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Formulation, *Ex-Vivo* and Preclinical *In-Vivo* Studies of Combined pH and Ion-Sensitive Ocular Sustained *In Situ* Hydrogel of Timolol Maleate for the Treatment of Glaucoma

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Abstract: The aim of the present research work was to develop safe, effective, and stable *in situ* hydrogel for the ophthalmic drug delivery using the combination of ion-responsive polymer gellan gum and pH-sensitive polymer carbopol 934P to treat glaucoma. Background: Timolol maleate is a BCS class I drug used as the first line of treatment in open-angle glaucoma. The rapid precorneal elimination of conventional formulation containing class I drugs exhibits poor therapeutic effect and bioavailability. So, *in situ* gelling system was formulated and characterized. Methods: Box-Behnken design was used to statistically optimize the formulation parameters and evaluate the effects of formulation attributes, namely concentration of gellan gum (X_1), the concentration of carbopol 934P (X_2) and concentration of benzododecenium bromide (X_3) on selected critical quality attributes (Y_1-Y_7). Trial run data were statistically analyzed using the polynomial equation and response surface plots. Optimized formulation was selected based on desirability function, design space, and was further characterized and compared with the marketed formulation. Results: The concentration of both polymers showed a synergistic positive impact on viscosity at the non-physiological and physiological conditions. Trial runs showed controlled drug release with diffusion-controlled mechanism and good mucoadhesive strength due to the presence of Carbopol 934P. The preservative benzododecenium bromide showed the ability to enhance trans-corneal permeation. The optimized formulation has appeared as a clear solution at the non-physiological condition and clear gel at the physiological condition with an acceptable pH range of 5-6. Other quality attributes like rheological properties, gelling capacity, texture analysis, Isotonicity, contact angle, sterility, antimicrobial efficacy, and stability were found in desires values for the ocular application. The safety of *in situ* gel for human use was confirmed by ocular irritation and histopathology studies in the rabbit eyes. The intraocular pressure (IOP) reduction with optimized formulation was found comparable and less fluctuating compared to ophthalmic gel-forming marketed solution of timolol maleate (TIMOPTIC-XE®). Conclusion: The cross-linking between Carbopol 934P with Gellan gum in the formation showed more viscous gelling at the physiological condition to provide long pre-corneal residence time. The optimized formulation exhibited all the desirable attributes of an ideal ophthalmic *in situ* gelling formulation, exhibited *in-vitro* controlled drug release, good gelling capacity, and was found to be stable and non-irritant to the eye.

Keywords: Glaucoma; Ion responsive; Box-Behnken design; Controlled release.

1. Introduction

Glaucoma is a common eye condition that can cause irreversible blindness if not diagnosed and treated within the early stage. Glaucoma is related to an increase in intraocular pressure (IOP). The conventional eye drops for the treatment is very useful but still have some problems like low bioavailability (1–5%), requires frequent instillation of drops, reflex tearing, and blinking[1,2]. The disadvantages of eye drop as a delivery system led to investigations of novel and alternative devices and delivery systems [3]. Also, due to the need to deliver the dose to the site by solution and resultant faster elimination of the drug, the patients have to suffer from many problems [4]. Due to these drawbacks, traditional methods of delivery of the drugs are replaced by alternative methods of delivery to fulfill the unmet needs [5,6]. Drug delivery in the form of *in-situ* gelling offers a substitute to eye drops as this concept decreases the dosing frequency. Such a delivery system provides phase transition *in-vivo* from sol to gel within the impasse of the eye when the polymer in eye drops gives a response to the stimuli and forms gel[7,8].

In situ forming gels are formulations applied as solutions, sols or suspensions that undergo gelation after installation due to physicochemical changes inherent to the physiological parameters. Parameters that can change and trigger the gel formation include pH, temperature, and ionic strength [4,9]. The present work describes the combination approach for gelling by pH and ionic strength. pH-sensitive *in situ* gelling is achieved by a change in pH. Most of the anionic pH-sensitive polymers (carbopol) swell as the external pH increases due to proton acceptance in the eye environment [10]. On the other hand, ion stimulated gelling is activated by a change in the ionic strength or due to the presence of ions in the tear fluid. Once it forms a gel, it can stand up to the drainage process and amplify residence time [11]. An effort was made using a combination of pH and ion stimulated gelling by Gupta *et al*. when they formulated in situ gel of Sparfloxacin with a combination of chitosan, which is pH sensitive and gellan gum which is ion-sensitive[12].

Glaucoma is an eye disease that results in damage to optic nerve and vision loss. Worldwide glaucoma is the second leading cause of blindness after cataracts. Currently, the treatment of choice of glaucoma is to reduce IOP [13]. Timolol maleate has been established as the first line of the drug in the treatment of glaucoma. Even after the advent of the latest drugs like prostaglandin analogs and alpha-2 agonists, timolol remains the first choice due to cost-effective reason. It is a beta-adrenergic blocker that is non-selective between beta-1 and beta-2 adrenergic receptors. It has no issue with solubility and permeability, and it effectively lowers the IOP, diminishes blood pressure by delaying both the receptors and reducing sympathetic discharge. It also develops an adverse chronotropic and inotropic movement. Lifelong treatment with topical drops is usually required in the treatment of glaucoma. Hence, reduction in its dosing frequency can improve patient compliance and therapy [14,15].

In order to study the combined effect of pH and ion stimulated gelling approaches, we have used carbopol 934 and Gelrite® Gellan gum for pH and ion-sensitive gelling, respectively. Carbopol 934 is a synthetic polyacrylic acid polymer which shows a sol to gel transition in aqueous solution as the pH is raised above its pKa of 5.5. Additionally, it interacts with mucin in the tear film to increase drug retention [16,17]. Gelrite® (deacetylated gellan gum) is one of the most promising ion-sensitive in situ gelling polymer and an approved ophthalmic excipient. It forms a clear gel in the presence of mono or divalent cations.

Rheological properties of gellan gum, such as thixotropy, pseudo-plasticity, and thermoplasticity, are advantageous for its use in ophthalmic formulations [18,19].

The aim of the present research work was to develop safe, effective, and stable *in situ* gel for the ophthalmic drug delivery using the combination of ion-responsive polymer gellan gum and pH-sensitive polymer carbopol 934P to treat glaucoma. The developed formulation was characterized and compared with ophthalmic gel-forming marketed solution of timolol maleate (TIMOPTIC-XE®). We have applied the quality by design approach for the optimization of the formulation. A complete characterization and assessment have been performed for developed formulation.

2. Materials and Methods

2.1. Materials.

Timolol Maleate was received as a gift sample from Centaur Pharmaceutical Limited (Mumbai). Gellan gum was procured from CP Kelco (Atlanta), Sodium Hydroxide was acquired from Merck KGaA (USA). Mannitol was attained from SD Fine-chem Limited (Mumbai). Carbopol 934P was purchased from Lubrizol Corporation. Benzododecinium Bromide was purchased from Vapi Care Pharma Pvt. Ltd. (Vapi). All other chemicals and reagents utilized were of analytical grade.

2.2. Methods.

2.2.1. Formulation development.

In the present investigation, Box-Behnken design as an optimization tool was applied for formulation development. This design is appropriate for three independent variables at their three levels. Through this design, the effect of three formulations attributes, namely concentration of gellan gum (X_1), the concentration of carbopol 934P (X_2), and concentration of benzododecenium bromide (X_3) was investigated on selected critical quality attributes. The independent and dependent variables for the delivery system are described in Table 1. As per Box Behnken design, total17 controlled experimental trial runs were conducted to observe respective dependant variables as described in Table 2. The independent variables selected with their low (-1), medium (0), and high (+1) levels were chosen based on the results from prior experience, preliminary experimentation, and literature survey. Data from designed trial runs were statistically analyzed using the polynomial equation, analysis of variance, and response surface plots utilizing Design Expert software (Version 9.0.0, Stat-Ease Inc., Minneapolis, MN). Optimized formulation was selected based on the desirability function and design space. The non-linear polynomial equation used for data analysis is shown in equation 1.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 \text{--- (Eq.1)}$$

Where Y is the measured response related to each factor level combination; b_0 is an intercept; b_1 to b_{33} are evaluated regression coefficients computed from the distinguished experimental values of Y; and X_1, X_2, and X_3 are the coded levels of independent variables. The terms X_1X_2 and X^2i (i = 1, 2, or 3) represent the interaction and quadratic terms, individually. The most impacts (X_1, X_2, and X_3) represents the average results of changing one factor at a time from its low to high value. The interaction terms(X_1X_2) indicates how the response alters when two or more factors are simultaneously changed [20].

Table 1. Description of independent and dependent variables for formulation development.

Translation of coded values in actual units			
Independent variables	**Levels used, actual (coded)**		
	Low	**Medium**	**High**
Concentration of Gellan gum (%w/v) = X_1	0.25**(-1)**	0.5**(0)**	0.75**(+1)**
Concentration of Carbopol 934P (%w/v) = X_2	0.15**(-1)**	0.30**(0)**	0.45**(+1)**
Concentration of Benzododecinium bromide (%w/v) = X_3	0.006**(-1)**	0.012**(0)**	0.018**(+1)**
Dependent variables	**Constraints**		
Y_1= Viscosity at non-physiological condition (25˚C±2, pH 5)	Y_1= 75 to 125 cps		
Y_2= Viscosity at physiological condition (35˚C±2, pH 7.4)	Y_2= 3200 to 3600 cps		
Y_3= Cumulative % drug release at 1 hr (Q_1 in %)	Y_3= 10 to 12 %		
Y_4= Time required to release 90% of drug (t_{90} in min)	Y_4= 1200 to 1250 minutes		
Y_5= Mucoadhesive Strength (gram(s))	Y_5= 22 to 25 gram(s)		
Y_6= Gel strength (seconds)	Y_6= 35 to 40 seconds		
Y_7= Rate of Permeability (sq.cm/sec.)	Y_7= 1.250 to 1.450 sq.cm/sec.		

Table 2. Box Behnken design layout with respective dependent variables ($Y_1 – Y_7$) for ocular *in situ* gelling system.

Batch No.	**Viscosity (cps)**		**Q_1 (Y_3)**	**$t_{90\%}$ (min) (Y_4)**	**Mucoadhesive strength (gm) (Y_5)**	**Gel Strength (Sec.) (Y_6)**	**Rate of permeation (sq.cm./sec)(Y_7)**
	Non-physiological (Y_1)	**Physiological (Y_2)**					
O 1	34.00	471.0	32	584	9.3	23.7	1.203
O 2	135.3	2860.3	23	664	12.3	47.7	1.180
O 3	151.3	2550.7	14	741	26.0	32.0	1.157
O 4	300.7	6500.0	12	1388	33.3	58.0	1.250
O 5	90.3	1700.0	16	817	14.0	26.0	0.625
O 6	174.3	4249.3	15	1187	18.0	51.0	0.578
O 7	76.7	1720.0	19	685	13.7	27.3	1.365
O 8	165.0	4300.0	16	1165	17.3	48.3	1.481
O 9	38.7	870.0	19	670	12.0	32.3	0.555
O 10	179.3	3200.0	13	1116	26.3	31.0	0.625
O 11	41.7	910.7	19	671	12.3	33.0	1.458
O 12	190.3	3149.7	11	1064	26.7	39.0	1.412
O 13	90.3	2600.0	13	1116	18.3	29.0	1.226
O 14	97.7	2589.3	12	1110	20.0	26.0	1.250
O 15	82.7	2450.7	13	1100	17.3	30.0	1.319
O 16	101.0	2709.0	13	1114	19.3	29.3	1.342
O 17	92.0	2851.7	12	1110	19.7	28.0	1.342

2.2.2. Composition of *in-situ* gel.

Preparations were formulated by mixing two phases. In the first phase, the required quantity of mannitol was dissolved in 45% of the total volume of batch size in deionized water and stirred for 30 min. In the above solution, the required quantity of gellan gum and/or carbopol was added with subsequent stirring for 60minutes at 70°C to 80°C. The second phase was prepared by dissolving the required quantity of Timolol maleate and benzododecinium bromide to form 45% of the total volume of batch size in deionized water. Upon cooling of the first phase, to room temperature, the second phase was added in it and mixed. The pH was adjusted between 5 and 6 by 0.1N Sodium hydroxide solution, and the final bulk volume was made up with deionized water. Bulk preparations were sterilized by autoclave (121°C, 15 psi for 20 minutes) and filled in LDPE (Low-density polyethylene) bottles for further study [12].

2.3. Evaluation of experimental design batches.

2.3.1. Determination of viscosity.

The viscosity of formulation was determined by Brookfield viscometer (LV DVII+PRO model) at 100 rpm utilizing spindle number 31 at room temperature (25±2ºC) and spindle

number 64at physiological condition (37±2ºC). This was done for comparative assessment of viscosity of the formulations at physiological and non-physiological conditions [21,22].

2.3.2. *In- vitro* drug release study of the ocular in-situ gelling system.

In-vitro drug release study was performed through the cellophane membrane using a modified USP XXIII dissolution apparatus [23]. It was performed using simulated tear fluid as a medium. The membrane used was previously saturated with a dissolution medium. Five ml of the formulation was accurately taken into this assembly. The glass cylinder was suspended in 50 ml of the specified dissolution medium at 37±0.5ºC so that the membrane just touches the receptor medium surface. The receiving medium was stirred at 50 rpm. A sample was placed evenly on the surface of the membrane in the donor compartment. Aliquots were withdrawn at hourly intervals till 24 hours and replaced by an equal volume of dissolution medium to maintain the sink condition. The aliquots were diluted with diluents medium (water and acetonitrile in the ratio of 60:40) up to 50 ml followed by sonication for about 15-20 minutes and analyzed for Timolol maleate concentration using optimized HPLC conditions against working standard area.

2.3.3. Measurement of mucoadhesive strength.

Mucoadhesive strength was determined by calculating the strength necessary to remove the preparation from mucosal tissue utilizing an adapted technique given by Yong *et al*. [24]. An area of corneal tissue membrane, along with part of the conjunctiva, was extracted from the eyes of a goat. The mucosal tissue was immediately tied onto each glass vial using a thread keeping mucosal side on the outer side. In another vial with a section of mucosal tissue was placed in an inverted position while the first vial was placed on a height-adjustable pan. The formulation gel was placed between the mucosal tissues of both vials. It was adjusted in such a way that the membrane surfaces of both the vials came in close contact. The mucoadhesive force is the minimum weight required to detach two vials (Eq. 2). The mucosal tissue pieces were changed for each measurement. All measurements were performed in triplicate.

$$\text{Detachment Stress}(\text{dyne/cm}) = \frac{(m*g)}{A} \text{---------- (Eq.2)}$$

Where m is the weight required for detachment in grams; g is the acceleration due to gravity taken as 980 cm/s^2, and A is the area of tissue exposed in sq.cm.

2.3.4. Gel strength Measurements.

Gel strength was measured by the gel strength gadget device [24]. The different formulations were converted into a gel at 37°C. Gel strength, i.e., the viscosity of the gel at physiological condition, was analyzed by the time(s) taken by the probe to drop down 5 cm over the gel (n = 3).

2.3.5. *In-vitro* trans-corneal permeation study.

In-vitro trans-corneal permeation study was carried out within the corneal eyeballs of a goat. Corneal tissue samples were embedded in Franz diffusion cell, which comprises of both donor and receptor compartments. The isolated cornea was fixed by sandwiching the neighboring scleral tissue between the donor and receptor compartments in a way that its

epithelial surface confronted the donor compartment. The upper compartment becomes a patron/donor chamber in which the formulation was positioned. The lower compartment assisted as a receiver chamber having 15 ml of simulated tear fluid (STF) kept at 37±0.5°C. The elutriate of 2 ml was collected at periodic time intervals for up to 4 hrs. The samples were analyzed for drug content by HPLC [25].

2.4. Characterization of optimized in-situ gel.

2.4.1. Clarity and pH.

The clarity test was performed by visually observing the optimized formulation alternatively against light and dark background. The pH of all ocular *in-situ* gel was measured with a standard calibrated digital pH meter at 25±1ºC. All measurements were done in triplicate [12].

2.4.2. Drug content.

The drug content was determined by taking 1 ml of the formulation sample and added into 50 ml of volumetric flask. The sample was diluted with diluents medium (water and acetonitrile in the ratio of 60:40) up to 50 ml followed by sonication for about 15-20 minutes and analyzed for Timolol maleate concentration using optimized HPLC conditions against working standard area [23].

2.4.3. *In-vitro* gelling capacity.

In-vitro Gelling Capacity test was carried out by two methods. In flowability method a test tube upsetting technique defined by Jeong *et al*., was utilized to unevenly decide the phase nature of formulation at three different storage temperature points viz. 5±1˚C (fridge temperature), 25±1°C (room temperature) and 37±1°C (physiological temperature). In the visual method, one ml of optimized formulation was added to a vial containing two ml of STF kept at 37±1 ºC temperature. As the formulation comes in contact with STF, it converts into a stiff gel, which was observed and graded according to its stiffness [22].

2.4.4. Isotonicity.

Isotonicity of the optimized formulation was measured by observing hemolysis in the blood. The formulation was mixed with few drops of blood and observed under the optical microscope at 45X magnification. The observation was compared with the effect on blood illustrated by hypotonic, hypertonic, and normal saline solution [12].

2.4.5. Texture analysis.

Texture analysis of optimized formulation at physiological condition (STF pH 7.4, 37°C) was carried out on Brookfield QTS Texture Analyzer. Texture analysis basically evaluates the mechanical properties where the optimized formulation was subjected to controlled force from which a deformation curve is generated. The analysis was performed in triplicate [21].

2.4.6. Measurement of contact angle.

The contact angle measurement of optimized formulation was conducted with CAM-101 contact angle optical goniometer (Attension Theta, KSV Instruments, Finland). The contact angle was measured in both hydrophilic and hydrophobic surfaces and compared with the marketed formulation of the drug. The contact angle at the hydrophilic and hydrophobic surface indicates the interactions at the ocular interface and spreadability of the formulation [26].

2.4.7. Histopathological evaluation of cornea.

The goat cornea was kept in contact with optimized formulation for 24 hours. These corneas were utilized for histopathological assessment. The cornea was placed in 10% buffered formalin (pH 7.4) and inserted in paraffin. Paraffin parts were pieced on the plates and blemished with hematoxylin and eosin (HE). Segments were inspected under the optical microscope to identify any impairment to the ocular tissue [27].

2.4.8. Sterility test.

The test for sterility was evaluated by Method-B Direct Inoculation as per Indian Pharmacopoeia. An optimized formulation was withdrawn from the test holder with a sterile pipette. An amount of 2 ml of the optimized formulation was inoculated directly into the culture medium and was incubated for 14 days. The cultures were observed for microbial growth during the 14 days of incubation. Negative control was also performed as described above to evaluate the sterility of media [28].

2.4.9. Antimicrobial effectiveness test.

Antimicrobial effectiveness test was carried out on the optimized formulation utilizing the agar diffusion method by cup plate method with standard organisms *Staphylococcus aureus* (ATCC 6538P) and *Escherichia coli* (ATCC 10536). The marketed sterile formulation of the timolol maleate was taken as a standard for the comparison with the optimized formulation. Both marketed and optimized formulations were diluted suitably to 5 and 30 µg/ml solution and were poured into cups of agar plates. After 2 hours of diffusion of the solution, the agar plates were incubated at 37ºC for 24 hrs. The zone of inhibition (ZOI) was measured and compared. The tests were carried in triplicate, and the mean inhibition zone ± S.D. were calculated. The positive and negative controls were implemented during the study [29]. The percentage efficiency for the optimized ocular *in situ* gelling systems was calculated using equation 3.

$$\% \text{ Efficiency} = \frac{\text{ZOI of test}}{\text{ZOI of standard}} \times 100 \text{ ---------- (Eq.3)}$$

2.4.10. Accelerated Stability study.

Accelerated stability study was conducted on optimized formulation according to ICH (International Conference on Harmonization) guidelines. An optimized formulation in its final primary packaging container was kept in stability chambers at 40°C±2°C/not more than (NMT) 25% RH. The samples were withdrawn at 0, 3, and 6 months interval and were analyzed for physical appearance, clarity, viscosity, related substances, pH, osmolality, *in-vitro* gelling capacity, *in-vitro* drug release, and assay. The logarithms of percent drug remaining were

calculated and plotted against time in days. The degradation rate constant was calculated with equation slope=K/2.303, where K is a degradation rate constant [12].

2.4.11. Ocular pharmacodynamic study.

Rabbits (New Zealand white, Male, 2.5 to 3.2 kg) were used for a comparative study of both optimized and marketed formulations. Animals were treated as prescribed in the NIH publication "Guide for the Care and Use of Laboratory Animals". All experiments conformed to the ARVO Resolution on the Use of Animals in Research. They were carried out under veterinary supervision, and the protocols were approved by the Ethical-Scientific Committee of the University. The animals were housed individually in standard cages in a room with normal controlled lighting, at normal room temperature (16-22°C) and humidity (30-70% relative humidity), with no restriction of food or water. During the experiments, the rabbits were placed in restraining boxes to which they had been habituated, in a room with dim lighting; they were allowed to move their heads freely, and their eye movements were not restricted [30,31].

Rabbits were divided into two groups (n=3) based on body weights. The optimized formulation was instilled in the left eye of group 1 rabbits, whereas the commercially available formulation was instilled in the left eye of group 2 rabbits. In all rabbits, the right eye was instilled with placebo in the form of a vehicle. The dosing was provided with an eyedropper (35-50µL). During the study of formulation, the rabbit eyes were assessed every day for tearing, discharge, blepharospasm (twitchy and forceful blinking of the eyelids), ptosis (eyelid drooping), and conjunctival redness, which are all signs of ocular discomfort. The assessment was carried as mentioned in OECD (Organization for Economic Co-operation and Development [OECD, 1987]) guidelines. At a predetermined time period, the IOP measurements were performed using a tonometer (TONOVET, Finland). The measurement was done in triplicate [25].

3. Results and Discussion

3.1. Evaluation of experimental design batches.

In the present investigation for formulation development, Box-Behnken design as an optimization design was used for three selected material attributes to study their effect on seven selected quality attributes, as shown in Table 1. All the responses (dependent variables) obtained for the 17 trial batches were at the same time fitted to the quadratic response surface model utilizing Design Expert (Version 9.0.0, Stat-Ease Inc., Minneapolis, MN). The observed responses for Y_1 to $Y_7$are revealed in Table 2.

3.1.1. Statistical analysis for Y_1 (Viscosity at non- physiological condition).

The obtained value for viscosity at the non-physiological condition for all 17 trial runs O1-O17 varied from 34 to 300 cps. The response (Y_1) observed at different levels of three independent variables were exposed to multiple reversion to give a quadratic polynomial equation as per mention in the above values are shown in Table 3. The non-linear model produced for viscosity at non-physiological conditions was found to be significant with an F-value of 49.62, p-value <0.0001, an R^2 value of 0.9845. Both X_1 (52.87) and X_2 (71.50) has a higher value of co-efficient. These two variables X_1 and X_2 were also found to be significant

in the prediction of Y_1. These two variables have a positive impact on viscosity at the non-physiological condition. The effect of carbopol 934P on viscosity at non-physiological atmosphere is about 1.4fold as compare to the effect of gellan gum. Thus, it can be said that if carbopol 934P is used at higher concentrations, then it would improve the consistency or gelling property of formulation at the non-physiological condition. It is also evident that other independent variables X_3- Benzododecenium bromide (BDB) did not show a significant effect on viscosity. The results are depicted in Figure 1.

3.1.2. Statistical analysis of viscosity at physiological condition (Y_2).

The obtained value for viscosity at the physiological condition for all 17 trial runs O1-O17 changed from 471 to 6500 cps. This result obviously demonstrates that viscosity in the physiological environment influenced by the independent variables chosen for examination. The response (Y_2) observed at different levels of three independent variables were exposed to manifold reversion to give a quadratic polynomial value shown in Table 3. The non-linear model produced for viscosity at physiological conditions was found to be significant with an F-value of 58.60, p-value <0.0001, and R^2value of 0.9869. The overhead calculation evidently replicates the wide variety of values of different co-efficient (b). Out of three independent variables, X_3 has a lower value of co-efficient. This variable X_3 (p>0.05) was found to be insignificant in the prediction of Y_2. Out of three independent variables, the X_1 (1433) and X_2 (1286) has a higher value of co-efficient. These two variables were X_1, and X_2 was also found to be significant in the prediction of Y_2. These two variables have a positive effect on viscosity at physiological conditions. Thus variable X_1, i.e., gellan gum, has a prominent effect on viscosity at physiological condition (Y_2). It is also apparent that other independent variables X_3-Benzododeceniumbromide (BDB) did not show an effect on viscosity and were non-significant. The results are depicted in Figure 1.

Table 3. Results of regression analysis for variables of ocular *in situ* gelling systems (Y_1-Y_4).

Independent variables	Viscosity at non-physiological = Y_1		Viscosity at physiological = Y_2		$Q_1 = Y_3$		$t_{90\%} = Y_4$	
	p value	Coefficients	p value	Coefficients	p value	Coefficients	p value	Coefficients
Intercept	< 0.0001	92.73	< 0.0001	2640.13	0.006	12.60	< 0.0001	1110.00
X_1	< 0.0001	52.88	< 0.0001	1433.50	0.061	-1.88	< 0.0001	197.13
X_2	< 0.0001	71.50	< 0.0001	1286.04	0.004	-5.38	< 0.0001	215.00
X_3	0.8106	-1.13	0.9338	7.63	0.775	0.25	0.0151	-25.63
X_{12}	0.1026	12.00	0.0169	390.00	0.186	1.75	< 0.0001	141.75
X_{13}	0.8702	1.08	0.9529	7.67	0.688	-0.50	0.0455	27.50
X_{23}	0.7635	2.00	0.8609	-22.75	0.688	-0.50	0.2802	-13.25
X_1^2	0.0005	38.34	0.0007	707.56	0.007	4.33	< 0.0001	-91.25
X_2^2	0.0059	20.70	0.0775	-252.19	0.024	3.33	< 0.0001	-174.50
X_3^2	0.4943	48.50	0.0226	-355.35	0.725	-0.43	0.0016	-55.25

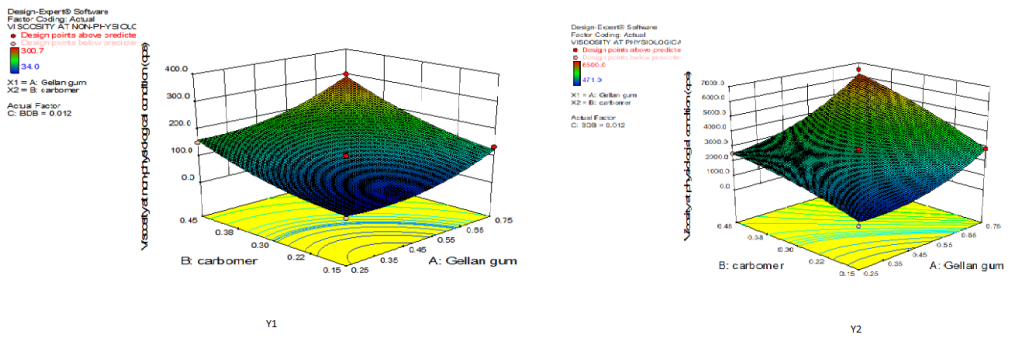

Figure 1. Response surface and Contour plots; Viscosity (cps) at the non-physiological condition (25 °C, pH 5) [Y_1], Viscosity (cps) at the physiological condition (35°C, pH 7.4 and STF) [Y_2].

3.1.3. Statistical analysis of % drug release at 1 hour – Q1 (Y_3).

The values obtained for Q_1 for all 17 trial runs O1-O17 were found, ranging from 12% to 32%. The outcome demonstrates that Y_3 is influenced by the independent variables nominated for the examination. The response (Y_3) observed at different levels of three independent variables were exposed to manifold reversion to give a quadratic polynomial value are shown in Table 3. The non-linear model produced for Q_1 was found to be significant with an F-value of 7.90, p-value 0.0062, an R^2 value of 0.9104. Among the independent variables, chosen, the X_1 and X_2showed negative value representing on significant effect on % drug release at 1 hour. The X_3 variable has a positive value of co-efficient (0.258) representative prominent favorable effect on Y_3. Out of three independent variables, the X_1 (-1.9) and X_2 (-5.4) has a higher value of co-efficient. The effect of carbopol 934P Q1 is about fivefold as compared to the effect of gellan gum. This indicates that carbopol 934P, if alone used at higher concentrations, would decrease initial release to the very low content of the drug, which would be below therapeutic concentration. The results are depicted in Figure 2.

3.1.4. Statistical analysis of the time required to release 90% of drug – t90% (Y_4).

The obtained values of $t_{90\%}$ for the 17 trial runs O1-O17 were found, ranging from 584 to 1388 minutes. The response (Y_4) observed at different levels of three independent variables were exposed to manifold reversion to give a quadratic polynomial value are shown in Table 3. The non-linear model produced for $t_{90\%}$ was found to be significant with an F-value of 208.4, p-value <0.0001, an R^2 value of 0.9962. Out of three independent variables, X_3(BDB) has a negative value of co-efficient (–25.6) and does not have a significant impact on sustaining the drug release. The variables X_1 and X_2 had a positive value of co-efficient (X_1=197 and X_2=215) and were also found to be significant in the prediction of Y_4. The results are depicted in Figure 2.

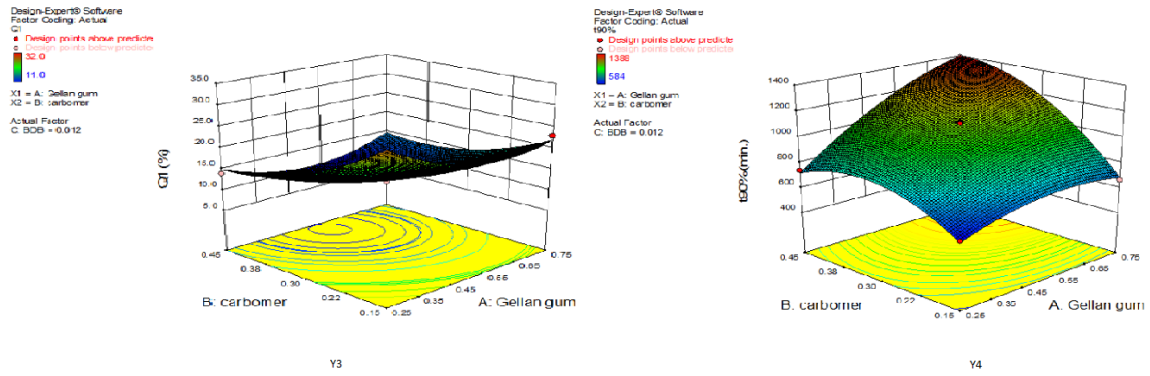

Figure 2. Response surface and Contour plots; Corrected Cumulative % drug release at 1 hour (Q_1 in %) [Y_3], the time required to release 90% of drug ($t_{90\%}$ in min) [Y_4].

3.1.5. Statistical analysis of Mucoadhesive strength (Y_5).

The obtained values for mucoadhesive strength for all 17 trial runs O1-O17 were found between 9.3 to 33.3 gm. The response (Y_5) observed at different levels of three independent variables were exposed to manifold reversion to give a quadratic polynomial value are shown in Table 4. The non-linear model produced for mucoadhesive strength was found to be significant with an F-value of 30.95, p-value <0.0001 and R^2 value of 0.9755. Out of three independent variables, X_3 has a negative value of co-efficient (-0.04), indicating an unfavorable

response on Y_5. The variable X_1 and X_2havea a co-efficient value of 2.3 and 8.3, respectively. These variables showed a significant effect while X_2represented the main causative effect on Y_5. The interaction between variable X_1 and X_2 had a positive value of co-efficient. Hence it can be concluded that a combination of X_1 and X_2 has a synergistic effect on Y_5. The results are depicted in Figure 3.

Table 4. Results of regression analysis for variables of ocular *in situ* gelling systems (Y_5-Y_7).

Independent variables	Mucoadhesive strength (Gm) (Y_5)		Gel Strength(Sec) (Y_6)		Rate of permeation(cm/sec) (Y_7)	
	p value	Coefficients	p value	Coefficients	p value	Coefficients
Intercept	< 0.0001	18.93	0.0001	28.46	< 0.0001	1.30
X_1	0.0041	2.25	< 0.0001	12.00	0.2749	0.02
X_2	< 0.0001	8.29	0.0167	2.91	0.6948	0.01
X_3	0.9403	-0.04	0.3583	0.97	< 0.0001	0.42
X_{12}	0.1964	1.08	0.7158	0.50	0.2049	0.03
X_{13}	0.9156	-0.08	0.4730	-1.00	0.0903	0.04
X_{23}	1.0000	0.00	0.2070	1.83	0.2049	-0.03
X_1^2	0.1692	-1.13	0.0004	8.10	0.0450	-0.05
X_2^2	0.0129	2.45	0.0220	3.76	0.0458	-0.05
X_3^2	0.0276	-2.05	0.2532	1.60	< 0.0001	-0.23

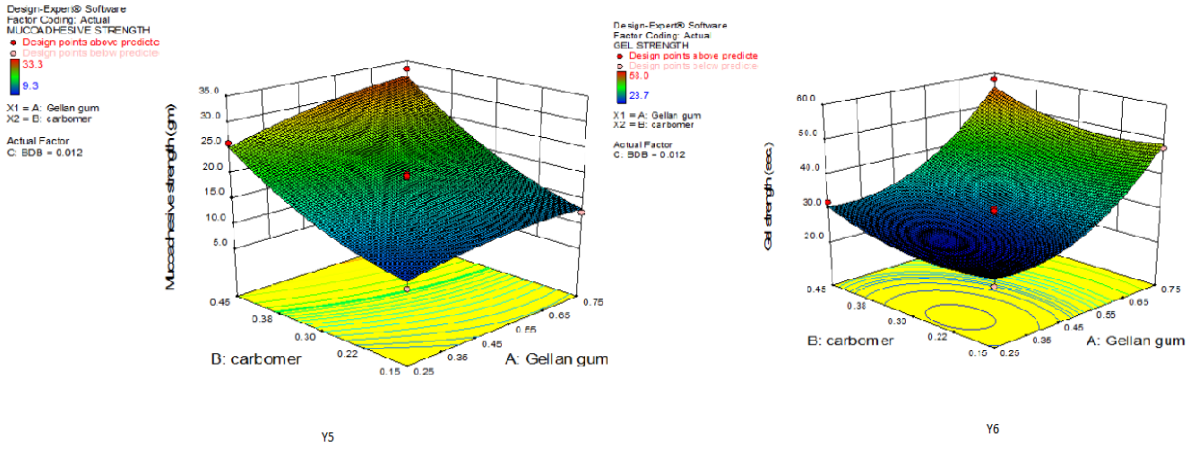

Figure 3. Response surface and Contour plots; Mucoadhesive strength (dyn/cm_2) [Y_5], Gel strength (sec.)[Y_6].

3.1.6. Statistical analysis of Gel strength (Y_6).

The obtained values for gel strength for all 17 trial runs O1-O17 were found between from 23 to 58 seconds. The response (Y_6) observed at different levels of three independent variables were exposed to multiple reversion to give a quadratic polynomial value are shown in Table 4. The non-linear model produced for gel strength was found to be significant with an F-value of 25.83, p-value <0.0001, an R^2 value of 0.9707. None of the independent variables had a negative value of co-efficient, indicating a favorable effect on Y_6. The variable X_1 and X_2 have a co-efficient value of 12 and 2.9, respectively. These variables were also found to be noteworthy, representing the main causative result of X_1 on Y_6. The results are depicted in Figure 3.

3.1.7. Statistical analysis of the permeability coefficient (Y_7).

The obtained values for the permeability coefficient for all 17 trial runs O1-O17 were found between from 0.56 to 1.46 (x 10^{-5} cm/sec.). The response (Y_7) observed at different levels of three independent variables were subjected to manifold reversion to give a quadratic polynomial value, as shown in Table4. The non-linear model produced for the permeability

coefficient was found to be significant with an F-value of 107.73, p-value <0.0001, an R^2 value of 0.9928. None of the independent variables had a negative value of co-efficient. The co-efficient value X_1 and X_2 were very low 0.01 and 0.006, respectively, representing a negligible effect on Y_7. These two variables were found insignificant. The variable X_3 Benzododecinium bromide was found to be significant with a coefficient value of 0.42representing the main causative effect of X_3 on Y_7. Thus benzododecenium bromide act as a trans-corneal permeation enhancer to improve corneal permeability of prepared *in situ* gelling trial runs. The results are depicted in Figure 4.

Results shown in Table 5 suggested that there is wide variability in values obtained for each response of formulations. It shows higher values of standard deviation (SD) and % coefficient of variation (CV). These results show that the chosen independent variable meaningfully affected independent variables. The good R^2 values (> 0.91) indicate a good correlation between the independent and dependent variables selected for the study.

Table 5. Results of regression analysis for responses of ocular *in situ* gelling systems.

Response/Dependent Variable	R^2	SD	% CV
Y_1= Viscosity at Non Physiological condition (25°C and pH 5)	0.9846	12.78	10.65
Y_2= Viscosity at physiological condition (35°C and pH 7.4)	0.9870	250.31	9.32
Y_3= Corrected Cumulative % drug release at 1 hour (Q_1 in %)	0.9104	2.39	14.93
Y_4= Time required to release 90% of drug ($t_{90\%}$ in min)	0.9963	22.64	2.36
Y_5= Mucoadhesive Strength (gm)	0.9755	1.52	8.16
Y_6= Gel strength (sec.)	0.9707	2.63	7.58
Y_7= rate of Permeability/Permeability coefficient (cm/sec.)	0.9928	0.04	3.64

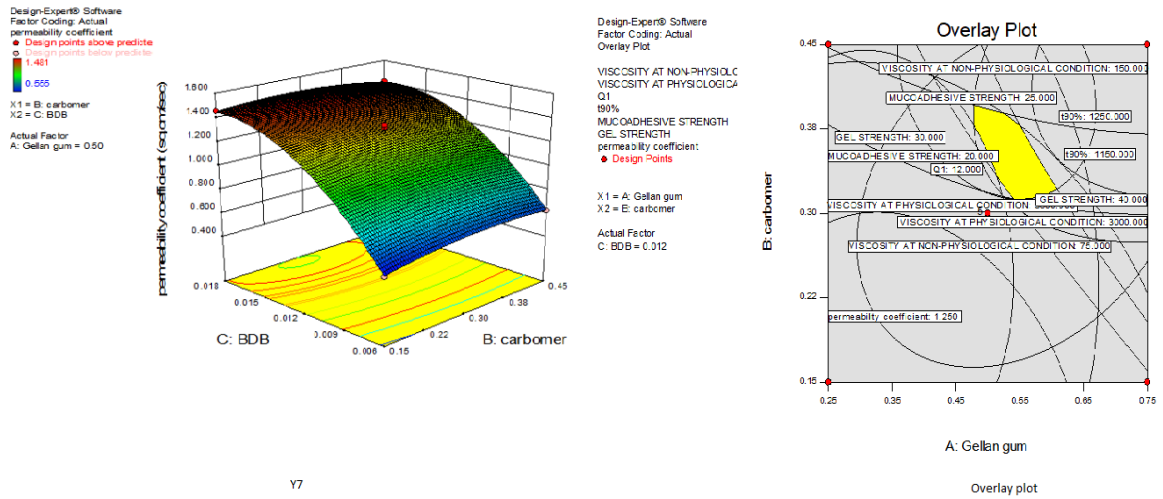

Figure 4. Response surface and Contour plot for Rate of Permeability/Permeability coefficient (cm/sec.)[Y_7] and Overlay Plot for all possible sets of variables.

3.2. Contour plots and response surface analysis.

The impact of independent variables on the response was further explained utilizing the contour plots. It was decided from the 3D surface plot that the desired non-physiological viscosity is 75 to 125 cps for ease of installation. It may be accomplished by an increase in the concentration of carbopol 934P. The viscosity remained at 125 cps at a concentration of carbopol 934P at 0.15% and of gellan gum at 0.45%.

The 3D surface plot (Figure 1) shows the impact of viscosity, which is due to phase transition from sol to gel at the physiological condition. The highest value of viscosity could be obtained at a higher concentration of both polymers. However, the desired viscosity range of $Y_2$is 3200 to 3600 cps. The increase in viscosity at physiological conditions is directly

proportional to the increase in the concentration of gellan gum. Hence the lines in contour plots are inclining towards the right side. The desired value of Y2 could be obtained in an area of 0.25-0.4% carbopol 934P and 0.4-0.7% gellan gum. The desirable value of cumulative % drug release at 1 hour is 10-12%, which could be observed in the region of 0.3-0.45% carbopol 934P and 0.2-0.65 % gellan gum.

The 3D surface plot (Figure2) also shows the prominent effect of gellan gum concentration (X1), carbopol 934P concentration (X_2), and BDB concentration (X_3) on time required for 90% release of Timolol maleate (Y_4). The optimum value of $t_{90\%}$ is1200 to 1250 minutes, which can be obtained by selecting 0.23-0.45% of carbopol 934P and 0.4-0.75 % of gellan gum, respectively.

Benzododecenium bromide (X_3) has been utilized as a preservative, and its permeability enhancement effect has been seen with non-linear curvature along with carbopol 934P (X_2). The mucoadhesive target strength (22 to 25 gm) effect can be attained in an area of 0.31-0.37% carbopol 934P and 0.55-0.67 % gellan gum.

The high value of gel strength (35 to 40 sec) in an area of 0.15-0.40% carbopol 934P and 0.50-0.65 % gellan gum. The plots clearly reflect that as the concentration of gellan gum rises with the value of gel strength (Figure 3).

The 3D surface plot Figure 4 shows the effect of factor gellan gum (X_1) and BDB (X_3) on the permeability coefficient (Y_7). The plots clearly show that as the concentration of BDB increases, the value of permeability also increases. There is more contributing effect of BDB. The desired value of Y_7 (1.250 to 1.450 sq.cm/sec) could be obtained with 0.011 – 0.015% of BDB.

3.3. Validation of response surface methodology.

To validate the Box Behnken Design model, three checkpoint batches were prepared and evaluated. The composition of checkpoint batches with predicted and experimental values are shown in Table 6. The prediction error of the predicted values from the experimental values varied between -8.33 and +7.69. The experimental values of viscosity (cps) at the non-physiological environment [Y_1], viscosity (cps) at the physiological environment [Y_2], Corrected Cumulative % drug release at 1 hour [Y_3] Time required to release 90% of drug[Y_4], Mucoadhesive strength (dyne/cm^2)[Y_5], Gel strength (sec.)[Y_6], permeability coefficient (cm/sec.)[Y_7] were found to be 123±8cps, 3300±90cps, 11±0.5, 1220 minutes, 23±2 dyne/cm^2, 37±2 and 1.350 sq.cm/sec. respectively, which are in close accordance with the predicted response by model. Thus, the preferred Box Behnken Design model was found fit and validated. It can be utilized for the optimization of the *in-situ* gelling system.

Table 6. Validation results of response surface methodology with checkpoint batches.

Checkpoint batch composition (X_1:X_2:X_3)	Response variable	Experimental value	Predicted value	% prediction error
0.37:0.23:0.012 (-0.52:-0.46:0)	Y1	48	51	-6.25
	Y2	1612	1525	5.39
	Y3	18	18.4	-2.22
	Y4	870	878	-0.91
	Y5	15	14.4	4
	Y6	26	24	7.69
	Y7	1.260	1.267	0
0.62:0.38:0.018 (0.48:0.53:1)	Y1	160	173	-8.12
	Y2	4090	3982	2.64
	Y3	11	11	0
	Y4	1180	1235	-4.66
	Y5	25	24	4

Checkpoint batch composition (X_1:X_2:X_3)	Response variable	Experimental value	Predicted value	% prediction error
	Y6	42	41	2.3
	Y7	1.460	1.476	-1.09
0.37:0.38:0.006	Y1	108	114	-5.55
(-0.52:0.53:-1)	Y2	2320	2258	2.67
	Y3	11	12	-8.33
	Y4	985	1005	-2.03
	Y5	20	21	-5
	Y6	27	26	3.7
	Y7	0.645	0.641	0.6
0.62:0.23:0.012	Y1	92	96	-4.3
(.48:-0.46:0)	Y2	2820	2748	2.55
	Y3	15	16	-6.66
	Y4	1030	1014	1.55
	Y5	15	16	-6.66
	Y6	37	35	5.4
	Y7	1.270	1.273	-0.23
0.3:0.2:0.015	Y1	39	42	-7.69
(-0.8:-0.6:+0.5)	Y2	1165	1104	5.2
	Y3	24	23	4.1
	Y4	750	715	4.66
	Y5	13	12	7.6
	Y6	27	25	7.4
	Y7	1.383	1.383	0

3.4. Optimization.

The experimental design, statistical analysis, and overlaying contour plot (Figure 4) revealed the optimal formulation composition, as shown in Table 7. This formula is expected to satisfy the most extreme essentials, considering the applied constraints on Y_1 to Y_7. The software suggested optimal formula is expected to give 99% desirability with predicted non-physiologic viscosity of 123±8 cps, physiological viscosity of 3300 ± 90 cps, cumulative % drug release at 1 hour (Q_1) of11±0.5%, time to release 90% of drug (t_{90}) of 1220 minutes, mucoadhesive strength of 23±2 dyne/cm^2, gel strength of 37±2 seconds and permeability coefficient of 1.350 sq.cm/sec. The optimized batch was prepared and evaluated. The results are discussed in the subsequent section.

Table 7. Optimized formulation composition.

Sr. No.	Ingredient	Quantity (%w/v)
1	Timolol maleate	0.50
2	Gellan gum	0.48
3	Carbopol 934P	0.37
4	Benzododecinium bromide	0.012
5	Mannitol	5.30
6	Double distilled deionized water q.s. to	100

3.5. Evaluation of optimized in-situ gel.

3.5.1. *In-vitro* drug release study.

The drug release profile after sol to gel transformation of *in situ* gelling showed linearity with the square root of time and followed 'Higuchi's equation. The transport technique was the same as obtained by the Korsmeyer- Peppas exponential Equation. It was moreover observed that 'Higuchi's plot and Peppas plot were more linear relative to zero-order and first-order plot. Hence these two model-dependent methods were considered for a deriving conclusion. The correlation coefficient (R^2>0.97) was obtained with 'Higuchi's law representing that release from the formulation is based on a diffusion method for all ion-sensitive *in situ* ocular gelling systems. The release was found by Fickian (n < 0.5) as well as non-Fickian (n > 0.5) diffusion

method as interpreted from the value of release exponent obtained from kinetic release data. The drug release profile of optimized formulation shows linear drug release, as shown in Figure 5. The drug release was found similar to marketed formulation with fewer fluctuations in % drug release.

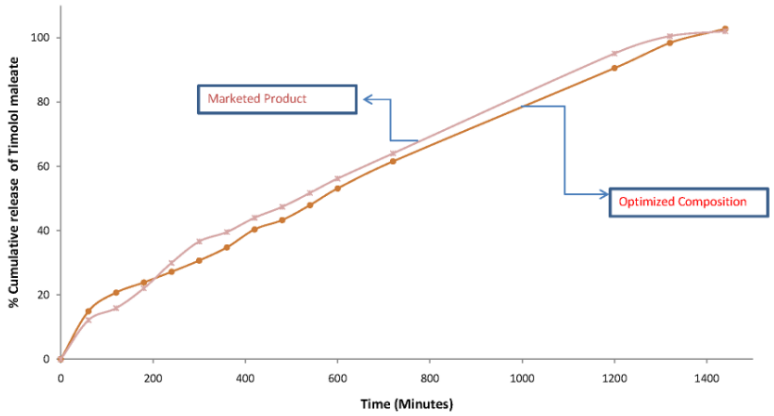

Figure 5. The comparative *in-vitro* drug release profile of optimized formulation with marketed Product.

3.5.2. *In- vitro* trans-corneal permeation study.

Trans-corneal penetration is believed to be the major route for ocular drug absorption. The quantity of the drug absorbed through the cornea can be optimized by controlling the drug release rate from polymer matrix and/or by decreasing drug loss through tear drainage and conjunctival absorption. Both tear drainage and diffusion across the conjunctiva are responsible for drug loss in the precorneal area. The rate of solution drainage decreases with higher viscosity and mucoadhesiveness. Drug diffusion across the conjunctiva is also another factor for drug loss. Hence, for the optimum drug absorption through the cornea, the drug release rate should be controlled along with the decrease in drug loss.

From optimization studies, it was clear that the drug permeation increases with a higher concentration of Benzododecinium bromide. From the Box Behnken design, the predicted value of the permeability coefficient was between 1.25-1.45 sq.cm/sec, while the experimental value was found out to be 1.35 sq.cm/sec for the optimized formulation, i.e., well within the desired range. Thus it can be said that the optimized formulation provides optimum trans-corneal permeation of the drug. The result is shown in Table 8.

Table 8. Characterization of the optimized formulation.

Sr.No.	**Dependent variables**	**Predicted values**	**Experimental values**
1	Y_1= Viscosity (cps) at non-physiological condition (25°C and pH 5)	Y_1= 75 to 125 cps	123 ± 8
2	Y_2= Viscosity (cps) at physiological condition (35°C and pH 7.4)	Y_2= 3200 to 3600 cps	3300 ± 90
3	Y_3= Cumulative % drug release at 1 hour (Q_1 in %)	Y_3= 10 to 12 %	11 ± 0.5
4	Y_4= Time required to release 90% of drug (t_{90} in min)	Y_4= 1200 to 1250 minutes	1220
5	Y_5= Mucoadhesive strength (gm)	Y_5= 22 to 25 gm	23 ± 2
6	Y_6= Gel strength (sec.)	Y_6= 35 to 40 seconds.	37 ± 2
7	Y_7= rate of Permeability/Permeability coefficient (sq.cm/sec.)	Y_7= 1.250 to 1.450 sq.cm/sec.	1.350
	Other evaluation parameters	**Experimental values**	
8	Appearance	Clear	
9	Clarity (%)	97	
10	pH	5.8	
11	*In-vitro* gelling capacity – by flowability	5-10 seconds	
	In-vitro gelling capacity – by visual method	+++	

3.5.3. Rheological study.

The viscosity of the optimized formulation was measured in physiological and non-physiological conditions. A significant increase in viscosity was observed of optimized formulation at physiological conditions (37°C pH 7.4) in the presence of simulated tear fluid. Figure 6 shows the viscosity versus angular velocity (RPM) flow curves of optimized formulation and marketed formulation at non-physiological and physiological conditions, respectively. Both formulations show shear-thinning pseudoplastic rheological behavior, which will allow uniform distribution of the formulation across the eye surface. The viscosity under physiological conditions was much higher compared to non-physiological conditions suggesting the phase transition from sol to gel. In the present investigation, the approach is to achieve the optimal viscosity by mixing carbopol 934P with gellan gum. When the non-physiological condition was changed into physiological condition with a pH 4-5 to 7.4 in the presence of cations, the viscosity of optimized formulation had a significant increase. This can be owing to the ion-sensitive gelling property of gellan gum.

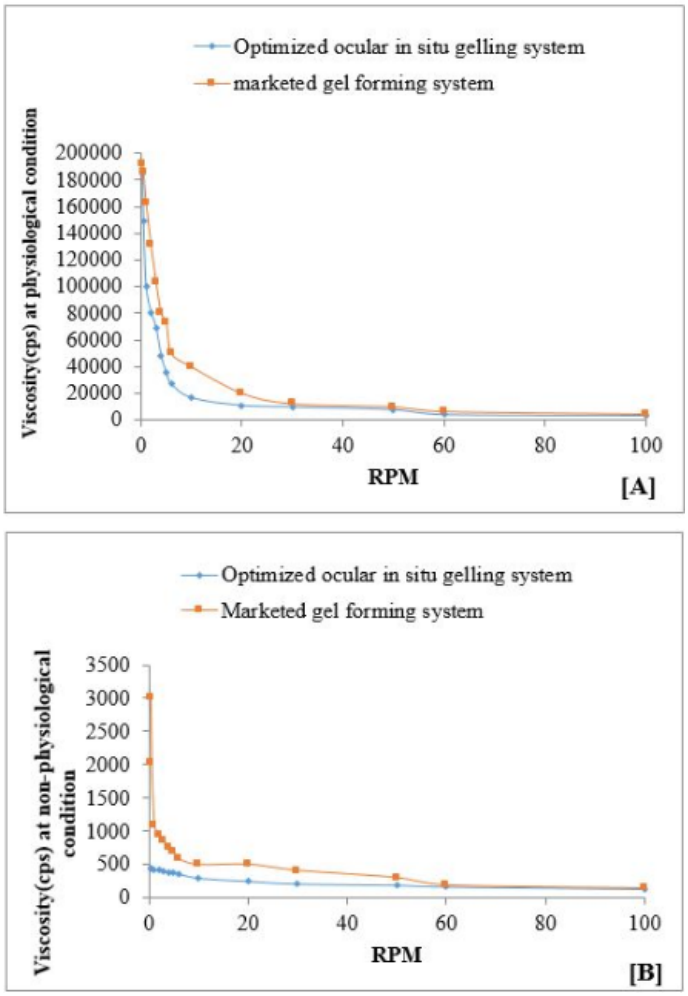

Figure 6. Rheological study at [A] Physiological condition [B] non-physiological condition.

Carbopol 934P shows a mucoadhesive and a pH-sensitive *in situ* gelling property and forms a stiff gel when the pH was raised above its pka value due to the increase in ionization. This leads to an increase in electrostatic repulsion between adjacent carboxyl groups and the subsequent expansion of the polymer network. The cross-linking between carbopol 934P with gellan gum may result in the formation of more viscous gel at the physiological conditions. The combined polymer solution may have enough strength to withstand the turnover and

provide a long precorneal residence time. This also shows that without increasing the concentration of individual polymer solution, the mixed vehicle may be administrated as eye drops and form stronger gel following the phase transition in the ocular cul-de-sac of the eye.

From the optimizations study, the predicted viscosity at non-physiological conditions was between 75-125 cps, while the viscosity of the optimized formulation was found to be 123 cps. Similarly, the viscosity at physiological condition was predicted to be 3200-3600 cps, and experimentally it was found to be 3300 cps for the optimized formulation. The results are shown in Table 8.

3.5.4. Clarity test and pH.

During the clarity testing, the optimized formulation was found clear and transparent visually, which shows the ease of application for treatment in day time use without affecting any visual acuity. The clarity doesn't get affected by the sterilization process. The result of clarity in the form of % Transmittance is shown in Table 8.

The pH of optimized formulation was obtained between the desired pH range of 5.5 and 5.8. The formulation was a clear transparent, free-flowing solution at the set range of pH when formulated. The result is shown in Table 8. Since the pH of the optimized formulation was found within an acceptable range, the formulation would not create irritation but will be well tolerated within the eye. The sterilization process doesn’t show any effect on the pH of the formulation.

3.5.5. *In-vitro* gelling capacity.

Gelling capacity is one of the most important requirements of in situ gelling formulations. The optimum viscosity of the formulation allows easy administration and rapid sol-gel transition at the physiological condition. The gelling capacity of optimized formulation was evaluated on the basis of flowability and visual evaluation of gel stiffness and its retention time. We assessed the gel capacity on a grading scale between – and ++++. The grades of gelation were recorded as: (-) No gelation, (+) weak gelation remains up to 10 min, (+ +) Immediate gelation remains for up to 5 hrs (less stiff gel), (+ + +) Immediate gelation remains for longer period up to 10 hrs (stiff gel), (+ + + +) Immediate gelation remains for extended period for more than 12 hrs (Very stiff gel).

The results showed in Table 8indicate the *in-vitro* gelling capacity of the optimized formulation by means of flowability and visual gelling observation. The authors could not see any change in flowability due to temperature change. The good flow at various temperatures shows the ease of installation in the eye and no gelling in the non-physiological state. However, during the physiological condition in the visual method, the optimized formulation showed immediate gelation within a period of 5-10 seconds. This short gelation time indicates that the formulation will not get drained due to eyelid blinking.

3.5.6. Isotonicity.

The results observed from isotonicity study are depicted in Figure 7. It was observed that the size of RBCs remained unchanged during the exposure with the optimized formulation and was found comparable with the exposure to the normal saline solution (0.9% sodium chloride). The results from the Isotonicity study shows that the optimized formulation is

isotonic with the physiological fluids, and the formulation will not cause any discomfort like irritation or inflammation in the eye.

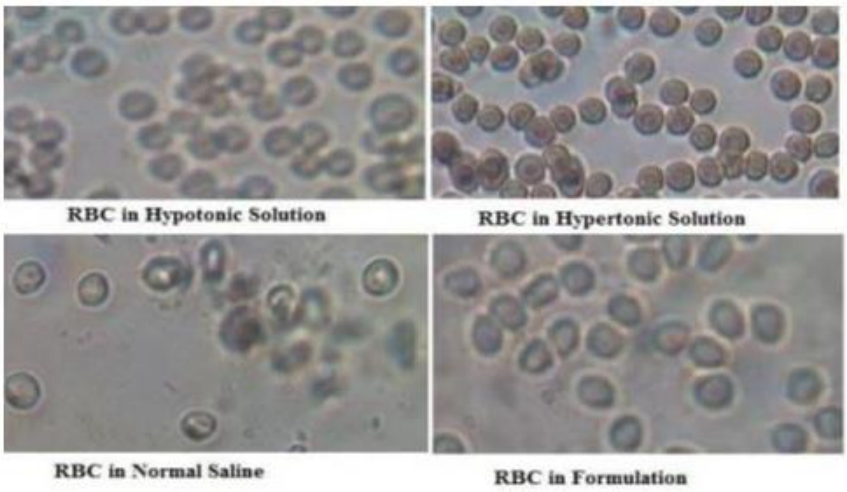

Figure 7. Isotonicity Study at 0.9% Saline at 60x magnification.

3.5.7. Measurement of texture analysis.

Texture profile analysis (TPA) characterizes the mechanical parameters like Gel strength (hardness), mucoadhesion force, gel rupture force, the force of adhesion, compressibility, and adhesiveness. The TPA graph and calculated mechanical properties of the ocular in situ gelling system are presented in Figure 8 and Table 9, respectively. The outcome of texture analysis confirms that Timolol maleate formulation had appropriate mechanical belongings for ophthalmic administration [22].

Table 9. Mechanical properties of ocular *in situ* gelling system.

Sr. No.	Parameter	Result
1	Gel Strength (hardness)	50798 gm
2	Adhesive/Mucoadhesion Force	-73.60 gm
3	Gel Rupture	50798 gm
4	Force of adhesion	0.7310 N
5	Compressibility (AUC)	63748
6	Adhesiveness	-57.46 gm

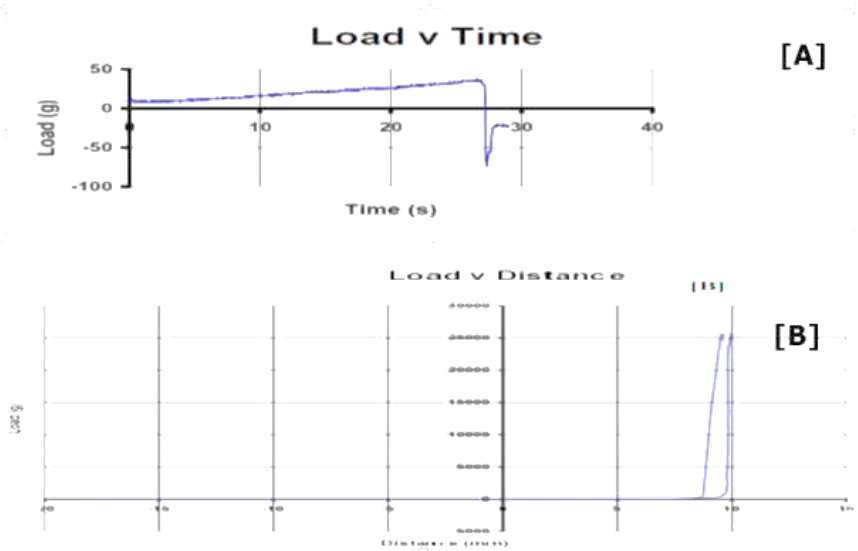

Figure 8. (A) TPA force-time plot of ocular *in situ* gelling system and (B) TPA force-distance plot of ocular *in situ* gelling system.

3.5.8. Measurement of contact angle.

Lower contact angle indicates ease of application and spreading on the ocular surface. The contact angle of the optimized formulation was found significantly lower compared to the marketed gel-forming system. The value of the contact angle on the hydrophilic surface was found to be lower than obtained on the hydrophobic surface. It can be interpreted from the result that the additional polymer in optimized formulation compared to marketed formulation would reduce the contact angle by reducing the surface tension. Hence it can be anticipated that the optimized formulation would give better spreading across the corneal surface compared to a marketed formulation, which would also enhance the permeation of drugs across cornea [26]. The results are shown in Table 10.

Table 10. Contact angle comparison of optimized formulation with marketed formulation.

Sr. No.	Sample details	Measurement surface	Average Contact Angle (θ) ± S.D.
1	Optimized *in situ* gelling formulation	Hydrophilic surface	24.6 ± 3.6
		Hydrophobic surface	92.19 ± 2.8
2	Marketed formulation	Hydrophilic surface	34.2 ± 6.1
		Hydrophobic surface	76.25 ± 5.4

3.5.9. Histopathological evaluation of cornea.

The microscopic observation of corneal structure after incubation with the optimized formulation is shown in Figure 9. It was observed that the epithelium film appeared unbroken and proper without any sign of inflammation and doesn't show any difference with the Phosphate buffer saline-treated cornea. Therefore, it is safe to administer the optimized formulation in the eye. It also shows that benzododecinium bromide is a safe alternative for the prevention of corneal damage compared to other conventional preservatives like benzalkonium chloride.

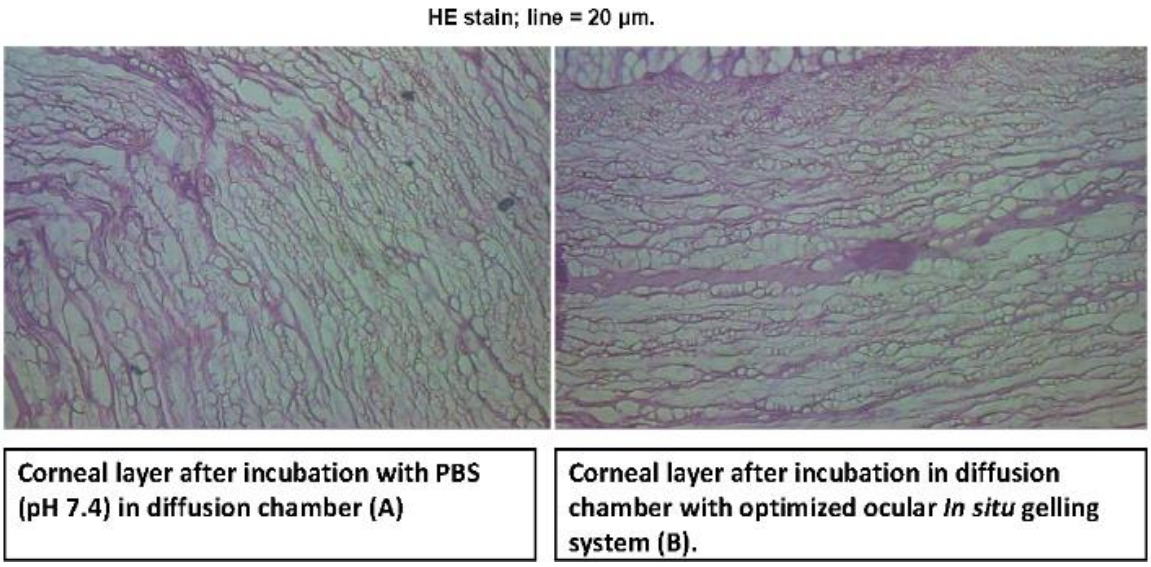

Figure 9. Histopathological evaluation of cornea.

3.5.10. Sterility test.

Sterility testing revealed that there was neither growth nor any evidence of turbidity observed in both media incubated for 14 days. Results assured the sterility of the optimized formulation, and the appropriateness of the sterilization method followed [28].

3.5.11. Antimicrobial effectiveness test.

The selected preservative for optimized formulation should have effectiveness to resist the growth of organisms during the entire shelf life and up to the end-use of the patient. The diameter of the zone of inhibition is shown in Table 11. The Zone of Inhibition (ZOI) values for the optimized *in situ* gelling systems were either similar or higher than the ZOI values of the marketed preparation. Also, the ZOI values against *S. aureus* were found higher than that against *E.coli*. The comparable ZOI values of the optimized formulation with the marketed formulation are due to the prolonged diffusion of the preservative, i.e., benzododecinum bromide from the *in situ* gelling system due to its higher viscosity. The results also suggest the effectiveness of benzododecinium bromide against the micro-organisms under study [29].

Table 11. Antimicrobial efficacy of *in situ* gelling systems against *E.Coli* & *S.Aureus*.

Sr. No.	Concentration (µg/ml)	Zone of Inhibition (cm)				Percentage efficiency (%)	
		Marketed formulation		Optimized *in situ* gelling system		Optimized *in situ* gelling system	
		After 18 hours	After 24 hours	After 18 hours	After 24 hours	After 18 hours	After 24 hours
(A) *E.Coli*							
1	5	16 ± 0.54	19 ± 1.0	16 ± 1.0	24 ± 0.0	100	126.3
2	10	18 ± 0.54	21 ± 1.0	18 ± 0.54	24± 0.54	100	114.3
3	30	21 ± 1.0	22 ± 1.0	21 ± 0.44	24 ± 1.0	100	109.0
(B) *S.Aureus*							
1	5	22 ± 1.0	24 ± 0.0	23 ± 1.0	29 ± 1.0	104.5	120.9
2	10	24 ± 0.44	25 ± 0.44	24 ± 0.54	32 ±1.0	100	128.0
3	30	26 ± 0.54	25 ± 0.54	27 ± 0.44	35 ± 1.0	103.9	140

3.5.12. Accelerated stability study.

During the accelerated stability study, no significant change was observed in optimized formulation after six months with respect to its drug content and viscosity. Accelerated stability studies revealed high stability with the shelf life of 2 years as per the ICH guidelines. The drug degradation rate for optimized ocular formulation was found very low (2.303×10^{-4} day^{-1}). Since the overall degradation was < 5%, the tentative shelf life of 2 years can be assigned to the optimized formulation. The results are described in Table 12.

Table 12. Accelerated stability study of optimized *in situ* gelling systems.

Sr. No	Testing parameters	Storage period (Months) at 40 ± 2°C temperature and NMT 25%RH		
		0 Month	3 Months	6 Months
1	Appearance	Clear	Clear	Clear
2	Clarity (%)	97.0	96.8	96.5
3	Viscosity (cps)	120	118	119
4	Assay of Timolol maleate (%)	98.71%	97.56%	96.73%
5	**Related substances**			
	Timolol related compound B (%)	0.005	0.015	0.081
	Timolol related compound D (%)	0.065	0.215	0.505
	Timolol related compound E (%)	Not Detected	Not Detected	Not Detected
	Timolol related compound C (%)	0.085	0.099	0.107
	Timolol related compound F (%)	0.56	0.68	0.95
	Any highest unspecified impurity (%)	0.001	0.098	0.18
	Total degradation products (%)	0.716	1.107	1.823
6	pH	5.8	6.1	5.9
7	Osmolality (mOsm/kg)	300	310	308
8	*In-vitro* gelling capacity	+++	+++	+++
9	*In-vitro* drug release	97.90%	97.34%	96.23%

3.5.13. *In-vivo* ocular irritation study.

In-vivo ocular irritation study revealed that the optimized formulation is non-irritant to the rabbit eyes. The formulation was very well tolerated and safe for use. Excellent ocular tolerance was noted. Therefore, optimized formulation was apparently as being appropriate and harmless for *in-vivo* utilization.

3.5.14. Intraocular Pressure Reduction studies.

The in-vivo pharmacodynamic study was carried out in an experimental model using two groups of normotensive Rabbits. The normal baseline for IOP was observed 15.05mmHg. No significant day to day variation ($p = 0.423$) was observed in the normal IOP measurement for each animal. There was no significant difference ($p = 0.348$) detected in both groups. The IOP reduction in both treated groups was found similar, as showed in Figure 10. To eliminate fluctuations due to diurnal IOP variations, the IOP values were expressed as the difference from the corresponding baseline values.

As described in the drug release study earlier, the *In-vitro* drug release profile showed sustained drug release, which is reassured by the *in-vivo* study, which showed a sustained therapeutic effect (reduction in IOP). The results suggest the potential of optimized formulation for sustained drug delivery. An IOP reduction study indicates that optimized formulation was equally efficacious with less variability in the reduction of IOP among the subjects when compared to marketed formulation. It also demonstrates that once-daily dosing is enough for the optimized formulation of Timolol maleate for ophthalmic delivery. It is noteworthy to mention that during the *in-vivo* pharmacodynamic study in rabbits, the eyelids, conjunctiva, and cornea were visually observed. The result of this test showed no opacity, conjunctival chemosis, redness, discharge, or no iris alteration. It can be said from the above observations that the optimized formulation is non-irritating.

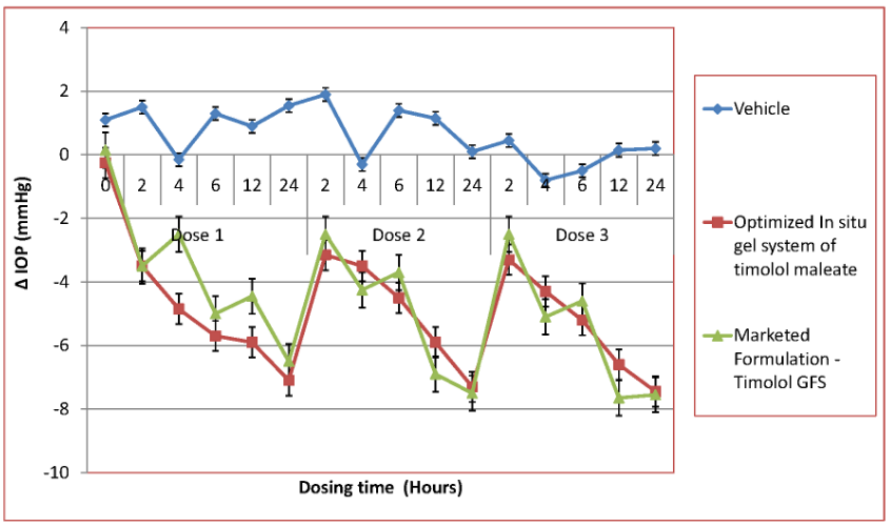

Figure 10. Graphical representation of data for the IOP study.

4. Conclusions

We have explored the development and optimization of *in situ* gelling systems of Timolol maleate for the ocular application using Box-Behnken design employing ion-sensitive gellan gum, mucoadhesive/pH-sensitive carbopol 934P and benzododecenium bromide as

corneal permeability enhancer and preservative. Upon administration into the eye, the formulation transforms from solution to gel with desired viscosity state by simultaneous dilution with tear fluid, which increases ocular residence time. The optimized formulation exhibited all the desirable attributes of an ideal ophthalmic formulation and was found to be stable and non-irritant to the eye. The *in-vitro* drug release studies demonstrated that the prepared system exhibits controlled drug release as compared to the marketed ophthalmic solution. *In- vivo* study indicated that the present formulation would be able to offer benefits, such as increased drug residence time, controlled drug release, reduction in dose frequency, and thereby improve patient compliance. The developed formulation using simultaneous pH and ion mediated gelling provides high gelling capacity without increasing individual polymer concentration. The formulation provides a long precorneal residence time with a mucoadhesive polymer. The investigation also suggests the effectiveness of preservative as a penetration enhancer.

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Conflicts of Interest

The authors declare no conflict of interest.

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