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Formulation and Evaluation of Niosomal *In-Situ* Gel for Ophthalmic Use

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ABSTRACT

Conventional liquid ophthalmic formulations are most convenient from patient point of view. But these formulation shows low bioavailability because of a constant lachrymal drainage in the eye which leads to frequent dosing. Moreover, the absorption of the drug drained through the nasolacrymal duct may result in undesirable side effects. To overcome these limitation different approaches has been applied such as ointment, gel, cream etc. These ophthalmic formulations also fails to show desired therapeutic responses because of their own disadvantages such as ointment makes blurred vision. So two different systems was combined together as niosomes and *in-situ* gel by incorporating niosomes in *in-situ* gel formulation so that it is easy to administered and retain at the site for prolong period of time. The Ofloxacin (OFL), a second generation fluoroquinolone derivative used in eye infections needs frequent dosing in its solution form. Vesicular system reported prolonged and controlled action at corneal surface but it has again limitation of drainage along tear produced. In this, first niosomes containing ofloxacin were prepared by applying 3^2 full factorial designs and evaluated for its vesicle size, percent entrapment, *in-vitro* drug release kinetics and their stability. Also *in-situ* gel formulation was prepared by dispersing the niosomes in solution of carbopol 940 and Hydroxy Propyl Methyl Cellulose (HPMC) K4M. *In-vitro* drug release kinetics from niosomal *in-situ* gel formulation indicates that the minimum inhibitory concentration (MIC) of drug (4 µg/ml) was achieved within 1-2 hrs (batch G1-G9).

Keywords: Niosomes, *in-situ* gel, ophthalmic, ofloxacin, factorial design

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INTRODUCTION

Ophthalmic drug delivery is one of the most interesting and challenging endeavors facing the pharmaceutical scientist. The anatomy, physiology, and biochemistry of the eye render this organ exquisitely impervious to foreign substances. The challenge to the formulator is to circumvent the protective barriers of the eye without causing permanent tissue damage[1]. The absorption of drug into the eye requires a prolonged precorneal residence time and good corneal permeation; however for most drugs corneal permeation is low[2, 3]. Frequent local instillation of solutions of antiglaucoma agents, antibiotics, antiviral and sulfonamide provide an unusually high drug and preservative concentration at the epithelial surface[4]. Improvement in dosing by mean of controlled and enhanced delivery, prolonged contact time and targeting within the globe will go a long way towards achieving safe and reliable ophthalmic delivery system. A number of drugs are available in the form of conventional dosage form such as eye drops, eye ointment, gels, etc. for the treatment of the eye disorders. However, from a biopharmaceutical point of view, their use has met some criticism over their efficiency as drug delivery systems. It has been reported that bioavailability particularly for ocular solutions ranges from 1 to 10% of the total administered dose. This was attributed to the rapid preclearance kinetics resulting from reflux tearing and blinking, where half-life of instilled isotonic solutions was found to be only 15 seconds in the humans[5]. Under normal conditions the human tear volume was about 7 µl with 1 µl in the precorneal tear film and about 3 µl in each marginal tear meniscus[6, 7]. From the patient acceptability point of view, an ideal ophthalmic liquid dosage form is one which can sustain drug release and remain in contact with the cornea of the eye for extended period of time. If the percorneal residence time of a drug could be improved from few min to a few hours, then improved local bioavailability and patient acceptability with reduced dose concentrations and dosing frequency may be achieved.

Moreover ofloxacin, a second-generation fluoroquinolone derivative used in external infections of the eye such as acute and sub-acute conjunctivitis, bacterial keratitis and kerato conjunctivitis needs frequent dosing of ophthalmic solution. The topical ophthalmic dose of ofloxacin is 1–2 drops of a 0.3% solution in the affected eye(s) every 3-4 hours or hourly in the case of severe infection[8].

Several approaches have been attempted to increase the bioavailability and the duration of therapeutic action of various ocular drugs. The typical pulse entry type drug release behavior observed with ocular aqueous solutions (eye drops), suspensions, and ointments can be replaced

by a more controlled, sustained, and continuous drug delivery, using a controlled release ocular drug delivery systems such as implantable systems, ocuserts and collagen shields. Such systems can achieve therapeutic action with a smaller dose and a fewer systemic and ocular side effects along with several limitations as poor patient compliance need of surgery, and difficulty in self-insertion. Therefore, a most pleasing dosage form for ophthalmic drug delivery can be designed with no or negligible vision problem, deliver the dose in solution form and having dosing frequency as once or twice daily.

Niosomal vesicular drug delivery system facilitate prolonged and controlled drug action at the corneal surface along with controlled ocular delivery through prevention of drug metabolism mediated by enzymes present at tear/corneal epithelial surface. However, disadvantage of precorneal and nasolacrimal drainage is more often associated with such niosomal system in the ophthalmic drug delivery. Therefore, several *in-situ* gel forming systems have been developed to prolong the precorneal residence time of a drug and improve ocular bioavailability. Such delivery systems consist of phase transition polymers that are in liquid form at the time of instillation into the eye and thereafter shift to the gel phase once it is in the *cul-de-sac* of the eye due to variations in physiological parameters. But the drug release rate from these systems was not found to be sustained release type.

So, to overcome the disadvantages associated with individual systems (niosomes and *in-situ* gel) and the usual barriers of conventional therapies along with controlled and patient complying drug delivery to the site of action, present research is an attempt to combine these two systems together[9, 10]. For this purpose ofloxacin containing non-ionic surfactant vesicles (niosomes) were prepared for ocular use. Prepared niosomes were then dispersed into the solution of carbopol 940 and HPMC K4M for *in-situ* gel formation showing better drug bioavailability and longer duration of action.

MATERIALS AND METHODS

Materials

OFL was a kind gift sample from NuLife Pharmaceuticals, Pune. Span 60 was purchased from Research Lab Fine Chem Industries, Mumbai. Carbopol 940 (polyacrylate) and HPMC K4M were purchased from Loba Chemie (Mumbai, India). All other chemicals used were of analytical grade.

Methods

Estimation of MIC of OFL

Different concentrations of OFL (1-10 µg/ml) were prepared and introduced into the series of nutrient broth tubes and inoculated with standard test organism *staphylococcus aureus*, to find out the MIC of drug. MIC was estimated in terms of the lowest concentration of drug that prevents growth of a particular pathogen. The lowest concentration of drug resulting in no growth of microorganisms indicated by no turbidity after incubation for 24 hours was considered as MIC of OFL for the above said organism.

Selection of method of preparation of niosomes

The method for the preparation of niosomes was selected by considering the criteria of highest entrapment efficiency and the release of drug. For this niosomes were prepared by ether injection method, ethanol injection method and thin film hydration method.

In ether injection method, the weighed quantity of drug was added to the ether solution of span 60 and cholesterol (1:1). This solution was then injected drop by drop through 20 gauge needle in warmed solution of the phosphate buffer solution pH 7.4 with continuous stirring. Stirring was continued until complete evaporation of ether and the buffer solution containing formed niosomes was then allowed to mature over night. Similarly, ethanol injection method was followed to prepare niosomes after replacing ether by ethanol as solvent.

Niosomes by thin film hydration method were prepared by using vacuum rotary evaporator. In this technique weighed quantity of drug was added to the solution of span 60 and cholesterol (1:1) dissolved in solvent system of chloroform: methanol (2:1). Prepared solution was transferred to round bottom flask maintained at 60 ± 2 ^{0}C with 10 Pa vacuum and attached to rotary vacuum evaporator. Rotation of round bottom flask was continued till complete removal of solvent leaving behind thin film of residue in the flask. The dried film was then hydrated using phosphate buffer pH 7.4 (60 ^{0}C) with continuous shaking of flask. The flask was then bath sonicated and kept for maturation overnight.

Preparation of OFL niosomes

A 3^2 full factorial design was used to study the effect of independent variables on quality attributes of niosomes. Independent variables have been selected on basis of highest entrapment and desired *in-vitro* drug release.

In present study, thin film hydration method was used to prepare niosomes containing OFL as it gives better entrapment supported by percent entrapment efficiency study[11, 12]. For optimization of span 60 and cholesterol ratio, 3^2 full factorial design was used and niosomal batches (N1-N9) were prepared as per runs obtained in design using Design Expert 7.0 software (Table 1). The independent variables selected were amount of span 60 (X_1) and amount of cholesterol (X_2).

The empirical second order equation for applied 3^2 full factorial design is as given below.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 \quad \ldots\ldots\ldots (1)$$

Where,

Y = Dependent variable

X_1 and X_2 = Independent variables

β_0 = Overall coefficient

β_1, β_2, β_{11}, β_{22}, β_{12} = The coefficients from the response of the formulation in design.

Table 1: Formulation composition of niosomal batches as per 3^2 Full Factorial Design*.

Batch code	N1	N2	N3	N4	N5	N6	N7	N8	N9
Span 60 (X_1)	30 (-1)	30 (-1)	30 (-1)	45 (0)	45 (0)	45 (0)	60 (1)	60 (1)	60 (1)
Cholesterol (X_2)	30 (-1)	40 (0)	50 (1)	30 (-1)	40 (0)	50 (1)	30 (-1)	40 (0)	50 (1)

*Values outside and inside bracket indicates actual and coded levels respectively. All actual values are in mg.

Preparation of OFL niosomal *in-situ* gel formulation

Prepared niosomal batches (N1-N9) were further processed to form *in-situ* gel formulation. For this combination of two polymers (carbopol 940 and HPMC K4M) was selected to overcome the drawbacks associated with single polymer (carbopol 940). These drawbacks are associated with higher carbopol concentration and include formation of stiff gel after instillation in the eye and increased risk of stimulation in the eye tissue due to acidic nature of carbopol. To overcome these drawbacks, the total content of single polymer must be reduced without compromising the gelling properties. This can be achieved with use of combination of two polymers in the formulation (Table 2). After comprehensive study the optimized ratio of these polymers was selected (combination F6, Table 2) on the basis of gelling capacity, gelling time and viscosity[13].

Table 2: Selection of Carbopol 940 and HPMC K4M ratio for *in-situ* gel formulation.

Batch code	Concentration (% w/v)		Gelling capacity*	Viscosity (cP at 20 rpm)
	HPMC K4M	Carbopol 940		
F1	0.5	0.2	—	889
F2	0.6	0.2	—	1013
F3	0.5	0.3	+	1151
F4	0.6	0.3	++	1221
F5	0.5	0.4	++	1292
F6	0.6	0.4	+++	1382
F7	0.7	0.4	+++	1955

*Where — no gelation, + gelation after few minutes, ++ immediate gelation retained for few hours, +++ immediate gelation retained for prolonged period.

Polymer solution was prepared by soaking the weighed quantity of carbopol 940 and HPMC K4M (Table 2) in phosphate buffer pH 6.2 for 24 hrs. The gelling capacity was determined by placing a drop of the polymer solution in a vial containing 2 ml of freshly prepared simulated

tear fluid (STF) equilibrated at 37 ^{0}C. Thereafter, visual assessment of the gel formation was done and time required for gelation and dissolution of gel formed was noted[8]. The viscosity of solution was measured using Brookfield viscometer (DV-II+ Pro Viscometer, Bangalore) in small sample adaptor at 20 rpm and 25±2^{0}C.

After removal of unentrapped drug, niosomal batches (N1-N9) were dispersed in optimized carbopol 940 and HPMC K4M polymer solution (combination F6, Table 2) to form *in-situ* gel niosomal formulations (G1-G9). Furthermore, benzalkonium chloride as preservative and sodium chloride to make gel formulations isotonic with tear fluid were added to the gel batches (G1-G9). Amount of sodium chloride to be added was calculated by using sodium chloride equivalent method[14].

Characterization of niosomes

Vesicle size

All batches of niosomes (N1-N9) have been characterized for vesicle size under Motic microscope (B1 Advanced) at 40× and 100× objective lens. The vesicle size and morphology of niosomes were determined by using motic software[15].

Entrapment efficiency

Entrapment efficiency of OFL niosomes was determined by centrifugation method[12, 16]. For removal of unentrapped drug, prepared niosomal suspension was centrifuged at 10000 rpm for 60 mins and supernatant was decanted. Niosomes settled at bottom of centrifugation tube were re-suspended in freshly prepared phosphate buffer solution, centrifuged and same process was repeated until last detection of OFL in the supernatant. Unentrapped drug content was estimated by analyzing supernatant decanted every time spectrophotometrically using UV spectrophotometer (Shimadzu, UV-1800, Thane). The amount of drug entrapped was determined by subtracting the unentrapped drug content from total drug added. The percent entrapment efficiency was determined by formula given in equation 2.

$$\% \text{ Entrapment efficiency} = \frac{\text{Entrapped amount of drug}}{\text{Total amount of drug added}} \times 100 \qquad \ldots\ldots\ldots (2)$$

***In-vitro* drug release kinetics from niosomes**

In-vitro release kinetics of niosomal suspension was carried out using dialysis bag (Hi media) method. Dialysis bag containing 0.5 ml of niosomal suspension was placed in STF in a beaker. The beaker was placed over magnetic stirrer and the temperature was maintained at 37±1°C. The aliquots of 1 ml were withdrawn after each hour interval and analyzed for drug content using UV-visible spectrophotometer (Shimadzu, UV-1800, Thane) at wavelength of 290 nm[17] using

STF as blank. Same amount of fresh STF kept at same temperature was used to replace the amount withdrawn from diffusion media to maintain sink condition throughout the study.

Stability studies of noisomes

Niosomal formulations were sealed in 20 ml glass vials and stored at refrigeration temperature (2-8°C) for period of 45 days. Samples from each batch were withdrawn at regular time interval and estimated for vesicle size, percent entrapment efficiency and *in-vitro* drug release kinetics[12,18].

Characterization of niosomal *in-situ* gel formulation

pH and appearance

The pH of the niosomal in-situ gel formulation was measured using pH meter (Equip-tronics, Mumbai). Visual inspection of the in-situ gel formulation was made to check the formation of any aggregates of noisomes.

Drug content

The drug content was determined by dissolving the 0.5 ml of in-situ gel containing dispersed niosomes in methanol to break the niosomal structure and release the entrapped drug. After suitable dilution with phosphate buffer pH 7.4, samples were analyzed spectrophotometrically using UV-visible spectrophotometer (Shimadzu, UV-1800, Thane) at wavelength of 287.4 nm and drug content was determined.

Viscosity

Viscosity of in-situ gel formulations (G1-G9) was determined using Brookfield viscometer (DV-II+ Pro Viscometer, Bangalore) at room temperature (25 ± 2 ^{0}C). From each formulation 3 ml solution was placed in small sample adaptor and the viscosity was measured using spindle s21 at 20 rpm.

Fourier transform infrared spectroscopy

FTIR spectra were obtained using FTIR spectrometer (Jasco, FT/IR-4100, Thane). Previously dried pure drug and gel samples were individually mixed thoroughly with potassium bromide in 1:300 (sample: KBr) ratio in a glass mortar. These samples were then placed in a sample holder and scans were obtained at a resolution of 2 cm^{-1} from 4000 to 400 cm^{-1}.

Differential scanning calorimetric analysis

DSC analysis was performed using a differential scanning calorimeter equipped with an intra-cooler. Inert atmosphere was maintained by purging nitrogen gas at a flow rate of 50 ml/min. Drug and gel samples (3-5 mg) were placed individually in a sealed aluminum pan, and the

samples were heated under nitrogen gas flow (20 ml/min) at a scanning rate of 10^0C/min from 30 to 300^0C. An empty aluminum pan was used as reference[19].

***In-vitro* drug release kinetics from in-situ gel formulation**

In-vitro drug release kinetics from *in-situ* gel formulation was determined by using dialysis membrane which was previously soaked overnight in diffusion medium. Niosomal dispersion measuring 0.5 ml *in-situ* gel was placed on membrane and assembly was made by filling receptor chamber with full capacity (17 ml) by STF. The temperature was maintained at 37 ± 1^0 C. The aliquots of 1 ml were withdrawn after each hour interval through sampling port and analyzed spectrophotometrically using UV-visible spectrophotometer (Shimadzu, UV-1800, Thane) at wavelength of 290 nm after proper dilutions. Same amount of fresh STF kept at same temperature was used to replace the amount withdrawn from diffusion media to maintain sink condition throughout the study.

Stability study of niosomal in-situ gel formulation

The optimized batch (G5) of niosomal *in-situ* gel formulation was subjected for stability studies for the period of 45 days at refrigeration temperature (2^0 to 8^0C). Samples were withdrawn at regular time interval of 15 days and evaluated for drug content, viscosity and *in-vitro* drug release kinetics[9, 12].

Validation of 3^2 full factorial design

Developed 3^2 full factorial design has been validated for 73 % entrapment efficiency target. Batch V1 was prepared as per composition suggested by experimental design for desired target of entrapment efficiency (Table 3) and same was evaluated for percent entrapment and *in-vitro* drug release kinetics.

Table 3: Validation of 3^2 full factorial design.

Batch code	Span 60 (mg)	Cholesterol (mg)
V1	43.62	42.36

RESULTS AND DISCUSSION

Estimation of MIC of OFL

MIC of OFL was found to be 4 µg/ml (Table 4) for *staphylococcus aureus*, where no growth of microorganisms has been observed as indicated by no turbidity method. Estimation of MIC will be helpful in confirming the time required for release of sufficient amount of drug necessary for achievement of MIC to exemplify the therapeutic action.

Selection of method of preparation for niosomes

The method for preparation was selected on the basis of better entrapment efficiency and sufficient amount of drug release to achieve MIC. A wide variation in entrapment efficiency of niosomes prepared by 3 different methods namely ether injection (24.7±0.45 %), ethanol injection (27.5±0.62 %) and thin film hydration method (68.59±0.71 %) has been observed. Ultimately, thin film hydration technique was selected for preparation of niosomes as it results in formation of multilameller vesicles (niosomes) with higher entrapment[20].

Table 4: Minimum inhibitory concentration of OFL.

Sr. no.	Volume of culture medium (ml)	Volume of test solution (ml)	Turbidity* (after 24 hrs)
1	9.5	0.5	+
2	9.0	1.0	+
3	8.5	1.5	–
4	8.0	2.0	–
5	7.5	2.5	–
6	7.0	3.0	–
7	6.5	3.5	–
8	6.0	4.0	–
9	5.5	4.5	–
10	5.0	5.0	–
11	10.0 (control inoculated)	0	+
12	10.0 (control uninoculated)	0	–

* – no turbidity; + turbidity.

Characterization of niosomes

Vesicle size

Vesicle size of niosomes (N1-N9) has been observed in between 0.35 µm to 3.21 µm (Table 5) which was in accordance with previously reported size for avoiding irritation to the eye[21, 22].

Table 5: Characterization of niosomes.

Batch code	Vesicle size range (µm)	Entrapment efficiency (%)*
N1	0.35 -2.69	63.631 ± 0.42
N2	0.35 – 2.08	64.892 ± 0.29
N3	0.35 – 2.72	62.595 ± 0.76
N4	0.35 – 2.39	66.144 ± 0.83
N5	0.35 – 2.08	73.405 ± 0.69
N6	0.35 – 2.11	68.586 ± 0.56
N7	0.35 – 2.34	65.946 ± 0.81
N8	0.35 – 3.07	66.126 ± 0.48
N9	0.35 – 3.21	69.108 ± 0.28

*indicates average ± standard deviation (n=3).

Entrapment efficiency

Entrapment or inclusion of hydrophilic drug (OFL) takes place in the hydrophilic core of noisome. An improvement in entrapment efficiency has been observed with increase in cholesterol content (batch N7 to N9; Table 5). This was due to addition of cholesterol molecule to niosomal system makes the membrane rigid and reduces leakage of drug from the core of noisome[25]. A profound effect of Span 60 (β_1 = +1.68) over cholesterol (β_2 = +0.76) has been observed. This was attributed to improved niosomal wall integrity in presence of Span 60 and hence leads to reduced drug loss. Span 60 showed maximum % entrapment at its middle content than extremes (batch N1:N4:N7 and N2:N5:N8; Figure 1a). It has been observed that hydrodynamic diameter and entrapment efficiency of niosomes get enhanced in presence of cholesterol in niosomal bilayer[23].

Reduced model equation for entrapment efficiency (R^2 = 0.7740)

% Entrapment efficiency = $70.81 + 1.68\ X_1 + 0.76\ X_2 — 4.00\ X_1^2 — 2.14\ X_2^2 + 1.05\ X_1X_2$ ….(3)

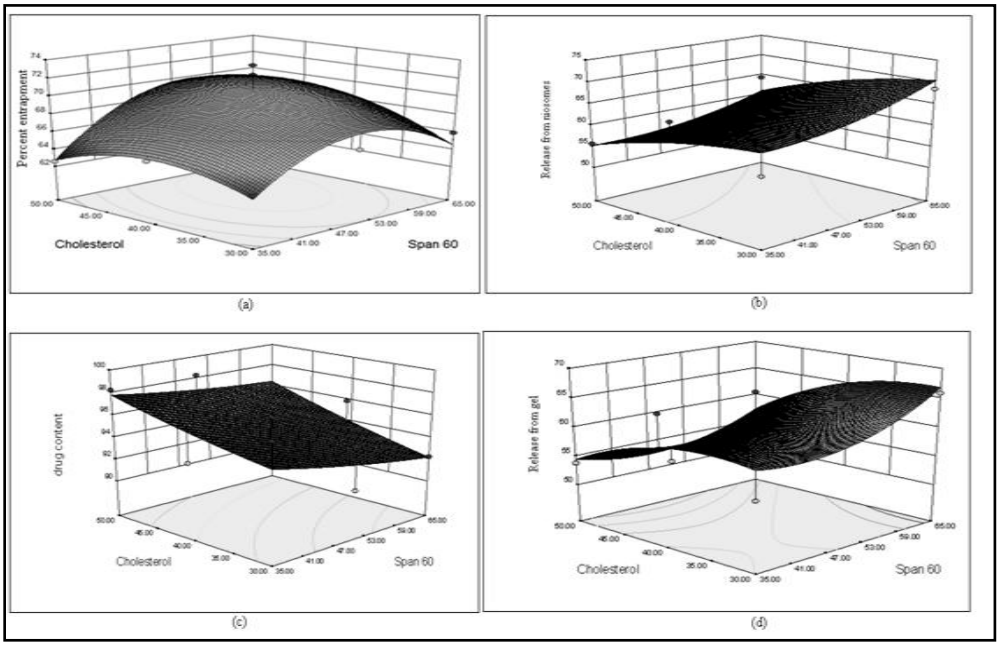

Figure 1: Response surface plot of (a) % drug entrapment, (b) % cumulative drug release from niosomes, (c) % drug content of gel formulations, (d) % cumulative drug release from *in-situ* gel formulation. *In-vitro* drug release kinetics from niosomes

In-vitro drug release kinetics from niosomes (batchs N1-N9) indicated the drug release in range of 63.061 % to 86.407 % at 10hrs (Figure 2). Reduced rate of drug release has been observed with increase in cholesterol content (batches N4:N5:N6 and N7:N8:N9). This was attributed to varying cholesterol content that acts as membrane stabilizing agent and gives rigidity to bilayer causing slower drug release[23]. Drug efflux outside niosomes was found to be reduced with

increase in cholesterol content. Cholesterol assimilation into vesicular bilayers seals the pores that causes formation of niosomes with very low or no drug leakage[24]. This improves stability of membrane and causes sustained drug release.

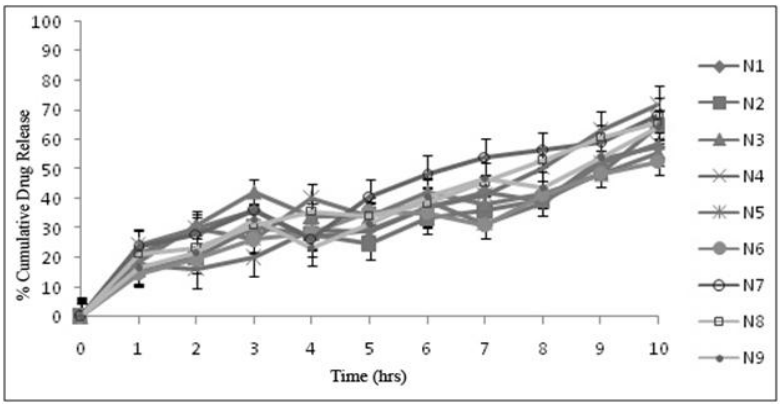

Figure 2: Plot of % cumulative drug release versus time (hrs) from niosomes (batch N1-N9

Response surface plot (Figure 1b) also states lowered drug release with increase in cholesterol content. However, higher rate of drug release has been observed with increased span 60 content. OFL release from niosomes (N1-N9) obeyed zero order release kinetics as the best fit model.

Reduced model equation for *in-vitro* drug release kinetics from niosomes ($R^2 = 0.5757$).

$$\% \text{ Cumulative drug release} = 61.99 + 3.33X_1 - 4.22X_2 + 1.69X_1^2 - 1.32X_2^2 - 0.53X_1X_2 \quad \text{.....} (4)$$

The regression coefficient ($\beta_2 = -4.22$) indicates profound negative effect of cholesterol on drug release than span 60 ($\beta_1 = +3.33$). Regression coefficients indicate reduced % drug release with increase in cholesterol content (batch N7 to N9).

Stability study of niosomes

Stability study was performed on samples stored at 2 to 8 ^{0}C at periodical intervals of 15, 30 and 45 days. All niosomal formulations tested (batch N1, N3, N5, N7 and N9) have shown good stability as indicated by no significant change in vesicle size, percent entrapment and *in-vitro* release from niosomes[25, 26].

Vesicle size

Stability data for vesicle size of niosomes (Table 6) indicates no any significant variation confirming stability of niosomal formulation over 45 days also. This indicates that cholesterol increased the rigidity of the vesicles.

Entrapment efficiency

Entrapment efficiency of the niosomes (Table 6) showed insignificant drug loss upto 5 % (batch SN3) after 45 days. This might be attributed to reduced membrane integrity with storage time period of 45 days. Additionally, higher cholesterol content resulted into niosomal formulation

with greater stability having superior drug retention properties and better entrapment efficiency. However, considerable % entrapment has been observed in samples stored for after 30 days indicating good stability of niosomal formulations. This confirms stability of niosomes following storage up to 30 days at 2-8 ^{0}C.

Table 6: Characterization of niosomes kept for stability study.

Batch code	Vesicle size range (µm)			Entrapment efficiency (%)*		
	After 15 days	After 30 days	After 45 days	After 15 days	After 30 days	After 45 days
SN 1	0.356-2.891	0.356-2.911	0.356-2.920	62.58 ± 0.34	61.37 ± 0.38	59.78 ± 0.29
SN 3	0.356-2.843	0.356-2.818	0.356-2.878	60.42 ± 0.47	58.98 ± 0.54	57.39 ± 0.47
SN 5	0.356-2.285	0.356-2.381	0.356-2.399	72.42 ± 0.28	71.62 ± 0.31	70.88 ± 0.38
SN 7	0.356-2.541	0.356-2.540	0.356-2.655	64.71 ± 0.48	63.57 ± 0.42	62.44 ± 0.45
SN 9	0.356-3.391	0.356-3.312	0.356-3.392	68.57 ± 0.42	67.72 ± 0.56	66.84 ± 0.51

*indicates average ± standard deviation (n = 3).

***In-vitro* drug release kinetics**

In-vitro drug release kinetics from stability samples stored upto 30 days at 2-8 ^{0}C have shown insignificant variation in drug release tested over 10hrs (batch N5, Figure 3). This was attributed to cholesterol mediated sealing of pores situated in vesicle bilayer that results into negligible drug leakage[23]. As a consequence, improved membrane stability and hence sustained drug release has been observed. However, samples stored for 45 days at 2-8 ^{0}C have shown considerable change in drug release kinetics as compared to samples at zero time (over 10hrs). This was attributed to increased drug leakage after storage for prolonged time period (45 days) due to loss of membrane integrity. These results were in accordance with stability studies for entrapment efficiency.

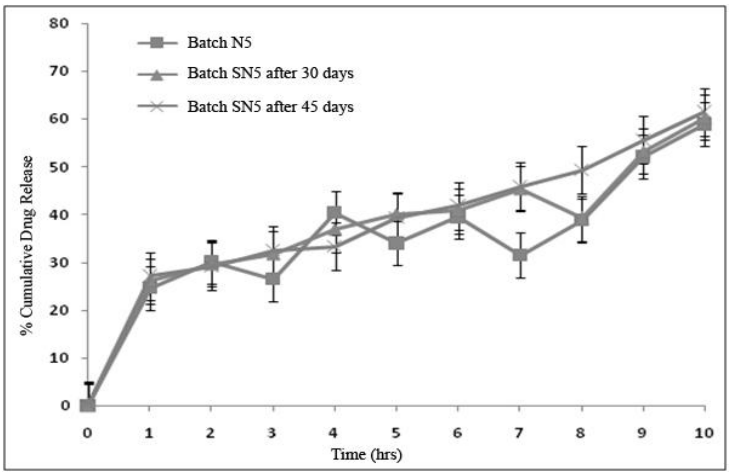

Figure 3: Plot of % cumulative drug release versus time (hrs) from niosomes kept for stability study at zero time (batch N5), after 30 and 45 days (batch SN5).

Characterization of Niosomal *In-situ* Gel Formulation

pH and appearance

Addition of carbopol 940 leads into a slight decrease in pH of buffer system due to acidic functional groups present in carbopol structure. The observed pH of in-situ gel formulation was within range of 6.01 to 6.08 (Table 7). Niosomal in-situ gel was appeared turbid when observed visually. This might be due to the dispersion of niosomes in polymer solution.

Drug content

Drug content of in-situ gel formulations (G1-G9) was found in range of 92.12 ± 0.73 to 97.89 ± 0.63 % (Table 7). Additionally, response surface plot (Figure 1c) clearly indicates profound effect of cholesterol in determining the drug content of in-situ gel. This may be due to increased rigidity of membrane with cholesterol content that leads to reduced drug leakage from noisomal system[23]. Improved drug content has been observed with increase in content of span 60 with cholesterol at its middle level (batch G2:G5:G8).

Reduced model equation for drug content ($R^2 = 0.5977$)

$$\% \text{ Drug Content} = 94.92 - 1.00X_1 - 1.80X_2 + 0.20X_1^2 + 0.20X_2^2 + 0.30X_1X_2 \quad \ldots\ldots\ldots (5)$$

From equation (5) and response surface plot (Figure 1c), a profound negative effect of cholesterol ($\beta_2 = -1.80$) has been observed.

Table 7: Characterization of niosomal *in-situ* gel formulation.

Batch code	pH	Drug content (%)*	Viscosity (cP at 20 rpm)
G1	6.02	97.47 ± 0.44	1290
G2	6.04	92.12 ± 0.73	1353
G3	6.03	97.89 ± 0.63	1377
G4	6.05	93.77 ± 0.57	1309
G5	6.01	94.70 ± 0.49	1288
G6	6.08	94.80 ± 0.74	1339
G7	6.02	96.32 ± 0.39	1362
G8	6.07	97.19 ± 0.58	1402
G9	6.02	94.08 ± 0.64	1295

*indicates average ± standard deviation (n=3).

Viscosity

Viscosity of all gel batches (G1-G9) varies between 1288 to 1402 cP (Table 7). It has been observed that cholesterol played a major role in deciding the viscosity of in-situ gel. Highest viscosity of gel leads to retarded drug release upto considerable extent. Intermediate viscosity of formulations has shown maximum retardation of drug release than for formulations with highest viscosity indication other factors (membrane integrity) were also responsible for retarded drug

release.

Reduced model equation for viscosity (R^2 = 0.7409).

$$Viscosity = 1063.56 + 14.83X_1 + 16.67X_2 - 3.83X_1^2 + 42.67X_2^2 + 24.00X_1X_2 \quad \ldots\ldots\ldots (6)$$

From reduced model equation (6), it has been observed that content of cholesterol (β_2= +16.67) has slight profound positive effect on deciding the viscosity of formulation than span 60 (β_1= +14.83). However, carbopol 940 and HPMC as polymer system have contributed majorly towards building viscosity of formulation.

Fourier transform infrared spectroscopy

The FTIR spectra of OFL and niosomal in-situ gel formulation (batch G5) were shown in Figure 4. After comparing the obtained peaks of OFL with standard peaks, it was confirmed that the drug molecule was (RS)-9-fluoro-3-methyl-10-(4-methylpiperazin-1-yl)-7-oxo-2,3-dihydro-7Hpyrido[1,2,3,-de]-1,4-benzoazeine-6-carboxylic acid that is OFL [17].

From the FTIR spectra for OFL and gel, it has been clearly observed that peaks of OFL were absent in FTIR spectra for gel (Figure 4). This was attributed to the inclusion of OFL in hydrophilic core of niosomes due to hydrophilic character of drug. This indicates no any structural and chemical changes and /or interaction between drug and polymer used in formulation.

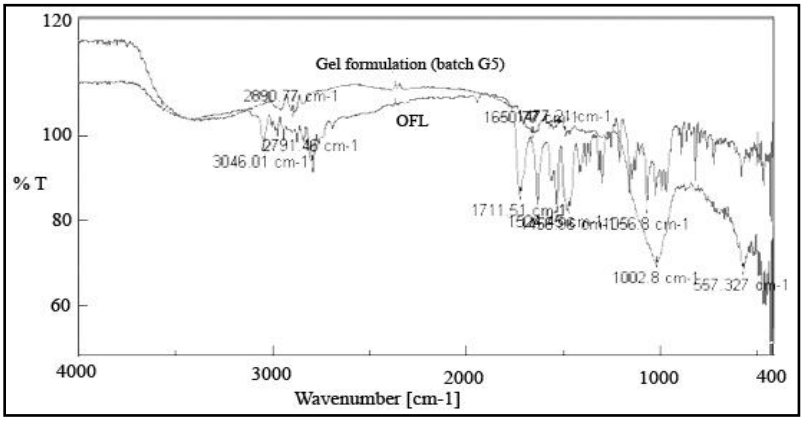

Figure 4: FTIR spectra of OFL and dried niosomal gel formulation (batch G5).

DSC thermo analysis

DSC thermogram of the OFL (Figure 5) shows sharp endothermic peak at temperature of 266.94 ^{0}C. This may be due to the melting of drug confirming melting point of OFL at about 266.94^{0}C. However, DSC thermogram of drug loaded niosomal gel formulation (Figure 5) showed a broad transition in peaks which are characteristic for lipid mixtures containing cholesterol, signifying good interaction of all components forming the bilayers of niosomes. A DSC thermogram of gel formulation shows disappearance of the melting endotherm of OFL and shifting and/or

broadening of the endotherms of surfactant bilayers of niosomes to 115.53^{0}C (Figure 5) suggest possible interaction of OFL with bilayer components. This may account for the enhanced entrapment or inclusion of OFL within hydrophilic core of niosomes which is in accordance with previously reported results[19, 20].

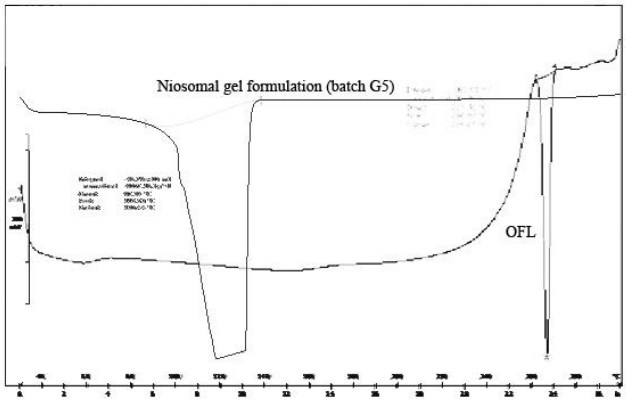

Figure 5: DSC thermogram of OFL, niosomal gel formulation (batch G5).

***In-vitro* drug release kinetics from in-situ gel formulation**

In-vitro drug release was found to be within range of 50.490 ± 0.43 to 66.150 ± 0.56 % over 10hrs for all gel formulation batches (batch G1-G9) as shown in Figure 6. Zero order kinetics was observed best fit model for most of the batches (G1-G9) as shown in Figure 6. Drug release was maximally retarded by batch G6 (50.490 ± 0.43 %) due to its higher cholesterol content than batch G4 with lower cholesterol content. Higher cholesterol content was acting as membrane stabilizer along with improved membrane or bilayer rigidity responsible for sustaining the drug release[21]. As like niosomal batches (N1-N9), the MIC of drug necessary to show therapeutic activity (4 µg/ml) has been achieved within 1 to 2 hrs from initiation of dissolution study (batch G1-G9). A slower rate of drug release has been observed with increase in cholesterol content (batch G4 to G6 and G7 to G9). Additionally, drug release was found to be more sustained in niosomal *in-situ* gel formulation than niosomal formulation attributed to drug diffusion first through niosomal bilayer and then through aqueous gel layer.

Reduced model equation for *in-vitro* drug release kinetics from *in-situ gel formulation* (R^2 = 0.5674)

$$\% \text{ cumulative drug release} = 59.40 + 2.44X_1 - 3.72X_2 + 2.99X_1^2 - 2.72X_2^2 - 0.99X_1X_2 \quad \text{.....(7)}$$

Regression equation (6) and response surface plot (Figure 1d) indicates the inverse relationship between rate of OFL release and content of cholesterol (batch G4 to G6 and G7 to G9). The regression coefficient (β_2 = -3.72) indicates profound negative effect of cholesterol content on rate of drug release than span 60 (β_1 = +2.44).

Stability study of in-situ gel formulation

Stability study was performed on samples from batch G5 (batch SG5) stored at 2 to 8 °C on periodical interval of 15, 30 and 45 days. Withdrawn samples were analyzed for drug content, viscosity and *in-vitro* drug release kinetics.

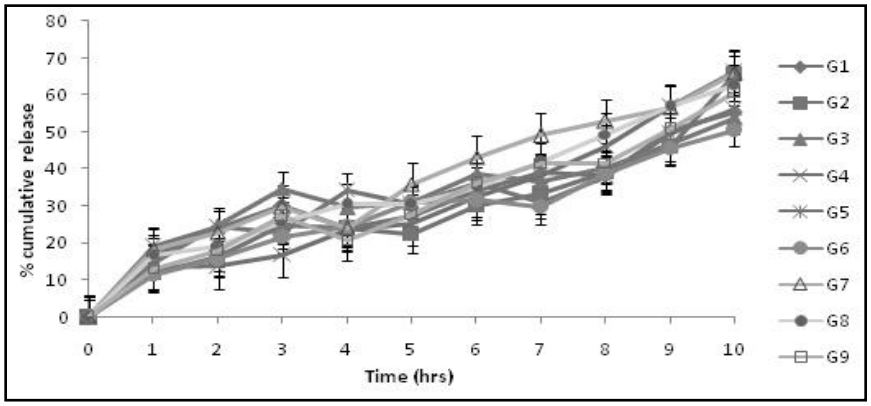

Figure 6: Plot of % cumulative drug release versus time (hrs) from niosomal *in-situ* gel formulations (batch G1-G9).

Drug content

Batch SG5 showed insignificant decrease in drug content after 45 days compared to batch G5 (Table 8), indicating better stability of niosomal *in-situ* gel formulation. This was attributed to higher cholesterol content that resulted into improved stability of niosomal gel with superior drug retention properties and better entrapment efficiency. Additionally, encapsulation of drug into niosomal vesicles dispersed in *in-situ* gel might results into avoidance of premature degradation of drug from tear enzymes. This could lead to retained drug content after long storage time also.

Table 8: Characterization of niosomal *in-situ* gel formulation kept for stability study.

Batch Code	Drug content (%)*			Viscosity (cP at 20 rpm)		
	After 15 days	After 30 days	After 45 days	After 15 days	After 30 days	After 45 days
SG 5	94.187 ± 0.42	93.533 ± 0.37	93.898 ± 0.52	1298	1234	1278

*indicates average ± standard deviation (n=3).

Viscosity

Viscosity of gel samples (batch SG5) kept for stability studies indicated no any significant variation after 45 days also (Table 8). This shows greater eye residence of niosomal *in-situ* gel formulation with prolonged drug-eye contact necessary for once daily dosing.

***In-vitro* drug release kinetics**

In-vitro drug release kinetics from gel samples (batch SG5) showed insignificant variation in %

cumulative drug release compared to batch G5 following 45 days storage indicating good stability of niosomal gel formulation (Figure 7). However, a slight variation in rate of drug release after 7 hrs of dissolution study has been observed after 15 days storage period might be attributed to drug leakage due to loss of niosomal membrane integrity.

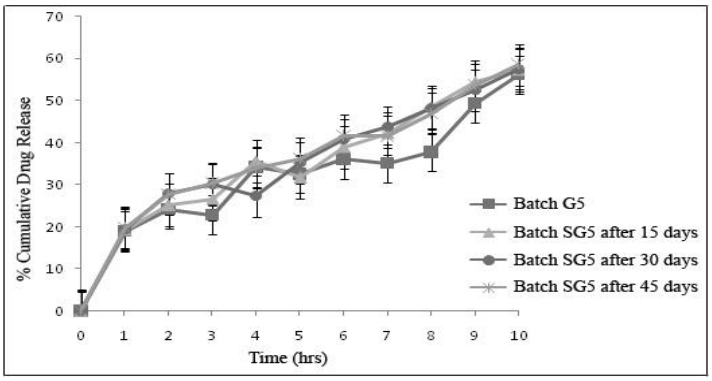

Figure 7: Plot of % cumulative drug release versus time (hrs) from niosomal gel samples kept for stability study at zero time (batch G5), after 15, 30 and 45 days (batch SG5).

Validation of 3^2 full factorial design

Developed 3^2 full factorial design has been validated for target entrapment efficiency.

Entrapment efficiency

The experimental value of percent entrapment (71.00%) was found very close to predicted value (73.00 %) for prepared batch V1 as suggested by experimental design (Table 3). The closeness of experimental and predicted values indicates validation of selected 3^2 full factorial design.

***In-vitro* drug release kinetics**

Prepared formulation (batch V1) showed *in-vitro* drug release kinetics (Figure 8) nearby to batch with matching composition (batch N5). The best fit model of drug release for batch V1 was matrix type with release exponent, rate of drug release and correlation coefficient values as 0.3862, 19.1668 and 0.9125, respectively.

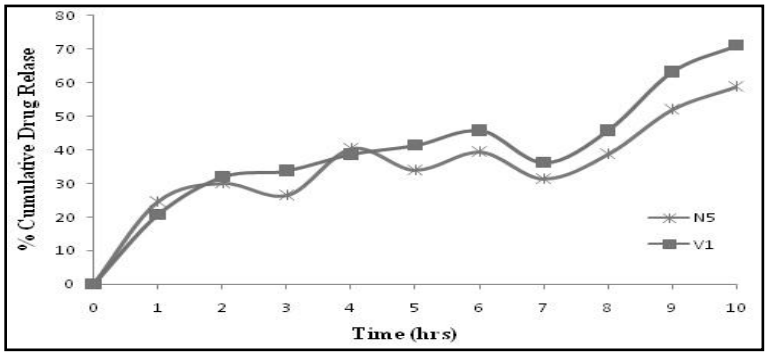

Figure 8: Plot of % cumulative drug release versus time (hrs) from niosomes (batch V1 and N5) for validation of experimental design.

CONCLUSION

Prepared ofloxacin niosomal *in-situ* gel formulation can be used for ophthalmic drug delivery with increased corneal retention time due to bioadhesive property of incorporated polymers. Additionally shorter period of time (4µg/ml within 1 to 2 hrs) to achieve MIC and total drug release extended up to 10 hrs ensures improved bioavailability with once daily dosing. This will overcome the drawbacks associated with conventional ophthalmic ofloxacin aqueous solution such as frequent dosage administration (due to the precorneal and nasolacrimal drainage systems), crystal deposition on cornea and limited bioavailability. Additionally, availability of niosomal formulation in liquid state at non-physiological conditions will help to improve the patient compliance. Encapsulation of drug in niosomal vesicles dispersed in *in-situ* gel avoids the premature degradation of drug from tear enzymes. The stability testing confirmed the stability of niosomal gel formulation.

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