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Optimization and Evaluation of Temperature Triggered *in situ* Gel Formulation using Design of Experiments (DoE) and HET-CAM Test

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Abstract

Purpose: The aim of the present work was carried out by the principles of quality by design approach to optimize thermo-responsive *in-situ* hydrogels. To recognize the effect of formulation variables (parameters) on the response of *in-situ* gels, a 3 factor, 3 level Box- Behnken design, was explored to expect the responses such as Viscosity (Y1), Temperature of gelling time (Y2) and % Cumulative drug release (Y3) when the concentration of poloxamer (X1), HPMC K200M (X2) and HPMC low viscous (X3) were selected as independent variables.

Methods: Using cold method, 17 formulations were prepared and their corresponding physicochemical parameters such as flow ability, gelling capacity, pH, rheological properties and transition of sol-gel were analysed.

Results: Based on *in-vitro* release studies and gelling capacity, it is inferred that F8 shows to be the best formulation. HETCAM study was also performed to indicate the formulation was non-irritant. Ex-vivo studies were done and compared with marketed formulation of ofloxacin.

Conclusion: These temperature triggered *in-situ* gel formulations can administered in a drop form and produce appreciably less inconvenience with vision.

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Introduction

Eye is one of the challenging organs for drug delivery because of its unique anatomy restricts drug absorption into the deep tissues. Although various ophthalmic drug delivery systems like inserts, ointments and suspensions have been developed to overcome this problem, it could not be accepted by patients due to several drawbacks i.e., difficult in administration of inserts, blurred vision due to the use of ointments and dosage heterogeneity of suspensions. Accordingly, more than 80-90% of marketed ophthalmic formulations are still in the form of eye drops specifically as water-soluble drugs. One of the main reasons of using eye drop is simple instillation onto the eye with accuracy in doses. However, this conventional system could not be considered as an optimum to treat eye disorders due to rapid precorneal elimination by protective mechanisms of the eye such as blinking reflex, lacrimal fluid dilution and nasolacrimal duct drainage. Nowadays, a major advancement in developing ophthalmic formulations has been achieved using ophthalmic gel technology in which droppable gels has been evolved known as "*in-situ* gel". *In-situ* gel is specifically made of certain polymers, which undergo sol-gel transition through changes in environmental conditions like pH, specific ions and temperature [1-5].

In particular, a thermo-responsive *in-situ* gel, an ophthalmic product vehicle responding to a shift in temperature, possesses liquid characteristic at low temperature and becomes gel when it comes to contact with a certain temperature defined as sol-gel transition temperature (GT). This transition could be determined through different techniques like UV spectroscopy, Fourier Transforms Infra-Red spectroscopy and through analyzing rheological properties. Researchers suggested that a good ophthalmic thermo-responsive *in-situ* gel should have sol-gel transition temperature higher than room temperature and it forms gel at precorneal temperature (35˚C) in order to avoid keeping it in a fridge before administration restricting eye irritation due to the use of cold eye drops [6,7].

One of the recognized polymer types possessing thermo responsive behaviour is Poloxamers. It is a triblock copolymer poly (ethylene oxide)-b-poly (propylene oxide)-b-poly (ethylene oxide) (PEO-PPO-PEO) showing amphiphilic behaviour due to the presence of both hydrophilic and hydrophobic domains such as ethylene and propylene oxide respectively. The gelation mechanism of poloxamer could be explained by the changes in micellar structure as a function of concentration and temperature. Poloxamer is widely used in ocular drug delivery system for their prolonged drug release which could present satisfactory inertia for eye tissues. However, a major disadvantage is their low mucoadhesive activity. Hence, some poloxamers based ophthalmic formulations have been improved by adding polymers providing mucoadhesive property such as carbopol and sodium hyaluronate [8,9].

Ofloxacin is a second-generation fluoroquinolone; an analogue of broad-spectrum antibiotic approved by US Food and Drug Administration for ocular usage. The functions of ofloxacin are inhibited by two bacterial enzymes such as type II topoisomerases and DNA gyrase. Topoisomerase IV is an enzyme necessary to separate the replicated DNA, thereby inhibiting bacterial cell division. An ophthalmic preparation of ofloxacin available commercially is as eye drops. The marketed formulation is used extensively by administering it nearly 3-4 times for ophthalmic inflammatory. Minimized frequency of administration either once or twice a day is crucial to increase patient compliance.

In developed ophthalmic solutions, sterilization is needed to make sure sterility of the finished products. It can be made either through filtration or autoclaving (steam sterilization). Although autoclaving is the first choice for most ophthalmic solutions because of its convenience for large-scale production, some products could not be sterilized by this method due to their physicochemical properties were altered under autoclaving conditions. Therefore, manufacturers must find the most appropriate method for sterilizing their ophthalmic products. Due to lack of evidence, the suitable sterilization method without affecting their physicochemical properties must be checked for the developed formulation.

The pharmaceutical Quality by Design (QbD) is a systematic approach in designing and development of the formulation along with manufacturing process that ensures predefined objectives, product and process understanding and process control. The significance of this approach is to comprehend how process and formulation parameters influence the product quality and ensuing optimization parameters in accordance to final specifications. QbD employs multivariate experiments to know process and product and to establish a design space through Design of Experiments (DOE). DOE is a mathematical tool used to define the importance of specific processing and/or product variables, and how to control them to optimize the system performance while maximizing properties. DOE uses statistical methodology to analyze data and predict product property performance under all possible conditions within the limits selected for the experimental design. In addition to understanding how a particular variable affects product performance, interactions between different process and product variables are identified.

In this study, the antibiotic ofloxacin was formulated using either poloxamer 188 or combination of poloxamer 188 and HPMC (low & High viscous) which act as a thermo responsive gelling agent and a solubility enhancer [10]. Formulations were prepared with different ratios in order to increase pre-corneal residence time and to decrease the frequency of administration of dosage forms. The suitable sterilization method without affecting their physicochemical properties for the developed formulation must be checked. The developed thermo-responsive ofloxacin ophthalmic *in-situ* gel was evaluated and optimized using design expert the formulation of thermo-responsive ofloxacin ophthalmic *in-situ* gels to evaluate their physicochemical properties, potential for being an eye irritant, and to determine the effect of autoclaving on their physicochemical properties.

Materials and methods

Materials

The drug Ofloxacin, polymers like poloxamer 188, HPMC (low & high Viscous) & Benzalkonium chloride were purchased from Sigma Aldrich chemicals-Bangalore. β-cyclodextrin, sodium chloride was purchased from Fischer chemicals Ltd-Chennai. Sodium bicarbonate, calcium chloride, citric acid was purchased from Fischer chemicals Ltd-Chennai. All other solvents used were of analytical grade.

Fourier transform infrared spectroscopy analysis (FT-IR)

The compatibility between the drug and poloxamer 188 was studied on FT-IR (Perkin Elmer) spectroscopy. Spectra of pure Ofloxacin, pure Poloxamer 188, pure HPMC(low & high viscous) and physical mixture of ofloxacin with poloxamer 188, HPMC

(low & high viscous) were compared at 400 to 4000 cm^{-1} [11-13].

Powdered X-Ray diffractometer analysis

X-ray Diffraction analysis was performed with a PANalytical Xpert Pro X-ray Diffractometer using Ni filtered Cu kα radiation. The powdered samples evaluated was taken on the glass slide and placed on the X-Ray Diffractometer. The scanning rate was 10 minutes over a 2θ range of 10 to 90°.

Preparation of thermo sensitive gel

The preparation of ophthalmic *in-situ* gel was done with slight modifications of "Cold Method". 0.3 gm of ofloxacin was dissolved in citro-phosphate buffer in aseptic condition. Further, preservative benzalkonium chloride (0.002%) was added at the same time. Separately the polymeric solution poloxamer 188 and HPMC (low & High Viscous) was prepared in the ratio as shown in the table 2 and kept aside for 24 hours for appropriate mixing. Finally, the drug and polymeric solution was well mixed together and further the isotonic agent (0.9% Sodium chloride) also added. Moreover the 50 µl of formulation was added into STF solution to get stiff gel when the solution gets raised to the temperature of 35°C [14].

Experimental design (Design of Experts)

The preferred design was Box-Behnken design engaged to optimize the *in-situ* gel formulation using Design-Expert software (Version 7.1.6. Stat-Ease, Inc., Minneapolis, MN). The design is suitable for exploring quadratic response surfaces and constructing second order polynomial models. The characterization of cubic design is done using a set of points available at the midpoint of each vertices of the multi dimensional cube which circumscribes the region interest is helpful to analyze interaction effects, main effects, and quadratic effects of the ingredients of prepared formulation in order to optimize the prepared formulation. The concentration of design matrix comprises of 17 experimental runs. The non-linear quadratic model equation of the design is given below,

$$Y= A_0+A_1X_1+A_2X_2+A_3X_3+A_4X_4+A_5X_5+A_6X_1X_3+A_7X_1^2+A_8X_2^2+A_9X_3^2 \quad \ldots\ldots\ldots (1)$$

where, A0: the intercept representing the arithmetic average of all of 17 runs; A1, A2, A3, A4, A5 A6, A7, A8, and A9: The regression coefficients estimated from the observed experimental values of response variable Y; X1, X2 and X3: the coded levels of the independent variables; X1X2, X2X3, and X1X3: the linear interaction terms; 2: quadratic terms; Factors evaluated in this design matrix were the percentage of poloxamer (X1), amount of HPMC (X2), and volume of solvent (X3) as the independent variables which were represented by 1, 0, and -1, analogous to the low, middle, and high values respectively. The responses obtained after the preparation of these 17 formulations were filled in the design [15,16].

Evaluation of *in-situ* gel

Clarity and pH

Clarity test was observed by visual inspection under a good light, viewed against a black and white background, with the contents set in motion with a swirling action. Also, it was observed for formation of turbidity or any unwanted particles dispersed in the solution. Each formulated batch, pH was measured using pH metre which was previously calibrated using standard buffers of pH 4 and pH 7 as per the established procedure [17-21].

Drug content uniformity

100 µl of the prepared ophthalmic *in-situ* gel formulation was taken and it was poured into 100 ml standard flask and the volume was made up to 100 ml using Simulated Tear Fluid (STF). The composition of STF is as follows: NaCl, 0.670 g; $NaHCO_3$, 0.200 g; $CaCl_2 \cdot 2H_2O$, 0.008 g and purified water was used to up to 100ml. Further the flask was shaken well for few minutes. The concentration (drug) of the prepared ophthalmic formulations was evaluated by using UV spectrophotometrically (Perkin Elmer Lambda 35) at particular 294 λ_{max}. The obtained results were measured in triplicates.

Gelation temperature measurement (GT)

About 1 gm of Formulation (F8) and 1 ml of water were taken separately in two test tubes of volume 10 ml. Both test tubes were kept in the water bath and temperature was checked in the water filled test tube by placing thermometer on it. Water temperature was gradually increased. Marking the temperature at which *in-situ* gel solution stopped flowing while tilting was marked as T1. Further, the temperature was gradually decreased in order to mark the temperature at which the gel started flowing again as solution as T2. The average temperatures of T1 and T2 were mentioned as the critical gelation temperature (Average °C). While using, the formulation was diluted using isotonic tears and the critical gelation temperature [22] was marked.

Gelling capacity

In order to determine the gelling capacity of ophthalmic formulations, about 2 ml of STF solution contains 100 µl of the prepared formulations. The visual examination of gelation was done. Further, the time taken for gelation and the complete dissolved state of the formed gel were noted [23]. In order to maintain the prolonged period of time, the optimum formulation would undergo a rapid sol-to-gel transition without dissolving or eroding of the gel. Gelling capacity increases with increasing concentration of gelling agent both at higher and lower concentration of viscosifying agent.

Determination of viscosity for temperature triggered in-situ gel formulations

In scrupulous, a thermo responsive *in-situ* gel have the characteristic of liquid at lower temperature and it undergoes gel when the necessitate temperature is reached. Among the rheological studies, the most important was determination of viscosity of *in-situ* gel, expressed in Centipoises (cP). It was then examined at different shear rates using viscometer fitted with spindle 63 (Brookfield Viscometer DV2T). After administration of the drug, the change in viscosity is measured and it is carried out in triplicates by drifting the temperature flow of 25°C and 35°C [24,25].

Sterilization using autoclaving

The group of representatives was autoclaved in order to revise the effect of sterilization on *in-situ* physiochemical parameters of ophthalmic *in-situ* gel using the procedure adopted by US Pharmacopeia 31 (The United States Pharmacopeial Convention, 2007). All the test tubes containing formulation were autoclaved at 121°C, 15 Psi for 20 min. Further, the *in –vitro* physiochemical parameters were evaluated such as % labelled

amount, flow ability, pH, viscosity and sol-gel transition temperature by using the previous autoclaved samples in order to find any changes in the physiochemical parameters after autoclaving [26].

In-vitro release Study for temperature triggered in-situ gel formulations F1-F17

In-vitro drug release study of ophthalmic *in-situ* gel formulations was done by using Franz diffusion cell. 1 ml of formulation of *in-situ* gel placed in donor compartment and freshly prepared Stimulated Tear Fluid in the receptor compartment. Among donor and receptor, dialysis membrane is positioned. After that whole set up is placed in thermostatically forced magnetic stirrer. The temperature of medium was maintained at 37°C ± 0.5°C. 3 ml of sample is withdrawn at programmed time interval of 1 to 6 hr and same volume of fresh STF solution is replaced. The withdrawn sample is diluted to 10 ml of volumetric flask with STF solution and analysed by UV spectrophotometer at respective 294 nm using blank at STF solution. The % cumulative drug release of the prepared ophthalmic formulation was calculated. The data was taken in triplicates and then they obtained is further subjected to curve fitting for drug release data [27-29].

Ex-vivo studies: Permeation experiment (temperature triggered *in-situ* gel)

The goat cornea was purchased from slaughter house and washed with water for several minutes. They were soaked in the STF fluid for few minutes. The goat cornea was preferred as the membrane for the Franz diffusion cell. Goat cornea was kept in between donor and receptor compartment. The formulations F8 was taken for the ex-vivo studies based on the release study data, showing maximum % cumulative drug release and it was taken in donor compartment. The studies were carried out for the period of 8 hours by withdrawing the sample and replacing 3 ml of STF fluid from the receptor compartment. Then the sample was analysed by UV visible spectrophotometer at 294 nm and the graph was plotted with % cumulative drug release vs. time.

In-vitro HET CAM test (hen's egg chorio allantoic membrane test)

At first, the fresh white Leghorn hen's fertilized eggs were incubated for 10 days at temperature and relative humidity of 37.5 ± 0.5°C and 66 ± 5% RH respectively. Subsequently after ten days, the incubated eggs were tapered to find embryo viability and to mark the air space with pencil. The shell around the air space on the embryo was amputated by surgical blade and discarded the defective embryos, if any. Further, the exposed inner embryo membrane was sprayed with 0.9% NaCl solution in order to be warm and wet till further use. During experimentation of HET CAM test, the inner membrane was removed using forceps to uncover the Chorioallantoic Membrane (CAM). About 0.3 ml of the optimized formulation, F8 was applied directly on CAM by concealing almost half of their surface area for 5 min [30]. Further, CAM membrane was examined for any vascular changes like hyperaemia, haemorrhage, lysis, clotting and coagulation of the vessels or albumen which appeared as white colour on CAM [31,32]. Any changes on CAM reflects the imminent nature of the test compound whether it damages the mucous membrane or not when applied to the eye. During the study, negative and positive controls used were 0.9% NaCl and 10% NaOH aqueous solutions. The examinations were carried out in triplicates.

The terms, hyperaemia is referred as the congestion based on increase in blood flow, haemorrhage and lysis are referred as red spots around the vessels due to their bleeding and contraction or disappearance of the vessels due to vessel spasm respectively. Clotting & Coagulation are defined as detected by dark spots either around (extra vascular coagulation) or inside (intravascular thrombosis).

Stability studies

To study the stability of different prepared formulations, the guidelines offered by International Conference on Harmonization (ICH) guidelines were adopted for this study. The stored *in-situ* gel was maintained at a temperature, relative humidity and refrigerator condition of 25°C ± 2°C/60% RH ±5% RH room temperature conditions and 40°C ± 2°C/75% RH ±5% RH elevated temperature conditions for a period of 6 months respectively. The stored vials were analyzed for every 30 days to study their gelling effect, appearance, drug content and pH.

Sterility test

The aerobic, anaerobic bacteria and fungi of the optimized best formulations underwent sterility test using thioglycolate and soya bean casein that was made by dissolving 500 mg of peptic digest of animal tissue. To obtain 100 ml of solution, it is adjusted with the buffer pH 7.1 ± 0.2. Then was drained, centrifuged, and disseminated into flask 7 of 10 ml quantities and was allowed to sterilize at 121°C for 20 min. The positive and negative control tests for growth promotion and sterility were performed respectively. The microorganisms like Bacillus subtilis (aerobic), Bacteroides vulgaris (anaerobic) and Candida Albicans (fungi) respectively were used as test organisms. The incubation was done, and growth was observed [33].

Antibacterial activity

Activity was determined by agar diffusion test employing a cup plate technique. The drug was allowed to diffuse through a solid agar medium. The standard Minimum Inhibitory Concentration (MIC 2 µg/mL) of control and developed formulations containing *in-situ* gel was prepared. A total of 100 ml of nutrient agar media was prepared and sterilized at 15 lb/sq-inch pressure for 20 min in an autoclave; 0.5 ml of microorganism suspension was poured into the above medium which is maintained at a temperature of 50°C to 58°C. This will be done in an aseptic condition. Immediately 20 ml of the microbial agar suspension was poured into each petri plate. After solidification of the media, sterile solutions of ofloxacin (standard solutions) and the developed formulations diluted suitably with sterile distilled water (test solutions) were poured into the cup of sterile nutrient agar Petri plates [34,35].

Results & discussion

FTIR spectroscopy analysis

From the FTIR spectrum the characteristics peaks of drug, polymer and combination of drug-polymer showed no interaction with each other as shown in the (Figure 1) and (Table 1). No interaction between the molecules of drug and polymer poloxamer 188, HPMC K200M and HPMC low viscous which confirms that the drug and polymer molecule binds with each other by physical bond or Vander Waal's force. So, that the bond can easily break and bind with any other molecule.

Powdered XRD spectrum analysis

To check the crystallinity of XRD spectral peak positions of drug, excipient and the mixture of excipients. XRD spectrum of ofloxacin indicates peak position (2θ) at 20.77°, 22.10°, 24.05°, 26.24°, 26.97°, 27.68° and 29.75° which is crystalline in nature. XRD spectrum of poloxamer 188 showed peak position (2θ) at 19.18° and 23.20° with crystalline nature. XRD spectrum of the HPMC K200M showed only two sharp peaks at 7.60° and 20.09° shows amorphous individually. Further the XRD spectrum of HPMC low viscous showed only two peaks at 7.76° and 19.92° shows amorphous separately. Finally, the combination of drug and polymers showed XRD peak position at 19.81° and 23.20° signals indicated that the prepared physical mixture formulation was in slightly amorphous state than the pure drug as shown in the fig 1. From the XRD peak signals, it was inferred that the prepared physical mixture formulation for temperature triggered *in-situ* gel was slightly amorphous in state when compared with the pure drug.

Experimental design for optimization of temperature triggered *in-situ* formulations:

Factors which is to be evaluated in this design matrix for optimizing the temperature triggered *in-situ* gel formulations were the percentage of poloxamer as X1, amount of high viscous HPMC as X2, and amount low viscous HPMC as X3 as the independent variables which were represented by 1, 0, and -1, analogous to the low, middle, and high values (low values18, 0.5 and 0.5. middle values as 19, 0.75 and 0.75 and high values 20, 1 and 1) as shown in the (Table 2). The dependent variables are Response 1 as viscosity (cP) and Response 2 as % drug release with constraints applied shown in (Table 2).

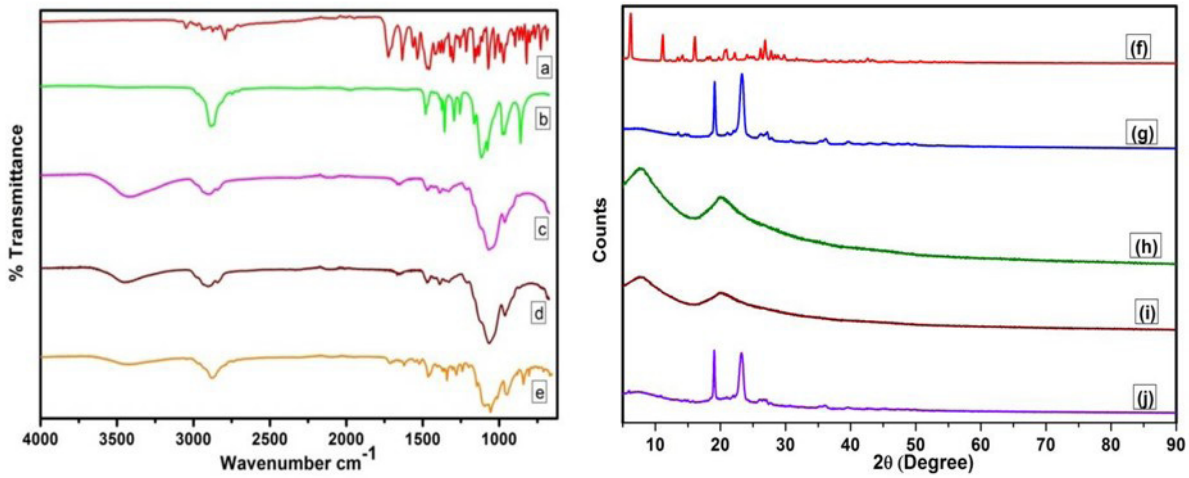

Figure 1: FTIR spectrum of (a) Ofloxacin, (b) Poloxamer 188, (c) HPMC K200M, (d) HPMC low viscous, (e) physical mixture of drug and polymers, (f) PXRD spectrum of the drug ofloxacin, (g) PXRD spectrum of the polymer poloxamer 188, (h) PXRD spectrum of the HPMC K200M (i) PXRD spectrum of HPMC low viscous (j) PXRD spectrum of the mixture of ofloxacin and the poloxamer with HPMC K200M and HPMC low viscous.

Table 1: Interpretation of FTIR spectrum of (a) Ofloxacin, (b) Poloxamer 188, (c) HPMC K200M, (d) HPMC low viscous and (e) physical mixture of drug and polymers.

Figure	Wave number (cm^{-1})	Vibrations
(a)	3042, 2787, 1709, 1620, 1520, 1454, 1287, 198, 1137, 1048, 1009, 954, 876, 798 and 704	N-H, C= H, C= O and C= N are stretching, N-H, C-H, C-N, C-H & C-C bending and N-H rocking
(b)	2875, 1466, 1341, 1276, 1230, 1100, 950 and 839	C-H stretching, C-H bending, OH bending, C-O stretching, C-C stretching, C-N stretching and C-H rocking.
(c)	3417, 2888, 1635, 1452, 1374, 1048 and 943.	O-H stretching, C-H stretching, C=N stretching, C-H bending, O-H bending, C-O stretching and C-C stretching.
(d)	3450, 2895, 1642, 1459, 1374, 1048 and 943	O-H stretching, C-H stretching, C=N stretching, C-H bending, O-H bending, C-O stretching and C-C stretching
(e)	3429, 2874, 1616, 1468, 1338, 1279, 1232, 1138, 1055, 943, 837, 801and 707.	O-H stretching, C-H stretching, C=N stretching, C-H bending, O-H bending, C-O stretching, C-C stretching, C-H rocking, C-C bending and N-H rocking.

Table 2: Optimization of temperature triggered *in- situ* gel formulations.

Formulation code	Factor 1 A: Poloxamer 188 (%)	Factor 2 B: HPMC K200M (%)	Factor 3 C: HPMC Low Viscous (%)	Response 1 Viscosity cPs	Response 2 Release (%)
F1	19	1	0.5	2016.27	87.62
F2	20	1	0.75	2316.6	89.58
F3	20	0.75	0.5	2282.4	89.14
F4	20	0.5	0.75	2668.42	85.09
F5	19	0.75	0.75	2078.9	83.89
F6	19	0.75	0.75	2091.1	83.85
F7	19	0.75	0.75	2012.3	84.21
F8	20	0.75	1	2331.6	90.89
F9	18	1	0.75	741.8	84.08
F10	19	1	1	4139.3	89.03
F11	19	0.5	1	2171.5	86.55
F12	18	0.75	0.5	979.84	87.17
F13	18	0.5	0.75	1087.7	85.61
F14	19	0.5	0.5	1998.08	85.43
F15	19	0.75	0.75	2088.46	85.97
F16	19	0.75	0.75	2059.87	4.01
F17	18	0.75	1	989.78	85.71

Effect of independent variables on viscosity (Y1)

ANOVAs test for the observed data of viscosity indicated that the quadratic model was significant with F value 4.11, P value 0.0378 respectively and fitting for the data. The resulting equation with coded values is as follows

Viscosity (Y_1) =2066.13+724.98*A+162.92*B+292.57*C+2.25*AB+6.06*AC+487.40*BC-648.93*A^2+290.21*B^2+224.96*C^2 ..... (2)

From the equation (1), it was observed that the viscosity is significantly affected by the concentration of variable A. The variables B and C have positive influence on viscosity but comparatively lesser than the variable C. The above equation suggests that factor A, the concentration of poloxamer 188 had significant effect on viscosity that the concentration of HPMC K200M and HPMC low viscous. The findings from this study substantiate the previous literature results. Increasing the concentration of poloxamer 188 from 3 to 5 resulted in the formation of more viscous formulation as shown in the figure 2 (a) to (f). Whereas when the concentration is increased further from 2(a) to 2(f) viscosity of the developed formulation was gradually decreased. Moreover, the more viscosity of formulation is necessary for the persistence of the drug in the cornea of the eye.

In addition, the combination of the concentration of HPMC K200M and HPMC low viscous has significant influence in improving the formulation viscosity than the combination of other factors. From the equation (1), it suggests that the concentration of HPMC K200M and HPMC low viscous is effective working as the better enhancer agents in improving the viscous nature of the formulation.

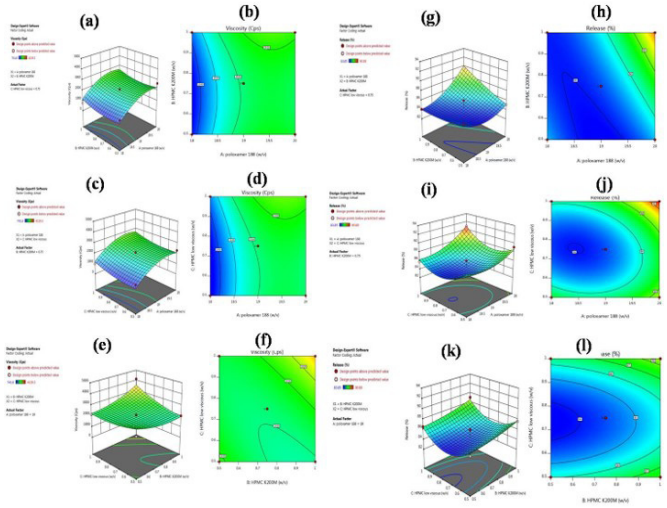

Figure 2: (a) and (b) represented the Optimized formulations F8 AB viscosity 3D and Contour plot, (c) and (d) represented the Optimized formulations AC viscosity 3D and Contour plot, (e) and (f) represented the Optimized formulations BC viscosity 3D and Contour plot, (g) and (h) represented the Optimized formulation AB release 3D and Contour plot, (i) and (j) represented the Optimized formulation AC release 3D and Contour plot, (k) and (l) represented the Optimized formulation BC release 3D and Contour plot.

Effect of independent variables on Release (Y2)

From ANOVA test, the observed data was found out that the release is significant with the quadratic model as shown in the figure 2 (g) to 2 (l). The coded values for the quadratic equation (3) is as follows

Release (Y_3) = 84.39 + 1.51*A + 1.12*B + 0.1787*C+1.83*AB - 0.4550*AC-0.0725*BC +1.38*A^2 – 0.6545*B^2 + 2.12*C^2 ..... (3)

The Model F-value of 11.70 implies the model is significant. There is only a 0.19% chance that an F-value this large could

occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, B, AB, A^2, B^2, C^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant.

Evaluation of In-situ gel

Clarity and pH

Clarity test was observed by visual inspection using the bright light to find any suspended particles. First the vial was placed before the white background and no visible dark or suspended particles were seen. And again, it placed before the dark coloured background under under a good light, with the contents set in motion of swirling action. No visible white particles are seen. Also, it was observed for formation of turbidity or any unwanted particles dispersed in the solution. The solution is clear and free from visible particles.

Each formulated batch was checked for pH measurement using pH metre which was previously calibrated using standard buffers of pH 4 and pH 7 as per the established procedure. The pH of all formulations was found as shown in the (Table 3).

Drug content uniformity

The concentration (drug) of the prepared ophthalmic formulations was evaluated by using UV spectrophotometrically (Perkin Elmer Lambda 35) at 294 nm. The drug content was checked for all the batches, by placing the STF as the blank. The obtained values were analyzed in triplicate and the pH range of all formulations F1-F17 was found to be 6.45 to 6.71, the clarity of the formulations were clear in solution and the drug content for the formulations are found to be in the range from 90.99±0.51 to 98.08 ± 0.42.

Gelation temperature measurement (GT)

The critical gel temperature of different temperature triggered in-situ formulations forms sol-gel systems. The mixture containing poloxamer 188 and copolymer/additives HPMC K200M with along HPMC low viscous showed the higher critical gel temperature at 28°C to 37.87°C before and after dilution with tear fluid respectively.

The critical gel temperature of all formulations were recorded as before and after dilution of tear fluid as follows were F1-25.24°C ± 0.4, 35.9°C ± 0.24, F2-26.34°C ± 0.51, 37.7°C ± 0.3, F3-27.24°C ± 0.14, 37.02°C ± 0.58, F4-25.47°C ± 0.23, 36.07°C ± 0.65, F5-26.09°C ± 0.17, 36.50°C ± 0.38, F6-27.12°C ± 0.22, 35.30°C ± 0.43, F7-26.54°C ± 0.57, 36.55°C ± 0.11, F8-25.06°C ± 0.16, 37.87°C ± 0.21, F9-25.33°C ± 0.38, 36.6°C ± 0.64, F10-26-.04°C ± 0.15, 36.41°C ± 0.27, F11-27.45°C ± 0.19, 37.54°C ± 0.43, F12-25.66°C ± 0.18, 36.35°C ± 0.56, F13-26.49°C ± 0.25, 36.47°C ± 0.24, F14-26.43°C ± 0.51, 37.07°C ± 0.29, F15-27.17°C ± 0.31, 36.9°C ± 0.11, F16-25.71°C ± 0.47, 35.74°C ± 0.26 and F17-25-.89°C ± 0.38, 36.42°C ± 0.27 respectively. A better thermo responsive system should be free flowing at room temperature and it should form gel after installation onto eyes. Poloxamers or Pluronics are triblock copolymers of Ethylene Oxide (EO) and Propylene Oxide (PO) it has amphiphilic properties dependent on their PEO/PPO weight ratio. In aqueous solutions, P188 molecules self-assemble themselves into micelles at the critical micellization temperature due to blockage of dehydration by PPO. Literature reports are available on the development of *in-situ* gel forming systems by mixing P188 and different polymeric additives such as cellulose derivatives. In this proposed work, Hydroxyl Propyl Methyl Cellulose (HPMC K200M & low viscous) was used as co-polymer in order to reduce the required P188 concentration due to their higher biocompatibility and better gel capacity.

Gelling capacity

An optimum gelling capacity is mostly necessitate for the formulation which could undergo transition from sol-gel state rapidly and it also maintain the integrity without dissolving completely for a longer time period. Gelling capacity increases with increasing concentration of gelling agent both at higher and lower concentration of viscosifying agent as shown in the (Figure 3(a) and (b)). The STF of 2 ml was taken in a vial and to this 50-100 µl of the formulation F1-F17 was added. All the formulation were clear at 25°C room temperature and from the ANOVA model F8 was found to fit the model. A translucent gel was formed at a temperature higher than room temperature i.e. 36.8°C of the human body temperature.

Determination of viscosity for temperature triggered *in-situ* gel formulations

The developed formulations F1-F17 of temperature triggered *in-situ* gels were measured using Brookfield Viscometer DV2T model attached with Helipath stand and were reported in (Figure 3(c)). The results showed that increase in concentration of polymer, viscosities of the respective formulations were found to be increased. Viscosities of the prepared formulations F1-F17 were in the range of 741.9 – 4139.3 cPs. Among all the formulations, formulation F10 showed the maximum viscosity of 4139.3 cPs whereas F9 formulations showed the minimum viscosity 741.9cPs. Remaining formulations (F1-F15) showed the viscosity range around 989.78 - 2668.42 cPs.

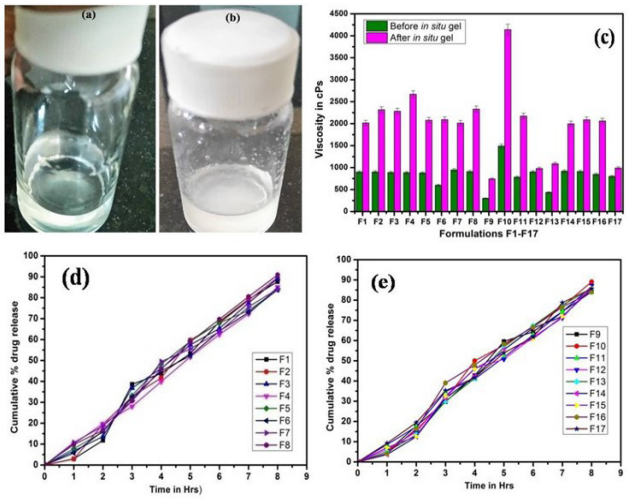

Figure 3: (a) clear formulation of F8 at 25ºC, (b) formulation F8 after conversion of sol into gel at 36.8ºC, (c) Viscosities of temperature triggered in situ gel of all formulations F1-F17 before and after gelling attained, (d) Cumulative drug release patterns of developed formulations F1-F8 and (e) Cumulative drug release patterns of developed formulations F9-F17.

Sterilization using autoclave

The ophthalmic *in-situ* gel was autoclaved as per US Pharmacopeia 31 (The United States Pharmacopeial Convention, 2007). All the test tubes containing formulation were autoclaved at 121°C, 15 Psi for 20 min. The *in-vitro* physiochemical parameters were evaluated for the flow ability and pH. The results

of flow ability was found to be easily pourable before and after autoclaved as mentioned above conditions. The prepared formulation was checked for pH, before and after autoclaved was found in the range of 6-7.

In-vitro release Study for temperature triggered _in-situ_ gel formulations F1-F17

The _in-vitro_ cumulative drug release of temperature triggered _in-situ_ gel of all the developed formulations F1-F17 was analyzed and calculated. The cumulative drug release percent of all formulations were as follows (F1-F8) F1-87.62%, F2-89.58%, F3-89.14%, F4-85.09%, F5-83.89%, F6-83.85%, F7-84.21% and F8-90.89% respectively as shown in the (Figure 3(d)). Further the drug release patterns of all other formulations of (F9 F17) are F9-84.08%, F10-89.03%, F11-86.55%, F12-87.17%, F13-85.61%, F14-85.43%, F15-85.97%, F16-84.01% and F17-85.71% respectively as shown in the (Figure 3(e)). Among all the formulations, F8 showed the maximum percent cumulative drug release of 90.89%. The pharmacokinetic studies revealed that F8 followed zero ordered and Hixson Crowell kinetics model (R^2 value = 0.9950) and the release mechanism confirms through the osmosis and erodible mechanism.

Ex-vivo Studies: Permeation experiment (temperature triggered _in-situ gel_)

The optimized formulation was subjected for permeation experiment as shown in the (Figure 4 (a) and (b)), F8 by plotting cumulative percent drug release with respect to time and as shown in (Figure 4(c)). The formulation F8 showed the maximum percent drug release of 89.76% at the end of 7 hours when compared with marketed formulations (Ofloxacin eye drops) showed 97.59% for 4th hour. The permeability coefficient of the developed formulation F8 was found to be Kp= 0.0069 cm/h.

Hen's Egg test on chorio allantoic membrane (HET-CAM): Temperature triggered in-situ gel formulation F8

HET-CAM test was specifically experimented to study the imminent nature of the optimized formulation, F8 through monitoring any possible irritation on CAM membrane upon their application. Positive control (10% NaOH) showed normal irritant which resulted in haemorrhage and normal hyperaemia as shown in the (Table 3). Negative control (0.9% NaCl) showed non-irritancy in the form of normal tissue vascularisation (Figure 5c). In comparison with positive and negative controls, it was observed that no inflammatory reaction was shown on ideal vascularisation of CAM tissue (arteries, veins and capillaries) as like rabbit's conjunctival tissue (Figure 5d). The optimized in-situ gel formulation F8 (Figure 5d) exhibited non-irritancy on CAM without any vascular damages. Through this study, it was confirmed that the optimized formulation F8 is safe and having non-irritant characteristic and could be a viable platform to deliver ofloxacin efficiently onto the ocular tissues especially to treat bacterial infections.

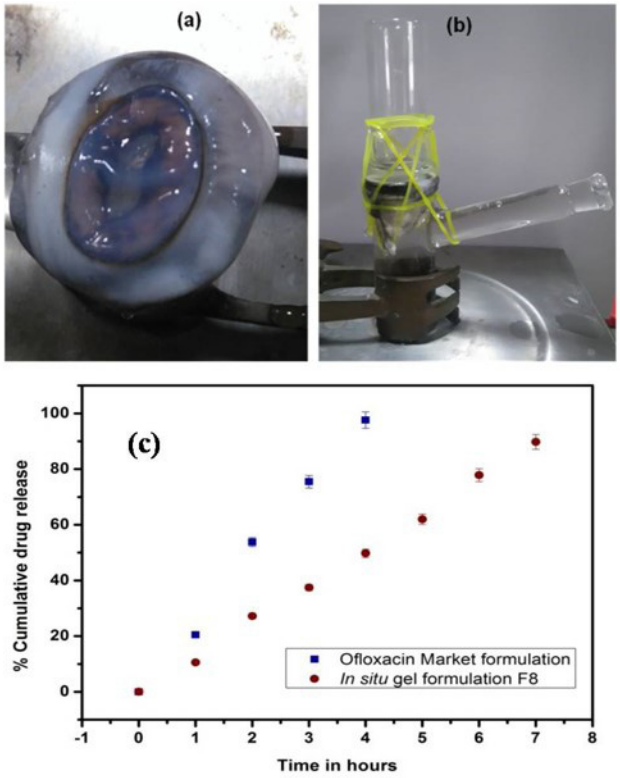

Figure 4: Morphology of Goat cornea, (b) Franz diffusion Cell with cornea membrane and (c) Ex- vivo studies of cumulative percent drug release for the optimized formulation F8 compared with ofloxacin market formulation.

Table 3: Score obtained in HET-CAM Test.

	Score Times (in minutes)								
Formulation F8	0	5	15	30	60	120	240	480	1440
***In situ* gelling system**									
Egg 1	0	0	0	0	0	0	0.5	0.5	0.5
Egg 2	0	0	0	0	0	0	0	0	0
Egg 3	0	0	0	0	0	0	0	0	0
Mean	0	0	0	0	0	0	0	0	0
Sodium hydroxide and normal Saline as Control (positive and negative)									
Egg 1	0	0	0	0	0	0	0	0	0
Egg 2	0	0	0	0	0	0	0	0	0
Egg 3	0	0	0	0	0	0	0	0	0
Mean	0	0	0	0	0	0	0	0	0

Score test – 0-3 is normal, 3-5 is Mild irritant, 5-9 is Moderate irritant and 9-11 is Severe irritant.

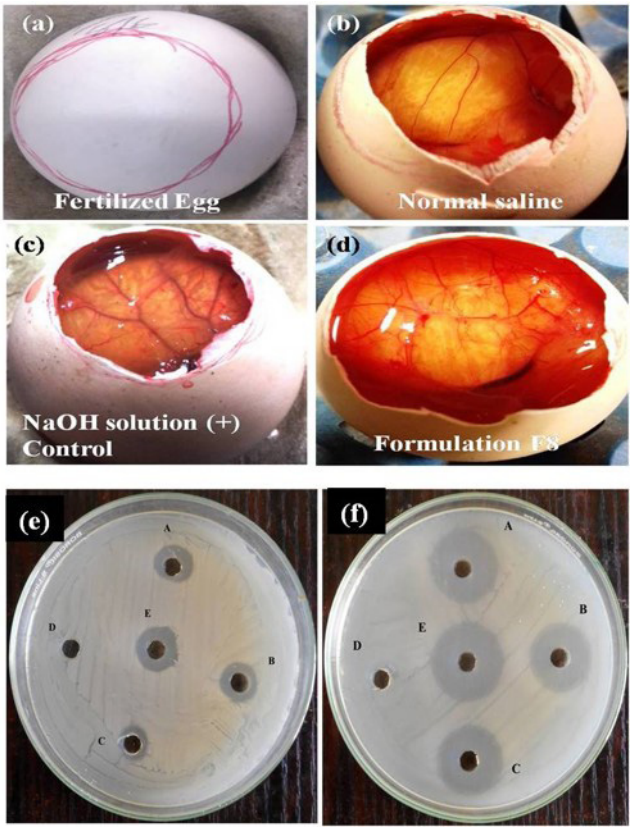

Figure 5: (a) Fertilized eggs, (b) Negative control using sodium chloride, d) Positive control using sodium hydroxide d) HET-CAM study using the best formulation, F8 and the CAM membrane shows that they have no damage or redness, (e) Anti-bacterial activity of Formulation F8 using (a) Escherichia coli sample labelled as A-30µg, B-20µg, C-10µg D- negative control and E- standard and (b) Bacillus subtilis sample labelled as A-30µg, B-20µg, C-10µg D- negative control and E- standard.

Anti-microbial activity

The developed pH based *in-situ* gel formulation F8 showed good antibacterial inhibition activity on microorganisms for *Escherichia coli* as concentration of 30, 20, 10 µg with zone of inhibition 13, 11, 10 mm and standard 10 µg of ofloxacin with 13 mm zone of inhibition. The microorganisms such as *Bacillus subtilis* was prepared with the concentration of 30, 20, 10 µg with zone of inhibition 21, 19, 19 mm and 10 µg of ofloxacin with 21 mm zone of inhibition as shown in the (Figure 5(e) and (f)).

Stability studies

The stability study was performed as per ICH guidelines and it is indicated that the developed temperature triggered formulation F8 were found to be most stable at room temperature than at higher temperatures. About 90% of the drug content was found to be stable as shown in the (Table 4). The sample was found to be translucent with the pH of 6.48 nearly neutral, with the drug content being of 97.55 ± 0.34. It has good gelling capacity with maximum drug release of 90.59% at the end of 6months.

Sterility test

The sterility test was preformed for the optimized formulation (for both pH based, and temperature triggered *in-situ* gel). Negative control shows no precipitation and the prepared growth medium is transparent which indicate that there is no growth of microorganisms. Whereas in the positive control, there is significant milky precipitate and also the inoculated growth medium is not transparent which reveals that there is remarkable growth of microorganism. The formulation F8 indicates that the growth medium was found to be sterile and no precipitates indicate that the test tube is free from microbes as positive and negative signs indicate presence or absence of micro organisms as shown in the (Table 5).

Table 4

Stability data at 40°C ± 2°C/ 75%RH of Formulation F8				Stability data at 25 ± 2°C/5%RH of Formulation F8		
Months	0	3	6	0	3	6
Appearance	Translucent	Translucent	Translucent	Translucent	Translucent	Translucent
pH	6.58	6.46	6.41	6.58	6.52	6.48
Drug content	98.04 ± 0.42	97.22 ± 0.18	96.87 ± 0.51	98.04 ± 0.42	97.86 ± 0.21	97.55 ± 0.34
Gelling studies	+++	+++	+++	+++	+++	+++
In-vitro release study	90.89%	89.05%	87.46%	90.89%	90.74%	90.59%

Table 5: Sterility test for temperature triggered in situ gel optimized formulations F8 respectively.

Sterility test	Results obtained		
	Negative control	Test Sample	Positive control
Test for aerobic bacteria	-	-	+
Test for Anaerobic bacteria	-	-	+
Test for Fungi	-	-	+

(+) indicates presence of micro-organism (-) indicates absence of micro-organism

Statistical analysis

The P value is less than 0.05 indicate the model terms are significant (ANOVA for linear model-DOE Design).

Conclusion

Temperature triggered *in-situ* formulations was optimized and developed by DoE software. These formulations were prepared as *in-situ* gel for easy instillation into the eyes as drops and on contact with the physiologic conditions of the eye like temperature, it get converted to a transparent gel on temperature gets increased. Thus prepared formulation was found to have improved contact time eventually leading to better bioavailability. Also the developed formulation was non-irritant which was substantiated by HET-CAM studies. This study provides concrete evidence alternate to conventional drops and ointments with respect to ease of administration, lesser frequency of administration and improved residence time.

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