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Original Article

NOVEL FLUCYTOSINE LOADED *IN-SITU* GEL FOR THE MANAGEMENT OF OCULAR INFECTIONS

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ABSTRACT

Flucytosine, a BCS class II drug, is indicated in fungal infection. Currently, flucytosine is available in tablet dosage form in the market. However, the drug is indicated for the ocular fungal infections. Thus, there is a need to develop a ocular formulation that can retain for longer duration with prolonged drug release. In the present work, in-situ ocular gel containing flucytosine was prepared for cryptococcosis and candidiasis fungal infection. Design of experiment was employed to obtain the optimized formulation. To determine the independent variables impacting the formulation preparation, preliminary trials were conducted using Poloxamer 407, Poloxamer 188, Carbopol 934, gellan gum, and HPMC K4M as polymer and it was optimized using Simplex lattice design. Amount of poloxamer 407, amount of gellan gum and amount of HPMC K4M were taken as independent variable while viscosity (cps), gelling time (sec), drug release in 6 h were taken as dependent variables. The prepared formulations were evaluated for clarity, pH, viscosity, gelling time, gelling strength, gelling temperature, in-vitro drug release, sterility test and stability studies. Results of the prepared in-situ ocular gel formulation of flucytosine demonstrated significant potential as a therapeutic breakthrough for enhancing treatment outcomes in candidiasis and cryptococcosis infections. Stability studies suggested formulation was stable for at least 6 months. Thus, a successful formulation of flucytosine was developed addressing critical challenges of conventional eye drops and offering prolonged drug release with improved retention in the eye.

KEYWORDS: Flucytosine, Poloxamer 407, Gellan gum, HPMC K4M, In-situ ocular gel, Simplex lattice design.

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1. Introduction

Conventional ocular drug delivery systems include various methods for eye medication administration, each with distinct advantages and limitations. Topical eye drops are practical, non-invasive, and safe but have rapid drug concentration decline, which additives can mitigate [1,2]. Emulsions, particularly oil in water (o/w), enhance solubility and bioavailability, improving drug residence and release [3]. Suspensions, with finely divided APIs, prolong medication duration and contact time, influenced by particle size. Ointments, made of semisolid hydrocarbons like paraffin, enhance bioavailability and drug release, melting at eye temperature [4]. However, challenges like poor bioavailability, limited corneal permeation, and non-creative absorption persist [5-7].

Novel ocular drug delivery systems aim to overcome these limitations via nanotechnology-based formulations such as nanoparticles, liposomes, dendrimers and others for targeted drug delivery to both anterior and posterior eye segments [8-10].

In-situ gelling systems allow transition from sol to gel upon administration, enhancing drug retention and bioavailability. These systems are easily administered, well accepted by patients, decrease dosing frequency, and work directly at the target site with fewer side effects [11-14]. Approaches for developing in situ gelling systems can be categorized into three main types: Physiological Stimuli-Based Systems involving temperature and pH [15], Physical Mechanism-Based Systems using swelling and diffusion mechanism [16], Chemical Reaction-Based Systems including ionic cross-linked [17], photo-polymerization method [18], and enzymatic cross-linking methods [19].

Flucytosine has a broad-spectrum antifungal activity. The drug is typically used to treat infections caused by *Candida* and *Cryptococcus*. The development of drug resistance in fungi and poor intraocular penetration of flucytosine limits its use in ophthalmic preparations [21]. Most ocular drug preparations should be able to pass through the tissue barriers of the eyes and reach the therapeutic targets within the space [22]. Investigators have reported in situ ocular gel of flucytosine using polymers from natural source. However, the associated disadvantage includes less stability and thus lower shelf life. Thus, a stable preparation employing a synthetic material may prove more beneficial.

Cryptococcus neoformans is a major opportunistic pathogen, particularly in immunocompromised individuals, often leading to AIDS-defining illnesses. It primarily infects the lungs and can disseminate hematogenously to the choroidal vasculature, causing choroiditis, which may indicate cryptococcal meningitis. Diagnosis in advanced AIDS patients requires distinguishing it from other opportunistic infections. Symptoms are typically mild and nonspecific, such as headaches, fatigue, fever, and intermittent vision problems [23].

Ocular candidiasis can arise through exogenous routes, involving direct eye infection, or endogenous routes, through bloodstream dissemination. Risk factors include *Candida albicans* infection, persistent candidemia, neutropenia, ocular symptoms, and corticosteroid use. Symptoms encompass reduced visual acuity, floaters, light sensitivity, and ocular pain. Diagnosis is based on ocular examination, revealing chorioretinal lesions, vitritis, vitreous haze, abscesses, corneal haze, anterior chamber cells, retinal hemorrhages, exudates, cotton wool spots, vascular sheathing, hypopyon, scleritis, and optic nerve involvement [24].

In the present work, in situ ocular gel of flucytosine is developed using poloxamer and optimization is carried out employing experimental design. Poloxamers are block copolymers composed of poly(ethylene oxide) and poly(propylene oxide), exhibiting amphiphilic properties. Key parameters, including the rate at which drugs are released and the stability of formulations, are closely associated with the associative and adsorption properties of poloxamers. The prepared formulation is evaluated for various parameters such as clarity, pH, viscosity, gelling time, gelling strength, gelling temperature, in-vitro drug release, ocular irritancy, sterility test and accelerated stability studies.

2. Materials and Methods

2.1. Materials

Flucytosine was a gift sample from Macleods Pharma, Sarigam, India. Benzalkonium chloride was obtained from SRL Chemicals, Mumbai, India. Poloxamer 407 was obtained from Ana lab Fine Chemicals, Mumbai, India, Carbopol 934 was obtained from Seva Fine Chemicals, Ahmedabad, Gujarat, India. Poloxamer 188 was obtained from BASF, Mumbai, India. Gellan gum and HPMC K4M was obtained from Loba Chemie Pvt.Ltd, Mumbai, India. Pancreatic digest of casein and peptic digest of soya bean were procured from Himedia Laboratory Pvt. Ltd., Mumbai, India, Sodium chloride, dibasic potassium phosphate, and dextrose were obtained from Rankem, Avantor, India. Distilled water was used throughout the investigation.

2.2. Methods

2.2.1. Preparation of in-situ ocular gel of Flucytosine

Polymeric solution was prepared by dispersing required quantity of Poloxamer as main polymer and gellan gum, HPMC K4M and Carbopol 934 as co-polymers in water using a magnetic stirrer to avoid lumps until the polymer was completely dissolved. Flucytosine (0.4 %w/v) was then added in to the polymeric solution with continuous stirring. Benzalkonium chloride was added as preservative to prevent microbial growth. Finally, pH of the formulation was adjusted using 0.02 M NaOH suitable for ocular administration.

2.2.2. Preliminary trial batches of flucytosine in situ ocular gel

In the preparation of in situ ocular gel containing Flucytosine, various polymers were screened such as Poloxamer 407, Poloxamer 188, Carbopol 934, gellan gum and HPMC K4M with varied concentration range. The prepared batches were added dropwise into 2 ml simulated tear fluid (STF, composition: NaCl 0.68 g, $NaHCO_3$ 0.22 g, $CaCl_2{\cdot}2H_2O$ 0.008 g, KCl 0.14 g, and distilled deionized water to 100 ml) [24] and evaluated for gelling time and viscosity (Spindle 91 and speed 100 rpm) at 37°C.

2.2.3. Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infrared (FTIR) spectroscopy was utilized to characterize the drug compound. The drug sample was mixed with dry potassium bromide (KBr) in a 1:1 ratio, and the resulting mixture was loaded into the sample holder cavity. The samples were then scanned in the wavelength region of 400-4500 cm^{-1} using pure KBr powder for baseline correction. This process allowed for the characterization and analysis of the drug's molecular identity based on its spectral features.

2.2.4. FT-IR spectroscopy studies

The interaction study between the drug and excipients was conducted using FT-IR spectrophotometry. The individual FT-IR spectra of flucytosine and formulated mixture were analyzed separately and subsequently correlated to identify any signs of interaction. This analysis aimed to assess the chemical interactions and compatibility between the drug and excipients within the formulations.

2.2.5. Optimization by using Simplex Lattice Design

In pharmaceutical formulation development, optimization is vital for achieving desired product characteristics efficiently. Experimental designs offer the optimization by employing critical attributes at critical levels [25]. Factorial design is the most commonly used experimental design for the optimization process. However, the selection of the experimental design mostly depends on the number of the critical attributes chosen and the type of the process or formulation [26]. Simplex Lattice Design offers a structured approach to this optimization process. The Simplex Lattice Design is used to study the blending process in the mixture formulation of in situ gel by simultaneously changing the concentrations of multiple factors while keeping the total

concentration constant [27, 28]. By systematically varying factors, it enables the exploration of complex interactions while minimizing experimental runs. The Quadratic Model within Simplex Lattice Design expands on this approach by incorporating quadratic effects, allowing for the detection of non-linear relationships between factors and responses. This model enhances the ability to capture curvature in the response surface, offering a more nuanced understanding of the formulation space. Through a series of experiments based on the Simplex Lattice Design with Quadratic Model, researchers can identify significant factors and interactions that influence product performance [29-30]. Statistical analysis techniques like ANOVA aid in discerning the most influential factors and optimizing the formulation accordingly. Using software from Stat Ease, Inc., an experimental design-expert version 13 a three-factor, three level, Simplex Lattice Design was employed. On the basis of primary trials, amount of Poloxamer 407 (X1), amount of gellan gum (X2) and amount of HPMC K4M (X3) were taken as independent variables as shown in (Table 1). Dependent variables, i.e. gelling time (Y1), viscosity (Y2) and % drug release at 6 h (Y3), were chosen. Table 2 demonstrated the Simplex Lattice design matrix layout. Total eleven formulation were developed as per the design and each batch was evaluated for various parameters.

Table 1. Independent variables

Translation of coded value in actual units		
Independent variables	Variable level	
	Low (0)	High (1)
Amount of Poloxamer 407 (gm) (X1)	1	2
Amount of gellan gum (gm) (X2)	0.3	0.5
Amount of HPMC K4M (gm) (X3)	0.3	0.5

Table 2. Matrix layout for simplex lattice design

Batch	X1	X2	X3
	Coded values		
F1	1	0	0
F2	0	1	0
F3	0	0	1
F4	0.5	0.5	0
F5	0.5	0	0.5
F6	0	0.5	0.5
F7	0.66	0.16	0.16
F8	0.16	0.66	0.16
F9	0.16	0.16	0.66
F10	0.33	0.33	0.33
F11	1	0	0

2.2.6. Evaluation of designed batches of in-situ gel

2.2.6.1. Gelling time

The gelling time was assessed by placing few drops of the formulation into a vial containing 2 ml of freshly prepared simulated tear fluid that had been equilibrated at 37°± 2 ºC. Gel formation was visually observed, and the time required for gelling was recorded [31].

2.2.6.2. Viscosity

It was essential to determine the residence time of the formulation in the eye by assessing the viscosity of the instilled formulation. The prepared solutions were allowed to gel at physiological temperature, followed by viscosity measurements using a Brookfield viscometer (DY-1 Prime, Mumbai, India) with spindle 91 at speed 100 rpm, recorded in centipoise (cps) [31].

2.2.6.3. In vitro drug release studies

A cellophane membrane was positioned between the donor and acceptor chamber of Franz diffusion cell (20 ml), which was used to measure the drug release from the gel (1 g). The acceptor chamber was filed with phosphate buffer (pH 7.4). A magnetic stirrer (37 ± 2°C with continuous stirring at 50 rpm) was used to provide thermostatic control over the entire assembly. Sample of 1 ml was taken at every h of the time period till the 6 h. Phosphate buffer was added after each samples were removed from the acceptor chamber to maintain the sink condition [32].

2.2.6.4. Contour plot and surface plot of the design

Surface plots (2-D) and contour plots (3-D) were used to optimize the formulation for all experimental dependent variables. Surface and contour plots were produced with the aid of the Design expert 13.1 software. These plots are helpful for examining the relationship between their concurrent influences on the response.

2.2.6.5. Optimized batch analysis

The optimal batch formulation was chosen based on the desirability function and overlay plot of the chosen dependent variables. The optimized batch was then further assessed for viscosity, gelling time, and drug release in 6 h. Following that, the prepared batch's result was compared to predicted (theoretical) values.

2.2.7. Evaluation of optimized batches of in-situ gel

2.2.7.1. Gelling strength

It was described as the amount of the time, measured in seconds, that 35 g of piston needed to penetrate five centimeters into 50 g of prepared gel that was placed inside a 100 ml measuring cylinder. A 100 ml measuring cylinder was filled with about 50 g of the optimized formulation and gelling was induced using artificial tear fluid. Next, a plunger was put in this measuring cylinder and a 35 g weight was fastened to it. The gel strength was obtained as the number of seconds required for the plunger to reach a depth of 5 cm [31].

2.2.7.2. Drug content estimation

The drug content was determined by diluting 1 ml of the prepared formulation with 100 ml of methanol and analyzing the solution using UV-visible spectroscopy at a wavelength of 290 nm.

2.2.8. Sterilization and sterility testing

Sterilization of the formulation was carried out using membrane filtration technique (0.45 µm) [33]. Sterility tests were based on the principle that if bacteria or fungi are placed in a medium which provided nutritive material, moisture, desired pH and kept at a favorable temperature, the organism will grow and their presence can be indicated by the growth in original medium. The tests for sterility were done by detecting the

presence of viable forms of bacteria, fungi and yeast in or on preparations. The tests were carried out under strict aseptic techniques to avoid accidental contamination of the preparation.

2.2.8.1. Culture media for anaerobic bacteria and fungi

Alternative Thioglycolate Medium, Soyabean Casein Medium. These media can be used for the detection of anaerobic bacteria and fungi [34].

2.2.8.1.1. Preparation of Alternative Thioglycolate Medium

Alternative Thioglycollate Medium is recommended for sterility testing of turbid or viscous biological products. About 29 g of thioglycolate medium was suspended in 100 ml distilled water. It was heated mildly to dissolve the medium completely, mixed well and dispensed into sterile tube.

2.2.8.1.2. Preparation of Soyabean Casein Medium

The ingredients: pancreatic digest of casein 17 g, peptic digest of soya bean 3 g, sodium chloride 2.5 g, dibasic potassium phosphate 2.5 g, and dextrose 2.5 g were dissolved completely in 1000 ml of distilled water, and the medium was boiled for 10 min. The pH was adjusted to 7.3 ± 0.2. Medium was distributed into 9 cm diameter petri dish.

2.2.8.1.3. Sterilization of medium

Anaerobic culture medium was sterilized by membrane filtration method at a temperature of 121°C for 15 min. Anaerobic culture medium was kept in room temperature before inoculation of the sample. The sterilized in-situ gel formulation and unsterilized in-situ gel formulation were placed into fluid Alternative Thioglycollate Medium and incubated at 20-25°C [35].

2.2.8.1.4. Control test

To support the above-performed test, a positive control (Inoculation of *S. aureus* and *A. niger*) and negative control (without inoculation of microbes) tests were also carried out for quality control maintenance. Negative control test was carried out to confirm that the media and the environment provided for incubation were suitable for the growth of microorganism [36]. In positive control test, inoculation of *Staphylococcus aureus* and *Aspergillus niger* were carried out.

2.2.9. Ocular irritation test

Modified hen's egg - chorioallantoic membrane (HET-CAM) test was employed for the ocular irritation study [37]. In brief, fertilized hen eggs weighing 50-60 g were acquired from the poultry farm and were incubated in humidified incubator at a temperature of 37°C ± 0.5°C for 3 days. The eggs were rotated manually in a gentle manner twice a day. On the 3rd day, 3 ml of the egg albumin was taken using sterile syringe from the pointed end of the egg. The hole was then sealed using cellophane membrane. To allow for the growth of CAM away from the shell, the eggs were maintained in the equatorial position. The 10th day of incubation involved creating a window of 2 cm x 2 cm on the equator of the eggs. Then, 0.5 ml of the formulation (sample) and 0.9%w/v solution of NaCl (control) were immediately injected onto the CAM surface and allowed to come into contact for 5 min. The membrane was subsequently assessed for vascular damage, and the duration of injury was quantified. The results were observed in terms of scores from the previously determined scorings (Table 8).

2.2.10. Accelerated stability studies

The optimized sterile ophthalmic formulation underwent stability testing by being filled into glass vials, closed with gray butyl rubber closures, and sealed with aluminum caps. These vials containing the optimized formulation were placed in a stability chamber maintained at 25 ± 2°C and 70 ± 5% relative humidity (RH) for a duration of one month [38]. Samples were withdrawn at intervals of 0, 3, and 6 month to assess various parameters including pH, visual appearance, gelling strength and gelling time.

3. Results and Discussion

3.1. FTIR Spectroscopy Study

The observed peaks of drug in FTIR spectra are in the range of standard peak of specific functional group such as C-N-H bending: 1336.89 cm^{-1}, C=C-N bending: 498 cm^{-1}, C-C=C bending: 636.18 cm^{-1}, C=C stretching: 1473.48 cm^{-1}, C=O stretching: 1640.84 cm^{-1}, N-H stretching: 3139.58 cm^{-1}, C-H stretching: 2620.91 cm^{-1}, which show the identity of the drug [39]. FTIR spectra of drug is shown in Figure 1A. FTIR spectra of flucytosine with excipients are shown in Figure 1B. The result exhibited that there were no significant changes in major peaks of flucytosine in sample in presence of excipients except mild broadening in later part of the wavenumber when compared to spectra of pure drug (Figure 1A), indicating absence of any remarkable interaction of drug with excipients [40].

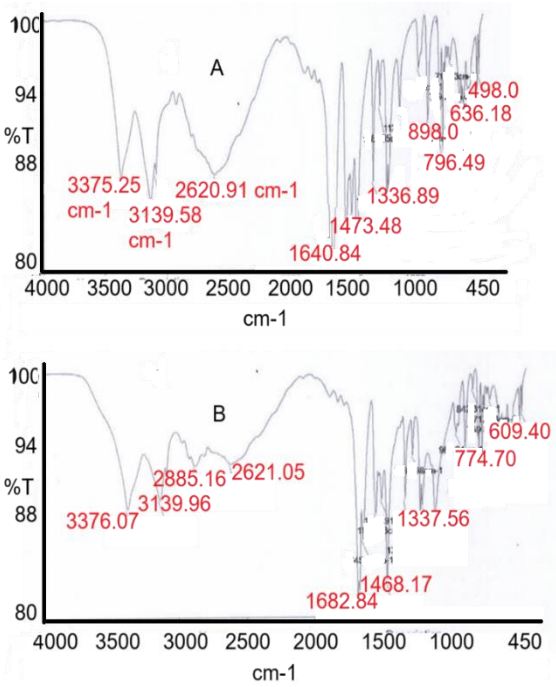

Fig. 1. FTIR spectra (A) FTIR spectra of flucytosine, (B) FTIR spectra of flucytosine + Poloxamer407 + gellan gum + HPMC K4M

3.2. Preliminary Trial Batches

Preliminary trials were carried out for screening of polymers and its concentrations. As demonstrated in Table 3, various polymers, namely gellan gum, HPMC K4M, Poloxamer 407 and Poloxamer 188 and Carbopol 934 in various concentrations were screened for their gelling property within varied concentration range and combinations. Preliminary trials were completed without

Table 3. Preliminary trials

Trial batches	Amount of Polymer (%)	Gelling time (sec)	Trial batches
Screening of concentration of Poloxamer 407 with Carbopol 934 (0.1%) constant			
T1	2.5 %	58.66 ± 0.5	91.66 ± 1.5
T2	5 %	34.66 ± 1.5	97.66 ± 0.5
T3	7.5 %	28.33 ± 0.5	97.33 ± 2.0
T4	10 %	25.66 ± 0.5	102.3 ± 1.5
Screening of concentration of Poloxamer 188 (P 188) with Carbopol 934 (0.1%) constant			
T5	2.5 %	95.33 ±2.51	126.06 ± 3.2
T6	5 %	81.66 ±3.21	139.63 ±3.0
T7	7.5 %	72.66 ± 3.0	156.73 ±2.7
T8	10 %	63.33 ± 3.0	177.33 ±3.7
Screening of concentration of of Poloxamer 188 and Poloxamer 407 - sum of these percentages equals 5%			
	P 188	P 407	
T9	2.5 %	2.5 %	91.66 ± 1.5
T10	3.5 %	1.5 %	64.33 ± 1.5
T11	1.5 %	3.5 %	82.33 ± 2.08
Screening of concentration of Carbopol 934 with Poloxamer 407 (P 407) (5 %) constant			
T12	0.1 %	37.33 ± 0.5	97.66 ± 0.5
T13	0.2 %	35.66 ± 0.5	96.40 ± 2.2
T14	0.3 %	35.33 ± 1.0	37.63 ± 2.3
T15	0.4 %	35 ± 1.0	76.46 ± 1.5
T16	0.5 %	34.66 ± 1.5	49.4
Screening of concentration of HPMC K4M with Poloxamer 407 (5 %) constant			
T17	0.1 %	57.66 ± 1.5	35.26 ± 1.9
T18	0.2 %	45 ± 1.7	46.50 ± 0.4
T19	0.3 %	44.33 ± 1.1	61.76 ± 0.6
T20	0.4 %	42.66 ± 2.0	68.73 ± 0.6
T21	0.5 %	40.66 ± 1.2	71.73 ± 0.8
Screening of concentration of gellan gum with Poloxamer 407 (5 %) constant			
T22	0.1 %	43.66 ± 2.0	39.06 ± 0.3
T23	0.2 %	42.66 ±1 .1	48.2 ± 0.3
T24	0.3 %	42.33 ± 1.1	50.73 ± 0.5
T25	0.4 %	41 ± 1.0	58.3 ± 0.6
T26	0.5 %	37.66 ± 1.5	60.7 ± 1.21
Screening of concentration of HPMC K4M with poloxamer 407 (5 %) and gellan gum (0.5 %) constant			
T27	0.1 %	24.66 ± 0.5	38.93 ± 0.5
T28	0.2 %	26.66 ± 0.5	47.53 ± 1.0
T29	0.3 %	25 ± 1.0	61.9 ± 1.1
T30	0.4 %	24 ± 1.0	68.33 ± 0.7
T31	0.5 %	22.66 ± 0.5	74.00 ± 1.2

the use of active pharmaceutical ingredient. Initially, Poloxamer 407 and Poloxamer 188 were screened in the presence of Carbopol 934 (1%w/v) in varying concentrations. It was observed that Poloxamer 407 with Carbopol 934 exhibited lower gelling time as compared to the later combination. Then, combinations of both grades of poloxamer were also tried in varying concentrations. It was found that gelling time was more. Later, Poloxamer 407 was kept fixed and tried in combinations with other polymers: Carbopol 934, gellan gum, HPMC K4M, with varying concentrations. Later, one combination of three polymers, i.e. Poloxamer 407, HPMC K4M and gellan gum, was screened with varying concentrations. The results obtained were satisfactory with lower gelling time. Thus, later the combination of three polymers was taken in the further optimization process.

3.3. Optimization using Simplex Lattice Design

The Simplex Lattice Design provides a structured approach to this optimization by systematically varying factors, enabling exploration of complex interactions while minimizing experimental runs. The Quadratic Model, an extension of the Simplex Lattice Design, incorporates quadratic effects to detect non-linear relationships between factors and responses. This model enhances the ability to capture curvature in the response surface, offering a more nuanced understanding of the formulation space. The analysis was conducted to achieve the optimized batch of in situ gel formulation. Based on preliminary trials, the independent variables — amount of Poloxamer 407 (X1), amount of gellan gum (X2), and amount of HPMC K4M (X3) — were considered. The dependent variables — gelling time (Y1), viscosity (Y2), and % drug release at 6 h (Y3) — were taken. A total of 11 formulations were developed according to the design, and each batch was thoroughly evaluated as shown in the Table 4.

Table 4. Responses of design batches

Batch	Viscosity (cps)	Gelling Time (sec)	D6 (%)
F1	42.7 ± 1.0	23.1 ± 1.4	74.1 ± 1.5
F2	92.7 ± 1.3	26.4 ± 0.5	72.6 ± 1.0
F3	41.0 ± 1.0	24.1 ± 0.6	73.7 ± 2.6
F4	89.7 ± 1.1	22.8 ± 0.1	84.3 ± 2.5
F5	55.0 ± 0.9	25.4 ± 0.8	83.9 ± 2.7
F6	85.4 ± 1.0	28.1 ± 1.5	83.1 ± 2.1
F7	70.4 ± 1.8	24.1 ± 0.5	84.1 ± 1.9
F8	94.7 ± 1.9	27.3 ± 1.3	85.4 ± 2.7
F9	77.7 ± 0.8	26.9 ± 0.4	84.9 ± 1.6
F10	78.7 ± 1.8	26.1 ± 1.7	89.7 ± 2.6
F11	42.9 ± 0.9	23.1 ± 0.4	74.1 ± 1.4

3.4. Viscosity Measurement

Rheological studies of the design batches were carried out using Brookfield Viscometer (Table 4). The viscosity was found to be dependent on the concentration of the polymeric solution. The viscosity was found to be in range of 41 – 94.7 cps indicating viscosity increase with increase in concentration of bio adhesive polymer (Poloxamer 407, gellan gum and HPMC K4M) [41].

3.5. Gelling time

The gelling time is defined as the time required for the transformation of liquid phase to a gel phase. It was observed that as the concentration of polymer increases

gelling time decreases (Table 4) [42]. Nevertheless, to facilitate administration and handling, the formulation is maintained in liquid form; however, upon instillation, it undergoes gelling. The thermo-gelling process is attributed to hydrophobic interactions between the chains of Poloxamer 407 copolymer. When the temperature is increased, the Poloxamer 407 copolymer begins to aggregate into micellar structures. The formation of these micellar structures is a consequence of hydrophobic interactions facilitated by hydration of polyoxypropylene (poly (propylene oxide)) repeat units resulting in gelling [38]. Gellan gum is a linear anionic polysaccharide made up of repeating units of tetrasaccharide. It is an ion-induced gel based system which uses ions of lacrimal fluid to transform into gel phase [43]. Thus, in this formulation, both Poloxamer 407 and gellan gum facilitate in situ gelling mechanism by dual process involved, whereas HPMC K4M provides the viscosity for the formulation

3.6. In vitro diffusion study

In vitro diffusion studies for all formulations were conducted using the Franz diffusion cell with phosphate buffer solution at pH 7.4 as media. The diffusion profiles of the batch series are detailed in Figure 2A. Analysis of the diffusion data reveals that increasing polymer concentration correlates with decreased drug release. Additionally, polymer concentrations influence the drug release profile through the dialysis membrane. The release profile of Flucytosine from all formulations indicates that the drug release is influenced by the concentration of polymer, which in turn affects viscosity [27].

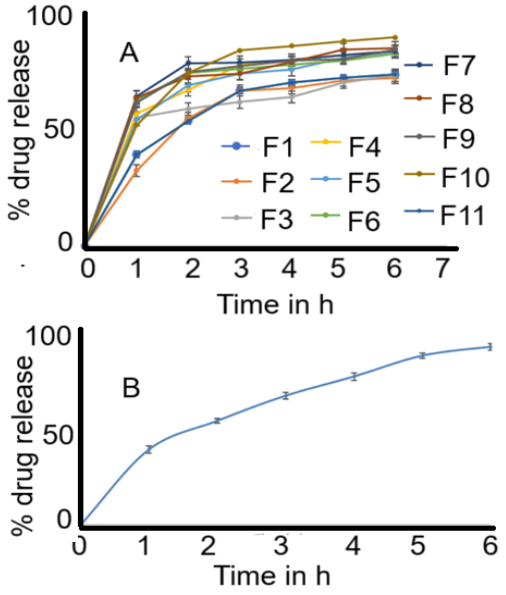

Fig. 2. Drug release of (A) design batches and (B) optimized batch

3.7. Statistical analysis of simplex lattice design

A simplex lattice design was used for the optimization process. All the responses observed for 11 batches developed were fit to a model utilizing Design Expert 13. The coefficients of the sums created using Design Expert software® for viscosity, gelling time and drug release at 6 h, of the ocular in-situ gel were reported. Since the values of R^2 are above 0.9 for all the responses, i.e. Y1 = 0.9817, Y2 = 0.9835 and Y3 = 0.9935, the polynomial equations seem excellent fits to the experimental data and are valid statistically. Table 5 displayes the Y1, Y2, and Y3 ANOVA results. Given that the response surface nonlinear quadratic model's P-value is less than 0.05 in the ANOVA result, it can be concluded that the chosen quadratic model is the best fit and significant.

Table 5. Results of analysis of variance (ANOVA)

Source	Sum of squares	DF	Mean Square	F value	P value
Y1 = Viscosity in cps					
Regression	4306.55	5	861.33	34.52	0.0007
Residual	124.75	5	24.95		
Total	4431.40	10			
Y2 = Gelling Time in sec					
Regression	33.72	5	6.74	26.34	0.0013
Residual	1.28	5	0.2560		
Total	35.00	10			
Y3 = drug release in 6 h					
Regression	367.59	5	71.31	59.75	0.0002
Residual	5.97	5	1.19		
Total	362.54	10			

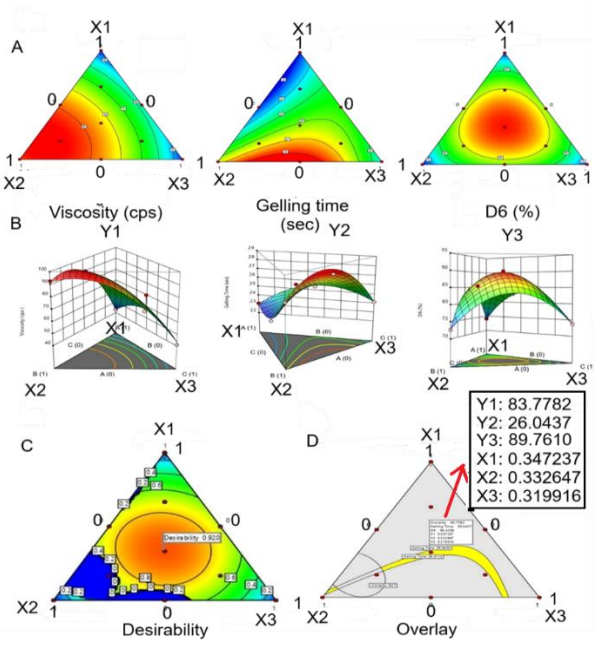

Fig. 3. Response surface plots: (A) Two-dimensional Counter plots showing of X1, X2 and X3 on Y1, Y2 and Y3, plots (B) Three-dimensional Counter plots showing of X1, X2 and X3 on Y1, Y2 and Y3, (C) Desirability of optimized formulation, (C) overlay plot of optimized formulation

The interaction effects of the factors on the responses could be studied with the help of 3-D response surface plots and two-dimensional contour plots. Plots of this kind were helpful in examining how two factors simultaneously affected a response while maintaining one factor constant (Figure 3A and Figure 3B). Results suggested the influence of the independent variables on the dependent variables which suggested increase in the viscosity and decrease in the gelling time with increase in the amount of polymers.

The optimized batch was generated employing Design Expert 13.1. The best batch of ocular formulation was made based on a predetermined selection process of in-situ gel based upon the criteria of amount of Poloxamer 407 = 0.34266 %w/v, amount of gellan gum = 0.332814 % w/v and amount of HPMC K4M = 0.324526 % w/v. The optimized batch was prepared as per desirability function with value of 0.920 (Figure 3C) and overlay plot (Figure 3D) and evaluated for various parameters. Table 6 shows the results of optimized batch with low relative error as compared to predicted values. Drug release profile as shown in Figure 2B demonstrated sustained release for upto 6 h.

Table 6. Results of optimized batch for response variables

Response variables	Optimized batch		
	Predicted value	Experimental value	% Relative error
Viscosity in CPS (Y1)	83.78	83.09 ± 1.31	0.8
Gelling time in sec (Y2)	26.1	25.66 ± 0.5	1.6
Drug release in 6 h (Y3)	89.761	91.2 ± 1.25	1.6
Gelling strength (sec)	-	38.1 ± 1.7	-
Drug content (%)	-	99.2 ± 2.2	-

3.8. Sterility test

Growth of microorganisms was not noticed during the 14 days of incubation period in sterilized optimized batch. Thus, sterilized optimized batch passed the test for sterility for anaerobic microorganism, and can be used for ophthalmic purpose (Table 7).

Table 7. Sterility test

Sample	Number of days						
	1	2	3	4	5	6	7
	Alternative thioglycolate medium						
Negative control	-	-	-	-	-	-	-
Positive control	+	+	+	+	+	+	+
Test sample	-	-	-	-	-	-	-
	Soyabean casein medium						
Negative control	-	-	-	-	-	-	-
Positive control	+	+	+	+	+	+	+
Test sample	-	-	-	-	-	-	-
	8	9	10	11	12	13	14
	Alternative thioglycolate medium						
Negative control	-	-	-	-	-	-	-
Positive control	+	+	+	+	+	+	+
Test sample	-	-	-	-	-	-	-
	Soyabean casein medium						
Negative control	-	-	-	-	-	-	-
Positive control	+	+	+	+	+	+	+
Test sample	-	-	-	-	-	-	-

(-) = No growth, (+) = bacterial growth, Test sample = optimized batch

3.9. Ocular irritation test

The hen's egg CAM test was used to assess the proposed formulation's ocular irritation. This test is quick, easy to use, and highly sensitive. The chorionallantoic membrane of the egg exhibits negative alterations upon exposure to test chemicals, indicating that the HET-CAM is a qualitative way of evaluating the possible irritancy of substances [44]. This score chart (Table 8) was utilised to test the developed formulation, and the results were compared with those obtained using normal saline (as a control; this solution is meant to be practically nonirritating). For normal saline, a mean score of 0 was attained. The in situ gel-based formulation showed zero irritation for the first six hours (mean score 0); nevertheless, after 24 h, the mean score increased to 0.33 (Table 8). According to the study, the formulation is well accepted and ranges from nonirritating to mildly irritating (score < 1).

Table 8. Ocular irritation studies

Effect	Scores	Inference			
No visible hemorrhage	0	Non-irritant			
Visible membrane color changes	1	Mild irritant			
Visible severe membrane disruption	2	Severe irritant			
Formulations	Scores (Time in h)				
Normal saline as control	1	4	6	12	24
Egg 1	0	0	0	0	0
Egg 2	0	0	0	0	0
Egg 3	0	0	0	0	0
Mean	0	0	0	0	0
Developed formulation					
Egg 1	0	0	0	0	0
Egg 2	0	0	0	0	0
Egg 3	0	0	0	0	1
Mean	0	0	0	0	0.33

3.10. Accelerated Stability Study

The stability study of the optimized batch showed non-remarkable changes in physical characteristics when stored under specified thermal condition and moisture conditions of 25°C ± 2°C/ 70% RH ± 5% RH for a duration of 6 month. Key parameters including viscosity, gelling temperature, gelling time, and drug release at 6 h remained consistent and did not exhibit significant deviations from the initial results. These findings indicate that the formulation maintained its stability under storage conditions, suggesting its suitability for further development and potential commercialization (Table 9).

Table 9. Accelerated stability study data of optimized batch of in-situ gel

Evaluations	Initial (0 day)	After 3 months	After 6 months
Clarity	Clear	Clear	Clear
pH	6.5 ± 0.05	6.7 ± 0.1	6.8 ± 0.1
Viscosity (cps)	83.09 ± 1.3	85.56 ± 1.0	87.54 ± 0.6
Gelling time (sec)	25.66 ± 0.5	27 ± 1.5	28 ±1.2
Gelling strength (sec)	38.1 ± 1.7	38.4 ± 1.8	38.5 ± 1.5
Gelling temperature (°C)	37 ± 1.0	38 ± 0.5	40 ± 2.0

4. Conclusions

Flucytosine, a fungal analog used for the treatment of ocular fungal infection, presents challenges in ophthalmic delivery due to factors like rapid tear overflow, short residence time, and poor ocular permeability. To overcome these obstacles, in-situ ocular gel was developed employing Poloxamer 407, gellan gum and HPMC K4M that undergo a sol-gel transition upon application, providing prolonged drug release and enhancing therapeutic efficacy. Simplex Lattice Design was applied for the optimization process. The optimized batch showed promising results for various parameters. Ocular irritancy studies demonstrated negligible irritation potential of the preparation. Results of sterility testing and stability studies demonstrated sterile and stable formulation suggesting a promising drug delivery approach for flucytosine.

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