OFLOXACIN ION PAIRING WITHIN SUBMICRON EMULSION: A POTENTIAL APPROACH FOR OCULAR DELIVERY

Pandey D.[a]*, Singh R.[a], Jain S.[b] and Jain D.[a*]

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ABSTRACT

The aim of the work was to improve the entrapment of ofloxacin within the submicron emulsion by ion pairing with sodium deoxycholate to improve antimicrobial activity and precorneal retention. Partition co-efficient of ofloxacin-sodium deoxycholate was found to be 3.788, compared to 0.113 for the drug alone. Formulation was characterized for globules size 0.143 ± 0.07 µm, viscosity 3.8 ± 0.2 cP and pH, 7.1 ± 0.3. The entrapment was 80 ± 1% for ofloxacin-sodium deoxycholate in submicron emulsion compared to 57 ± 2% for the drug alone. More than 90% drug remained after 90 days in optimized formulations and was found stable. SEM confirmed droplets size to be 200 nm and spherical. Drug released 53.16% after 24 h from optimized formulation. *In vitro* antimicrobial efficacy improved against *S. aureus* as compared to free drug. No toxicity of optimized formulation on HET-CAM test was observed. Designed formulation may hold some promise for severe ocular infections where frequent dosing is required.

Keywords: Complexation, Entrapment, Prolonged drug release, Ofloxacin, Submicron emulsion, Antimicrobial activity.

INTRODUCTION

Fluoroquinolones are broad spectrum antimicrobials. Clinically, they have been used to treat a variety of infections due to their broad spectrum activity against Gram-positive and Gram-negative bacteria[1]. The drugs show zwitterionic behavior[2,3] and formion pair complexes[4] with suitable complexing agents. Aqueous solubility of these compounds is low at a pH close to 7[5]. Retention of ofloxacin (OF) is low in the internal phase of submicron but due to their zwitterionic behavior it can form a complex with surfactants like sodium deoxycholate (SDC). Their ion paired complex results in improved entrapment within the vesicular system like submicron emulsion and controlled release behavior is observed. In this work, we prepared ion pair complex of OF with SDC and evaluated it for entrapment and drug release behavior. Formulation was characterized for viscosity, pH, and globules size. Several authors reported use of submicron emulsion for ocular and parenteral applications which were prepared with biocompatible materials and comfortable due to their smaller droplets[6-8]. Ocular bioavailability of conventional eye drops is very low due to tear turnover[9]. As a consequence, frequent dosing of drug is required to achieve therapeutic effect which results in systemic absorption of the drug and may result in undesirable effects[10,11]. Only corneal absorption is responsible for the drug entry into the aqueous humor and for beneficial therapeutic effects[12].

Quinolones complexed with metals exhibited higher activity than free drugs[13]. Ciprofloxacin ion has been paired with arylsulfonates to form a complex in precipitated form and entrapped in liposomes[14]. Ofloxacin is a broad-spectrum antibiotic that is active against both Gram-positive and Gram-negative bacteria. It acts by inhibiting DNA gyrase, a type II topoisomerase, and topoisomerase IV, which is an enzyme necessary to separate replicated DNA, thereby inhibiting bacterial cell division[15].

MATERIALS AND METHODS

Soya oil, egg lecithin, Poloxamer 188 (Pluronic F-68), sodium deoxycholate (SDC) and dimethylsulphoxide (DMSO) were purchased from Sigma. Ofloxacin was kindly provided by Astam Health Care Pvt. Ltd. Baddi, India. All other reagents and chemicals were of analytical grade.

[a] School of Pharmaceutical Sciences, Rajiv Gandhi Proudyogiki Vishwavidyalaya, Airport Road Gandhi Nagar, Bhopal - 462 033, Madhya Pradesh, India

[b] Sagar Institute of Research and Technology - Pharmacy, Bhopal - 462 041, Madhya Pradesh, India

* For Correspondence: E-mail: durga.pandey9@gmail.com deepti2515@gmail.com

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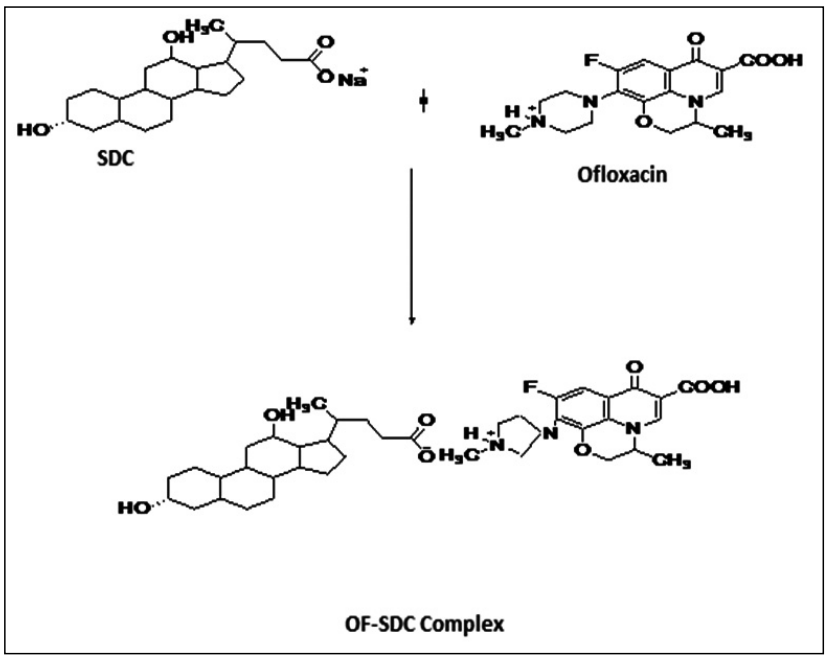

Fig. 1: Ion pair complex formation between sodium deoxycholate and ofloxacin

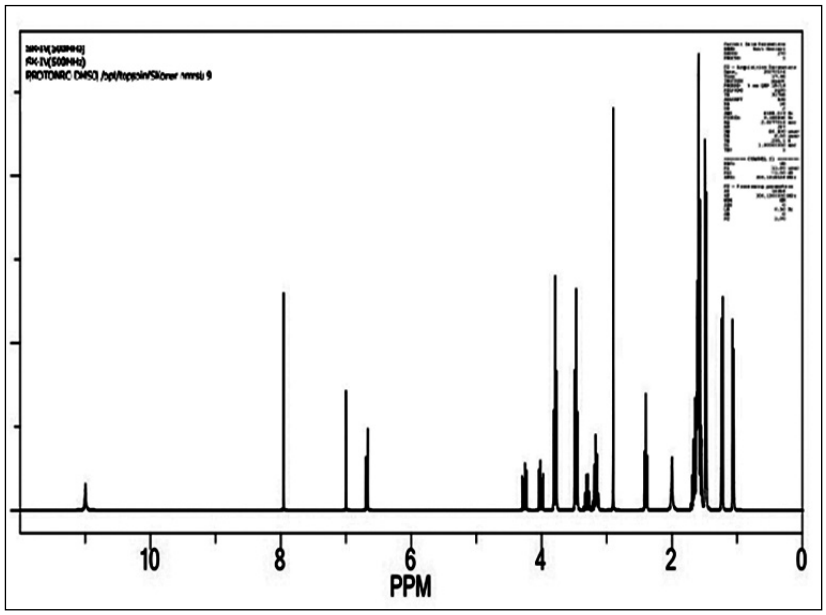

Fig. 2: NMR spectra of OF-SDC complex

Preparation and optimization of ofloxacin and sodium deoxycholate ionic complexes

The ionic complexes of ofloxacin with sodium deoxycholate were prepared by dissolving appropriate quantities of sodium deoxycholate in distilled water and gradually added this solution into ofloxacin solution. The formation of ionic complex was optimized by preparing at different pH values and different molar ratio of both the components (Fig. 1).

Effect of pH on complex preparation

To study the effect of pH on complex formation, OF was added drop wise into SDC solution at pH 3.5, 7.4 and 9.2. Ofloxacin ionic complex formation was indicated by precipitation in aqueous phase. The tubes were centrifuged and amount of drug left uncomplexed in the supernatant was measured using UV/visible spectrophotometry on a SHIMADZU 1700 spectrophotometer at 286 nm.

Effect of molar ratio

The concentration of SDC added in OF solution at pH 3.5, was adjusted keeping SDC/OF molar ratio in the range of 0.5 to 10 molar. The formation of water insoluble ion paired complex between OF and SDC at different molar ratios was traced by monitoring the transmittance of solution at 500 nm using spectrophotometer (UV-SHIMADZU 1700).

Characterization of OF-SDC complex

Ionic complex of OF and surfactant SDC was prepared at pH 3.5 using 1:1 molar ratio and characterized to ascertain the formation of complexes.

NMR spectra of drug complex

^{1}H-NMR spectra of drug complex was determined by BRUKER AVANCE II 400 advance at 500MHz by dissolving the complex in DMSO solvent.

FTIR spectra of drug complex

FTIR spectra of drug complex was determined by Prestige spectrophotometer in KBr disc and reported as wave number (cm^{-1}).

Partition coefficient or log P

The hydrophobic ion pair complex formed using optimized condition was separated by centrifugation, the supernatant was discarded and precipitate was immediately freeze dried. Complex was added for partition study in an equal volume of aqueous/octanol mixture and was allowed to stir for a period of 6 h and then kept in separating funnel overnight for partitioning. After complete partition, the mixture was separated into two phases (layers), and the concentration of OF was measured using UV SHIMADZU 1700.

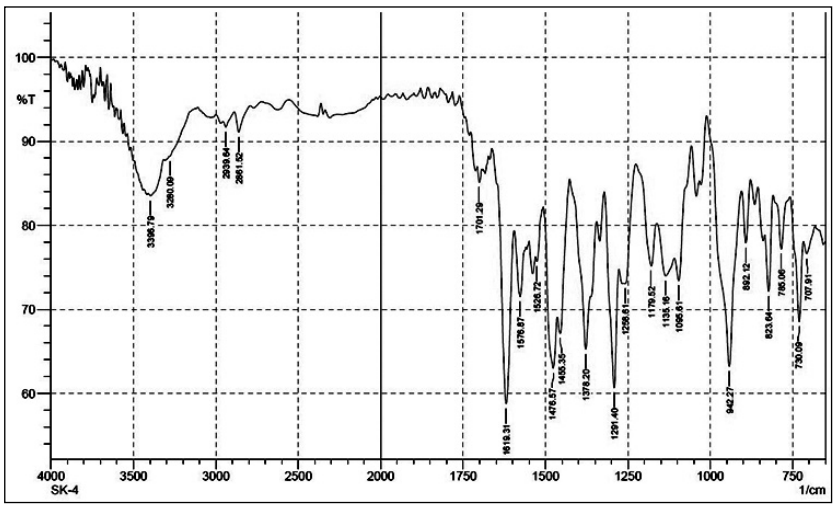

Fig. 3: FTIR spectra of OF-SDC complex

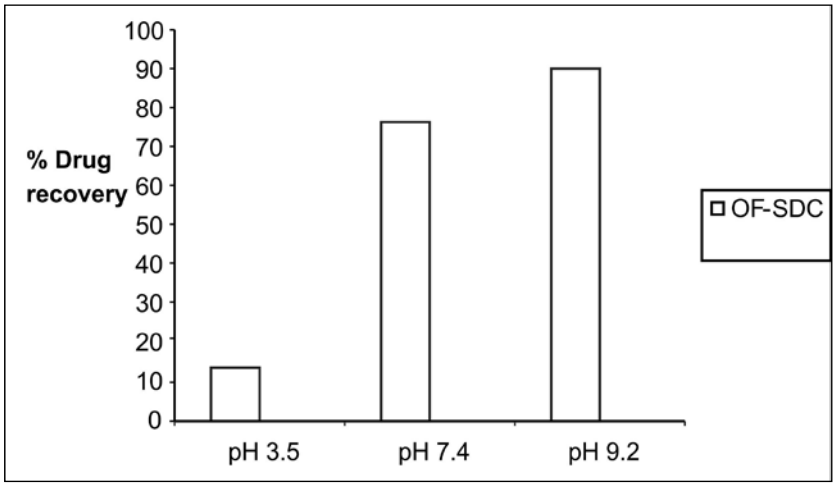

Figure 4: Effect of different pH on ionic complex formation of OF-SDC

% drug recovery was very less at pH 3.5 indicates maximum drug complexed with complexing agent.

Table I: Composition (%) of optimized formulation

Ingredients	SE-OF	SE-OF-SDC
Soya oil (ml)	10.00	10.00
Pluronic (F-68)	2.5	2.5
Lecithin	1.25	1.25
OF	0.3	
OF-SDC	-	0.3
Water	100.0	100.0

Preparation of lipid based submicron emulsions

Different types of lipid based submicron emulsion containing OF-SDC ionic complex were prepared following standard procedure with minor modifications[16]. Lipid/oil phase was prepared by heating soya oil at 70°C containing lecithin. The OF-SDC ionic complex (equivalent to 0.3% OF) was added to the lipid mixture separately. The lipid phase was added gradually to aqueous phase containing Pluronic F-68 at 70°C and magnetically stirred. The primary emulsion was prepared by stirring for 20 min using high shear mixer at the speed of 18000 rpm, and subsequent emulsification was accomplished by sonication using ultrasonic probe at 20% amplitude for 5min. The pH of the resulting solution was adjusted to 7.0 and filtered (0.45 µm filter) to separate the non-soluble accompanying fibers (Table I).

PHYSICOCHEMICAL CHARACTERIZATION OF FORMULATIONS

Particle size

The particle size was measured by a Zetasizer 2000 (Malvern Instruments). For study, sample was extemporaneously diluted in distilled water to an appropriate concentration before measurement at room temperature. The measurements were carried out in the fully automatic mode and each sample was analyzed thrice.

SEM

Scanning Electron Microscopy was performed on model SEM Ultra Plus using software ZEISS (Germany).

pH

The pH of the submicron emulsions was determined by digital pH meter at room temperature (25^{0}C ± 2).

Table II: FT-IR Interpretation of OF-SDC Complex

Peaks (cm^{-1})	Groups and Peak assignment
3280 cm^{-1}	OH stretching
3396.79 cm^{-1}	N-H stretching
1701.29 cm^{-1}	Carbonyl group
1619 cm^{-1}	N-H bending vibration
2983.1 and 2864 cm^{-1}	CH stretching vibration of SDC and OF
1550-1500	Alkyl groups stretching CH_3 and CH_2
1450-1400	Methylene group in benzoxazine stretching vibration of CH_2
1400-1350	Hydroxyl group O-H bending vibration
1250-1258	C-O-C stretching vibration (ether)

Table III: NMR interpretation of OF-SDC complex

S.No.	Proton in chemical groups	Chemical shift value in ppm of OF-SDC complex and peaks	Chemical shift value in ppm of alone OF
1	H-OH	11(s)	11 (s)
2	H-CH	7.96(s)	7.96(s)
3	H-CH	3.30(m)	3.30(m)
4	3H-CH_3	1.23(d)	1.23(d)
5	2H-CH_2(s)	4.26(d)	4.26(d)
6	CH (s)	6.68(s)	6.68(s)
7	2H-CH_2	3.79(t)	3.45(t)
8	2H-CH_2	3.47(t)	2.59(t)
9	H-NH	7.0(s)	2(s)
10	3H-CH_3	2.90(s)	2.27(s)
11	2H-CH_2	2.40(t)	-
12	2H-CH_2	1.58(q)	-
13	H- CH	1.64(m)	-
14	3H-CH_3	1.06(d)	-
15	H- CH	1.48(m)	-
16	H-CH	1.49(q)	-
17	H-CH	3.16(t)	-
18	H-OH	3.0(s)	-
19	H-CH	1.41(m)	-
20	2H-CH_2	1.52(q)	-
21	H-CH	1.42(m)	-
22	2H-CH_2	1.67(t)	-
23	H-CH	3.17(m)	-
24	2H-CH_2	1.72(q)	-

Table IV: log Po/w value of drug and complex.

Drug /ion pair complex	Partition coefficient (log Po/w)
OF	0.113
OF-SDC (1:1)	3.788

Zeta Potential

The zeta potential was measured by a Zetasizer 2000 (Malvern Instruments). A sample was extemporaneously diluted in Millie-Q (Millipore Corp., USA) water and injected in the apparatus. The measurement was carried out in the fully automatic mode and each sample was analyzed thrice.

Drug entrapment[17]

The OF loaded emulsion was centrifuged at 18000rpm for 30 min at 4°C in an ultracentrifuge (5810 R) in order to separate the incorporated drug from the un-incorporated drug. The supernatant was analyzed by UV spectroscopy for the un-entrapped drug (A1) concentration to determine the encapsulation percentage from total amount of drug (A2). Entrapment efficiency was calculated by using the equation:

$$EE\ (\%) = \frac{(A2-A1)}{A2} \times 100$$

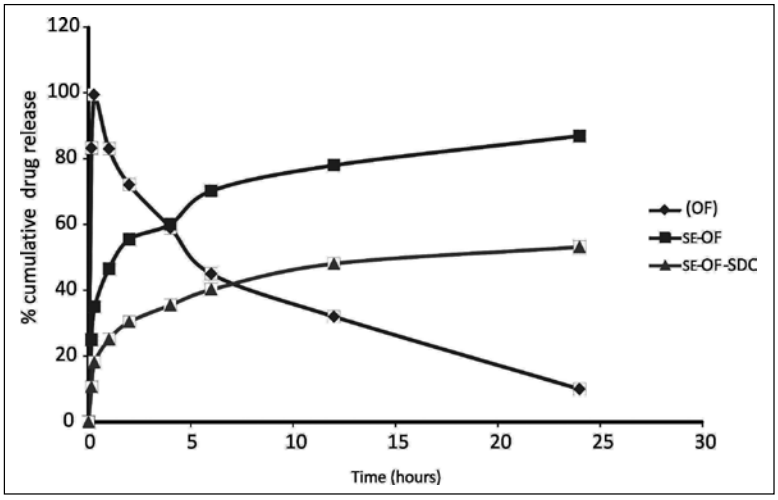

Fig. 5: *In vitro* drug release profile of different submicron emulsion formulations

Prolonged dug release observed as compared to control. SE-OF-SDC drug release was found 53.16% in 24 hours. Data represented as mean average value of three determinations (N=3)

OF- Plain drug dispersed in aqueous media.

SE-OF- Submicron emulsion contained plain drug.

SE-OF-SDC- Submicron emulsion contained OF-SDC complex in internal phase.

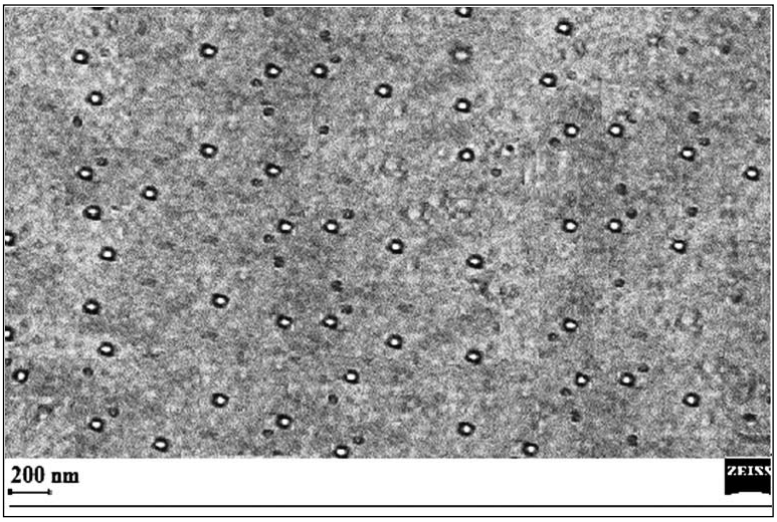

Fig. 6: SEM of SE-OF-SDC (Spherical shape & size 200 nm)

In vitro drug release (Dialysis method) [18]

In vitro release studies were carried out using bulk equilibrium reverse dialysis bag technique at 37°C. For the release experiment, 1mL of SE containing OF and its ionic complex was pipetted into a dialysis bag. The dialysis bag was kept in 100 mL of stirred sink solution (PBS pH 7.4) for 24 h, temperature was maintained at 37°C. The samples were collected at different time intervals i.e. 0, 0.15, 0.30, 1, 2, 4, 6, 12 and 24 h the release medium was exchanged with equal volume of fresh PBS solution and concentration of drug was analyzed by UV spectroscopy.

Stability studies

Formulations were placed for 3 months and evaluated for the any change in viscosity, globules size, pH, and drug content at different time intervals 0, 15, 30, 45, 60, 90 days[19].

In vitro antimicrobial activity

Minimum inhibitory concentration tests were performed using the broth dilution method according to NCCLS standards. Bacterial culture of *S. aureus* was obtained from SIFC SIRT- P, Bhopal India. Strain was inoculated onto agar plate incubated for 18 hrs at 35^{0}C and then diluted in PBS to make its optical density equal to McFarland No. 0.5. 110 µL of bacterial culture was diluted 1:10 and added to the microtitre wells containing the drug solution and incubated at 35^{0}C. After 3 h, 100 µL Mueller Hinton II Broth (USA) was added to each well. The final concentration of micro organism was 5×10^{5}cfu/mL. The plates were incubated for 18 h at 35^{0}C. Positive control (growth) consisted of bacteria in broth and bacteria with the empty formulation in broth. Negative control consisted of uninoculated broth and each of the drug/formulation dilutions in broth [20].

HET-CAM test

As per ICCVAM recommended test protocol, published in 2010 (NIH publication no. 10-7553), vascular response of formulation was measured by the onset of hemorrhage, coagulation and vessel lysis over a period of 5 min. The irritancy potential of the compound detected by observing toxicity on the chorioallantoic membrane of the 9 days old incubated chicken eggs after exposure to the test compound. These eggs were obtained from commercial sources. In this study, we applied 0.9% sodium chloride as a negative control, de-ionized water as a solvent control, 1% sodium dodecyl sulphate (SDS) as a positive control and SE-OF-SDC as the test compound[21].

RESULTS AND DISCUSSIONS

NMR spectra showed protons of both components and confirms the formation (Fig. 2) of complex due to the

Table V: Characterization of formulations

Formulation	Average particle size (µm)	Viscosity (cP)	pH	% Encapsulation	Cumulative % drug release in 24 hrs
SE-OF-SDC	0.143 ± 0.07	3.8 ± 0.2	7.1 ± 0.3	80 ± 1	53.16
SE-OF	0.227 ± 0.08	3.1 ± 0.5	7.2 ± 0.4	57 ± 2	86.93
Plain drug, OF	-	-	-	-	99 (within 2 hrs)

Table VI: Long term stability studies at 37°C for 3 months

Formulation	pH		Viscosity (cP)		Average particle size (µm)	
	Initial	After 3 months	Initial	After 3 months	Initial	After 3months
SE-OF-SDC	7.1 ± 0.3	6.91 ± 0.7	3.8 ± 0.2	3.1 ± 0.1	0.143 ± 0.07	0.191 ± 0.05
SE-OF	7.2 ± 0.4	7.16 ± 0.4	3.1 ± 0.5	2.0 ± 0.6	0.227 ± 0.08	0.280 ± 0.09

Table VII: % Drug content in control and optimized formulation during stability studies

Formulation	% drug content Initial	% Drug content after 90 days
SE-OF	92 ± 1	91 ± 0.9
SE-OF-SDC	91 ± 2	90 ± 1

Table VIII: Antimicrobial activity of formulations

Formulation	*S. aureus*
OF	2
SE-OF	1.9
SE-OF-SDC	0.11

MIC nominal (mcg/mL)

Table IX: CAM Irritation test

Test Substance	Irritation score	Irritation severity	Overall observed effects
0.9% w/V sodium chloride	0	0	No reactions
0.3 mL SE-OF-SDC	0	0	No reactions
Solvent Control	0	0	No reactions
1%SDS	14	4	Severe reaction

Severity of score: 0→No reaction; 1→ slight reaction; 2→ moderate reaction; 4→ severe reaction.

change in chemical shifts value and spin-spin coupling of protons in piperazinyl ring when compared with ofloxacin alone (Table II).

FTIR spectroscopy of OF-SDC showed main bands of ofloxacin (Table III) and SDC but COO stretching vibration of SDC disappeared in complex FTIR spectra that was found at 1564 cm^{-1} in individual spectra of SDC (Fig. 3). This indicated that the carboxyl group of the anionic sodium deoxycholate interacted with the cationic amino group of ofloxacin and formed an ionic complex between the two compounds.

Ofloxacin exists in the protonated form at acidic pH, which can form ion pair complex with negatively charged counter ions. Based on this assumption, we prepared OF ionic complex with another amphipathic molecule having an opposite charge such as like SDC. It has been observed that the formation of ion pair increases the lipophilicity of ofloxacin. SDC was chosen for ion paring because it is negatively charged with a wide range of pH. Partition coefficient increases after complexation as compared to free drug, indicating increased lipophilicity (Table IV).

The hydrophobic ion pair complex formation depends upon the pH of the solvent and ionic strength of the solutes; therefore it was mandatory to study the complex formation at different pH and molar ratio of SDC and OF at room temperature (25°C). The amount of OF recovered in the supernatant was less at lower pH (3.5) which gradually increases as the pH of the buffer approached to neutral (7.4) or basic pH (9.2) (Fig. 4). This may be attributed to the fact that as the pH of the medium decreases, the

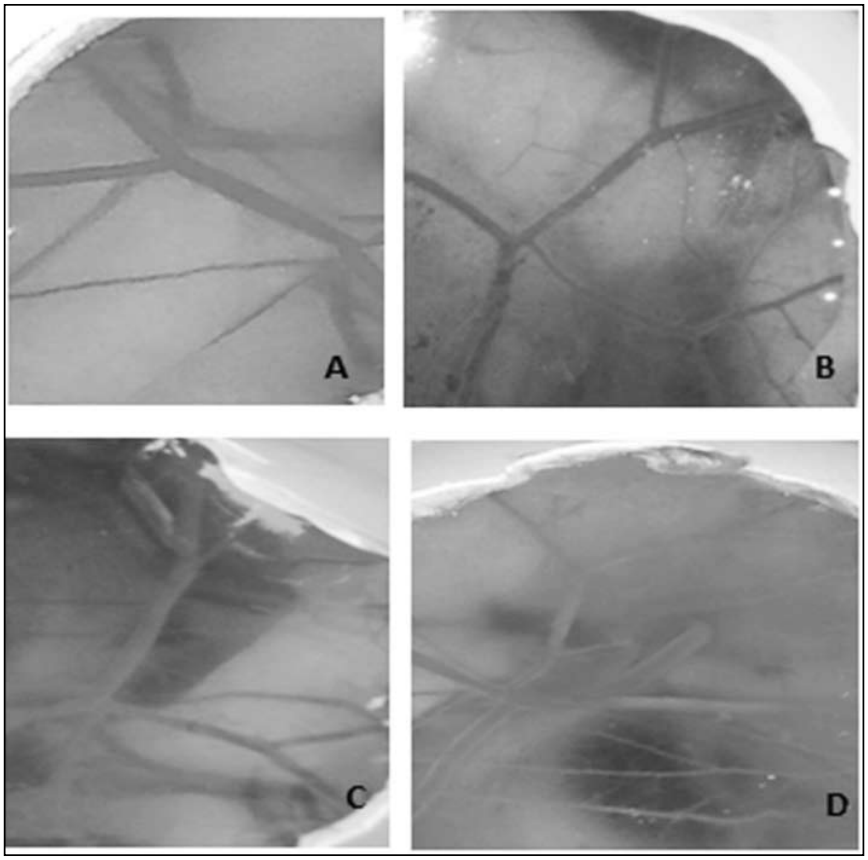

Fig. 7: A- 0.9% NaCl, B- Solvent Control water, C- SE-OF-SDC, D- 1% SDS

proportion of protonated OF increases, and thus available for complexation with negatively charged surfactant. As the pH increased, the zwitterionic and an anionic form of OF become more dominant, which do not favor complex formation. Further complexation was, therefore, carried out at acidic pH.

The value of transmittance decreases sharply as the molar ratio of surfactant/drug increases from 0.5 to 2 which is indicative of complex formation; however, there was an abrupt increase in transmittance when the molar ratio exceeds 2. However, at 8 molar ratios, complete clear solution was observed. This clearly indicated saturation of binding sites of OF with negatively charged amphiphilic surfactant i.e. reduced aqueous solubility. Further addition of surfactant beyond the binding saturation point resulted in dissociation of hydrophobic ion pair complex in to individual micelles which are completely soluble; therefore equal molar ratio (1:1) of OF-SDC was chosen for complex formation.

Optimized formulation was characterized for globules size, viscosity and pH. All parameters were found satisfactory for parenteral and ocular applications (Table V). The zeta potential value was -24.6 mV with SE-OF-SDC whereas it was -28.73 mV for SE with plain SDC without complexation. It has been revealed from the graph that the drug release through SE-OF-SDC was in a sustained manner compared to control, the control group was a dispersion of drug in aqueous media. 53.16 % drug was released from SE-OF-SDC in 24 h (Fig. 5). SEM confirmed droplet size less than 500 nm and spherical shape globules (Fig. 6).

Stability studies for 3 months, at 4°C and 37°C

During stability studies, formulations were analyzed for creaming and cracking and assessed visually at sufficient time intervals. Change in viscosity, pH, globules size (Table VI) and drug content were also determined at the sufficient time interval. More than 90% drug content was found in all optimized formulations after storage of three months (Table VII)[22]. Polaxamer and lecithin concentration in formulations is very important to produce a stable emulsion. Lecithin alone cannot produce a stable emulsion a co-surfactant like Poloxamer was mandatory to produce a stable emulsion[23, 24]. Viscosity of formulation stored at 37°C slight decreased with time. It has been observed that the pH and viscosity were not influenced at 4°C & 37°C up to 3 months of storage. No significant creaming and cracking was observed.

Antimicrobial activity

MIC was improved for SE-OF-SDC compared to free OF which may be due to the detergent action of anionic surfactant SDC (Table VIII) MIC nominal for OF was 2 mcg/mL, SE-OF was 1.9 and MIC for SE-OF-SDC was reduced up to 0.11 mcg/mL.

HET-CAM test

HET-CAM test was done on hen egg cells SE-OF-SDC was found to in non-irritant to the chorioallantoic membrane. This hinted that the drug vehicle will not irritate the ocular system, thereby, making the drug available to the eyes for prolonged duration. No hemorrhage, Coagulation, and vascular lysis reaction were observed, with the solvent control (water), 0.9% sodium chloride (positive control) and SE-OF-SDC (0.3 ml) applied directly

to the chorioallantoic membrane (Table IX, Fig. 7) and observed for 5 min, whereas 1% SDS (0.3 mL) caused immediate blood vessel lysis followed by hemorrhage and coagulation on CAM. All ingredients, as well as the ion pairing agent, were found safe during experimental work.

CONCLUSION

Thus, it can be concluded that ofloxacin retention, improved in the oil phase of SE due to the complexation with SDC. The prolonged drug release pattern observed and 1:1 molar ratio were found best for complexation. Antimicrobial activity improved as compared to control or marketed formulation of the same drug. HET CAM test supported suitability of formulation for ocular applications.

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