BSF (liquid suspension form) is already available in two different market brands: Besivance and Besix. Both of these commercialized drug products (DP) have lower mucoadhesive characteristics, therefore they spend less time in contact with various ocular tissues (cornea and conjunctiva). This research work emphasized the design, development, and optimization of BSF-loaded solid-lipid nanoparticles (BSF@SLN) to increase antibacterial activity by enhancing drug absorption through passive diffusion techniques in the ocular organ compared to conventional dosage forms. The SLN was prepared through a very simple process hot homogenization process using glyceryl monostearate and polysorbate-80 along with poloxamer-188 and water for injection. Various characterization techniques like FTIR, DSC, and XRD were performed to check the compatibility between DS and excipients. The preliminary formulation of SLN was characterized through zeta potential, polydispersity index, and particle size techniques. The developed SLN was optimized following 3^2 factorial designs. Surface morphology and size of optimized BSF@SLN ophthalmic DP were confirmed by using advanced microscopic techniques such as SEM. *In-vitro* release of optimized BSF@SLN ophthalmic DP was performed by using two chambers of Franz diffusion cell. Stability studies of optimized BSF@SLN ophthalmic nanoformulations were tested for up to 6 months at 25°C ± 2°C/60% ± 5% RH. The antimicrobial activity of optimized ophthalmic nanoformulation was found 2.76 times more effective compared to the Besix eye drop. *In-vivo* study of BSF@SLN ophthalmic nanoformulations was carried out with an eye irritancy test and compared with marketed DP (Besix). and found significant pharmacological.

***Keywords*:** Besifloxacin hydrochloride (BSF) drug substance (DS); BSF DS-loaded solid-lipid nanoparticles (BSF@SLN); Besix eye drop; antibacterial efficacy; ophthalmic nanoformulation

1. Introduction

Conjunctivitis, a typical eye disease, occurs due to inflammation (swelling), irritation through infection and allergies of the conjunctiva (transparent membrane) which appears on the eyelid and warps the white part of the eyeball. It is visible whenever the small blood vessels of the eyes are inflamed due to the white portion of the eyes becoming reddish or pink. Acute conjunctivitis is a common ocular disease that can influence all age groups. Due to the colour change of the white eye into red, conjunctivitis is also pronounced as pink eye. Conjunctivitis can

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affect one or both eyes at one time. It can be divided into three main types based on the infection: viral, bacterial, and allergic. Among all viral conjunctivitis is nearly quite common however bacterial conjunctivitis is responsible for infecting 78% of children and 50% of adults [1]. The gram-positive microorganisms i.e., *Staphylococcus aureus (S. aureus)*, *S. epidermidis*, and *S. pneumonia* and the gram-negative microorganisms i.e., *Haemophilus influenzae (H. influenzae)* are the most responsible bacterial pathogen for conjunctivitis. However, in all of these bacteria, *S. aureus* and *H. influenzae* frequently infect adults while *H. influenzae* is most responsible for infecting children [2]. BSF is a fourth-generation antibiotic which is identical to fluoroquinolone. It has potent antibacterial efficacy against various gram-positive and gram-negative microorganisms in dominant and drug-resistant pathogens. It also demonstrates potent bactericidal activity against various pathogens that are responsible for conjunctivitis in human beings [3,4]. The physicochemical properties of BSF DS are mentioned in **Table S1**.

BSF is used for curing conjunctivitis and is approved by the United States Food and Drug Administration (USFDA) and also by the Health Canada regulatory authority as Besifloxacin ophthalmic suspension 0.6% under the brand name "Besivance^{TM}", manufactured by: Bausch & Lomb Inc., USA. This DP was approved by the US FDA. Besivance^{TM} (besifloxacin ophthalmic suspension) 0.6%, is a quinolone antimicrobial indicated for the treatment of bacterial conjunctivitis caused by susceptible isolates of the following bacteria: CDC coryneform group G, *Corynebacterium pseudodiphtheriticum (C. pseudodiphtheriticum)*, *C. striatum*, *Haemophilus influenzae*, *Moraxella lacunata*, *Staphylococcus aureus (S. aureus)*, *S. epidermidis*, *S. hominis*, *S. lugdunensis*, *Streptococcus mitis group*, *Streptococcus oralis*, *Streptococcus pneumonia*, *Streptococcus salivarius* [3].

Besivance (manufactured and marketed by: Bausch & Lomb Inc., USA) and Besix (manufactured and marketed by: Ajanta Pharma Ltd, India) (0.6% drug products) are two different market-available eye drops in 5 ml of sterile packing. In contrast to other antibiotics of the same class, besifloxacin is distinctive in its category. It is not used for systemic infections and lowers the chance of resistance. **Table S2** shows the orange book available detail about Besivance (Besifloxacin ophthalmic suspension 0.6%) DP [5,6].

However, the approved marketed DP is responsible for providing several eye discomfort and side effects like burning, itching, redness, swelling, pain, temporary blurred vision, headache, nausea, diarrhoea, fever, etc. in the eyes. In addition to these discomfort and side effects, it gave some other troubles also that are accountable for reducing the therapeutic efficacy of Besivance and Besix such as less hold time in the eye, drug loss through tears, and irritation in the eyes due to the large size of particles of suspension.

The SLN are alternative nano-sized drug vehicles, for delivering lipophilic pharmaceuticals with distinctive properties like forming smaller size particles (50 to 1000 nm), higher drug loading capacity, high surface area, reduced toxicity, enhanced biodistribution and improved patient compliance compared to other nanoformulations such as emulsions, liposomes and polymeric micro and nanoparticles [7]. Additionally, the ophthalmic formulation of SLN is a very good eye-catching colloidal drug delivery system for topical applications. Thus to improve the performance of pharmaceuticals, SLN can be administrated through various routes such as parenteral [8], ocular [9], oral [10], rectal, pulmonary [11], topical [12], transdermal, etc. Currently, SLN can be fabricated by several techniques as it can various homogenization techniques: high-pressure homogenization (HPH), hot homogenization [13], cold homogenization [14], various emulsions (w/o/w double emulsion, solvent emulsification-evaporation, oil-in-water microemulsion), spray drying [15], ultrasonication [16], supercritical fluid method, etc.

In the present research, we prepared an optimized and stabilized BSF@SLN by an easy-to-prepare, fast and cost-effective hot homogenization technique and evaluated the efficient antibacterial activity against the conjunctivitis-responsible pathogens. The poorly aqueous soluble BSF-loaded ocular SLN can enhance ocular drug delivery through increased ocular absorption, bioavailability, drug efficiency, patient acceptability, and prolongation of the existing drug substance life cycle and reduced side effects compared to market-prescribed formulations [17].

The therapeutic effectiveness of ophthalmic nanoformulation depends not only on the action of the drug itself but also on other factors related to the delivery system improved holding time in the eye, improvement of ocular bioavailability, reduction of drug loss through tears, decreased irritation due to very small size and extended release of BSF and thus increase therapeutic activity.

2. Materials and methods

2.1 *Materials*

BSF was purchased from Indoco Remedies Limited, Mumbai, India. Glyceryl monostearate (GMS) lipid was purchased from Gattefossé, France. Polysorbate 80 and Poloxamer 188 were purchased from BASF, Germany.

Besix eye drop was purchased from Ajanta Pharma Ltd, India. Sodium chloride (NaCl), mannitol, hydrochloric acid (HCl), sodium hydroxide (NaOH), benzalkonium chloride and edetate disodium (EDTA) were purchased from Merck, Germany. Gellan Gum (Gelrite) was purchased from CP Kelco U.S., Inc. Casein enzymic hydrolysate, Peptic digest of soya bean meal and agar were purchased from Hi-media India Pvt. Ltd. 0.45 µm Polyvinylidene fluoride (PVDF) 47 mm, Durapore® white, plain, cut disc membrane filter (item code: HVLP04700) was purchased from Merck Millipore. Water for injection (WFI), USP were taken from our research laboratory. low-density polyethene (LDPE) and high-density polyethene (HDPE) plastic bottles were purchased from SSF Plastics India Pvt. Ltd., Mumbai.

2.2 *Methodology*

2.2.1 *Methodology for fabrication of SLN*

Drug substance-loaded SLN was prepared by a simple and cheap hot homogenization technique. This technique is used to formulate the non-temperature-sensitive formulation. It is a 2-step process. In the initial step, the lipid was melted above its melting point and 6.6 mg poorly water-soluble BSF DS was added to it. In the later step, an aqueous solution of emulsifier/surfactant was heated almost at the same temperature and both mixtures were stirred at high speed and high temperature. Based on this procedure, initial 3-different lipids (GMS, glyceryl palmitostearate and glyceryl behenate) were selected and every lipid (3.3 mg of each lipid) was heated between 60°C to 70ºC temperature on a hot plate. In the melted lipid solution, BSF was slowly added with continuous stirring of high-speed homogenization (HSH) (IKA T 50 digital ULTRA-TURRAX®, IKA) at 10,000 revolutions per minute (RPM) for 5 mins. Later, an aqueous solution of mixed emulsifier (Poloxamer 188: Polysorbate 80) was dissolved in hot purified water at about 80ºC temperature. The hot aqueous surfactant solution was added to the warm lipid phase and mixed uniformly by using HSH at 10,000 RPM for 10 minutes at a temperature between 60°C to 70°C. The above bulk dispersion was passed through HPH (EmulsiFlex-C55, Avastin, U.K.) at 1000 bar pressure for 5 cycles. After completion of the cycle, the bulk was cooled at controlled room temperature as per USP 20°C to 25°C (68°F to 77°F). The cold solution was centrifuged (Remi KPR-70 PLUS, India) for 10 minutes at 10,000 RPM which was further diluted with purified water as per requirement [18].

Preliminary formulation development of BSF@SLN:

In the present study, the composition details and process parameters that were used for manufacturing ophthalmic BSF@SLN are mentioned in **Table S3**.

2.2.2 *Optimization of BSF@SLN ophthalmic drug product*

2.2.2.1 *3^2 full factorials experimental design*

BSF@SLN were prepared following the 3^2 factorial design of the experiment (Stat-Ease, Inc., Minneapolis, MN, USA) [19]. This experimental design helped to evaluate the combined effect of ingredients and process conditions using the design expert software as shown in **Table S4**. In the experimental design, two factors: process parameter (X_1) and the quantity of lipid concerning drug (X_2) were evaluated and mean particle size (M_D) (Y_1), zeta potential (ZP) (Y_2) and % entrapment efficiency (% EE) (Y_3) were tested. The results were analyzed statistically through the Analysis of variance (ANOVA test) and the level of significance of the tested factors on the selected responses was evaluated.

2.2.2.2 *Optimization of drug substance and lipid ratio*

After the selection of GMS lipid, the DP was optimized against the BSF DS and GMS lipid ratio. For optimization of ratio, three different ratios (1:1, 1:0.75, 1:0.5; BSF DS: GMS lipid) were selected and the HPH processes assessed were evaluated at 1000 bar for 5 cycles; 1000 bar for 10 cycles and 1000 bar for 20 cycles. The composition and process parameters are mentioned in **Table S5**.

2.2.3 *Optimization of freeze-drying (lyophilization) process*

The optimized BSF@SLN (BSF~6.6 mg) ophthalmic solution was filled in clear USP type-1 glass vials, half-stoppered by using with two-slotted rubber stopper and immediately frozen at -70°C and then was immediately transferred to the lyophilizer (FreeZone 4.5 Liter -50°C Benchtop Freeze Dryer, Labconco). The vacuum was allowed to reach 100 mTorr. The lyophilization cycle was carried out for 24 Hrs. After completion

of the cycle, lyophilized vials were fully stoppered with a back pressure of nitrogen and the stoppered vials were sealed by using flip-off aluminium seals.

2.2.4 *Preparation of optimized BSF@SLN ophthalmic drug product*

Initially, the bulk solution was prepared by dissolving the NaCl, mannitol, poloxamer, benzalkonium chloride, EDTA, and polysorbate 80 in the 70% cooled WFI of the batch volume. The pH of the bulk solution was adjusted between 6.0 to 6.5 by using 1.0N NaOH solution and the solution was heated between 60°C to 70°C temperature.

Later, the required amount of GMS lipid was melted by heating between 60°C to 70ºC temperature on the hot plate. The DS BSF (6.6 mg) was dispersed in the melted lipid (drug: lipid-1:1) solution with continuous high-speed stirring by using HSH (Ika-t50) at 10,000 RPM for 5 mins. The initial aqueous phase bulk solution was mixed with the lipid phase by HSH at 10,000 RPM for 10 minutes between 60°C to 70°C temperature. The resulting dispersion was passed through HPH at 1000 bar pressure for 5 cycles. The bulk solution was then cooled at room temperature. At last, gelrite (gellan gum) was dissolved in the remaining batch volume of WFI and mixed in the above step. The unfiltered bulk solution was aseptically sterilized through membrane filtering by using a 0.45 µm hydrophilic PVDF membrane filter. The filtered sterilized DP was filled in 5 mL soft, flexible, lightweight, opaque low-density polyethene (LDPE) plastic bottles, closed with LDPE nozzles and sealed with high-density polyethene with polypropylene tamperproof lock caps (SSF Plastics India Pvt. Ltd., Mumbai). The finalized ingredients and their quantity (mg/mL) for preparing optimized ophthalmic BSF@SLN are shown in **Table S6**.

2.3 *Determination of entrapment efficiency and assay of BSF*

The percentage of entrapment efficiency (% EE) was calculated by measuring the amounts of free BSF in the aqueous phase against the total BSF DS taken in the drug product. BSF@SLN were centrifuged through Centrifuge, Remi, K-70 and the filtrate was analyzed by high-performance liquid chromatography (Agilent Technologies, 1100/1200 series) after appropriate dilution. % EE was calculated as below given equation:

$$\% EE = \left(\frac{W1 - W2}{W1}\right) \times 100 \quad (1)$$

where W1 is the initial weight of the BSF drug substance; W2 is the weight of the unloaded BSF drug substance in the supernatant [20,21].

The assay of BSF was estimated by measuring the concentration of BSF drug substance by using the below equation:

$$\% Assay\ of\ BSF = \left(\frac{A + B}{C}\right) \times 100 \quad (2)$$

where A: Amount of BSF DS in SLN; B: Amount of BSF DS in the supernatant after centrifugation; C: Total weight of BSF DS.

2.4 *Analytical Method Development*

BSF was analyzed using high-pressure liquid chromatography (Agilent's 1100 series HPLC system, USA) and supplied with a very sensitive diode array detector (DAD).

Column and Chromatographic conditions

The chromatographic separation was tested on Zodiac C18 5µ column, (150 × 4.6) mm, (Zodiac Life Sciences). The samples were monitored using UV detection with a 10 µL injection volume at 295 nm at 2 mL/min flow rate with a run time of 6 mins.at 30°C. The retention time was about 2.5 minutes.

Mobile phase and stock solutions preparation

The buffer solution was prepared by adding 3.45 gm of ammonium dihydrogen phosphate and 1.0 mL triethylamine in 1.0 L water and pH was adjusted to 3.0 ± 0.05 with diluted orthophosphoric acid. The mobile phase was prepared as acetonitrile: methanol (3:7). The test sample was prepared by adding diluent and 3.3 mL of BSF Ophthalmic suspension 0.6% and made up the volume 100 mL with diluent. The simulated tear was prepared by dissolving 0.68 g of sodium chloride, 0.22 g of sodium bicarbonate, 0.008 g of calcium chloride, and 0.14 g of potassium chloride in 100 mL water. The standard solution in a diluent, simulated tear and buffer solution was prepared by mixing 50 mg of BSF in 250 mL diluent, simulated tear and buffer solution separately.

Identification and peak purity of BSF peak

The working condition for the HPLC method was established with BSF standard preparation, a chromatogram obtained after injection of 10 µL of working standard solution. the retention time for BSF DS was observed at about 2.5 minutes.

Specificity, Accuracy, Linearity and Precision

The specificity of the stock solution was checked in a diluent, simulated tear, and phosphate buffer media to determine the retention time in different media.

Accuracy was analyzed by percentage recovery of 140.10 to 260.19 µg/mL drug solution. A standard stock solution was prepared by dissolving 200 mg of BSF in 100 mL of diluent in a volumetric flask and preparing the final concentration of 140.0, 200.0 and 260.0 µg/mL. Similarly same concentration samples were also prepared using simulated tears and phosphate buffer media.

The linearity was established across different concentration samples 140.0, 160.0, 200.0, 240.0 and 260.0 µg/mL of standard stock solution in different media like diluent, simulated tear and phosphate buffer.

Precision was calculated by analyzing six sets of known concentrations of Besifloxacin Hydrochloride solution. The repeatability precision was expressed as %RSD.

2.5 *Characterization of BSF@SLN ophthalmic drug product*

The optimized ophthalmic BSF@SLN was characterized by advanced techniques that can be divided into two parts: microscopic technique and spectroscopic techniques. In microscopic technique, scanning electron microscopy (SEM) image was acquired on the Jeol JSM 6360 with 5.0 kV energy, 100X magnification and 100 µm scale. By using the SEM, we got information about the surface morphology and size of the BSF@SLN in the dispersed medium. For drying the aqueous dispersion of SLNs, the lyophilization method was employed. However, in spectroscopic techniques, the functional groups present on the BSF (drug substance), GMS (lipid carrier), GMS-BSF conjugate, and dried optimized BSF@SLN were recorded by using Fourier-transform infrared (FTIR) spectrophotometer (FT-IR spectrophotometer, Spectrum GX, Perkin Elmer, USA) in the mid-infrared region between 4000–400 cm^{-1} by using the KBr disc method [22]. Visual appearance was observed under the white light and recorded to compare the stability. The X-ray diffraction (XRD) spectra were collected by applying a Cu-Kα radiation source in a 2θ range of -3° to 60° with step size and time 0.02° and 2θ/s, respectively and operated at 40 kV and 40 mA (Ultime IV, Rigaku, Japan). The thermal analysis of various substances was done by using differential scanning calorimetry (DSC) (DSC60, Shimadzu Corporation, Japan). The 2.0 to 4.0 mg samples were kept in an aluminium pan and sealed and DSC spectra were collected by melting the samples in the heat range between room temperature (25°C) to 400°C with a heat flow of 10°C/min. and cooled with nitrogen gas (50 mL/min) [23]. The mean particle size (M_D) and zeta potential (ZP) of the SLN were determined by using dynamic light scattering (DLS) (Zetasizer Nano ZS, Malvern Instruments Ltd., UK). DLS analysis was performed at a scattering angle of 90° at 25°C in the auto-measuring mode [24]. Stability studies of optimized ophthalmic BSF@SLN were carried out with the sealed LDPE Bottles containing the final formulation placed in a stability chamber (NEC221RT10I, Newtronic, India) based on the International Council for Harmonisation (ICH) guidelines Q1A (R2) [30,31], and analyzed followed by USFDA's draft guidance for industry on abbreviated new drug applications (ANDAs): Stability Testing of Drug Substances and Products [32].

2.6 *In-vitro drug release testing and its release kinetics*

The *in-vitro* drug release testing (IVRT) of BSF DS from the optimized ophthalmic BSF@SLN (BSF~ 6.6 mg) was carried out by "Franz diffusion cell apparatus". The samples of BSF ophthalmic SLN were placed on the tyffrin membrane (M/S Pall Inc.) between the donor chamber and receptor chamber of the Franz diffusion cell **(Figure 1)**. The receptor chamber was filled with simulated tear fluid (STF) at 37°C ± 0.5ºC with continuous stirring with a magnetic stirrer for 12 Hrs. The testing samples were taken at 2 Hrs. intervals and the withdrawal volume from the chamber was restored with the same volume of media. STF was prepared by dissolving potassium chloride 0.14 gm, calcium chloride 0.008 gm, sodium bicarbonate 0.22 gm, and NaCl 0.68 gm in 100 mL of WFI. The correlation coefficients (r^2) for all models in STF media are shown in SI **Table S7**. The criterion for selecting the most appropriate model was chosen based on the goodness-of-fit test.

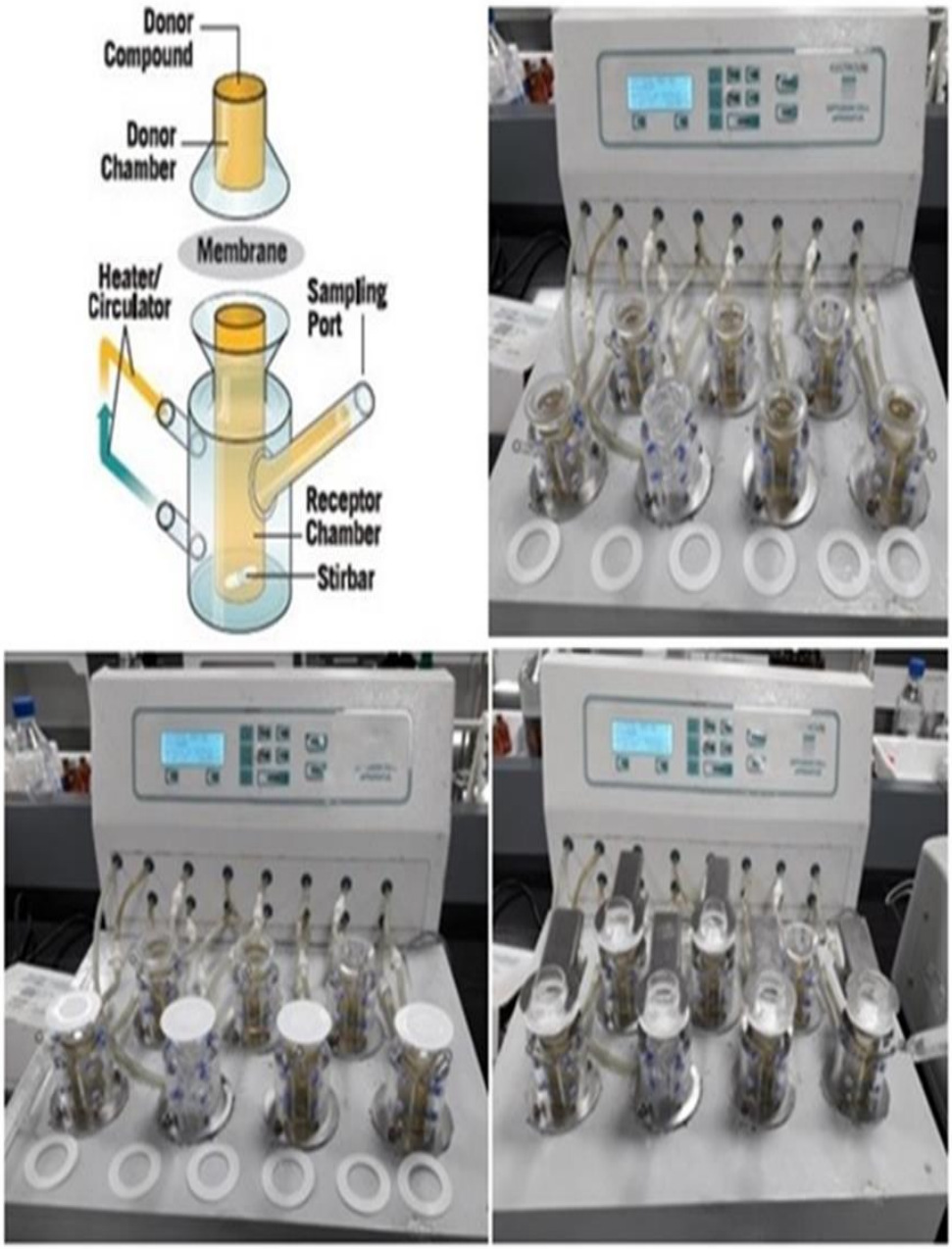

Figure 1. Schematic figure of the Franz diffusion cell apparatus for in-vitro drug release testing (IVRT) of the optimized ophthalmic drug product

2.7 *Comparative antimicrobial efficacy of BSF@SLN and Besix marketed eye drops*

The antimicrobial efficacy of prepared ophthalmic BSF@SLN and Besix marketed eye drops was tested by cup-plate method against organism's *S. aureus* (a Gram-positive spherically shaped bacterium), Bacillota family with the agar well diffusion method in the Soyabean casein digest media [25,26]. The ingredients and their concentrations were taken to prepare the agar media to test the antimicrobial activity against the *S. aureus* bacterium. They are given in **Table S8**.

The ingredients mentioned in **Table S4** were suspended in 1 litre of boiling water. The prepared media was poured into conical flax followed by moist heat sterilization in an autoclave at 15 psi pressure at 121ºC temperature for 15 minutes. After sterilization, 15 ml media was poured into previously sterilized Petri dishes and the *S. aureus* organism was spread on the Petri dishes with the help of a loop. These plates were kept in the bio-oxygen demand incubator at 37ºC for 24 Hrs. The filter paper was dipped into optimized ophthalmic BSF@SLN and compared with the Besix-marketed eye drop. The plates were kept in a bio-oxygen demand incubator maintaining the same condition after being treated with formulations as per USP ⟨71⟩ sterility tests [27].

2.8 *In-vivo study*

The *in-vivo* study was performed by using the *"Draize rabbit eye Irritation Test"* method. The safety of the new formulation was tested following the Draize Irritancy test. It is an acute ocular toxicity test for evaluating the effects of chemicals, substances and mixtures of test samples in terms of their potential to cause eye irritancy or damage to the eye. The study was supported by an intensive examination of the rabbit's eye to establish the safety of the developed ophthalmic formulations [28,29]. The animal experiment was approved by the Institutional Animal Ethics Committee (Registration no. IAEC/05/17/BS/1) and the test was carried out at Deshpande Laboratory, Bhopal, Madhya Pradesh, India. The test was performed following the guidance of care and handling of laboratory animals.

Rabbit eye test:

An eye irritation test was carried out to predict the eye irritancy of the ophthalmic formulation. Here, all three rabbits' eyes were treated with ophthalmic nanoformulation, a placebo of BSF@SLN, BSF@SLN and Besix-marketed eye drops. Several symptoms like irritation, swelling and redness in all three rabbit's eyes were observed for 24 Hrs.

Procedure:

i) Three rabbits (weight between 2.5 to 3.0 Kg) were used for the study.
ii) Rabbits were kept in different cages and provided sufficient food and water.
iii) Rabbits were grouped into three batches for the repetition of the test to lessen test errors.
iv) Animals were then put separately into rabbit holders and marked.
v) Test parameters like irritation, swelling, and redness were checked in all animals before the study.
vi) The test formulation of 100 µL was dropped into the conjunctival pocket of the left eye and the untreated right eye was considered as a control.
vii) The eyes of each animal were observed visually with an ophthalmic torch to check the ocular reactions like redness, swelling or oedema, and itching at various time intervals (1 Hr., 6 Hrs., 12 Hrs., and 24 Hrs.).

2.9 *Statistical analysis*

The analytical results were presented as mean ± standard deviation. Statistically, ANOVA was carried out on the data sets and differences were considered significant for $p < 0.05$.

3. Results and discussion

3.1 *Preliminary developmental batches of SLN*

The hot homogenization technique was used to prepare BSF@SLN. The prepared nanoformulation was evaluated by using various characterization techniques to determine various physicochemical properties like mean diameter, polydispersity index, morphology (size and shape), the surface charge, available and conjugated functional groups, and the crystallinity of the DS and DP.

3.2 *Preliminary developmental and optimization of BSF@SLN nanoformulation*

The initial results of Mean Diameter (M_D), polydispersity index (PDI) and ZP of the preliminary development batches of SLN are mentioned in **Table 1**.

Batch No. SLN-A, SLN-B and SLN-C were manufactured initially to select the lipid and to check the process feasibility. The M_D of SLN-A was found lower than the other two batches (SLN-B and SLN-C), PDI and ZP of the formulation were found acceptable. Therefore, further batches with GMS lipids were planned to optimize the formulation and process variables.

The concentration of finalized lipid (GMS) was decreased from 5 mg/mL to 0.5 mg/mL and the process parameter was evaluated by changing the HPH process parameters. The initial and the 6-month stabilized at 25°C ± 2°C/60% RH ± 5% RH conditions result of the optimized batches of SLN are mentioned in **Table 2**.

As the lipid concentration increased, the % EE also increased. % EE was increased from 61.77 ± 1.33% to 72.38 ± 1.63% by increasing lipid concentration from 0.5 mg/mL to 1.0 mg/mL. Also % lipid content is responsible for the size distribution of the SLN. By optimizing the homogenization cycle number, the M_D was found <100 nm with PDI <1. The % EE was 72.38 ± 1.63% in formulation BSF@SLN-A9 which may be due to

the highest content of lipids. In the optimized BSF@SLN-A9 formulation, PDI and the ZP were obtained at 0.262 and −45.58 ± 0.85 mV, respectively. Therefore, batch no BSF@SLN-A9 was finalized and to be taken forward to optimize the final freeze-drying process. Results are shown in **Table 2**.

Table 1. Results of the preliminary development batches of solid lipid nanoparticles drug products

Sr. No.	Batch No	Lipid carrier	M_D (nm)	PDI	ZP (mV)
1.	**SLN-A**	**GMS**	**90.78 ± 1.2**	**0.278 ± 0.2**	**−41.07 ± 0.64**
2.	SLN-B	GPS	191.49 ± 2.4	0.454 ± 0.3	−38.25 ± 0.44
3.	SLN-C	GB	499. 3 ± 2.7	0.313 ± 0.3	−43.42 ± 0.76

Results are expressed as mean ± SD (n = 3)

Abbreviation: *GMS: Glyceryl monostearate; GPS: glyceryl palmitostearate; GB: glyceryl behenate; SLN: Solid lipid nanoparticles*

Table 2. Initial and 6-month stability results of results mean diameter (MD), polydispersity index (PDI), zeta potential (ZP), and percentage entrapment efficiency (% EE) of various besifloxacin hydrochloride (BSF)-loaded solid-lipid nanoparticles (BSF@SLN-A) ophthalmic drug products

Batch no	Initial results				6 months at 25°C ± 2°C/60% RH ± 5% RH condition			
	M_D (nm)	PDI	ZP (mV)	% EE	M_D (nm)	PDI	ZP (mV)	% EE
BSF@SLN-A1	58.45 ± 3.2	0.258 ± 0.4	−28.13 ± 0.22	61.77 ± 1.33	60.15 ± 7.6	0.274 ± 1.4	−34.41 ± 0.51	60.57 ± 1.43
BSF@SLN-A2	46.11 ± 2.5	0.324 ± 0.6	−30.17 ± 0.57	62.17 ± 2.45	49.77 ± 8.2	0.365 ± 2.1	−38.55 ± 0.37	48.40 ± 2.25
BSF@SLN-A3	41.27 ± 4.2	0.252 ± 0.5	−30.55 ± 0.44	62.85 ± 2.97	46.43 ± 8.6	0.224 ± 2.4	−42.12 ± 0.71	49.61 ± 3.18
BSF@SLN-A4	65.29 ± 4.3	0.376 ± 0.7	−34.11 ± 0.25	64.18 ± 1.98	69.67 ± 6.8	0.364 ± 2.7	−46.32 ± 0.55	52.05 ± 2.51
BSF@SLN-A5	53.28 ± 3.5	0.314 ±0.6	−34.75 ± 0.12	64.75 ±1.35	57.43 ± 7.2	0.325 ± 1.5	−46.12 ± 0.33	52.71 ± 3.66
BSF@SLN-A6	51.05 ± 3.3	0.364 ± 0.6	−36.21 ± 0.65	65.41 ± 1.55	56.78 ± 7.0	0.361 ± 2.1	−47.65 ± 0.44	54.21 ± 2.33
BSF@SLN-A7	90.78 ± 4.6	0.278 ± 0.5	−41.07 ± 0.64	68.03 ± 1.07	104.65 ± 6.4	0.269 ± 1.8	−50.41 ± 0.45	57.44 ± 3.41
BSF@SLN-A8	93.31 ± 3.1	0.334 ± 0.7	−45.22 ± 0.30	69.33 ± 2.14	97.43 ± 7.8	0.341 ± 1.5	−53.64 ± 0.41	61.10 ± 3.55
BSF@SLN-A9	**91.24 ± 4.0**	**0.262 ± 0.5**	**−45.58 ± 0.85**	**72.38 ± 1.63**	**96.11 ± 8.1**	**0.285 ± 1.2**	**−54.74 ± 0.61**	**62.82 ± 3.11**

Results are expressed as mean ± SD (n = 3)

3.3 *Freeze-dried batch BSF@SLN-A9 drug product*

The BSF@SLN-A9 batch was subjected to a lyophilization process in a benchtop lyophilizer. The batch was dried for 24 Hrs. and the water content of the dried SLN was found to be 4.6 ± 0.5%. The water content result was well within the target limit (not more than 5%). Hence, the BSF@SLN-A9 batch was selected and taken for further analysis.

3.4 *Statistical analysis*

Here two independent factors were used as Process (X_1), drug and Lipid ratio (X_2) and three response factors such as PS, ZP and EE were selected. Three factorial levels are coded as −1 (low), 0 (medium), and +1 (high) and the experimental run design is presented in **Table 3**.

The ANOVA of M_D, ZP and EE indicates a narrow and homogenous size distribution and values of "Prob > F" found less than 0.05 indicate the model was significant. The regression model was used to generate the contour plots, for M_D (nm), ZP (mV) and % EE are shown in **Figure 2** for analysing interactions of the independent factors.

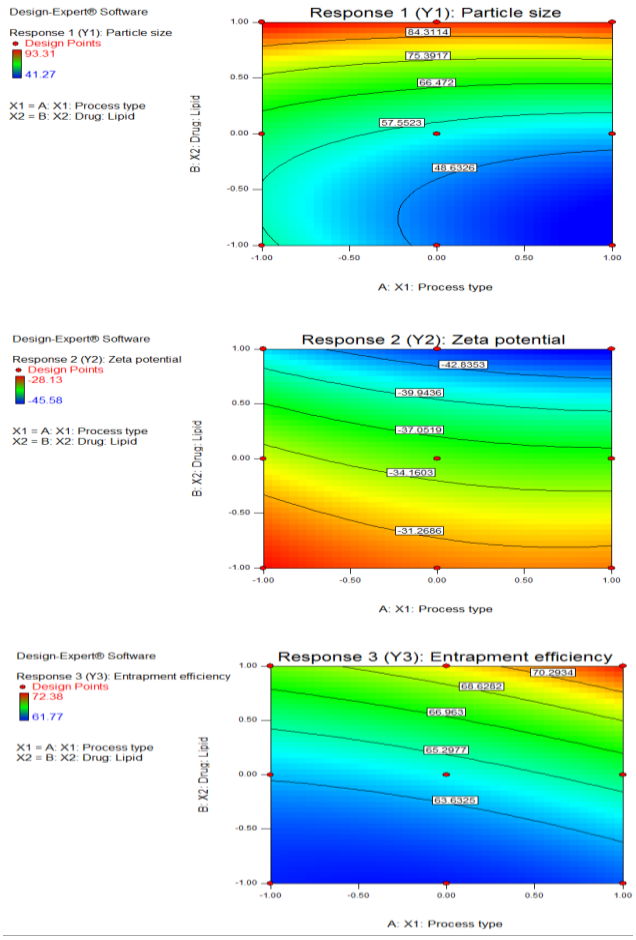

Figure 2. Counter plot for Response 1 (Y1): mean particle size, Response 2 (Y2): zeta potential and Response 3 (Y3): entrapment efficiency through Design–Expert® statistical software

Table 3. Experimental run with two different factors (process and drug: lipid) and their responses mean particle size (MD), zeta potential (ZP) and % entrapment efficiency (% EE) of besifloxacin hydrochloride drug substance

Standard	Run	Factor 1	Factor 2	Response 1	Response 2	Response 3
		X_1: Process	X_2: BSF: Lipid	Y_1: M_D (nm)	Y_2: ZP (mV)	Y_3: EE (%)
2	1	0.00	-1.00	46.11	-30.17	62.17
6	2	1.00	0.00	51.05	-36.21	65.41
9	3	1.00	1.00	91.24	-45.58	72.38
5	4	0.00	0.00	53.28	-34.75	64.75
7	5	-1.00	1.00	90.78	-41.07	68.03
1	6	-1.00	-1.00	58.45	-28.13	61.77
8	7	0.00	1.00	93.31	-45.22	69.33
4	8	-1.00	0.00	65.29	-34.11	64.18
3	9	1.00	-1.00	41.27	-30.55	62.85

Abbreviations: *BSF: Besifloxacin hydrochloride; M_D: Mean particle size; ZP: Zeta potential; EE: Entrapment Efficiency*

3.5 *Evaluation of optimized BSF@SLN-A9 nanoformulation*

The optimized ophthalmic BSF@SLN-A9 was evaluated in terms of various physicochemical parameters like appearance, pH, weight per mL, and an assay of BSF, PDI, ZP, and M_D results at initial and 6 months of long-term stability are presented in **Table 4**. The optimized batch showed M_D 91.24 ± 4.0 nm, PDI 0.262 ± 0.5, ZP

−45.58 ± 0.85 mV and entrapment efficiency 72.38 ± 1.63% in the lipid phase and 27.62% of the drug was in aqueous phase.

Table 4. Physico-chemical results of stabilized optimized besifloxacin hydrochloride (BSF)-loaded solid-lipid nanoparticles (BSF@SLN-A9) ophthalmic drug product under long-term condition (25°C ± 2°C/60% ± 5% RH) and accelerated condition (40°C ± 2°C/75% ± 5% RH) stability conditions for 6-months and 1 month, respectively

Test Parameter	Target Specification	Initial	40°C ± 2°C/75% ± 5% RH	25°C ± 2°C/60% ± 5% RH		
			1 Month	1M	3M	6M
Appearance	White colour suspension	Complies	Complies	Complies	Complies	Complies
pH	5.7- 6.7	6.5	6.4	6.5	6.4	6.3
Density (g/mL)	0.99 -1.02	1.00	1.01	1.00	1.00	1.00
Osmolality (mOsm/Kg)	270-330	291	289	293	293	290
Assay of BSF (%)	90.0-110.0	104.5	102.8	103.2	103.4	101.7
M_D (nm)	NMT 500.0	203.2	364.4	227.5	243.1	292.4
PDI	NMT 0.4	0.177	0.361	0.189	0.196	0.235
ZP (mV)	NLT -30.0	-62.2	-77.5	-63.1	-64.5	-68.1

Abbreviations: *BSF: Besifloxacin hydrochloride; M_D: Mean particle size; NLT: Not less than; NMT: Not more than; PDI: Polydispersity index; ZP: Zeta potential*

3.6 *Characterization of optimized BSF@SLN*

Scanning Electron Microscopy:

The surface morphology, size distribution and structure of the optimized ophthalmic BSF@SLN-A9 was observed under SEM. SEM was performed to capture for morphological evaluation at a magnification of 100X and spatial resolution of 100 µm. The SEM image BSF@SLN-A9 has a spherical texture and is uniformly distributed throughout the matrix as shown in **Figure 3**. The SEM image also revealed the smooth surface of ophthalmic SLN. Based on the SEM images, the BSF@SLN-A9 have an average particle size of <100 µm. The obtained mean particle diameter size was also compared with the results derived from the DLS.

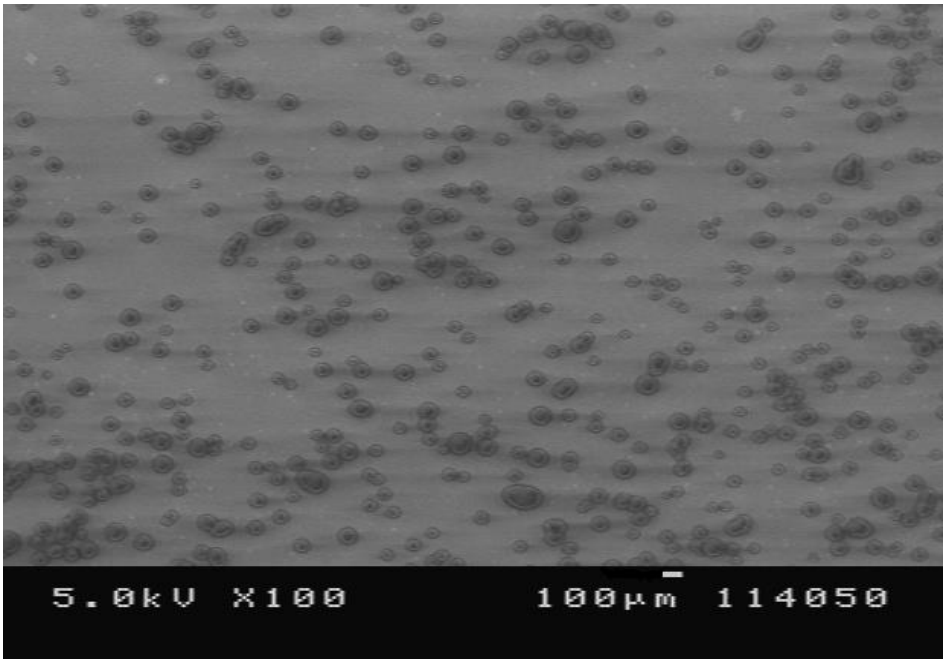

Figure 3. Scanning Electron Microscopy micrograph of optimized besifloxacin hydrochloride (BSF) drug substance-loaded solid-lipid nanoparticles (BSF@SLN-A9) ophthalmic lyophilized drug product at a magnification of 100x and spatial resolution of 100 µm

Fourier transform infrared spectroscopy:

The FTIR spectra of all the compounds are shown in **Figure 4**. The absorption spectrum bands of BSF at 1620 cm^{-1} correspond to C=C stretching; 1640 cm^{-1} is attributed to amide-I; 1690-1730 cm^{-1} is corresponding to –C=O stretching of COOH; 1440-1500 cm^{-1} is corresponding to –CH bending and 1050-1250 cm^{-1} is corresponding to C–O stretching **(Figure 4A)** [33–35]. The typical infrared characteristic absorption bands include 3473 cm^{-1}, stretching vibration of broad –OH group; 2956–2850 cm^{-1}, symmetric and asymmetric stretching vibration of the $–CH_2$ and $–CH_3$ group; 1467 cm^{-1}, bending vibration of the $–CH_3$ group; 1731–1734 cm^{-1}, stretching vibration of –C=O group present in ester linkages; 1160–1169 cm^{-1}, bending vibration of –C–O band; 720–721 cm^{-1}, long chain band **(Figure 4B)** [36,37]. The FTIR results of BSF@SLN clearly show that the sharpness of symmetric and asymmetric stretching vibrations at 2956–2850 cm^{-1}, was decreased. It may be due to the mixing of a low amount of BSF. Additionally, other absorption peaks were also shifted compared to the GMS. It is proven that the BSF DS is properly mixed in the GM's matrix **(Figure 4D)**. The concentration of the BSF DS is much lower while the GMS lipid concentration is too high. So, the drug is properly entrapped in a lipidic core and enhances the drug entrapment and loading capacity. This also shows the storage stability of the system.

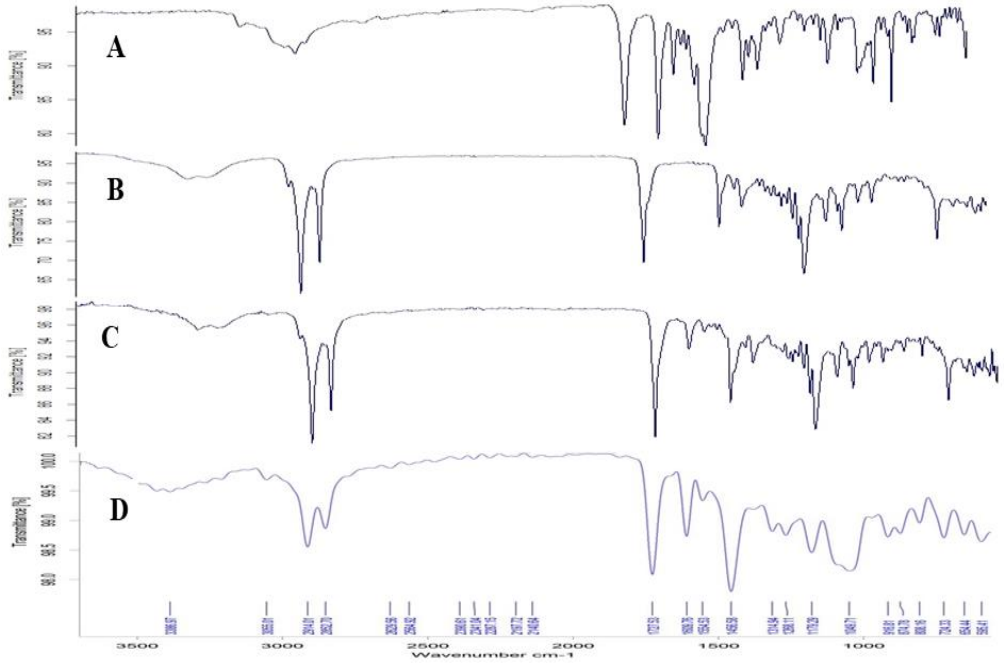

Figure 4. FTIR spectra of (A) Besifloxacin hydrochloride drug substance (BSF) (B) Glyceryl monostearate (GMS) (C) BSF and GMS mixture (D) Optimized BSF-loaded solid lipid nanoparticles (BSF@SLN-A9) ophthalmic lyophilized drug product

X-ray diffraction spectroscopy:

The XRD of drug substance, lipid and BSF@SLN are shown in **Figure 5**. Diffraction from different planes of atoms within the crystalline material produces a pattern of sharp diffraction peaks due to the atomic arrangements of BSF. The amorphous solids show the disappearance of the sharp peaks present in the spectrum. XRD of final formulations showed that characteristic peaks of BSF were decreased in intensity and the number showed that the crystalline nature of the drug was reduced and the drug was completely encapsulated in the SLN.

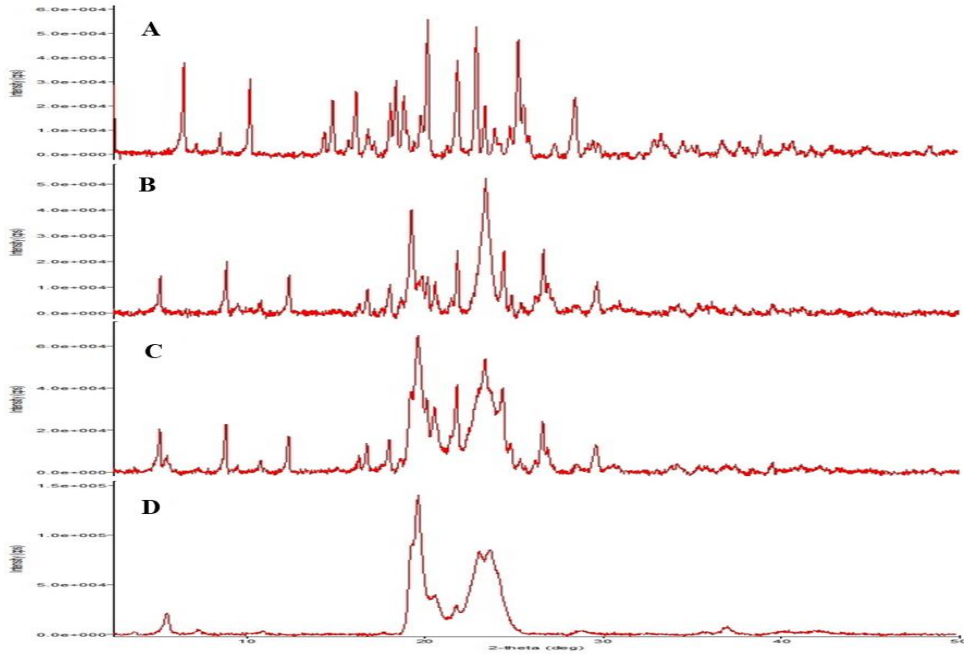

Figure 5. XRD spectra of (A) Besifloxacin hydrochloride (BSF) (B) Glyceryl monostearate (GMS) (C) BSF and GMS (D) Optimized BSF-loaded solid lipid nanoparticles (BSF@SLN-A9) ophthalmic lyophilized drug product

Differential scanning calorimetry:

The DSC curve was running between room temperature (25°C) to 400°C. The DSC thermogram shows that BSF does not show thermal events in the temperature range up to 200°C [38]. It is a crystalline powder with a strong and sharp exothermic peak onset temperature at 304.33°C, weak endothermic peak at 308.68°C and heat of fusion at 106.17 J/g **(Figure 6A)** [39]. Bulk glyceryl monostearate melted and showed a strong and sharp endothermic peak at around 70°C **(Figure 6B)** [40]. The thermogram of BSF@SLN shows the onset temperature initially as endothermic peaks at 57.91°C, another strong and sharp endothermic peak was obtained between 100°C to 150°C and finally as 304.96°C **(Figure 6D)**. The thermal behaviour of SLN suggested that the BSF DS was entrapped in the vesicle.

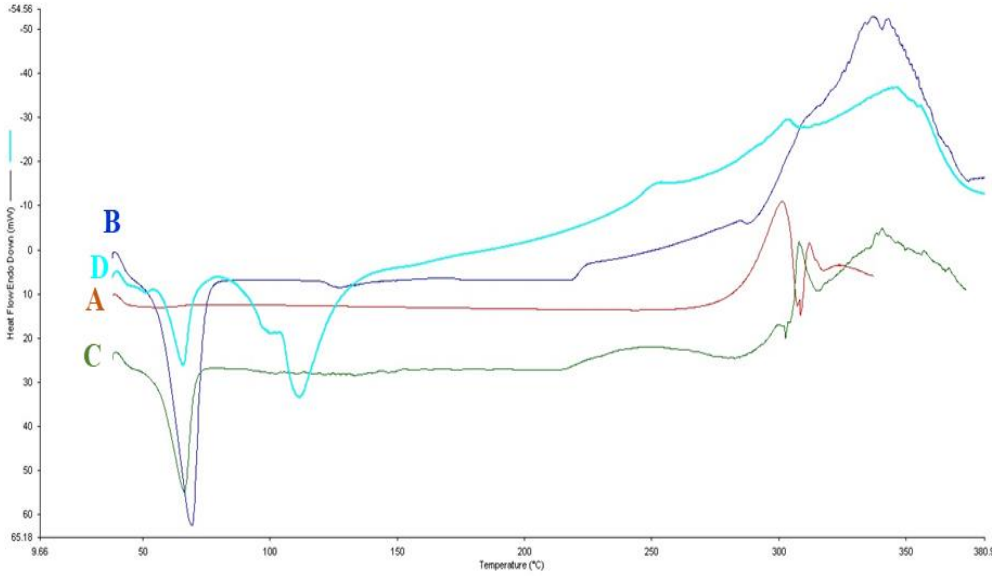

Figure 6. DSC spectra (A) Besifloxacin hydrochloride (BSF), (B) Glyceryl monostearate (GMS), (C) BSF and GMS, and (D) Optimized BSF-loaded solid lipid nanoparticles (BSF@SLN-A9) ophthalmic lyophilized drug product

3.7 *In-vitro drug release testing and its release kinetics*

Figure 7A shows the IVRT of BSF DS from optimized ophthalmic BSF@SLN-A9 for 12 Hrs. in the STF at 37°C ± 0.5ºC. From this release profile, it can be assumed that the optimized ophthalmic BSF@SLN-A9 (drug: lipid = 1:1) shows the long-term release for increasing the hold time in the eye.

In the initial 4 Hrs., a release of 19.68% ± 0.254% BSF from BSF@SLN was observed. However, 60.45% ± 0.366% BSF was released from optimized BSF@SLN-A9 after 12 Hrs. study. The result clearly showed that BSF@SLN-A9 was responsible for the controlled release of BSF from BSF@SLN-A9. The IVRT of BSF was performed by using the Franz diffusion cell apparatus. The release kinetics results suggest that BSF is released from BSF@SLN-A9 by a Korsmeyer–Peppas mechanism and follows a Higuchi pattern **(SI Table S7)**.

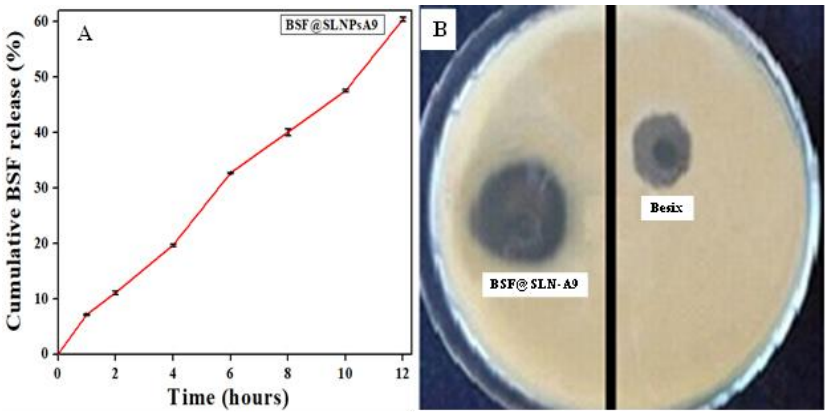

Figure 7. (A) In-vitro release of optimized Besifloxacin hydrochloride drug substance-loaded solid lipid nanoparticles (BSF@SLN-A9) ophthalmic drug product by using IVRT. Data shown in the line graph denote the mean ± standard deviation of n=3 replicates. (B) Comparative antimicrobial activity of optimized BSF@SLN-A9 ophthalmic DP and market Besix eye drop against *S. aureus* after 24 hours incubation

3.8 *Comparative antimicrobial efficacy of BSF@SLN-A9 and marketed DP*

The antibacterial activities of the BSF@SLN-A9 compared to the marketed DP, Besix eye drop were calculated against *S. aureus*. The zones of inhibition of Besix marketed eye drop and the developed BSF@SLN-A9 were found at 16.35 ± 2.14 mm, and 45.15 ± 1.48 mm, respectively. From the above results, it was found that the BSF@SLN-A9 nanoformulation is 2.76 times more effective than the Besix eye drop. The zones of inhibition were evaluated after 24 Hrs. and microorganism growth reduction was presented in **Figure 7B and Table 5**. The result clearly showed that BSF@SLN-A9 has better antimicrobial activity than the marketed Besix eye drop.

Table 5. Zone of inhibition results of market formulation and developed nanoformulations

Sr. No.	Sample details	Zone of inhibition (mm)
1.	BSF@SLN-A9	45.15 ± 1.48
2.	Besix (Marketed eye drop)	16.35 ± 2.14

The result is expressed as mean ± SD (n=3)

3.9 *Sterility testing*

The sterility testing of optimized BSF@SLN-A9 ophthalmic DP was also performed for fungi, anaerobic bacteria and aerobic by using Fluid Thioglycollate medium for aerobic & anaerobic bacteria and Soyabean casein digest medium for aerobic bacteria and fungi through Methods A: Membrane Filtration as per the IP'07 procedure.

Test for anaerobic bacteria: The test organism used was *Clostridium sporogenes* (ATCC 19404). No proof of growth was found in the test samples.

Test for aerobic bacteria: The test organisms used were *Bacillus subtilis* (ATCC 6633) and *Pseudomonas aeruginosa* (ATCC 9027). No proof of growth was found in the test samples.

Test for fungi: The test organism used is *Candida albicans* (ATCC 10231). No proof of growth was found in the test samples.

The results showed that optimized ophthalmic BSF@SLN-A9 passed the sterility test. The above formulation was considered to be sterile and used in an *in-vivo* study.

3.10 *In-vivo study*

The photographs of Rabbit eyes in **Figure 8**, showed, that after 24 Hrs. of administration of the above formulations, no evidence of eye irritation, swelling, redness, discharge or opacity was observed. Optimized ophthalmic BSF@SLN-A9, placebo of BSF@SLN-A9 and Besix-marketed eye drops were found compatible with experimental animals after 24 Hrs.

The physiology of experimental animals (Rabbit eye) is closely related to the human eye. Therefore, it can be concluded that the prepared ophthalmic BSF@SLN-A9 nanoformulation does have not any irritant effect on human eyes too, and can be used safely as an ophthalmic preparation.

The ocular safety studies of the optimized ophthalmic BSF@SLN-A9 were found safe at the end of 24 Hrs. Therefore, the final formulations were considered non-irritating. No significant effects were observed in the eyes of experimental animals during the study. The effects of different formulations in rabbit eyes after 24 Hrs. are presented in **Table 6 and Figure 8**.

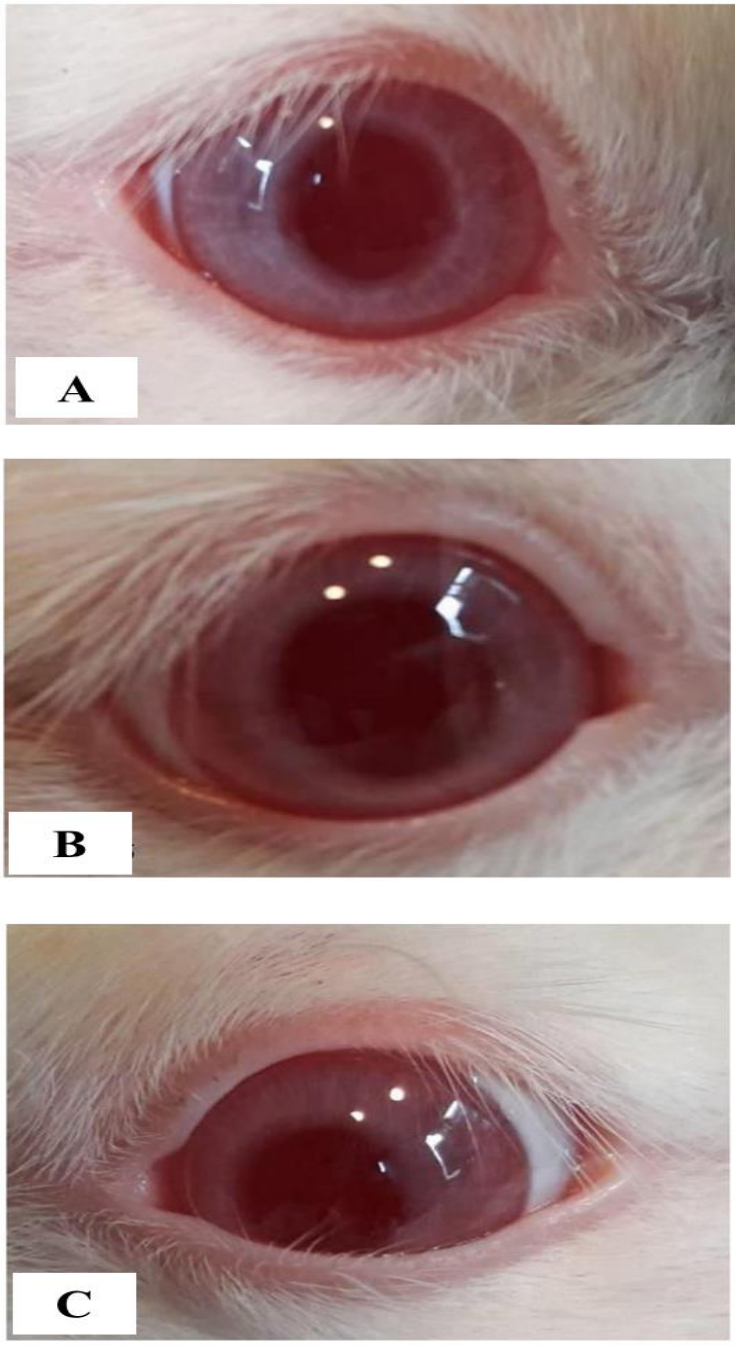

Figure 8. Photograph of Rabbit eye after 24 hours of instillation of the samples (A) Optimized BSF@SLN-A9 ophthalmic drug product, (B) Placebo sample of BSF@SLN-A9 ophthalmic drug product (C) Besix marketed eye drop

Table 6. Effects of different formulations such as optimized besifloxacin hydrochloride (BSF)-loaded solid-lipid nanoparticles (BSF@SLN-A9) ophthalmic drug product (DP), placebo of BSF@SLN-A9 ophthalmic DP, and marketed Besix eye drop on the Rabbit eye at various periods

Sample name	Time after treatment	Cornea	Conjunctiva			Iris
Test parameters		Opacity	Redness	Swelling	Discharge	Swelling
Optimized BSF@SLN-A9 ophthalmic drug product	1 hour	×	×	×	×	×
	6 hours	×	×	×	×	×
	12 hours	×	×	×	×	×
	24 hours	×	×	×	×	×
Placebo of BSF@SLN-A9 ophthalmic drug product	24 hours	×	×	×	×	×
Market Besix eyedrop	24 hours	×	×	×	×	×

×: No effect observed

4. Conclusion

In the present investigation, the SLN and BSF@SLN ophthalmic DP were successfully fabricated. The optimized formulation shows the desired quality target product profile and high entrapment efficiency of BSF was also observed for all BSF@SLN nanoformulations. In SEM, the spherical-shaped NPs were found uniformly distributed throughout the matrix of a polymer. The stability data shows that there is no significant difference in the optimized ophthalmic BSF@SLN-A9 after 6 months at long-term conditions (25°C±2°C/60%±5% RH). The high drug-loaded BSF@SLN-A9 showed a sustained-release ocular delivery system for up to 12 Hrs. and was found to be better than marketed available eye drops. The study opens the chances of manufacturing the BSF@SLN at a competitive cost at the commercial level.

The lipid-based drug delivery system may have been subjected to a passive diffusion process, which might be responsible for better penetration and the entrapped formulation was able to provide a sustained release of drugs from lipidic cores. Moreover, the formulations were hydrophilic enough to be easily wetted by tear and adhere to the corneal surface. The antimicrobial activity of optimized BSF@SLN-A9 ophthalmic DP was found 2.76 times more effective compared to the Besix eye drop. At last, based on the *in-vivo* ocular safety test it was concluded that BSF@SLN-A9 can be a promising controlled-release formulation one day. From the development, optimization and characterization of BSF@SLN, it is concluded that BSF@SLN showed satisfactory outcomes for long-acting ocular nanoformulation and suggested scale-up in future.

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Conflict of interest

There is no conflict of interest for this study.

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