Original Article

Optimization of *in-situ* Nanoparticulate Gel of Ofloxacin using Factorial Design to Improve Treatment Strategy for Conjunctivitis and Corneal Ulcers

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ABSTRACT

Background: One of the main problems in ophthalmic drug delivery is the rapid elimination of conventional liquid eye drops from the eye due to rapid tear turnover resulting pre-corneal loss because of irritation caused by the drug preparation from large volume of the administered eye drop (~50 µl), a very small amount i.e. <5% of the dosage actually penetrates and is able to reach intraocular tissues. Ofloxacin (OFL), a fluoroquinolone drug is used for the treatment of conjunctivitis and corneal ulcers and it is given quarterly in a day because its bioavailability is very low. The present study was designed to increase the ocular residence time of drug by formulating nanoparticulate *in-situ* gel for ensuring low irritation, better release and compatibility with ocular tissue. **Materials and Methods:** Initially, OFL nanoparticles were prepared using single emulsion solvent evaporation method and evaluated for various evaluation parameters. Based on the results, an optimized batch (B3) was selected by applying factorial design; which was further converted into *in-situ* gel. **Results:** Results of evaluation parameters revealed that optimized batch (B3) showed significant ($p=0.0001$ and $p=0.0090$) increased in particle size and entrapment efficiency in comparison to rest batches. Moreover, nanoparticulate *in-situ* gel showed satisfactory results. **Conclusion:** Hence, Ofloxacin nanoparticulate *in-situ* gel thus formulated showed controlled drug release with lesser dosing frequency.

Key words: Ofloxacin, Nanoparticle(s), Nanoparticulate *in-situ* gel, Conjunctivitis, Factorial design.

INTRODUCTION

Controlled drug delivery provides the drug at a predetermined rate, locally or systemically for a specified period of time.[1] Ocular drug delivery is one of the most appealing and arduous endeavours faced by the pharmaceutical scientists. Usually less than 5% of the topically applied drug penetrates the cornea and attains the posterior segment of the eye. The bioavailability of an instilled conventional drug onto the ocular surface is usually low due to physiological mechanisms like tear drainage, protein binding, systemic absorption, enzymatic degradation and complex penetration barriers. Moreover, a bigger portion of it is absorbed systemically through the nasolacrimal duct which may give rise to systemic adverse effects.[2] Nanotechnology deals with nanometer sized objects e.g. nanoparticles which are used for various reasons.[3,4] As a drug delivery system, the *in-situ* gel has an advantage related to the gel or polymer network being formed (*in-situ*) providing sustained release of the drug.[5] Ofloxacin (OFL) ((±)-9-fluro-2, 3 dihydro-3-methyl-10 [4-methyl-1-piperazynyl-7-oxo-7H-pyrido [1, 2, 3-de]-1, 4-benzoxacine-6-carboxilic acid, stops or prevents ocular bacterial infections by either

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killing or inhibiting their growth.[6,7] It is reported that maximum serum concentrations after ten days of topical ophthalmic dosing were approximately 1000 times lower than standard oral doses of OFL.[8] That's why, the present work aimed at formulation of an optimized Nano particulate *in-situ* ocular gel of Ofloxacin that would augment its ocular bioavailability and thus therapeutic adequacy which is vital for legitimate treatment of Conjunctivitis and Corneal Ulcers as well as such other ailments.

MATERIALS AND METHODS

Materials

Various materials i.e. drug sample, additives, reagents etc. were obtained from different reputed companies. Ofloxacin-Vivan Life Sciences, Mumbai, Ethyl cellulose (EC)- Fine Chem Labs, Mumbai, Dichloromethane-Rankem, Ethanol- Jiangsu Huaxi International, Span -80- Rankem, Mannitol- Local source suppliers, Poly Vinyl Alcohol (PVA)- West coast laboratories, Mumbai, Gellan gum- Hi media GGM2388, Benzalkonium chloride, Sodium chloride, Sodium bicarbonate and Calcium chloride dihydrate- CDH Delhi, respectively.

Methods

Estimation of λ_{max}

A sample (5 µg /ml) was scanned at the range of 200-400 nm to access the λ_{max} value for OFL which was reported and confirmed by obtaining the overlain UV spectra of the drug using different concentrations (5-25 µg/ml).

Drug-excipient compatibility study

Compatibility study using FTIR technique

Drug-excipient compatibility study was carried out by FTIR (Shimadzu, Affinity-1) spectrophotometry. The mixture of drug and KBr (Potassium bromide) was ground into fine powder using mortar pestle and then compressed into discs in a hydraulic press at a pressure of 75 kg/cm^2. Each KBr disc was scanned 45 times at a resolution of 2 cm^{-1}. The characteristic peaks were recorded and compared with that obtained with individual excipient used.

Thin Layer Chromatographic (TLC) method

The drug-excipient compatibility was also studied by densitometric TLC evaluation. The spots of drug and different excipients were obtained on pre-coated silica gel 60GF^{254} plates against n-butanol: ethanol: ammonia in the ratio of 5:5:4 (%v/v) as mobile phase. The densitometric evaluation of separated spot was performed at 295 nm.[9]

Method of Preparation of Ofloxacin loaded nanoparticles

Ofloxacin (OFL) loaded nanoparticles were prepared using single emulsion solvent evaporation method as given by Huyang Yan *et al*. with some modifications. Drug and polymer in different proportions were weighed and co-dissolved at room temperature into a mixture of ethanol and dichloromethane (2:1% v/v) with magnetic stirring. This was slowly poured drop wise into the dispersion medium consisted of 20 ml of 1% (w/v) aqueous PVA and 1.5% (w/v) span 80. Sonication was done by probe sonicator (PCI analytics, Mumbai (DP 120), MHA) for 2 min. on the ice bath. Later on, the system was put on magnetic stirrer (overnight) for complete evaporation of organic solvents. The prepared suspension was centrifuged (Remi, Mumbai, MHA) at 15000 rpm for 1 hr. The supernatant was removed and the sediment was freeze dried in the presence of 5% mannitol as cryoprotectant for 48 hrs to stabilize the nanoparticles. The obtained particles were kept in dehydrated condition.[10]

Evaluation of Nanoparticles

The nanoparticles thus prepared were evaluated for various physicochemical parameters i.e. Percentage yield, Drug entrapment efficiency, Surface morphological study, Particle size and Zeta potential, *in-vitro* release and release kinetic studies.

In-vitro release studies

Drug-loaded nanoparticles were suspended in pH 7.4 phosphate buffer in a glass vial which was placed in a mechanical shaking bath (100 cycles/min) at a temperature adjusted to 37°C. At selected time intervals, samples were removed, replaced with fresh buffer medium and centrifuged at 15,000 rpm. The obtained supernatant was analyzed using U.V.-visible spectrophotometer at every transaction.[10]

Factorial design

Full factorial design was used in the present study. In this design 2 factors namely amount of EC (X1) and the polyvinyl alcohol (X2) were evaluated, at 3 levels (-1,0,1) and experimental trials were performed at all 9 possible combinations. The amount of EC (X1) and PVA (X2) were chosen as independent while particle size and % entrapment, as dependent variables. The simplified models were then utilized to produce three dimensional response surfaces and contour plots to analyze the influence of independent variables.[11]

Release kinetic

The *in-vitro* dissolution data of Ofloxacin thus obtained was fitted into various pharmacokinetic models i.e. zero order, first order, higuchi and korsemeyer-peppas models to assess the release mechanism of drug.

Preparation of Nano particulate *in-situ* gel

Based on the results obtained from statistical studies, optimized batch was converted into gel with selected gelling agent.

Determination of gelling capacity of selected gelling agents

Aqueous solution of different concentration of polymer (s) i.e. Gallen gum, HPMC and Carbopol 940 with formulation codes F1, F2.....F6 were prepared and evaluated for their gelling capacity and viscosity in order to identify the compositions most suitable for use in *in-situ* gelling system. The gelling strength of prepared formulation (*in-situ* gel) was determined by placing 100 ml of the system in a vial containing 2 µl of simulated tear fluid (STF) freshly prepared and equilibrated at 37°C and visually assessing the gel formation and noting the time for gelation including the time taken for the formed gel to dissolve (Table 1).

Preparation of Nano particulate *in-situ* gel of Ofloxacin

Nano particulate *in-situ* gel of Ofloxacin was prepared by using gellan gum because it showed highest viscosity that persists for longer duration of time and thus selected for further work. Gellan gum was dispersed in deionized water, heated to 90°C while stirring and then cooled to room temperature. Later on, drug loaded nanoparticles (equivalent to the prescribed dose of the drug) were dissolved in purified water and allowed with the addition of benzalkonium chloride. Then this solution was added to the above solution. The pH was adjusted in the range of 5.0-6.0 using 0.1 M NaOH Solution. The volume was made-up to 100ml with purified water. Prepared formulations were sterilized in an autoclave at 121°C and 15psi for 20 min. (Table 2).[12]

Evaluation of Nano particulate *in-situ* gel of Ofloxacin

Clarity

The clarity before and after gelling was determined by visual examination of the formulations under light, alternatively against white and black backgrounds.

pH

Formulations were taken in a beaker and pH was checked using digital pH meter.

Gelling strength

The gelling strength of prepared formulation (*in-situ* gel) was determined by placing 100 ml of the system in a vial containing 2 µl of Simulated Tear Fluid (STF) freshly prepared and equilibrated at 37°C and visually assessing the gel formation and noting the time for gelation including the time taken for the formed gel to dissolve.

Rheological evaluation

Viscosity of formulation was determined after gelation using Brookfield viscometer.

Ocular irritancy test

The prepared formulation i.e. *in-situ* gel was evaluated for ocular irritancy in Albino Rabbits (n=3). They were housed and maintained according to protocol. Eyes were marked as test and control respectively. The control eyes received no sample while the test eyes received the formulation (0.5ml) and observed for the ocular irritancy including the macroscopic observation of cornea, iris and conjunctiva. The protocol was approved by IAEC of the institute in accordance with CPCSEA norms (Lic No.- (1205/C/08/CPCSEA/21.04.08).

Ex-vivo drug release study of Nano particulate *in-situ* gel

Goat cornea was used for the present investigation to study permeation across the corneal membrane. Whole eyeballs of goat were procured from a slaughterhouse and transported to laboratory in cold condition in normal saline maintained at 4°C.

The cornea were carefully removed along with a 5–6 mm of surrounding scleral tissue and washed with cold saline. The washed cornea were kept in cold and freshly prepared solution of STF (pH 7.4). The study was carried out by using Franz-diffusion cell in such a way that corneal side continuously remained in an intimate contact with the formulation in the donor compartment. The receptor compartment was filled with STF (pH 7.4 at 34°C ± 0.5°C). The receptor medium was stirred on a magnetic stirrer. The samples were withdrawn at different time intervals and analyzed for drug content. Receptor phase were replenished with an equal volume of STF (pH 7.4) at each time interval.[13]

Accelerated stability studies

Formulations were packed in amber colour vials and sealed with aluminum foil and stored at 40± 2^{0}C and 75 ±5% RH for 3 months as per ICH guidelines. Sample was analyzed at specific time intervals for clarity, pH and *in-vitro* dissolution.[14]

Table 1: Gelling capacity of different polymers.

S. No.	Drug	Batch code	Polymer (mg)		Gelling capacity	Viscosity (cP)
1.	Ofloxacin		HPMC K100M	Carbopol 940		
		F1	50	450	+	413
		F2	100	650	++	668
		F3	150	850	+++	860
			Gelrite			
		F4	500		++	643
		F5	750		+++	862
		F6	1000		+++	1080

Notice: (-) no gelation, (+) gel after a few minutes dissolved rapidly, (++) immediate gelation that remained for few hours and (+++) immediate gelation that remained for extended period.

Table 2: Formulation design of nanoparticulate *in-situ* gel (100ml, 0.3%).

Ingredients (mg)	Amount (w/v)
Ofloxacin nanoparticles (mg)	1020.1
Gellan gum (mg)	750
Benzalkonium chloride (%)	0.1
NaOH (0.1M)	q.s for pH adjustment
Purified water	q.s upto 100 ml

RESULTS AND DISCUSSION

In the present study, Nano particulate *in-situ* gel of Ofloxacin was prepared to increase the residence time of drug on ocular surface which is a major drawback of majority of conventional dosage forms i.e. eye drops, ointments and gels etc. Fluoroquinolones are commonly used antimicrobials (effective for both gram negative and gram positive bacteria) in the treatment of various bacterial infections and are generally well tolerated.[15] Ofloxacin, a fluoroquinolone drug is used for the treatment of conjunctivitis and corneal ulcers and it is given quarterly in a day because its bioavailability is very low. Hence, nanoparticles of Ofloxacin were firstly prepared using EC to improve the bioavailability of drug. The stock solution of Ofloxacin (5µg/ml) was prepared using pH 7.4 phosphate buffer and scanned between 200-400 nm which concluded λ_{max} of 295nm (Figure 1). Compatibility of drug with different excipients was checked by FTIR and TLC densitometric studies. The IR spectra, of pure drug alone and in its combination with the polymers used, were obtained and compared and depicted in following Figures (Figure 2). FTIR spectra of the pure OFL and the drug polymer mixture showed characteristic bands at 3000 cm^{-1} (stretching vibration of OH group, NH stretching), 2520.51 cm-1 and 2135.78 cm^{-1}(υCH3 gp), 1803.12 cm^{-1}(acidic carbonyl C=O stretching) and 1464.67 (stretching vibration of CH2), indicating the chemical stability OFL in the chosen polymeric mixture. Similar results were presented by Kumar M, *et al*.[16] The R_f values of pure Ofloxacin and with various excipients ranged between 0.62-0.729 (Table 3 and Figure 3).

Prepared nanoparticles were evaluated for Percentage yield, Drug entrapment efficiency (EE), Particle size analysis, Zeta potential analysis and *ex-vivo* permeation (Table 4). Results of *in-vitro* dissolution profile in Simulated Tear Fluid (STF) have depicted (Figure 5). The *in-vitro* release studies conducted on nanoparticles indicated that the OFL loaded ethyl cellulose nanoparticles provided sustained drug release over a period of 12 hr.

Whole experimental data was fitted into 3^2 full factorial design and results of regression analysis interpreted that both independent variables i.e. the amount of EC (factor A) and percentage of PVA (factor B) had positive effect on dependent variables i.e. particle size and entrapment efficiency with *p* value of 0.0001 and 0.0090 respectively. Results of factorial design were as shown in following table (Table 5, 6). It was observed from equation (1) (Table 6) that A and B factors had significant positive effect on Particle size, which indicated that when the values of A and B increased, the response increased (p=0.0001) i.e. as the amount of EC and percentage of PVA was increased, Particle size and Entrapment efficiency were also increased. Elaissari A, *et al*.[17] reported that there was no considerable influence of different concentrations of PVA on the mean particle size. However, after a certain limit of concentration of PVA (above 0.1 mg/mL) a large increase in particle size was observed. This sudden increase in size could be attributed to deposition of extra PVA onto the surface of particles.

It was observed from equation (1) (Table 6) that both A and B factors also had significant positive effect on Entrapment efficiency, which indicated that when the values of A and B increased, the response increased

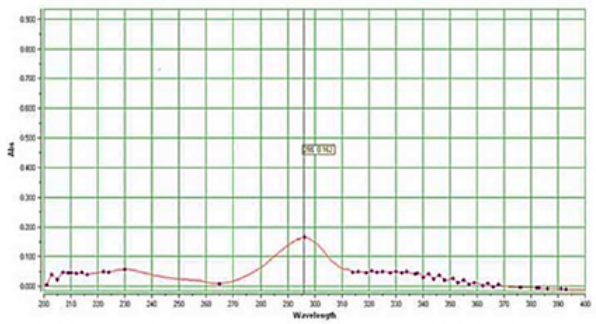

Figure 1: UV scan of Ofloxacin showing characteristic wavelength.

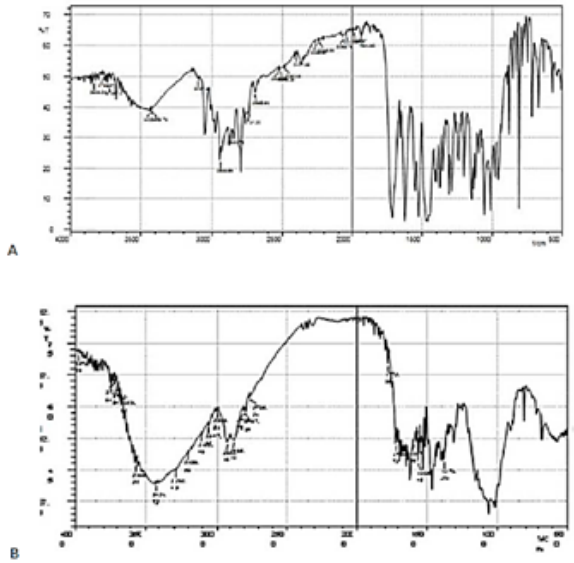

Figure 2: FTIR spectra of A) Ofloxacin (pure drug), B) Ofloxacin with Gellan gum.

Figure 3: Photographic representation of TLC with different drug polymer combination.

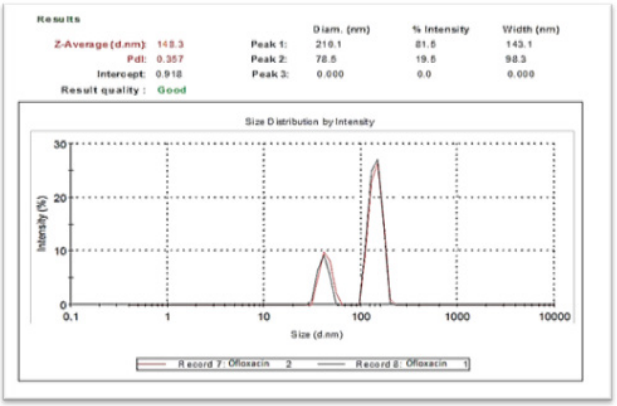

Figure 4: Particle size analysis of Ofloxacin nanoparticles (Optimized batch B3).

(*p*=0.0090) and hence, both dependent variables were increased with increase in independent variables.

From Response surface plots (Figure 6, 7), it was concluded that both particle size and entrapment efficiency increased from blue to orange region owing to increase in amount of EC and PVA.

Optimization study thus conducted, has given a predicted solution. Predicted solution had the similar composition (factor A: 300 mg; factor B: 2%) and showed identical responses as that of batch B3 and thus it was selected as optimized batch with a desirability of 1. The optimized batch (B3) showed particle size (148 nm, Figure 4), EE (57.22%) and drug release (52.5 % and 51.3%) in pH 7.4 phosphate buffer and simulated tear fluid, respectively. Release kinetic study showed that drug release had followed korsemeyer peppas model. The values of R^2 and n were found to be 0.949 and 0.646 respectively that showed anomalous transport as a mechanism of drug release.

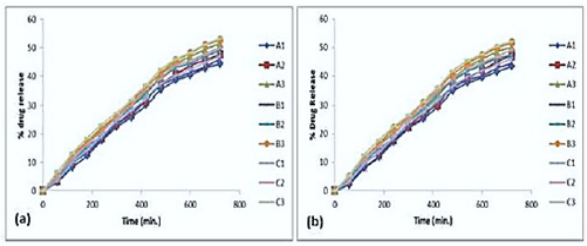

Figure 5: *In-vitro* dissolution of nanoparticles in (a) pH 7.4 phosphate buffer and (b) simulated tear fluid.

Table 3: R_f value of different combination of drug and polymer.

S. No.	Ingredients	R_f-value
A	Ofloxacin	0.720
B	Ofloxacin: Ethyl Cellulose	0.729
C	Ofloxacin: Carbopol	0.623
D	Ofloxacin: Gallen gum	0.722

Table 4: Various evaluative parameters of Ofloxacin nanoparticles.

Formulation code	Evaluation Parameters			
	Percentage yield	Drug entrapment efficiency (%)	Particle size analysis (nm)	Zeta potential analysis
A1	35.5	40.52	152	-20.65
A2	45.73	49.69	156	-19.72
A3	58.67	56.92	160	-14.35
B1	36.25	43.75	139	-17.59
B2	48.1	51.55	144	-8.78
B3	59.10	57.22	148	-22.8
C1	37.06	48.37	168	-11.55
C2	46.83	55.14	174	-7.56
C3	58.96	58.74	180	-2.83

Table 5: 3^2 Full Factorial Design Layout*.

Formulation Code	Variable levels in coded form		Particle size (nm)	Entrapment (%)
	X1 (mg)	X2(%)		
A1	-1	-1	152	40.52
A2	0	-1	156	49.69
A3	1	-1	160	56.92
B1	-1	0	139	43.75
B2	0	0	144	51.55
B3	1	0	148	57.22
C1	-1	1	168	48.37
C2	0	1	174	55.14
C3	1	1	180	58.74
Coded value	**Actual value**			
	X1(mg)	X2 (%)		
-1	100	1		
0	200	2		
1	300	3		

Table 6: Summary of regression analysis results.

For particle size						
Polynomial equation	$Y=143.78+4.83A+9.00B+1.00AB-0.17A^2+21.33B^2$ eq (1)					
Response	**Bo**	**A**	**B**	**AB**	**A^2**	**B^2**
FM	+143.78	+4.83	+9.00	+1.00	-0.17	+21.33
p-value	0.0001					
For entrapment efficiency						
Polynomial equation	$Y=51.67+6.73A+2.52B-1.51AB-1.19A^2+0.69B^2$ eq (2)					
Response	**Bo**	**A**	**B**	**AB**	**A^2**	**B^2**
FM	+51.67	+6.73	+2.52	-1.51	-1.19	+0.69
p-value	0.0090					

*(FM) indicated full model.

Table 7: Evaluation Parameters of Optimized batch (B3).					
S. No.	Drug	Evaluation Parameters			
		Percentage yield	% Entrapment Efficiency	Particle size (nm)	Zeta Potential
1.	Ofloxacin	59.10	57.22	148.0	-22.8

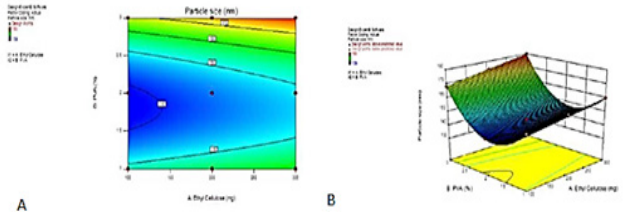

Figure 6: 3D Response Surface plot showing the influence of ethyl cellulose and PVA on Particle size.

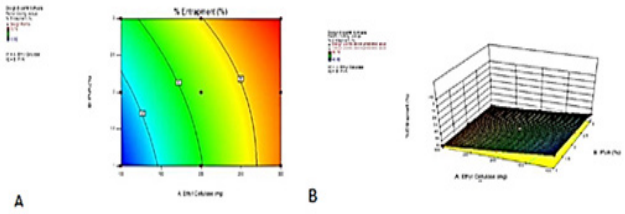

Figure 7: 3D Response Surface plots showing the influence of ethyl cellulose and PVA on Entrapment efficiency.

Table 8: Evaluation parameter of nanoparticulate *in-situ* ocular gel of Ofloxacin.		
S. no.	Parameter	Inference
1	Clarity	Clear
2	pH	7.4
3	Viscosity (Cp)	865
4	Gelling strength	+++
5	*In-vitro* diffusion study	62.19% (pH 7.4 PBS) 61.82% (STF)

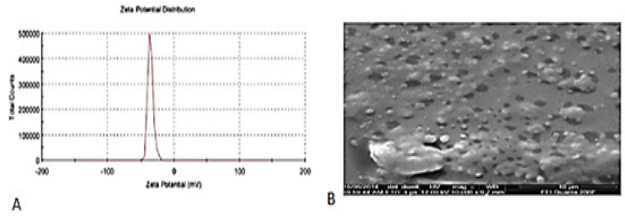

Figure 8: A) Zeta potential of optimized batch (B3), B) SEM image of nanoparticles of Optimized formulation (B3) (5000X magnification).

Table 9: *Ex-vivo* diffusion study of nanoparticulate *in-situ* ocular gel of Ofloxacin.		
Time (min.)	pH 7.4 Phosphate buffer Saline (PBS)	Simulated Tear Fluid (STF)
0	0	0
60	9.57	8.62
120	16.55	15.74
180	20.66	19.32
240	24.35	23.85
300	28.38	27.36
360	34.65	33.62
420	39.49	38.49
480	44.32	43.33
540	48.83	47.82
600	52.79	51.77
660	55.71	54.61
720	62.19	61.82

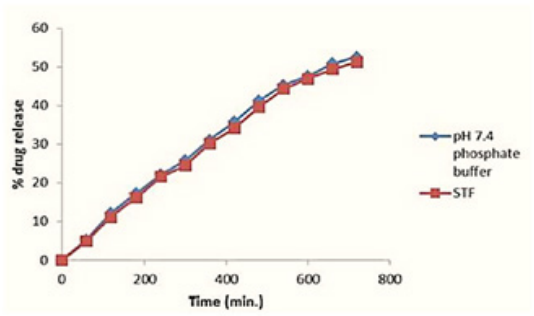

Figure 9: *In-vitro* release study of optimized batch of Ofloxacin nanoparticles (B3) in pH 7.4 Phosphate buffer and Simulated Tear Fluid (STF).

Various evaluative parameters obtained for optimized batch (B3) were as follows (Table 7 and Figure 8-10). Zeta potential analysis of ofloxacin nanoparticles belonging to optimized batch i.e B3 peformed on Malvern zeta sizer were as follows (Figure 8). The surface morphology of ofloxacin nanoparticles belonging to the significant batch B3 was examined by SEM (Figure 8). *In-vitro* dissolution study was conducted on optimized batch i.e. B3 in pH 7.4 Phosphate buffer and Simulated Tear Fluid (Figure 9). Results of kinetic studies were presented in Figure 10.

Optimized batch was then used to formulate *in-situ* gel using gellan gum, selected by comparing gelling capacities of different gelling agents. The mechanism of gelation by gellan gum involves the formation of double helical junction zones followed by aggregation of

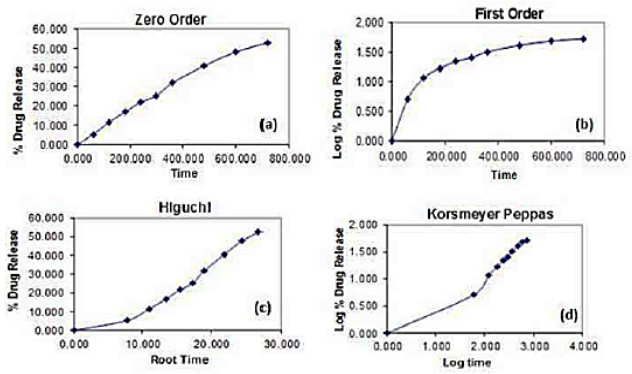

Figure 10: Release kinetics graphs- a. Zero order, b. First order, c. Higuchi model and d. Korsemeyer peppas models of optimized batch (B3) in Simulated Tear Fluid (STF).

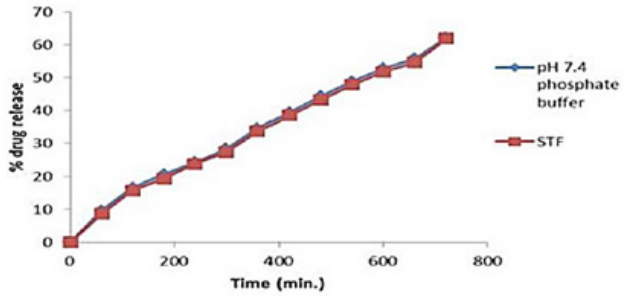

Figure 11: Comparative *ex-vivo* release of Ofloxacin nanoparticulate *in-situ* ocular gel in pH 7.4 phosphate buffer and Simulated Tear Fluid (STF).

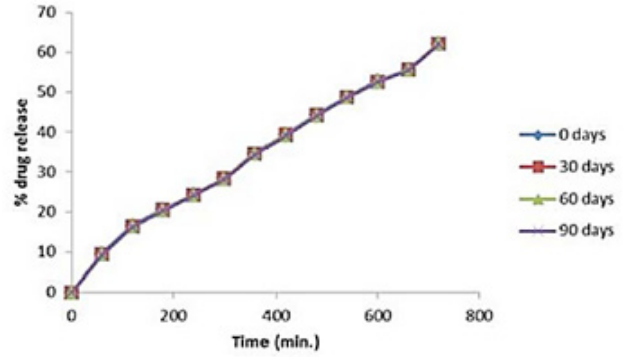

Figure 12: Comparative release profile of Ofloxacin nanoparticulate *in-situ* ocular gel on stability studies

double helical segment to form three dimensional networks by complexation with cations and hydrogen bonding with water which consequently prolongs the residence time of drug at absorption site and increases the bioavailability of drug. It is widely used in ophthalmology because of its thixotropic, thermo plasticity and pseudo plasticity.

Nano particulate *in-situ* gel of Ofloxacin thus prepared was evaluated for various parameters i.e. clarity, pH, viscosity, gelling strength, *in-vitro* diffusion and stability studies (Table 8, 9 and Figure 11, 12). The *in-vitro* drug release from Nano particulate *in-situ* ocular gel was found to be 62.19% and 61.82% in pH 7.4 phosphate buffer and simulated tear fluid (STF), respectively, over a period of 12 h. Results of *ex-vivo* diffusion study were depicted in Table 9 and Figure 11. During stability studies, samples were collected at specified time intervals and evaluated for release characteristics (Figure 12).

CONCLUSION

In the present study, Nano particulate *in-situ* gel of OFL was prepared for the treatment of conjunctivitis and corneal ulcers by utilizing the previously prepared nanoparticles. Initially, nanoparticles of Ofloxacin were prepared and evaluated for various evaluation parameters. Full factorial design was adopted to optimize the formulation and based on the predicted solution given by the software a checkpoint batch (B3) was selected and considered as an optimized batch. Nano particulate *in-situ* gel was prepared with the optimized batch and prepared gel was further evaluated for various parameters and satisfactory results were obtained. Nano particulate *in-situ* gel passed the irritancy test and found compatible. It was observed that an adequate amount of gel was permeated through the ocular tissue as confirmed by the *ex-vivo* study. Hence, it was concluded that the research work, in reference, comprised of quite novel approaches of investigations.

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CONFLICT OF INTEREST

The authors report no conflicts of interest.

ABBREVIATIONS

OFL: Ofloxacin; **STF:** Simulated Tear Fluid; **TLC:** Thin Layer Chromatography; **FTIR:** Fourier Transmission Infra-Red; **EC:** Ethyl Cellulose; **PVA:** Poly Vinyl Alcohol; **SEM:** Scanning Electron Microscopy; **ICH:** International Conference of Harmonization; **PBS:** Phosphate buffer Saline; **RH:** Relative humidity, **R_f:** Retention factor.

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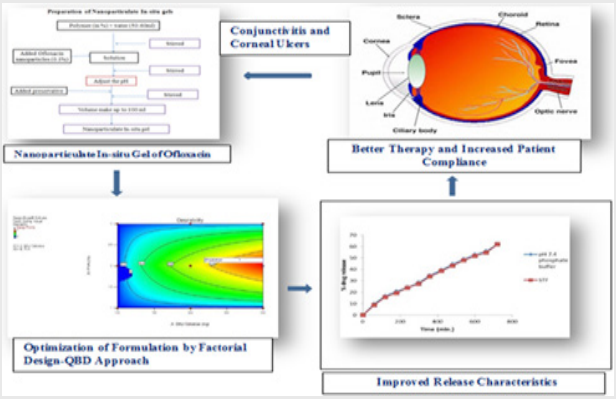
PICTORIAL ABSTRACT



SUMMARY

- Ofloxacin (OFL) stops or prevents ocular bacterial infections by either killing or inhibiting their growth
- A serum concentration of Ofloxacin after 10 days of topical dose is very much less than the standard oral doses of the same
- Prepared *in-situ* gel would prolong the residence time of drug at corneal surface which is a crucial need for legitimate entry of drug in interior segment of eye for complete mending action.

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