BMC Pharmacology and Toxicology

RESEARCH Open Access

In-situ gel bases ocular delivery system of Ganciclovir, in-vivo and in-vitro investigation

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Abstract

Ocular drug delivery is challenging due to the eye's unique anatomy and physiology, which limit drug absorption and distribution. Traditional methods like eye drops have poor bioavailability and often require frequent dosing. In-situ gel systems, liquid formulations that transform into a gel upon contact with physiological conditions (pH, temperature, or ions in tear fluid), offer several advantages for ocular drug delivery. Ganciclovir was incorporated into a thermoresponsive in situ gel base. Nine formulations were prepared using the cold technique and a 3^2 full factorial design. The physical properties of ganciclovir gel, including clarity, pH, viscosity, gelation temperature, and gelation time, were scrutinized. In vitro drug release was assessed using a dialysis membrane method, and ocular toxicity was evaluated in a rabbit model. Poloxamer 407 and HPMC E-50 LV were used to prepare the gel, with the 3^2 factorial design analyzing the effects of varying concentrations on the gel's viscosity, gelation temperature, and gelation time. The optimized in-situ gel formulation (Batch B5) contained 15% w/v poloxamer 407 and 1% w/v HPMC E-50 LV, achieving a viscosity of 64.81 cPs, with a gelation temperature of 39.0 °C and a gelation time of 183 s. This formulation demonstrated better permeability and sustained ganciclovir release than a commercial formulation. Ocular toxicity studies confirmed that the formulation was non-irritating and well-tolerated. Overall, the gel showed suitable physical properties, sustained drug release over 12 h, and significant potential for enhancing drug bioavailability and treatment efficacy for ocular infections. These findings confirm that the optimized ganciclovir in situ gel preparation improves eye permeation and prolongs ocular retention time, thus enhancing its therapeutic efficacy. Its sustained release and prolonged retention time make it a promising alternative to conventional eye drops, offering better patient compliance and efficacy.

Keywords In situ gel, Poloxamer 407, Hydroxypropyl methylcellulose, Factorial design, Ganciclovir, Ocular drug delivery

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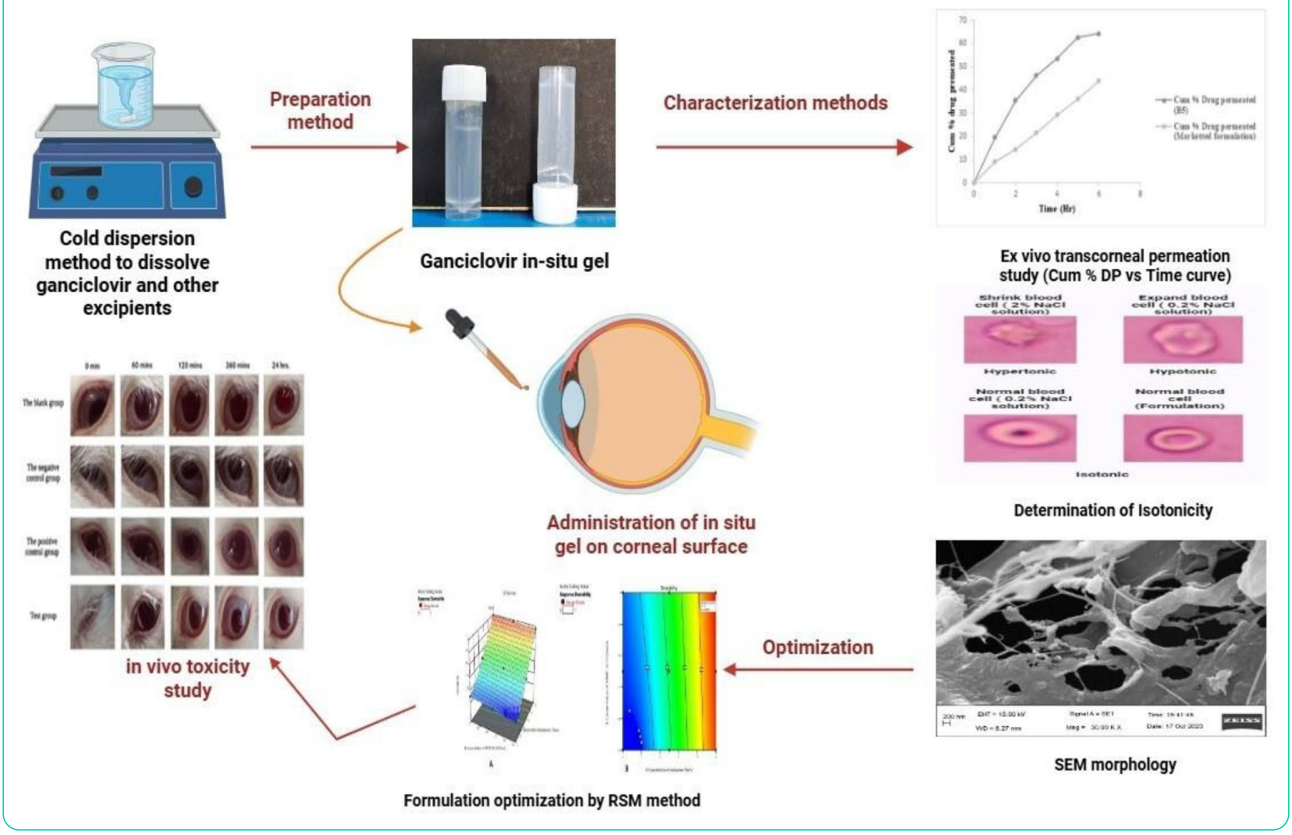
Graphical Abstract



Introduction

Ocular drug delivery systems represent a compelling yet highly challenging area of pharmaceutical research [1]. The eye's complex anatomical and physiological design poses significant barriers to the entry of exogenous substances, necessitating innovative strategies to overcome these defensive mechanisms. Achieving optimal bioavailability at targeted ocular sites remains a substantial challenge due to extensive drug loss before corneal absorption, which limits therapeutic efficacy. Thus, the researcher faces the complex task of overcoming the various protective barriers of the eye, such as the corneal epithelium, tear film, blood-ocular barriers, and high enzymatic activity, without inducing lasting tissue damage [2]. Eye drops are the most used drug delivery approach due to their low price and modest usage. The drawbacks of these conventional formulations are shorter residential periods, limited bioavailability, and quick precorneal drainage [3]. The primary cause of the poor bioavailability of eye drops is precorneal drug loss caused by nasolacrimal drainage. Frequent dosing is necessary for the drug administered topically and often has a poor therapeutic effect due to its quick absorption into the eye [4]. Ophthalmic semisolid formulations such as ointments and gels can improve the bioavailability of active therapeutics by extending the contact time, reducing tear dilution, and limiting nasolacrimal drainage. However, they can cause blurred vision, restricting use to night-time or periocular application. Liquid formulations such as suspensions and drops are also common, as they allow particles to linger in the conjunctival sac, minimizing precorneal drug loss [5]. Additionally, diffusion-controlled, non-erodible polymeric inserts can further reduce drug loss [6, 7]. Ganciclovir (GCV) is a synthetic guanosine analog and a potent antiviral agent commonly used for the treatment of cytomegalovirus (CMV) infections, particularly CMV retinitis, a sight-threatening condition often seen in immunocompromised patients, such as those with AIDS or undergoing organ transplants. Its selective antiviral activity arises from phosphorylation by viral thymidine kinase (UL97 in CMV), converting it into the active ganciclovir triphosphate form, which inhibits viral DNA polymerase and disrupts viral replication by causing premature termination of the DNA chain. Despite its efficacy, ganciclovir exhibits poor ocular bioavailability due to its low aqueous solubility, rapid precorneal clearance, and limited corneal permeability, necessitating frequent dosing when administered as conventional eye drops [8]. These limitations make it an ideal candidate for in situ gel formulations designed to enhance

ocular retention and improve therapeutic outcomes. In situ gels, which undergo sol-to-gel transformation upon contact with physiological conditions like temperature or pH, can prolong drug residence time on the ocular surface, facilitating sustained drug release and reducing the need for frequent administration. Ganciclovir's primary sites of action include the cornea and conjunctiva, particularly for herpetic keratitis, and the posterior segment of the eye, such as the vitreous and retina, in CMV retinitis cases [9]. The development of ganciclovir-loaded ocular in situ gels offers advantages like prolonged drug retention, controlled release, reduced systemic exposure, and improved patient compliance, making it a promising approach for effective ocular antiviral therapy. Poloxamer 407, a thermosensitive triblock copolymer of poly (ethylene oxide)-poly (propylene oxide)-poly (ethylene oxide) (PEO-PPO-PEO), has gained considerable attention for its application in ocular in-situ gel formulations. It exhibits temperature-sensitive gelation, transitioning from a low-viscosity solution at room temperature to a gel at physiological ocular temperature (35–37 °C), thereby ensuring enhanced drug retention and controlled release. This unique sol-to-gel transformation enhances ocular residence time, minimizes drug loss, and improves therapeutic efficacy. Additionally, Poloxamer 407 offers excellent biocompatibility, mucoadhesive properties, and ease of administration, making it a promising vehicle for ophthalmic drug delivery. The goal of this work was to prepare and optimize a GCV in situ gel to determine the optimal composition of poloxamer 407 and HPMC E-50 LV. In this study, a three-level factorial experimental plan was used to optimize the composition of poloxamer 407 and HPMC E-50 LV. Physical characteristics (viscosity, gelation temperature, and gelation time) were used to evaluate the optimized formulas [10]. All factors were assessed for the optimization of the formula, however, only the optimized formulation batch was characterized for further studies, such as in-vitro drug release investigations, ex-vivo transcorneal investigations, and ocular toxicity studies.

Experimental

Materials

Ganciclovir (Test Assay - ≥ 99.58%) was bought from Yarrow Chem. (Mumbai, India). Poloxamer 407 (M.W – 12600, Oxyethylene – 74.9 ± 1.7%) and hydroxypropyl methylcellulose (HPMC E-50 LV, Methoxy content: 28.0–30.0%, Hydroxypropoxy content: 7.0 – 12.0%, Viscosity – 50.3 mPa.s in 2% aqueous solution at 20 °C) were purchased from Lepid Life Sciences Pvt. Ltd. (Rajasthan, India). The analytical grade of the additional reagents and chemicals was procured via SD Fine Chem Ltd. (Mumbai, India). The purity of the ganciclovir and other excipients was determined according to the standards outlined in the United States Pharmacopeia (USP).

Methods

Spectrophotometric estimation of ganciclovir

A standard stock solution of ganciclovir was prepared by accurately weighing 10 mg of pure ganciclovir and dissolving it in 100 mL of STF (simulated tear fluid, pH 7.4) to obtain a concentration of 100 µg/mL. The solution was sonicated for 10 min to ensure complete dissolution. 10 µg/mL solution was scanned at 200–400 nm using a UV-Vis spectrophotometer (Shimadzu UV-1800, Shimadzu, Japan) to determine the maximum absorbance (λ_{max}). The wavelength corresponding to maximum absorbance (λ_{max}) was determined and recorded [11]. Serial dilutions of the stock solution were prepared to obtain concentrations of 5, 10, 15, 20, 25, and 30 µg/mL. The absorbance of each dilution was measured at the determined λ_{max} using a quartz cuvette with a path length of 1 cm. A calibration curve was made by plotting the absorbance against concentration.

Preformulation study

Preformulation investigations were performed to determine the compatibility of ganciclovir, poloxamer 407, and HPMC E-50 LV. The functional groups present in ganciclovir, poloxamer 407, HPMC E-50 LV, and their physical mixture were qualitatively estimated and identified using an FTIR spectrophotometer (Spectral Range – 350 to 8,000 cm^{-1}, Alpha-II, Bruker, Germany) [11].

The KBr pellet approach for FTIR analysis involves accurately weighing 1–2 mg of ganciclovir, poloxamer 407, HPMC E-50 LV, and the physical admixture of ganciclovir and poloxamer 407, mixed with approximately 100 mg of dry potassium bromide (KBr). The mixture is finely ground using a mortar and pestle to ensure uniform distribution of the sample in the KBr matrix. The ground mixture is then compressed using a hydraulic press at a pressure of 5–10 tons to form a transparent pellet appropriate for infrared analysis. The pellet is placed in the FTIR spectrophotometer, and the spectra are recorded in the range of 4000 cm^{-1} to 400 cm^{-1} with a resolution of 4 cm^{-1}. A background scan using a blank KBr pellet is performed before the sample scan for baseline correction. The obtained spectrum is analyzed for the presence of characteristic functional groups and compatibility studies.

Optimization of the ophthalmic ganciclovir in situ gel

A 3^2 factorial design optimized the formulation process involving poloxamer 407 and HPMC E-50 LV. In this experimental design, different concentrations of poloxamer 407 (X_1) and HPMC E-50 LV (X_2) were designated as independent factors, while viscosity (Y_1), gelation

temperature (Y_2), and gelation time (Y_3) served as dependent variables. For poloxamer 407 (X_1), concentrations of 5%, 10%, and 15% were assigned to the lower, middle, and higher levels, respectively. Similarly, for HPMC E-50 LV (X_2), concentrations of 0.5%, 0.75%, and 1.0% were selected for the lower, middle, and higher levels, respectively. This factorial conception was acceptable for a comprehensive exploration of the effects of individual factors and their connections on the response values, thereby facilitating a more detailed analysis compared to simpler experimental models [9, 10]. Consequently, nine distinct formulation variants were generated through optimization using a three-level factorial design, as tabulated in Table 1.

Preparation of ganciclovir in situ gel

The cold dispersion process was employed to formulate the in situ gel. In a beaker, 50 mL of distilled water (4 °C) was added, and accurately measured amounts of poloxamer 407 (5, 10, and 15% w/v) were blended slowly into the distilled water by using a magnetic stirrer at 80 rpm. Throughout the preparation, the temperature of the distilled water was held constant. The solution was then refrigerated overnight at 4 °C. HPMC E-50 LV (0.5, 0.75, and 1.0% w/v) was then added to the poloxamer 407 solution. A preservation solution containing 0.1% w/v methylparaben was prepared by mixing methylparaben in hot water. After cooling, the preservative solution was included in the poloxamer solution. Subsequently, ganciclovir (0.15% w/v) was included in the prepared poloxamer 407 solutions. 0.1% v/v of triethanolamine was mixed to tailor the pH of the final solution. Table 1 lists the components of the in-situ gel [11–16].

Physical evaluation of ganciclovir in situ gel

Assessment of appearance

The appearance of the in-situ gel involved examining it optically using a fluorescent light source projected onto a black-and-white screen. This method allowed visual inspection of the appearance of the formulated in situ gel. Additionally, a sensory assessment was conducted on the day of preparation, focusing on the gel's colour, odour, and clarity to evaluate its sensory attributes [16–18].

Determination of viscosity

The viscosity of the developed preparations was measured using a temperature-controlled viscometer (Brookfield TC150MX, Ametek, USA). For the evaluation, a spindle number of 62 was utilized because the geometry of the spindle 62 also fits well within standard sample containers used in viscometers, ensuring uniform and reproducible measurements [19–21]. The viscosity of the preparations was also determined at 37 °C ± 0.5 °C. During the study, the small sample holder containing the gel under investigation was positioned within the viscometer, and the spindle was dropped perpendicularly into the gel. The spindle was then rotated at 60 rpm to assess viscosity [22–26]. The results of the rheological examinations of the formulations are tabulated in Table 2.

Determination of gelation temperature

The term "gelation temperature" refers to the temperature at which the preparation's meniscus ceases to flow. The gelation studies were conducted using Miller and Donovan's method [27]. A test tube containing a sufficient quantity of the formulation was put in a water bath to determine the gelation temperatures. The water bath temperature was then incrementally elevated by 5ºC every two minutes.

Determination of gelation time

The gelling time for the preparations was determined using the methods developed by Miller and Donovan [13]. Before administration, the delivery systems were in a sol form, but upon administration, they underwent gelation to form a gel. The point at which gelation was first detected was recorded as the gelling time.

To determine the sol-gel alteration temperature ($T_{sol-gel}$) of the in-situ gel preparations, a test tube (10 ml) with a diameter of 1.0 cm was loaded with 2 ml of the

Table 1 3 [3] factorial design optimization outcomes for ganciclovir ophthalmic in situ gel optimization

Formulation code	Concentration of Ganciclovir (%w/v)	Concentration of poloxamer (%w/v)	Concentration of HPMC E-50 LV (%w/v)	Concentration of Methyl paraben (%w/v)	Concentration of Triethanolamine (%v/v)
B_1	0.15	05	1.00	0.1	0.1
B_2	0.15	10	0.75	0.1	0.1
B_3	0.15	05	0.75	0.1	0.1
B_4	0.15	15	0.50	0.1	0.1
B_5	0.15	15	1.00	0.1	0.1
B_6	0.15	15	0.75	0.1	0.1
B_7	0.15	10	1.00	0.1	0.1
B_8	0.15	10	0.50	0.1	0.1
B_9	0.15	05	0.50	0.1	0.1

Table 2 The results of experiments conducted with different formulations of a gel containing poloxamer 407 and HPMC E-50 LV at variations in concentrations. The parameters measured included viscosity, gelation temperature, and gelation time

Formulation code	Concentration of poloxamer 407 (%w/v)	Concentration of HPMC E-50 LV (%w/v)	Viscosity (cPs)	Gelation temperature (˚C)	Gelation time (Seconds)
B_1	5	1	6.78	45.29	598
B_2	10	0.75	16.93	41.02	422
B_3	5	0.75	4.45	45.34	618
B_4	15	0.5	60.98	39.15	191
B_5	15	1	64.81	39.03	183
B_6	15	0.75	61.7	39.46	189
B_7	10	1	22.34	40.76	428
B_8	10	0.5	10.87	41.45	432
B_9	5	0.5	2.33	46.23	630

prepared formulation. The tube was then wrapped with parafilm and placed in a water bath with circulation set at 37 °C. A ten-minute equilibration period was allowed after each temperature setting. Finally, the test tube was positioned horizontally to assess gelation and evaluate the condition of the sample [27–29].

Determination of pH

The pH of the prepared ocular in situ gel can be determined using a calibrated digital pH meter to ensure ocular compatibility and minimize irritation. The pH meter should be calibrated using standard buffer solutions of pH 4.0, 7.0, and 9.2 for accuracy. The electrode should be rinsed thoroughly with distilled water and blotted dry between each calibration step. 5 mL of the prepared ganciclovir in situ gel formulation was transferred to a beaker. The pH meter electrode was then immersed directly into the sample, stirring gently to ensure uniform contact, and the reading was recorded [30].

Data analysis

Design Expert® 13 executed the data analysis. Based on the optimization results, the response surface method's three-level factorial design showed data analysis. The viscosity, gelation temperature, and gelation time were estimated for formula optimization. These constituents were chosen for data analysis because they have interval scales for measurement, and their organoleptic qualities were descriptively optimized.

In vitro release studies

The in vitro release study of ganciclovir from both the formulated in situ gel and a commercial ophthalmic gel (Gancigel, Entold Pharmaceuticals Ltd., Mumbai, India) was evaluated utilizing a dialysis bag method [31, 32]. Simulated Tear Fluid (STF) was prepared by dissolving 7.0 gm of sodium chloride (NaCl), 0.2 gm of potassium chloride (KCl), 0.08 gm of calcium chloride dihydrate ($CaCl_2{\cdot}2\ H_2O$), 0.02 gm of magnesium chloride hexahydrate ($MgCl_2{\cdot}6\ H_2O$), 1.0 gm of sodium bicarbonate ($NaHCO_3$), and 0.2 gm of dipotassium hydrogen phosphate (K_2HPO_4) in 1000 mL of distilled water. The pH of the solution was adjusted to 7.4 using 0.1 N hydrochloric acid (HCl) or 0.1 N sodium hydroxide (NaOH), and the prepared STF solution was then filtered through a 0.45 µm filter. The prepared STF was stored in a sealed container at room temperature [33]. First, the optimized formulation was positioned in pre-treated dialysis bags, which were then immersed in 100 ml of simulated tear fluid (STF) adjusted to pH 7.4 and maintained at 37 °C with constant stirring at 50 rpm. In vitro drug release studies were conducted over 6 h. At fixed-time intervals (0, 1, 2, 3,..., 6 h), 2 mL of aliquots were withdrawn and immediately replaced with an equivalent volume of fresh Simulated Tear Fluid (STF) to maintain sink conditions. The samples were scanned using a UV spectrophotometer (Shimadzu UV-1800, Japan) at a wavelength of 256 nm. The same procedure was followed for the marketed formulation.

Release kinetics

The data obtained from the in vitro drug release investigations were analyzed by fitting them to various release kinetic models, including zero-order, first-order, Higuchi, and Korsmeyer–Peppas models. This analysis aimed to elucidate the drug release mechanism from the in situ gel [33].

Zero-order kinetics describes a release mechanism in which the drug is released at a constant rate, independent of its concentration.

$$Q_t = Q_0 + k_0 t \quad (1)$$

Where Q_t is the cumulative amount of drug released at time t, Q_0 is the initial drug concentration, and k_0 is the zero-order release rate constant.

The first-order model assumes that the release rate is dependent on the drug concentration remaining in the dosage form.

$$log\, C \;=\; log\, C_0 - Kt/2.303 \tag{2}$$

Where C_0 is the initial concentration of drug, k is the first-order rate constant, and t is the time.

The Higuchi model is based on Fickian diffusion and applies to systems where drug release is controlled by diffusion through a polymeric matrix.

$$Q_t = k_H \sqrt{t} \tag{3}$$

Where Q_t is the cumulative percentage of drug released at time t, and k_H is the Higuchi release rate constant.

To determine the mechanism of drug release, the Korsmeyer-Peppas model was applied, expressed as:

$$Q_t/Q_\infty = kt^n \tag{4}$$

where Q_t/Q_∞ is the fraction of drug released at time t, k is the release rate constant, and n is the diffusional exponent, which indicates the release mechanism.

Ex vivo ocular permeation studies

An ex vivo corneal permeation study was conducted using freshly excised goat corneas. Whole eyeballs were procured from the slaughterhouse and kept at 4 °C in a normal saline solution (0.9% NaCl solution). The corneas were sensibly extracted, along with the surrounding 5–6 mm of scleral tissue, using forceps and scissors [34]. After extraction, they were wetted with saline solution. The cornea was then mounted onto a Franz diffusion cell (Orchid Scientific Pvt. Ltd, Mumbai, India), with the surrounding scleral tissue clamped between the upper and lower chambers. The lower chamber, serving as the receiver compartment, was continuously infused with simulated tear fluid (STF) adjusted to pH 7.4, at a rate of 20 ml/min and maintained at 37 ± 0.5 °C. Perfusate samples were collected at regular intervals over 6 h in Eppendorf tubes. These samples were subsequently analyzed for ganciclovir quantification using a UV- spectrophotometer (Shimadzu UV-1800, Shimadzu, Japan) at a wavelength of 256 nm [35].

Table 3 Modified Draize irritation scores of different formulations

Formulation	Different observing time (hrs.)				
	0 h.	1 h.	2 h.	6 h.	24 h.
Blank group (Blank in situ gel)	2	0	0	0	0
Negative group (0.9% w/v NaCl solution)	0	0	0	0	0
Positive group (1% w/v SDS solution)	3	2	1	0	0
Test group (Ganciclovir in situ gel)	1	0	0	0	0

Ocular irritation studies

The modified Draize irritancy test was utilized to assess ocular tolerance to the prepared in-situ gel formulation. The study involved two healthy New Zealand white rabbits weighing between 1.5 and 2 kg in each group. The New Zealand white rabbits used in this experiment were procured from a CCSEA-registered breeder (Saha Enterprise, Kolkata, India, Registration No.– 1828/PO/Bt/S/15/CPCSEA). The rabbits were housed in cages at a constant temperature of 22–25 °C and were fed at regular intervals before the experiment. Approval for the experiment was obtained from the Institutional Animal Ethical Committee of the NSHM Knowledge Campus, Kolkata Group of Institutions (approval number NCPT/IAEC-011/2023). The modified Draize irritancy test was used for the following groups (Table 3). Each rabbit received an instillation of the sterile optimized formulation, 0.9% w/v NaCl solution, and or 1% w/v sodium dodecyl sulphate (SDS) solution into the conjunctival sac of their respective eyes. Symptoms of redness, inflammation, or ocular edema were monitored regularly [36]. A volume of 100 µL of the optimized ganciclovir in-situ gel, 0.9% w/v NaCl solution, and 1% w/v SDS solution were administered to the eyes of the rabbit as part of the test. The animals were examined by observing the cornea and conjunctiva tissue after 1, 2, 6, and 24 h of sample instillation [17, 18, 37]. Following the completion of the investigations, by the established protocols, the rabbits were observed throughout the rehabilitation period, and any observations were meticulously noted. Post-experimentation care was provided to ensure the well-being of the animals, and all necessary steps were taken to ensure a controlled environment. As per the protocol of the study, there was no requirement for anesthetizing and/or euthanizing the experimental animals. The objective of the in-vivo study was to assess the ocular tolerance to the prepared in situ gel formulation, which does not necessitate anaesthesia and/or euthanasia of the experimental animals. The Rabbits were rehabilitated in the Animal House facility of the institute and were utilized to carry out other experimental protocols after the required washout period.

Data collected from the examinations was thoroughly analyzed to ensure accuracy and reliability. The integrity of the examination process was always upheld to ensure the validity of the results.

Determination of isotonicity

The ophthalmic products must be isotonic to avoid any potential harm or irritation to the eye. To ensure this, the optimized formulation was mixed with a small amount of blood, and the shape of the blood cells was observed under a microscope at 45x magnification. The results were then compared with a control solution. Isotonic

solutions help maintain the integrity of blood cells, while hypertonic solutions cause cells to shrink, and hypotonic solutions cause them to swell [38].

Surface morphology

The prepared in situ gel was dried using a freeze dryer (Triad Labconco, MI, USA) and subsequently stored in a desiccator until analysis. The surface topography of the dried samples was assessed using a scanning electron microscope (SEM) (FEI Quanta-200 MK2, **Netherlands**) operating at an accelerating voltage of 15 kV [39].

Results and discussion

Spectrophotometric estimation of ganciclovir

The UV spectrophotometric method developed for the estimation of ganciclovir was found to be precise and accurate. The λ_{max} of ganciclovir was observed at 256 nm, which is consistent with reported values. The calibration curve demonstrated excellent linearity with an R^2 value of 0.9801 (Fig. 1).

Preformulation study

Figure 2(A) exhibits the FTIR spectra of pure ganciclovir, showing characteristic peaks at 3320 cm^{-1} and 3420 cm^{-1}, corresponding to the stretching vibrations of the –OH group and the N-H group, respectively. Moreover, the aromatic C-H stretching is observed at 3147 cm^{-1}, while the aliphatic C-H stretching is observed at 2942 cm^{-1} and 2893 cm^{-1}. These peaks are consistent with the molecular structure of ganciclovir, confirming its chemical identity. Figure 2(B) shows the FTIR spectrum of poloxamer 407, which exhibits major absorption peaks at 2893.02 cm^{-1} (aliphatic C-H stretch), 1355.86 cm^{-1} (in-plane O-H bend), and 1124.42 cm^{-1} (C-O stretch). In Fig. 2(C), the FTIR spectrum of pure HPMC E-50 LV reveals peaks at 3427.61 cm^{-1} and 2978.55 cm^{-1}, which are attributed to the stretching of the –OH group and $–CH_3$ groups, respectively. Figure 2(D) shows the FTIR spectrum of the physical admixture of ganciclovir, HPMC E-50 LV, and poloxamer 407. The spectra of the physical admixture display absorption bands at 3436.79 cm^{-1}, 2973.22 cm^{-1}, and 1283.01 cm^{-1}, which correspond to the OH, CH_2, and C-N stretching vibrations, respectively. Notably, the absorption bands of the physical admixture are in close agreement with those of the individual components, suggesting no significant chemical relations between ganciclovir and the excipients. These results imply that the functional groups in ganciclovir stayed unaffected in the physical admixture, indicating that no chemical or physical changes developed during the preparation of the formulation. This observation is critical as it ensures that the active drug retains its original structure and activity, which is necessary for its therapeutic efficacy.

Physical evaluation of ganciclovir in situ gel

Clarity

The organoleptic properties of the prepared ganciclovir ophthalmic in situ gel formulations are presented in Table 4. The observations revealed no detectable changes in colour, clarity, or odour across all prepared formulations throughout the study period. The formulations asserted a transparent appearance with no signs of turbidity or precipitation, demonstrating the absence of physical instability or incompatibility between the excipients and the active pharmaceutical ingredient.

The consistent clarity observed is specifically significant for ocular preparations, as visual transparency is a critical requirement to ensure patient comfort and prevent visual disturbances upon administration. Similarly, the absence of any evident odour indicates the stability of the excipients and the lack of chemical degradation or volatilization of constituents, which is necessary for patient compliance and safety in ophthalmic drug delivery systems.

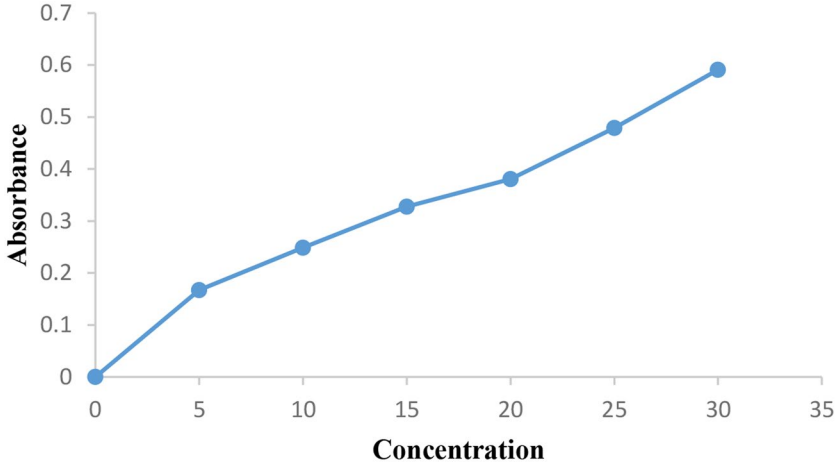


Fig. 1 Calibration curve of pure ganciclovir

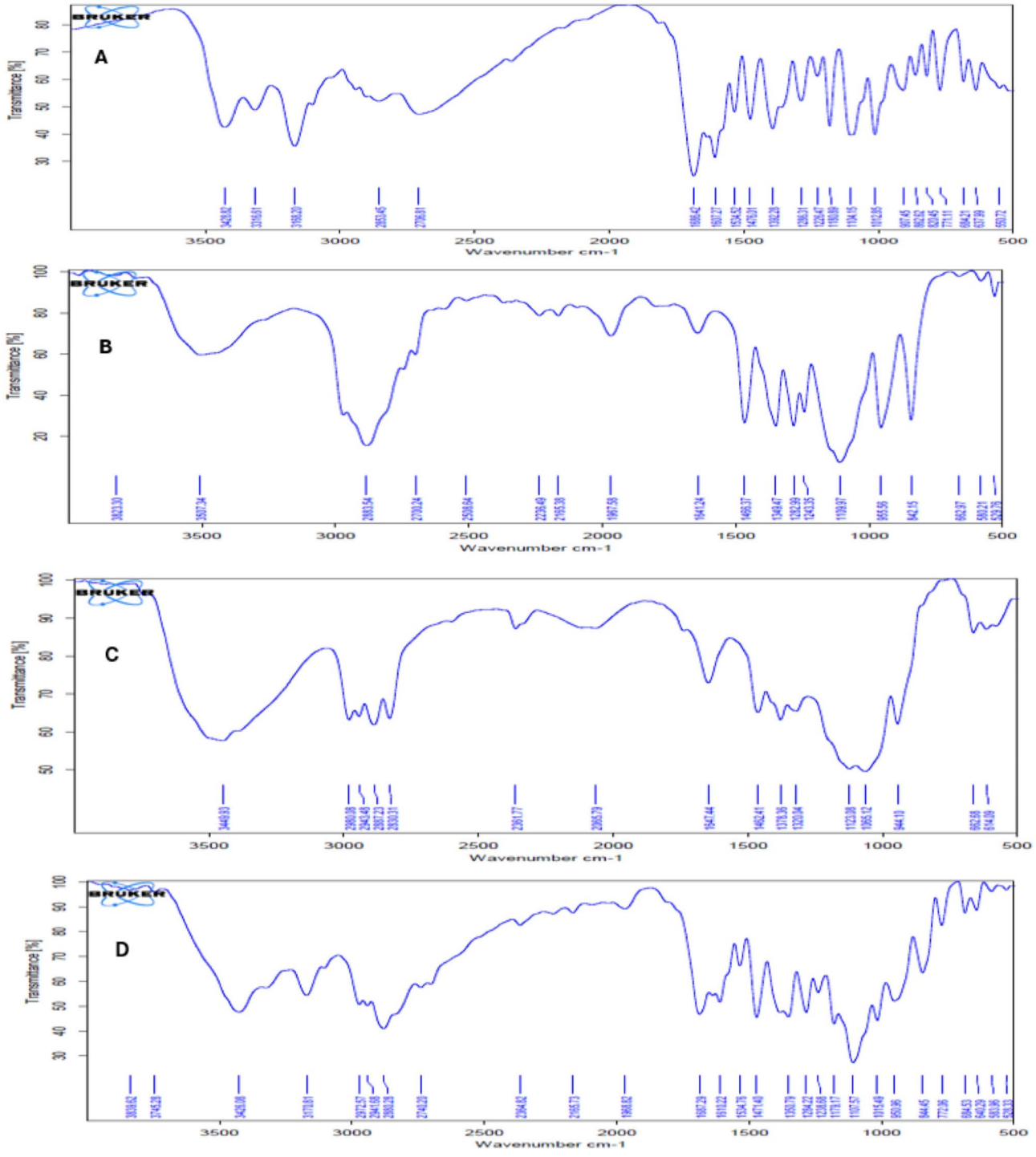


Fig. 2 (**A**) FTIR spectrum of pure ganciclovir, (**B**) FTIR spectrum of poloxamer 407, (**C**) FTIR spectrum of HPMC E-50 LV, (**D**) FTIR spectrum of the physical admixture of ganciclovir, poloxamer 407, and HPMC E-50 LV

These results indicate that the formulated ganciclovir ophthalmic in situ gel formulations meet the fundamental organoleptic criteria for ocular preparations, emphasizing their suitability for ocular administration. Furthermore, the stability in physical appearance aligns with regulatory standards, reinforcing the quality and reliability of the developed preparation for sustained ocular drug delivery [40, 41].

Determination of pH

The pH of the tested ocular in situ gels ranged from 6.50 to 6.87, with an average value of 6.60. This pH range is

Table 4 Organoleptic properties of different batches of ganciclovir in situ gel

Formulation code	Organoleptic properties			pH
	Clarity	Colour	Odour	
B_1	Clear	Colourless	Odor less	6.68
B_2	Clear	Colourless	Odor less	6.5
B_3	Clear	Colourless	Odor less	6.66
B_4	Clear	Colourless	Odor less	6.87
B_5	Clear	Colourless	Odor less	6.72
B_6	Clear	Colourless	Odor less	6.84
B_7	Clear	Colourless	Odor less	6.61
B_8	Clear	Colourless	Odor less	6.74
B_9	Clear	Colourless	Odor less	6.80

appropriate for ocular administration, ensuring comfort and compatibility with the physiological conditions of the eye, thereby making these formulations suitable for therapeutic use. The results have been tabulated in Table 4.

Viscosity

The viscosity, gelation temperature, and gelation time of the ganciclovir ophthalmic in situ gel are presented in Table 2. The viscosity of the ganciclovir ophthalmic in situ gel was evaluated at 37 °C. According to the findings presented in Table 2, increasing the concentration of poloxamer 407 leads to increased viscosity and gelation temperature. For instance, comparing batches B_1, B_3, and B_5, where the concentration of poloxamer 407 was enhanced from 5 to 15% w/v, there was a noticeable increase in both viscosities. Like poloxamer 407, increasing the concentration of HPMC E-50 LV generally results in a higher viscosity. This can be observed by comparing B_2, B_7, and B_9, where the concentration of HPMC E-50 LV increased from 0.5 to 1% w/v. The viscosity results indicated that all the prepared formulations, excluding B_3 and B_9, fulfilled the viscosity criteria for ophthalmic in situ gels, which should be within the range of 5–100 cPs [42, 43].

Gelation temperature

A perfect in situ gel should have the ability to rapidly convert into a gel at normal body temperature. The gelation temperature was tested to ascertain the temperature required for the transition from the sol to gel phase [42]. The gelation temperature and gelation time did not show a consistent trend with variations in the HPMC E-50 LV concentration. However, there might be a slight reduction in gelation temperature as the concentration of HPMC E-50 LV rises, as observed between batches B_2 and B_7. Formulation batches B_4, B_5, and B_6 transform into gels at 39.15, 39.03, and 39.46, respectively, which is near to the normal human body temperature. The highest gelation temperature was 46.23 °C for batch number B_9.

Gelation time

The primary objective of evaluating gelation time was to determine the duration necessary for the transformation from the sol phase to the gel phase, a critical parameter for ocular in situ gels aimed for immediate ocular administration [38–40, 43]. The results signify relatively consistent gelation times across varying concentrations of poloxamer 407, with slight variations observed among different batches.

Batch B_3, containing 5% w/v poloxamer 407 and 0.75%w/v HPMC E-50 LV, exhibited a marginally longer gelation time compared to batch B_1, while batch B_5 (15% w/v poloxamer 407) demonstrated a shorter gelation time. The gelation times were recorded for formulations B_5 and B_6, with gelation occurring at 191 and 183 s, respectively. Conversely, batches B_3 and B_9 needed the longest duration to transition from sol to gel.

For an ideal ophthalmic in situ gel, rapid gelation upon contact with ocular tissues is essential to ensure prolonged retention, minimize pre-corneal drainage, and enhance therapeutic efficacy. Immediate gelation also contributes to patient comfort by reducing the risk of premature elimination due to tear turnover and blinking. Therefore, the slight delay observed in some batches underscores the importance of optimizing the poloxamer concentration to balance gelation time with sustained retention.

Data analysis

Using Design Expert® 13 (StatEase®, USA), a three-level factorial design was employed to analyze data and identify the optimal formulation. The software evaluated viscosity, gelation temperature, and gelation time as response variables, determining the factors with the greatest impact and their interrelationships [44].

The first investigation examined the relationships between viscosity, gelation temperature, and gelation time of poloxamer 407 and HPMC E-50 LV. Figure 3 presents the correlation between the concentrations of poloxamer 407 and HPMC E-50 LV and viscosity, Fig. 4 shows the correlation with gelation temperature, and Fig. 5 illustrates the correlation with gelation time.

Additionally, the viscosity, gelation temperature, and gelation time were determined for all the formulas (B_1–B_9) via 3-dimensional response surface plots, contour plots, and regression equations. Figure 6 displays the contour plot and the three-dimensional response surface plot displaying the relationships among viscosity, poloxamer 407 concentration, and HPMC E-50 LV concentration.

The viscosity-concentration relationship of poloxamer 407 and HPMC E-50 LV follows a quadratic model, with the highest viscosity point, as shown in Fig. 7(a). Figure 7(b) presents a contour map with poloxamer 407 on the x-axis, HPMC E-50 LV on the y-axis, and viscosity

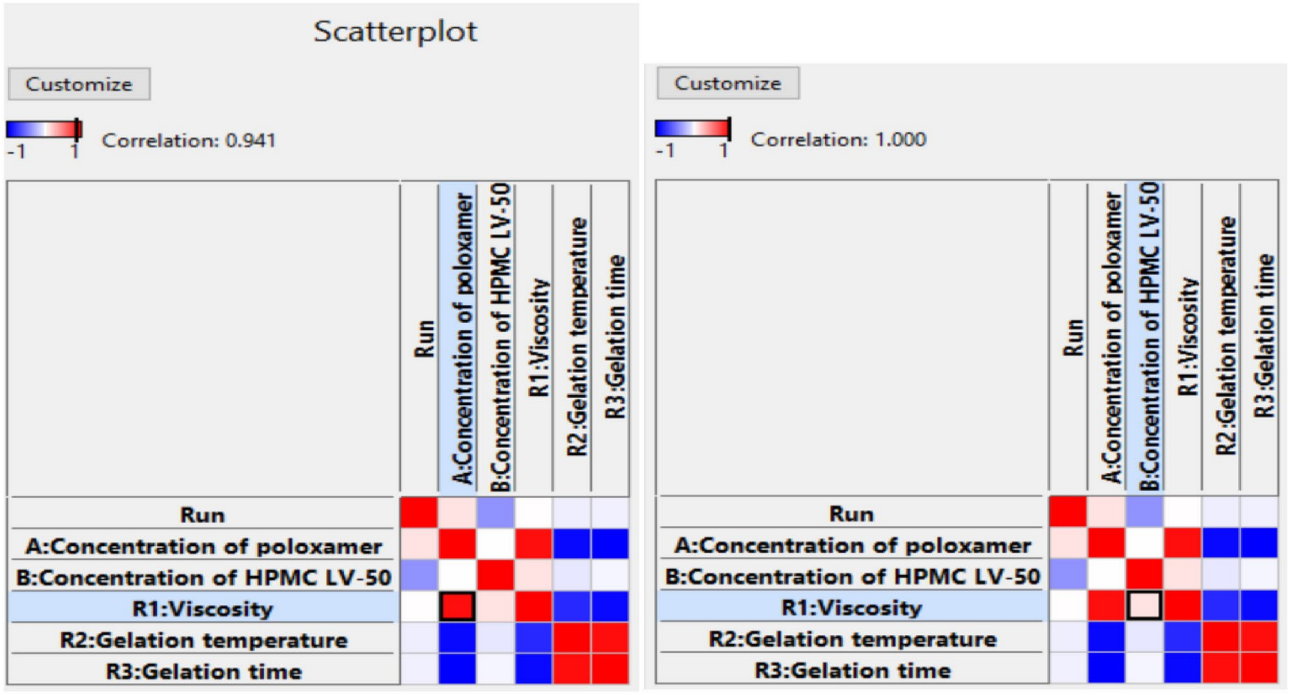


Fig. 3 Correlation of the viscosity of poloxamer 407 and HPMC E-50 LV

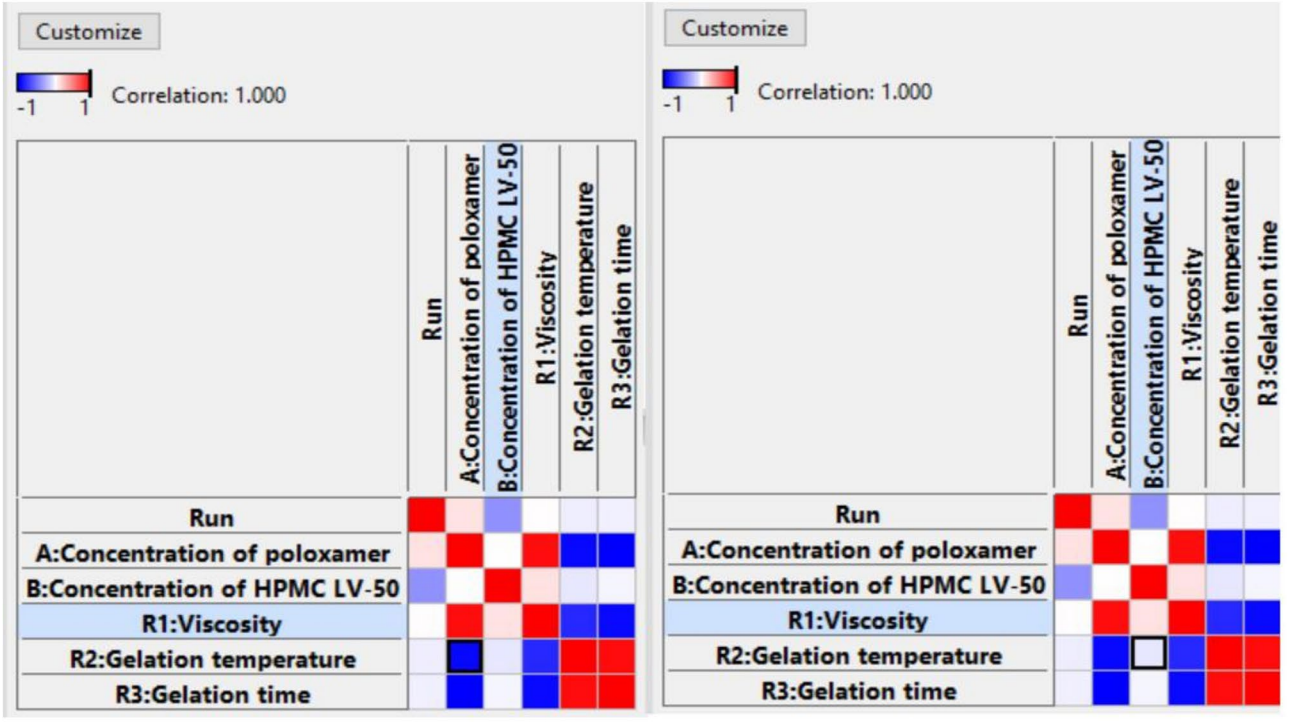


Fig. 4 Correlation of poloxamer 407 and HPMC E-50 LV with gelation temperature

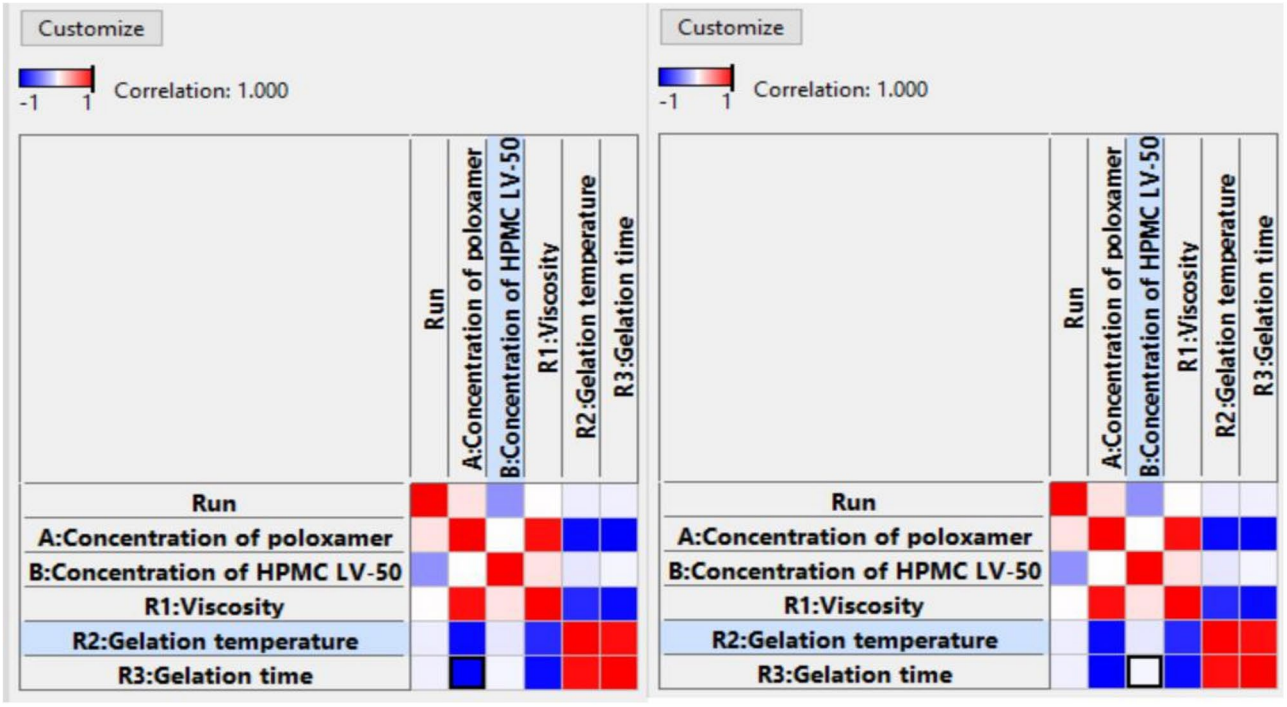


Fig. 5 Correlation of poloxamer 407 and HPMC E-50 LV with gelation time

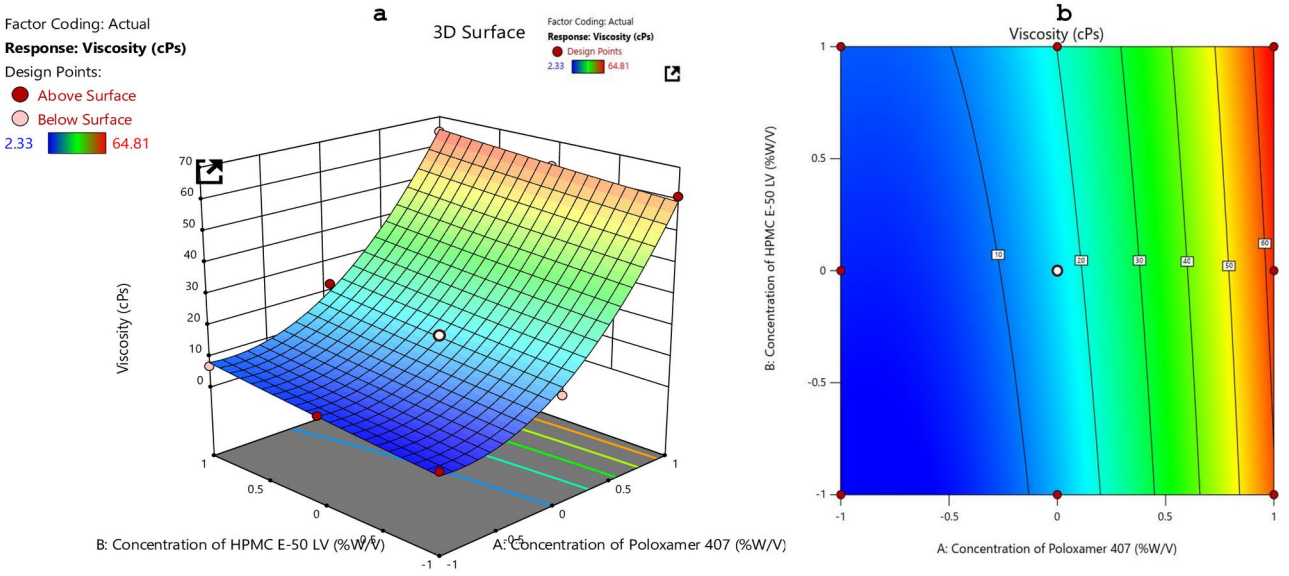


Fig. 6 (**A**)Three-dimensional response surface plot for viscosity and (**B**) contour plot for viscosity response

levels represented by color gradients. The red zone indicates the highest viscosity, suggesting that increasing the concentrations of poloxamer 407 or HPMC E-50 LV toward this region results in higher viscosity.

The associations between viscosity and poloxamer 407 and HPMC E-50 LV concentrations were represented by the following quadratic equation model:

$$Viscosity = 17.82 - 7.54\,A + 6.60\,B - 0.12\,AB + 0.68\,A^2 + 5.20\,B^2 \quad (5)$$

Where A is the amount of poloxamer 407, and B is the amount of HPMC.

A quadratic model represents the relationship between gelation and varying concentrations of poloxamer 407 and HPMC E-50 LV, highlighting the lowest viscosity point, as shown in Fig. 8. The viscosity response contour

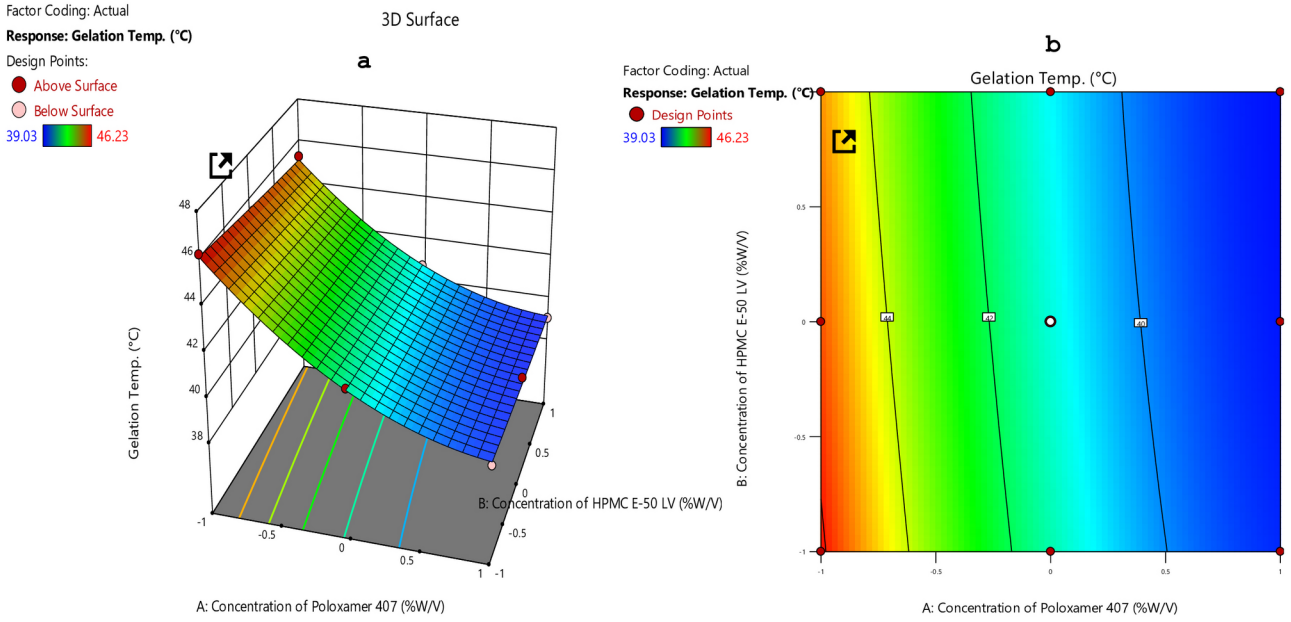


Fig. 7 (**A**) Three-dimensional plot and (**B**) contour plot of the gelation temperature response

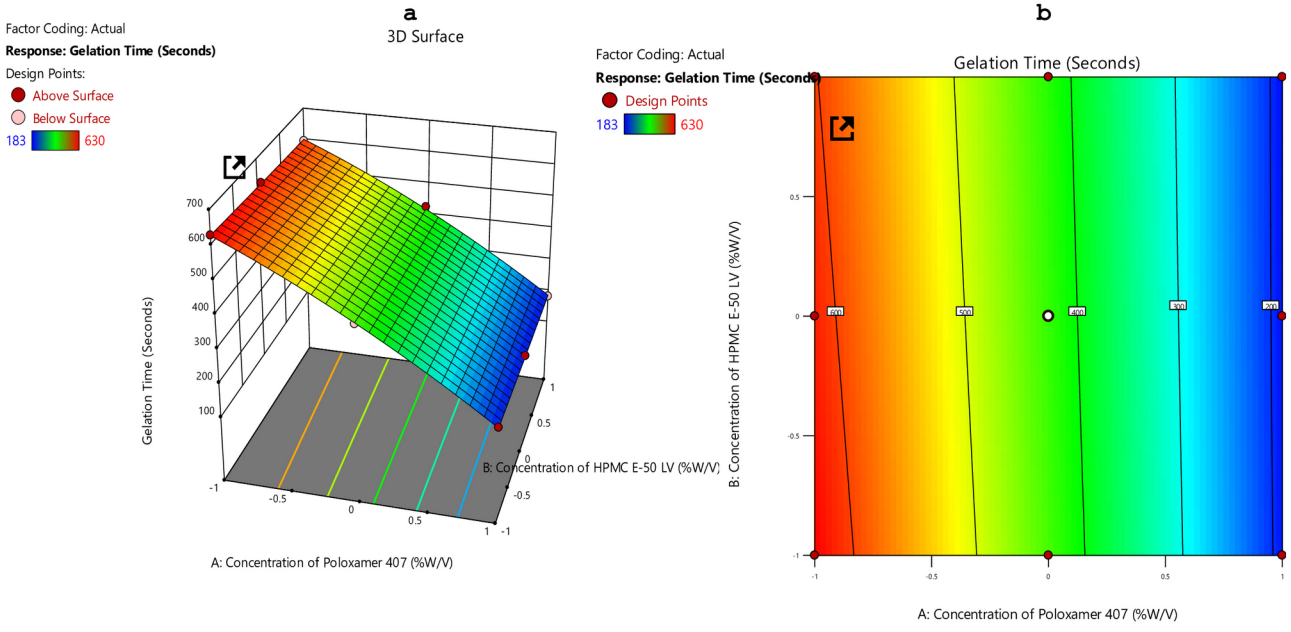


Fig. 8 (**A**) Three-dimensional plot and (**B**) contour plot of the gelation time response

plot graph displays poloxamer 407 on the x-axis, HPMC E-50 LV on the y-axis, and gelation temperature as the contour. The gelation temperature response control table indicates that as the concentration of poloxamer 407 and HPMC E-50 LV rises closer to the red area, where the greatest gelation temperature is produced, the gelation temperature value will increase. It is projected that the gelation temperature of the formula suggested by the Design Expert® 13 program will be 39.03° **C**.

The link between gelation temperature and poloxamer 407 and HPMC E-50 LV quantities was represented by the following quadratic equation model:

$$\begin{aligned} Gelation\ temperature = {} & 41.05 - 3.20\,A \\ & - 0.2917\,B + 0.2050\,AB \\ & + 1.34\,A^2 + 0.0450\,B^2 \end{aligned} \quad (6)$$

Where A is the amount of poloxamer 407, and B is the amount of HPMC.

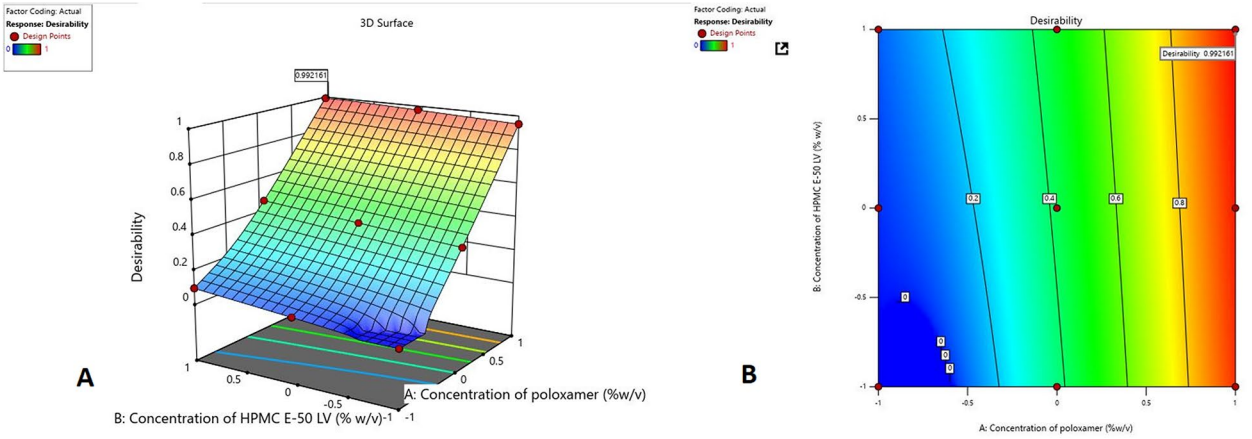


Fig. 9 (**A**) Three-dimensional response surface plot and (**B**) contour plot for the desirability value

Table 5 The viscosity, gelation temperature, gelation time, and desirability of formulations B_1– B_9, and B_{pred}

Formulation code	Concentration of poloxamer 407 (%w/v)	Concentration of HPMC E-50 LV (%w/v)	Viscosity (cPs)	Gelation temperature (°C)	Gelation time (Seconds)	Desirability
B_1	5	1	6.78	45.29	598	0.095
B_2	10	0.75	16.93	41.02	422	0.420
B_3	5	0.75	4.45	45.34	618	0.045
B_4	15	0.5	60.98	39.15	191	0.953
B_5	15	1	64.81	39.03	183	0.992
B_6	15	0.75	61.7	39.46	189	0.976
B_7	10	1	22.34	40.76	428	0.465
B_8	10	0.5	10.87	41.45	432	0.374
B_9	5	0.5	2.33	46.23	630	0
B_{pred}	15	0.992	65.62	39.14	187	0.992

A quadratic equation model with a minimal gelation time point is shown in Fig. 7 when seen from a 3-dimensional perspective, together with the contour plots of gelation time responses. This model shows the link between gelation time and the amounts of poloxamer 407 and HPMC E-50 LV. Poloxamer 407 as mapped on the x-axis, HPMC E-50 LV was mapped on the y-axis, and the gelation time data were plotted as contours on the viscosity response contour plot.

The link between gelation time and poloxamer 407 and HPMC E-50 LV quantities was represented by the quadratic equation model:

$$\begin{aligned} Gelation\,time = {} & 426.88 - 213.83A \\ & - 7.33B + 6.00AB \\ & - 25.83A^2 + 0.66B^2 \end{aligned} \quad (7)$$

Where A is the amount of poloxamer 407, and B is the amount of HPMC.

Based on these physical factors, response surface methodology (RSM) will be able to determine the optimal solutions for poloxamer 407 and HPMC E-50 LV composition (B_{pred}). This software will offer a strategy based on desirability after establishing the viscosity, gelation temperature, and gelation duration required for the ophthalmic in situ gel. Table 2 shows the pH, viscosity, and attractiveness of B_1–B_9 and B_{pred}.

The desirability value, as demonstrated in Fig. 9(a), explains why the response value produced by the factors X_1 and X_2 is close to the intended requirement values (viscosity, gelation temperature, and gelation time). The value of desirability falls between 0 and 1. The software's capacity to generate ideal formulas improves as the desirability approaches.

The contour plot in Fig. 9(b) and Table 5 shows that the software's suggested prediction formula, B_{pred}, provides a reasonably decent result, with desirability levels ranging from 0 to 1. Because the desirability value is near zero, the response does not satisfy the ideal viscosity, gelation temperature, or gelation time fulfillment criterion if it approaches the blue contour. Because the red contour and the resulting response are getting closer to the desired value of 1, they will meet the satisfactory optimal viscosity, gelation temperature, and gelation time criteria as the response approaches. With a desirability score of 0.992, Design Expert® 13's results for this study fall within

the green region. This indicates that the experimental R^2 value of 0.992 reflects an excellent correlation with the predicted values, signifying high model reliability and minimal deviation between observed and projected results.

Table 6 shows that, out of all the formulas, B_5 had the greatest desirability value (0.992). The Design Expert® 13 program (B_{pred}) suggested the use of 15% w/v poloxamer 407 and 0.992% w/v HPMC E-50 LV, with a desirability value of 0.992. Figure 9 displays the results of the contour plot and three-dimensional response surface based on the desirability value.

The data analysis performed using Design Expert® 13 involved a three-level factorial design under the response surface methodology (RSM). Statistical significance was assessed using p-values and F-values for each factor and interaction term.

For viscosity, the model was significant ($p = 0.0006$), with the concentration of poloxamer 407 ($p = 0.0001$) and its quadratic term ($p = 0.0025$) showing the most substantial impact. The concentration of HPMC E-50 LV was also statistically significant ($p = 0.0483$), indicating its influence on the response. However, the interaction term (AB) was non-significant ($p = 0.9091$).

For gelation temperature, the model was significant ($p = 0.0006$). The concentration of poloxamer 407 exhibited a highly significant effect ($p < 0.0001$), while the quadratic term (A^2) was also significant ($p = 0.0059$). However, the concentration of HPMC E-50 LV and the interaction terms were not statistically significant ($p > 0.05$).

For gelation time, the model was highly significant ($p < 0.0001$). Poloxamer 407 concentration had a highly significant effect ($p < 0.0001$), as did its quadratic term ($p = 0.0128$). The effect of HPMC E-50 LV and the interaction term were non-significant ($p > 0.05$).

The robustness of the models was further validated by the high F-values and low p-values, indicating the reliability of the regression equations. To ensure model validity, replicate experiments were performed, confirming the reproducibility of the results and supporting the statistical conclusions.

In vitro drug release study

The cumulative release (%) of ganciclovir from the in-situ gel reached 63.76% over 6 h, with 14.44% released in the first hour. In contrast, the marketed gel exhibited a release of 98.45% within 6 h, with 18.58% released during the first hour (Fig. 10). The coefficients of correlation (R^2) for the zero-order, first-order, Higuchi, Hixon–Crowell, and Korsmeyer–Peppas models for the in situ gel was 0.9163, 0.9405, 0.9612, and 0.9519, respectively. Subsequently, the coefficient of correlation (R^2) for the Higuchi model was closest to unity (0.9612), and it was carefully chosen as the best-fit model (Table 7). The Higuchi model describes drug release from a matrix system based on Fickian diffusion, which can be mainly relevant for ocular drug delivery. Sustained release is often needed in ocular applications to ensure prolonged therapeutic drug levels,

Table 6 An ANOVA summary for quadratic response surface models

Foundation	Sum of square	Degree of freedom	Mean square	'F'- value	'P'- value	
Viscosity (cPs)						
Model	5671.40	5	1134.28	181.7	0.0006	Significant
A – Concentration of poloxamer 407	5041.94	1	5041.94	807.6	0.0001	
B – Concentration of HPMC E-50 LV	65.01	1	65.01	10.41	0.0483	
A B	0.09	1	0.09	0.01	0.9091	
A^2	564.14	1	564.14	90.37	0.0025	
B^2	0.211	1	0.21	0.03	0.8658	
Gelation temperature (°C)						
Model	65.84	5	13.17	181.08	0.0006	Significant
A – Concentration of poloxamer 407	61.57	1	61.57	846.62	< 0.0001	
B – Concentration of HPMC E-50 LV	0.5104	1	0.5104	7.02	0.0770	
A B	0.1681	1	0.1681	2.31	0.2258	
A^2	3.59	1	3.59	49.38	0.0059	
B^2	0.0041	1	0.0041	0.0557	0.8286	
Gelation time (Seconds)						
Model	2.762	5	55230.09	1179.76	< 0.0001	Significant
A – Concentration of poloxamer 407	2.743	1	2.74	5860.29	< 0.0001	
B – Concentration of HPMC E-50 LV	322.67	1	322.67	6.89	0.0786	
A B	144.00	1	144.00	3.08	0.1777	
A^2	1334.72	1	1334.72	28.51	0.0128	
B^2	0.8889	1	0.8889	0.0190	0.8991	

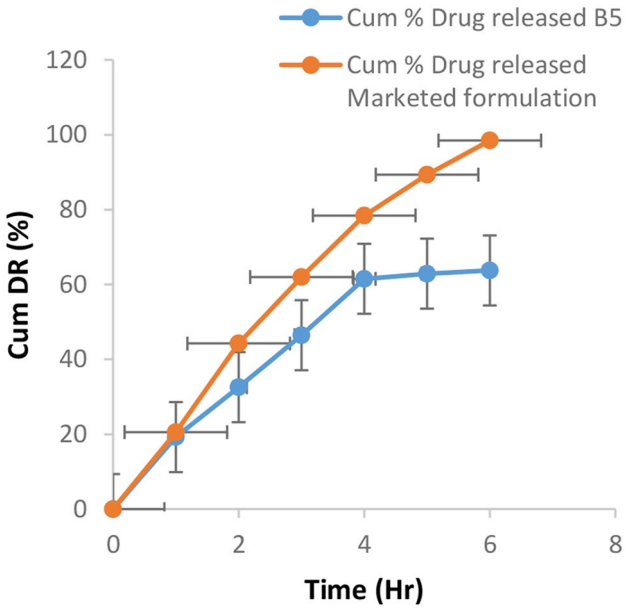


Fig. 10 In vitro drug release profile of the in situ gelling system and marketed formulation of ganciclovir

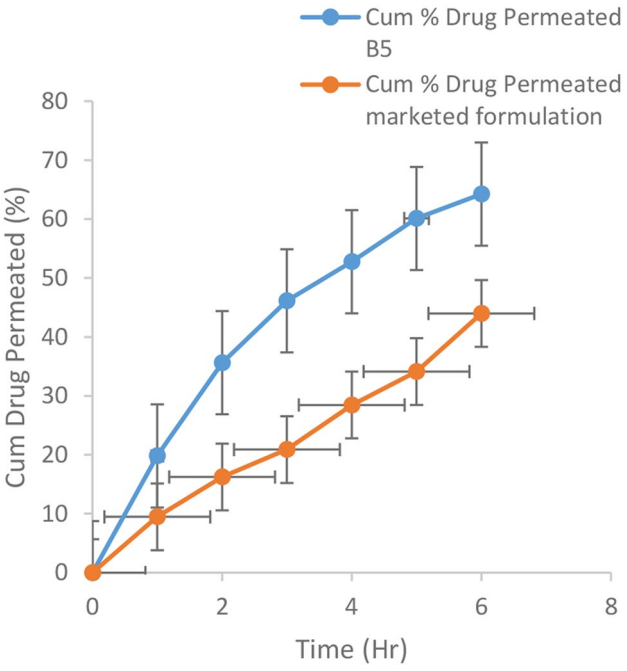


Fig. 11 Ex vivo transcorneal study of the in situ gelling system and marketed formulation of ganciclovir

minimize dosing frequency, and improve patient compliance. The Higuchi model's diffusion-controlled release profile can help accomplish these goals by providing a steady release of the drug over a prolonged period, which is significant for effective ocular treatment. The linear relationships observed, combined with the slow dissolution rate, suggest that in vitro release of ganciclovir from the in situ gel formulation under physiological situations primarily occurs through diffusion [45].

Ex vivo transcorneal permeation studies

Ex vivo transcorneal permeation studies were conducted on the optimized in situ gel formulation B_5. This formulation was selected based on its viscosity, gelation temperature, and gelation time characteristics. The degree of permeation of the optimized formulation was assessed to that of a marketed ganciclovir gel [46]. Formulation B_5 showed a cumulative percent permeation of 64.23% over 6 h. In comparison, the marketed ganciclovir gel exhibited 43.98% permeation during the same period (Fig. 11). The higher permeation of the in-situ gel formulation can be attributed to the bioadhesive and permeation-enhancing properties of poloxamer 407. The improved permeation is necessary for topical ocular delivery of ganciclovir, as it confirms effective drug transport transversely to the cornea to reach intraocular tissues, mainly the anterior chamber, where the drug can exert its antiviral effect against cytomegalovirus retinitis and herpetic keratitis. Optimized corneal permeation improves therapeutic efficacy while reducing frequent dosing requirements and adjusting patient compliance. Additionally, poloxamer 407's bioadhesive properties extend corneal residence time, further supporting sustained drug delivery and therapeutic action. Importantly, improved permeation does not imply systemic absorption, as the focus remains on localized drug action within the ocular tissues.

Ocular irritation studies

The normal saline solution group did not exhibit any signs of irritation in the cornea, iris, or conjunctival tissues, serving as a negative control. In contrast, the 1% w/v SDS group confirmed severe ocular irritation, including discharge, redness, and chemosis, within 1 h of administration (Fig. 12) [45, 46]. Over time, the discharge subsided, and chemosis and redness showed slight reductions over the 6-hour observation period, implying prolonged irritation caused by the SDS solution. After

Table 7 Kinetic release model data of the optimized formulation (B_5) and marketed formulation

Formulation name	Zero-order		First order		Higuchi model		Korsmeyer- Peppas model	
	R^2	K_0	R^2	K_1	R^2	K_H	R^2	K_{KP}
Optimized formulation (B_5)	0.9163	10.736	0.9405	0.07	0.9612	27.96	0.9519	0.84
Marketed formulation	0.9949	16.65	0.8092	0.26	0.9351	41.48	0.9976	0.94

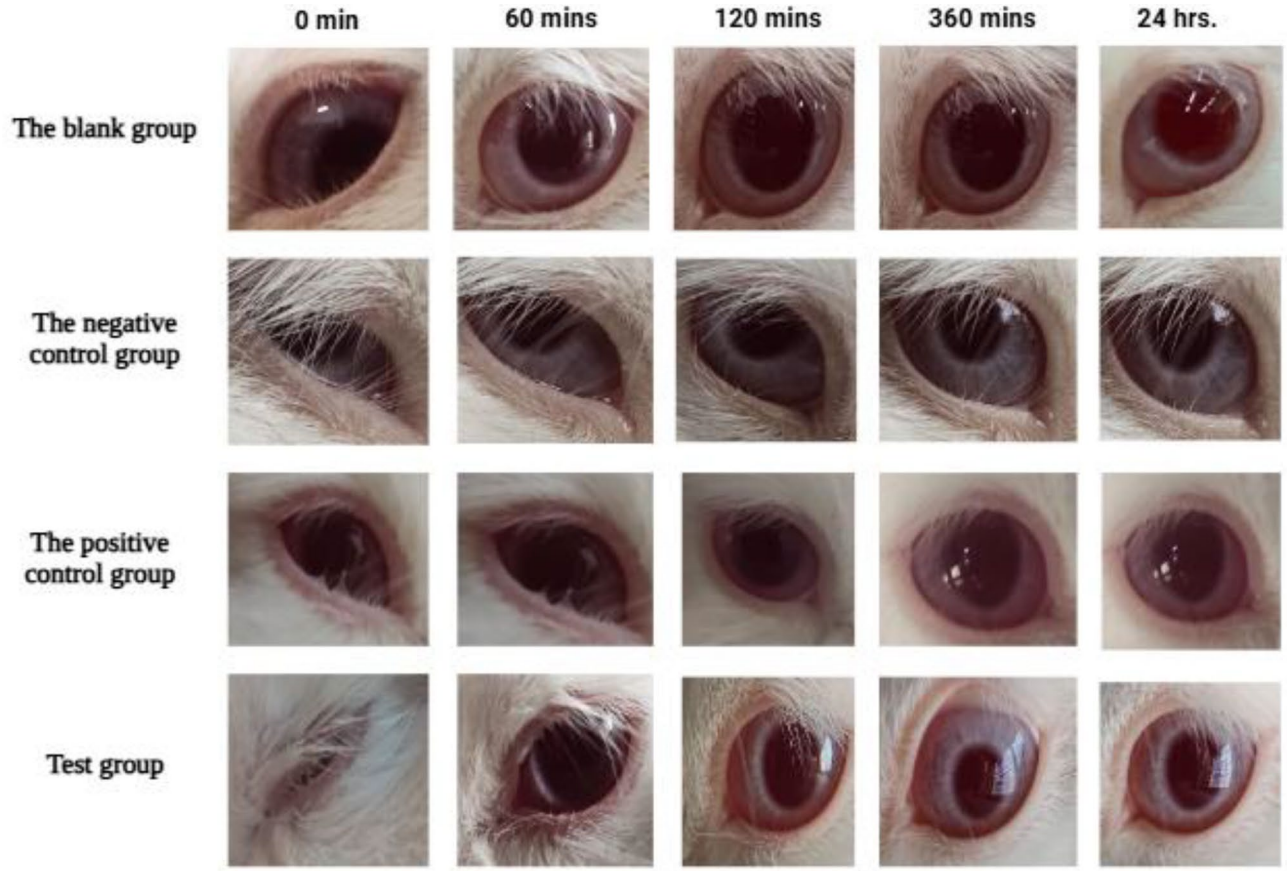


Fig. 12 Draize Ocular Irritation Test: Evaluation of the ocular irritation potential of the optimized ganciclovir in situ gel using the Draize test, assessing redness, swelling, and discharge in the rabbit eye model over a 24-hour period

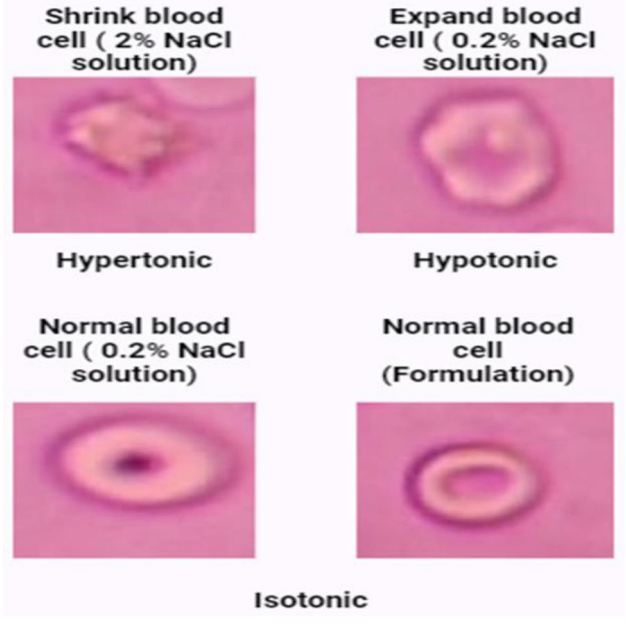


Fig. 13 Observation results of the isotonicity test

the instillation of the blank in-situ gel formulation and ganciclovir in-situ gel into the lower cul-de-sac of the conjunctiva, mild local redness was detected within the first 5 min. This redness gradually diminished and disappeared completely within 60 min, suggesting a transient irritation response. Importantly, the redness observed was comparable for both the blank formulation and the ganciclovir-loaded gel, indicating that the transient irritation was likely due to the formulation components, such as poloxamer 407, rather than ganciclovir itself. Tear flow and natural ocular clearance mechanisms ensured that the formulation was effectively washed away within 60 min of administration. Based on these findings and the absence of persistent adverse effects, the designed ganciclovir in situ gel preparation can be classified as non-irritant and well-tolerated.

Determination of isotonicity

Microscopic examination revealed that the four preparations produced normal blood cells with characteristics like those in the isotonic control solution. This is illustrated in Fig. 13, which shows observations using an isotonic control solution (0.9% NaCl), a hypertonic control solution (2% NaCl), and a hypotonic control solution

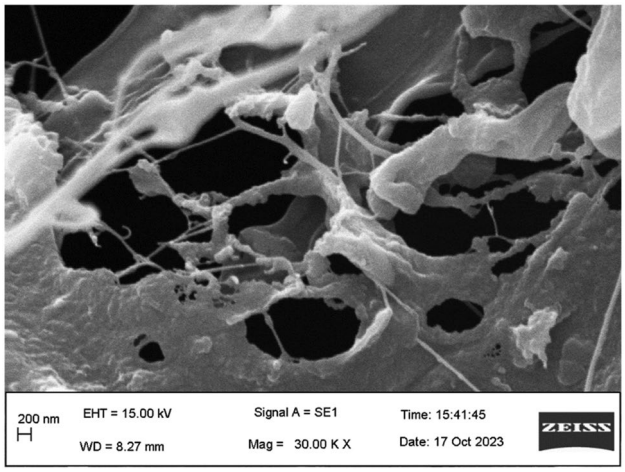


Fig. 14 SEM image of the poloxamer 407 thermosensitive gel

(0.2% NaCl). These results confirm that the necessary measures were taken to achieve isotonicity [47].

Surface morphology

Scanning electron microscopy (SEM) images show the gel formations containing ganciclovir, as presented in Fig. 14. SEM images of the optimized formulation revealed a sponge-like topology with a surface that appeared to be porous. The formation of large pores was attributed to rapid phase separation and continuous breakdown of the polymeric matrix [47].

Discussion

The bioavailability of ocular formulations is less due to the defensive mechanisms of the eye, such as nasolacrimal drainage, tear dilution, and enzymatic degradation of active pharmaceutical ingredients (API) [48]. Minimizing the drawbacks of conventional ocular formulations and improving bioavailability can be done by reducing the rate of elimination of the API and enhancing the absorption of the API, which are common strategies. The primary objective of the study was to prepare an in situ gelling system to deliver ganciclovir for ocular administration. For the preparation of in situ gels, poloxamer 407 was used as a smart polymer that underwent gelation with increasing temperature. HPMC E 50 LV was used as a thickening agent [49]. The secondary objective of the work was to optimize the preparation to achieve the best formulation that can improve the ocular bioavailability of the GCV by enhancing the ocular contact time [50].

Poloxamers are triblock copolymers containing hydrophilic and hydrophobic blocks that form micelles beyond the critical micelle concentration. Enhancing the temperature decreases the critical micelle concentration. Upon further heating, it transforms into a gel [51]. The benefits of using poloxamers include less toxicity and decent tolerability. The mucoadhesion property of poloxamer is not up to the mark. Improving the mucoadhesion property of the poloxamer by adding HPMC E-50 LV was our aim. It is important to choose the excipients of eye drops for safe ophthalmic administration. HPMC E-50 LV was utilized mainly in preparation as a viscosity enhancer, which allows the poloxamer to transform into a gel in lesser amounts. FTIR analysis was performed to determine the compatibility of ganciclovir, poloxamer, and HPMC E-50 LV [52]. The results displayed that there was no probable interaction between the active drug substance and the polymers used for the formulation. The inverse proportionality between the concentration of poloxamer 407 and the gelation temperature is well recognized in the previous works. However, the existence of other polymers can modify the gelation temperature and time, as determined by the poloxamer 407 concentration [53]. To explore this approach, the combined outcome of poloxamer 407 and HPMC E-50 LV on viscosity, gelation temperature, and gelation time was studied. The optimized formulation was also characterized by an in vitro drug release investigation, ex vivo transcorneal permeation investigations, and ocular toxicity investigation. Batch number B_5 was chosen as the best-optimized preparation in terms of viscosity, gelation temperature, and gelation time. It was observed from the study that the concentration of HPMC E-50 LV can enhance the viscosity and reduce the gelation temperature of a poloxamer 407-based in situ gel. The optimized preparation B_5, with its higher concentration of poloxamer 407 (15%), exhibited favourable viscosity and gelation characteristics suitable for ocular use. The in vitro drug release study showed that the in-situ gel released 63.76% of ganciclovir over 12 h, with an initial release of 14.44% in the first hour. This indicates a sustained release profile, which is beneficial for prolonged therapeutic effects and reduced dosing frequency. The marketed formulation of ganciclovir gel showed a cumulative release of 98.45% within 6 h, with 18.58% released in the first hour. This result suggested a faster release profile, which may require more frequent application to maintain therapeutic levels. The ex vivo transcorneal properties were also compared with those of a conventional gel of ganciclovir. The extent of permeation of the B_5 was compared with that of a marketed ganciclovir gel [54]. Over 6 h, batch number B_5 demonstrated a cumulative percent permeation of 64.23%, significantly greater than the 43.98% achieved by the marketed ganciclovir gel [54]. The Draize ocular irritation study confirmed that the formulated ganciclovir in situ gel formulation is non-irritant and well tolerated, with only minimal transient redness observed. This indicates a good safety profile, making it a promising ophthalmic formulation for further development. The SEM images showed that the optimized formulation batch had a sponge-like structure. This kind of structure

is characterized by a network of interconnected pores, which can significantly influence the release dynamics of the drug [55, 56].

Conclusions

This research work aimed to formulate an in-situ gel delivery system to increase the ocular residence time of ganciclovir at the site of action, the eye. Nine in-situ gel formulations were prepared using the cold technique, based on the Response Surface 3^2 full factorial design. The results revealed that the optimized formulation batch (B_5) comprised 15% w/v poloxamer 407 and 1% w/v HPMC E-50 LV, resulting in an in-situ gel with a viscosity of 64.81 cPs. The gelation temperature and gelation time were determined to be 39.0 °C and 183 s, respectively. The optimized formulation demonstrated superior permeability and continual release of ganciclovir compared to the commercial formulation.

Acknowledgements
The authors are thankful to the Researchers Supporting Project Number (RSPD2025R940), King Saud University, Riyadh, Saudi Arabia for supporting this study. Mukherjee would like acknowledge Department of Pharmaceutical Technology, NSHM Knowledge Campus, Kolkata, West Bengal, India for providing the research and library facilities to complete the research works. Dr.Prajapati, extends his sincere appreciation to the Faculty of Pharmacy, Silpakorn University, Thailand, for their generous financial support that enabled the completion of this work.

Author contributions
Conceptualization, SM, BP and MC; data collection: SP, NS and SM1 writing—original draft preparation, SP, NS, BP, SN; analysis and interpretation of results, SP, NS, SM1 and MC; writing—review and editing, BP, NA, AA and SM2; Supervision, SM1. All authors have read and agreed to the published version of the manuscript.

Funding
The research was funded through Researchers Supporting Project Number (RSPD2025R940) King Saud University, Riyadh, Saudi Arabia.

Data availability
The results/data/figures in this manuscript have not been published elsewhere, nor are they under consideration (from you or one of your Contributing Authors) by another publisher. All generated research data is included in this manuscript.

Declarations

Ethical approval
This study involving the use of animals was conducted by the ethical guidelines and regulations set forth by the Institutional Animal Ethics Committee (IAEC) of NSHM Knowledge Campus, Kolkata, India. The experimental protocol was reviewed and approved by the IAEC under approval number [NCPT/IAEC-011/2023]. All procedures performed in the study were in strict adherence to the ethical standards for the care and use of laboratory animals. Efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments.

Consent for publication
All authors have participated significantly in the research, writing, and review process, and each has read and approved the final version of the manuscript.

Clinical trial number
Not applicable.

Competing interests
We declare that the authors have no competing finical interests as defined by BMC, or other interests that might be perceived to influence the results and/or discussion reported in this paper. However, Dr.Prajapati declares that he is guest editor of this special issue.

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Received: 14 November 2024 / Accepted: 23 April 2025
Published online: 13 May 2025

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