Journal of Pharmaceutical Research International

33(28A): 58-77, 2021; Article no.JPRI.67993
ISSN: 2456-9119
(Past name: British Journal of Pharmaceutical Research, Past ISSN: 2231-2919, NLM ID: 101631759)

Formulation, Optimization and Evaluation of Ion Triggered Ophthalmic *in Situ* Gel

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Authors' contributions

This work was carried out in collaboration among all authors. Amol Tagalpallewar designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Rai Prajvita managed the analyses of the study. Satish Polshettiwar, Manish Wani, and Akshay Baheti. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JPRI/2021/v33i28A31511
Editor(s):
(1) Dr. Syed A. A. Rizvi, Nova Southeastern University, USA.
Reviewers:
(1) Naida Omerović, University of Sarajevo, Bosnia and Herzegovina.
(2) Hanitrarimalala Veroniaina, China Pharmaceutical University, China.
Complete Peer review History: http://www.sdiarticle4.com/review-history/67993

Original Research Article

Received 22 February 2021
Accepted 29 April 2021
Published 04 May 2021

ABSTRACT

Topical eye drop is the convenient and patient compliant route of drug administration, particularly for the treatment of anterior part diseases. Transport of drugs to the targeted ocular tissues is limited by various precorneal, active and stationary ocular barriers. The aim of developed, optimized and evaluated ion sensitive brimonidine tartrate *in situ* gel is patient compliance and maximum therapeutic activity in the treatment of glaucoma. The effect of independent variables that are polymer concentration on dependent variables like the percent drug release, gelling time and viscosity was studied. The optimized formulation was further evaluated for ex-vivo study and histopathology study. Experimental study showed that optimized *in situ* gel formulation (F6) showed *in vitro*, *ex vivo* sustained release with polymer sodium alginate and hydroxypropyl methyl cellulose (HPMC) K4M. The optimized formulation F6 presented increased retention time upto 8 hours. The developed *in situ* gel can be a promising ophthalmic formulation to increase retention time of

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formulation and hence it will reduce the intra ocular pressure. The histopathology studies reveals the safety of prepared formulation. The stability studies revealed no significant change in the drug content and physical properties.

Keywords: Glaucoma; brimonidine tartarate; sodium alginate; hydroxy propyl methyl cellulose; factorial design.

1. INTRODUCTION

The eyes are one of the most important and complex sensory organs; they act as a gateway to collect external images and transmit them to the brain as signals through the optic nerve. By this process eyes maintain a connection between the body and surroundings. Various diseases, such as inflammations or bacterial and viral infections, affect the function of the eye. Most of the diseases affecting anterior eye tissues can be easily treated with high doses of drugs. However, diseases affecting posterior tissues are difficult to reach and treat. Age related macular degeneration, glaucoma and endophthalmitis are some of the common posterior eye diseases that may lead to vision loss if not treated. The complex anatomy, physiology and biochemistry of the eye render this organ highly impervious to drugs. To provide an effective treatment of diseases affecting both anterior and posterior ocular tissues, a close examination of ocular anatomy, physiology and barriers is of great importance [1]. Glaucoma is a serious eye disorder characterized by an increase in intra ocular pressure, which gradually leads to vision loss of due to damage of the optic nerve with no symptoms. Glaucoma development may be observed due to imbalance between aqueous humor secretion and drainage processes within the ocular chamber. There are several drugs available to treat these conditions, most commonly used dosage form is eye drops. Economical cost, ease of production, patient compliance and tolerability are the features that made eye drops wide acceptable for glaucoma patient. However, eye drops have major problems such as short residence time, poor bioavailability, poor permeability and rapid precorneal drainage [2].

Due to several disadvantages of conventional eye drops, long acting ophthalmic drug delivery systems are needed for better patient compliance, improved local bioavailability, reduced dose and dosing frequency. It is believed that *in situ* gel drug delivery system can address some of the problems associated with conventional eye drops. *In situ* gel offers several advantages such as sustained prolonged action, simpler production techniques with low cost of manufacturing as compared to conventional drug delivery system [3-4]. *In-situ* forming gels refers to polymer solution that can be administered as liquid upon instillation and undergo phase transition in the ocular *cul-de-sac* to form viscoelastic gel and this responds to environmental changes. Gelation can be triggered by temperature, pH and ions. An *in-situ* gel system should be a low viscous, free flowing liquid to allow for reproducible administration to the eye as drops and the gel formed following phase transition should be strong enough to withstand the shear forces in the *cul-de-sac* and demonstrated long residence times in the eye. This may increase residence time of in situ formed gel along with its ability to release drugs in sustained manner which will enhance the bioavailability, reduce systemic absorption and the need for frequent administration leading to improved patient compliance [5].

The common types of *in situ* gels are: temperature dependent *in situ* gels, Ion sensitive *in situ* gels, pH sensitive *in situ* gels.

In situ gel based drug delivery systems consist of active pharmaceutical ingredients, polymer and excipients. Sodium alginate, family of linear unbranched polysaccharides, the sodium salt of alginic acid, is a natural hydrophilic polysaccharide containing two types of monomers, β-D-mannuronic acid (M units)and α-L glucuronic acid (G units) residues. This polymer undergoes instantaneous gel formation due to formation of calcium alginate by of its interaction with divalent cation(Ca^{+2}) present in lachrymal fluid (pH 7.4).Alginates can be crosslinked ionically in the presence of divalent cations. Hydroxy Propyl Methyl Cellulose(HPMC) is in corporated as a viscosity enhancer to further aid in accomplishment of sustained drug delivery. HPMC is semisynthetic, inert, viscoelastic polymer which is non-ionic, nontoxic, a good carrier for pharmaceutical application that exhibits high swellingcapacity [2,5]. Brimonidine tartrate is commonly used drug in glaucoma therapy, which is a selective alpha - 2 adrenergic

agonist. It is one of the most important drug widely used in glaucoma treatment. It mainly acts by reducing aqueous humor production and increasing uveoscleral outflow.

2. MATERIALS AND METHODS

2.1 Materials

Brimonidine tartrate was obtained from FDC Ltd (FDC Ltd. Mumbai, India)and Protanal CR 8133fromFMC. The HPMC K4M was purchased from Sigma Aldrich chemicals, Bangalore. All other ingredients and reagents were of research grade.

2.2 Methods

2.2.1 Preparation of *in situ* gels

Sodium alginate, an ophthalmic gel-forming mucoadhesive polymer was chosen as the polymer and hydroxyl propyl methyl cellulose (HPMC) as a viscosity modifier. Cold method was used to prepare Brimonidine tartrate *in situ* gel. Half of the desired volume of distilled water, containing accurately weighed HPMC K4M, was kept in the refrigerator (for cooling) to get a clear solution. The desired amount of Sodium Alginate was added to HPMC K4M solution with continuous stirring and solution was stored at 4℃ to obtain clear solution. The solution of the desired amount of drug, sodium chloride and benzalkonium chloride was prepared in distilled water. This drug, sodium chloride and benzalkonium chloride solution was mixed with the polymeric solution under constant stirring to get clear solution [7]. Purified water was then added to make up the volume to 100 ml. The formulations were then subjected to terminal sterilization by autoclaving at 121°C, 15 p.s.i. for20 mins.

3. CHARACTERIZATION OF BRIMONIDI-NE TARTRATE

3.1 Organoleptic Properties

The sample of brimonidine tartrate was studied for organoleptic characteristics such as color, odor and appearance.

3.2 Melting Point

Melting point of brimonidine tartrate was determined by the capillary method. Brimonidine tartrate was filled into the one sided sealed capillary then the capillary in oil bath. The temperature at which the drug melts was recorded.

3.3 FT-IR Spectroscopy

The representative sample of brimonidine tartrate was mixed with IR grade KBr in 1:100 ratios and triturated to obtain uniform blend. This blend was dried for 10 min under IR lamp and then subjected to Fourier transform infrared spectroscopy (FT-IR) (Jasco FTIR 4100) scan in range of 400- 4000 cm^{-1}.

3.4 DSC Study

Differential scanning calorimetry (DSC) analysis was performed by using (DSC METTLER TOLEDO DSC 1 STARe). Approximately 5 mg of brimonidine tartrate was weighed into an aluminium pan and sealed hermetically. DSC scan was recorded from 30°C to 250°C at a heating rate of 10°C/min under nitrogen purge.

3.5 HPLC Method Development

3.5.1 Estimation of λ_{max}

To determine the λ_{max} of brimonidine tartarate a solution of 100 µg/ml was prepared in distilled water and simulated tear fluid. The absorbance were scanned over a wavelength range of 200-400 nm on ultraviolet –visible (UV-Vis) spectrophotometer **(Jasco V-630)**. The λ_{max} of brimonidine tartarate was found to be 253 nm.

3.5.2 Solutions preparation for HPLC method development

i) Preparation of stock solution

Standard stock solution of brimonidine tartrate was prepared by dissolving 100 mg drug in 100 ml methanol (1000 ug/ml).

ii) Preparation of citric acid buffer

Citric acid buffer: 0.01 M Citric Acid Monohydrate Buffer. Accurately weighed citric acid monohydrate (1.05 gm) was transferred to a beaker and dissolved in double distilled water (500 ml). pH adjusted to 3 by using trimethylamine.

Determination of λ max **:** 1ml of each stock solution (100 µg/ml) was further diluted to 10 ml with distilled water and ATF to produce 10

µg/ml solutions. The resulting solution (10µg/ml) was scanned over range of 200 – 400 nm on UV spectrophotometer.

4. FULL FACTORIAL EXPERIMENTAL DESIGN

A 3^2 randomized full factorial design was used for optimization of brimonidine tartrate *in situ* gel and to study the effect of concentration of Sodium alginate and HPMC K4M on the gel formulation. The amount of sodium alginate and HPMC K4M was selected as independent variables, in this study. These two factors were evaluated, each at three levels. The actual units of higher, middle and lower levels of factor X1(sodium alginate concentration) were 0.5 %, 1 % and 1.5 % and for factor X2 (HPMC K4M concentration) 0.2, 04 and 0.6%. The coding was +1, 0 and -1 respectively for higher, middle and lower levels of each factor. The percent cumulative drug release at 8 h. (Y1), viscosity at R.T. (Y2) and gelling capacity (Y3) were dependent variable or response. Design expert(Version 10.0 Stat Ease Inc., Minneapolis ,MN) 10.0 software was used for studying effect of independent variables on responses. Various models such as Linear, 2 FI and Quadratic were fitted to the data and the model which fit well was suggested by software and was tested for analysis of variance (ANOVA). Regression polynomials were calculated for the individual independent variables and then counter plots and three -dimensional (3D) surface plots were obtained for each individual dependent variable.

5. VALIDATION OF MODEL

The optimized compositions of Brimonidine tartrate *in situ* gel required to obtain desired responses. Any composition with desirability near to one can be chosen as optimized formulation.

6. PHYSICOCHEMICAL EVALUATION

i) Clarity: Clarity is one of the most important characteristic feature of an ophthalmic preparation. The clarity of formulations before and after gelling was determined by visual inspection under a black and white background [5].

ii) pH: The developed formulations were evaluated for pH value by preparing a1% aqueous solution of prepared gel using calibrated Equip- Tronics digital pH meter model EQ- 610 [8].

iii) *In vitro* gelation study: Gelling capacity was determined by placing a drop of formulation in vial containing 2 ml of freshly prepared ATF pH 7.4 and equilibrated at 37°C. The gel formulation was visually evaluated for gelation time and time taken for gel to dissolve was recorded. All prepared formulations were evaluated for gelling capacity to check *in situ* gelation. The prepared formulation should remain as gel for extended period [9].

B. Viscosity study: The viscosity of the formulation was determined by using Brookfield DV II +pro viscometer equipped with a spindle number D. Viscosity of sample solutions was measured at different angular velocities at a R.T. A typical run comprised of changing the angular velocity from 5 to 100 rpm at a controlled ramp speed. After 10 seconds at 5 rpm, the velocity was increased up to 100 rpm with a similar wait at each speed. The hierarchy of angular velocity was reversed (100 rpm to 5 rpm) with a similar wait of 10 seconds. Evaluations were conducted in triplicate [10].

C. *In vitro* drug release: *In vitro* drug release was carried out by using Franz diffusion cell in triplicate. Freshly prepared ATF (NaCl 0.67g, $NaHCO_3$ 0.20g, $CaCl_2.H_2O$ 0.008g and deionized water to produce 1000ml) was placed in receptor compartment. Previously soaked dialysis membrane of pore size 0.22µm was placed between receptor and donor compartments. The whole assembly was kept on the thermostatically controlled magnetic stirrer and the temperature of medium was maintained at $37^oC \pm 0.5^oC$. The medium was continuously stirred at 50 rpm. One ml of formulation was placed in the donor compartment. Samples (0.5ml) were withdrawn at predetermined time interval of 1h to 8h and same volume was replaced. The withdrawn samples were diluted to 10 ml by ATF and analyzed by RP-HPLC. Each of the *in vitro* release tests was repeated three times. The per cent of cumulative drug release was calculated [2,11].

D. Release kinetic models: *In vitro* drug release data analyzed with various kinetic models. Such as zero order, first order, Higuchi model, Hixson-Crowell model and korsmeyer Peppas model. The zero order rate describes the systems where the drug release rate is independent of its concentration. The first order describes the

release from system where release rate is concentration dependent. Higuchi model describes the release of drugs from the insoluble matrix as a square root of time dependent process based on Fickian diffusion. The Hixson-Crowell cube root law describes the release from systems where there is a change in surface area and diameter of particles. Korsmeyer Peepas model derives a simple relationship which described the drug release from a polymeric system [2].

E. Content uniformity: Uniform distribution of an active ingredient is important to achieve dose uniformity. The drug content was determined by diluting 1 ml of the formulation to 10 ml with ATF. Aliquot of 1 ml was withdrawn and further diluted to 10 ml. Brimonidine tartrate concentration was then determined at 253nm by using RP-HPLC [10].

F. Sterility testing *as per I.P 2014:* Sterility testing was performed as per I.P. 2014 by using direct inoculation method. The test was carried out to detect presence of viable forms of microorganisms in the sterilized formulation. The test is based on the principle that if the nutrient media is provided to sterilized formulation and and favorable condition for growth is maintained and microbes grow, the presence is indicated by turbidity in medium.

7. CULTURE MEDIA

FTM was used as culture media for bacteria while soyabean casein digest medium was used for fungi. Media were prepared and sterilized in test tubes by autoclaving at 121°C at 15 lb/inch gauge pressure for 20 minutes.

7.1 Test Sample Preparation

two ml of the optimized formulation were taken for the sterility test and inoculated into FTM and 2 ml into soyabean casein digest medium.

7.2 Positive and Negative Control

Pseudomonas aeruginosa microbial suspension in media acted as positive control. Sterile media without test sample acted as negative control

7.3 Incubation

The inoculated culture media for bacteria and fungi were incubated at 30 -35°C and 20 -25°C respectively in BOD incubator for 14 days [12].

G. Preservative efficacy study *as per I.P. 2014:* Preservative efficacy study of brimonidine tartrate *in situ* gel was performed as per I.P.2014 by challenging the optimized formulation with *Staphylococcus aureus.* Benzalkonium chloride was used in 0.01 % v/v concentration as a preservative in the optimized formulation.

7.4 Procedure

The serial dilution method was used to adjust the colony count to about 1×10^5 to 1×10^6 with sterile saline solution, 0.1 ml microbial suspension mix with 20 ml formulation. Inoculated containers were incubated at 20°C to 25°C. The viable count (by plate count method) at 7, 14, 21 and 28 days was determined.

Preservative is effective in the product if –

a) Concentration of viable bacteria is not more than 0.1 % of initial concentrations by the 14th day
b) Concentration of each test microorganism remains at or below these designated levels during the remainder of 28 day test period.

H. *Ex vivo* permeation study: Transcorneal permeability of the drug was evaluated by using goat cornea. The fresh whole eyeballs of goat were obtained from local slaughter shop and transported to a laboratory in normal saline solution (4°C). The cornea was then carefully excised along with 2-4 mm of surrounding sclera tissue and washed with saline solution.The excised cornea was placed between the donor and receptors compartment of the Franz diffusion cell in a way that epithelial surface faced the donor compartment. The receptor compartment contained freshly prepared ATF [13]. The whole assembly was placed on a thermostatically controlled magnetic stirrer at 37°C ± 0.5°C and 50 rpm stirring rate was maintained. One ml of prepared formulation was placed in donor compartment. The samples were withdrawn at different time intervals and analyzed for drug content with the help of RP-HPLC. The receptor compartment was replenished with an equal volume of ATF after withdrawing the sample. The percent drug released was plotted against time to get the dissolution rate curve [2,14].

I. Histopathology study: To evaluate the effect of *in situ* formulation on the corneal structure and the irritation potential, corneas were removed from the eyes of freshly sacrificed goat and

incubated at 37°C for 5 h in the formulation. Sodium dodecylsulfate (SDS) solution in phosphate buffer saline (PBS) 0.1% (w/w) was used as a positive control. After incubation, corneas were washed with PBS and immediately fixed in formalin (8% w/w). Tissues were dehydrated in an alcohol gradient, placed in melted paraffin and solidified in block form. Cross sections were cut, stained with haematoxylin and eosin (H&E) and observed microscopically for any modifications [15].

J. Stability studies as per ICH Guideline (Q1A R2): The optimized formulation was stored in a stability chamber (Bio Technics, India.) at 40°C and 75% RH for 3 month and samples were evaluated for physicochemical parameters like appearance, pH value , drug content and the drug release at 1 month interval for 3 months.

8. RESULTS AND DISCUSSION

8.1 Characterization of Brimonidine Tartrate

8.1.1 Organoleptic properties

The sample of brimonidine tartrate was found to be a clear, colorless to slightly yellow powder.

8.1.2 Melting point

Melting point of brimonidine tartrate was determined by the capillary method and was found to be in the range of 209°C to 210°C which comply with reported melting point (209°C) [16].

8.1.3 FTIR spectroscopy

FTIR studies were used to get unique structural information extracted from the spectral data. IR absorption spectrum of brimonidine tartrate was taken in the range of 400–4000 cm^{-1} with KBr using IR spectrophotometer (Jasco FTIR-4100). Major functional groups present in brimonidine tartrate showed characteristic peaks in IR spectrum. The major peaks are identical to functional group of brimonidine tartarate. Hence, the sample was confirmed as brimonidine tartrate Fig.1, Table 1. The FTIR spectrum of brimonidine tartrate and excipients is shown in Fig. 2. Characteristic functional groups of brimonidine tartrate were observed in spectra. There was no significant change observed in FTIR spectra of blend which indicates that the drug may be compatible with excipients [6].

8.1.4 DSC study

Thermal behavior of brimonidine tartrate was studied by using DSC. The characteristic endothermic peak of the pure drug and drug with excipients was observed at 96.73°C and 214°C Fig.3 and Fig. 4 which corresponded to its melting point which indicated the purity of the sample and its crystallinity [17].

8.2 Development of Analytical Method for Brimonidine Tartrate by HPLC Method

A. Determination of λ max of brimonidine tartrate: The sample of brimonidine tartrate shows the λmax about 253nm which complied with the reported literature. The UV spectra of drug the in various media are shown in Fig. 5 and Fig.6.

B. HPLC Method development: The aim was to develop simple RP-HPLC method which can be used to analyze drug in the process of product development. The method development was carried out according to a literature survey. Different trials were carried out by using different combinations of citric acid buffer (pH = 3) and methanol. The optimized condition of HPLC analysis is shown in Table 2.

Table 1. FT-IR spectra of brimonidine tartrate

Functional groups	IR frequencies reported peak (cm^{-1})	IR Frequencies observed peak (cm^{-1})
C-Br (S)	960-515	960.37
C-C (S)	1200-1385	1287
N-O	1345-1385	1351
C=N	2220-2400	2324

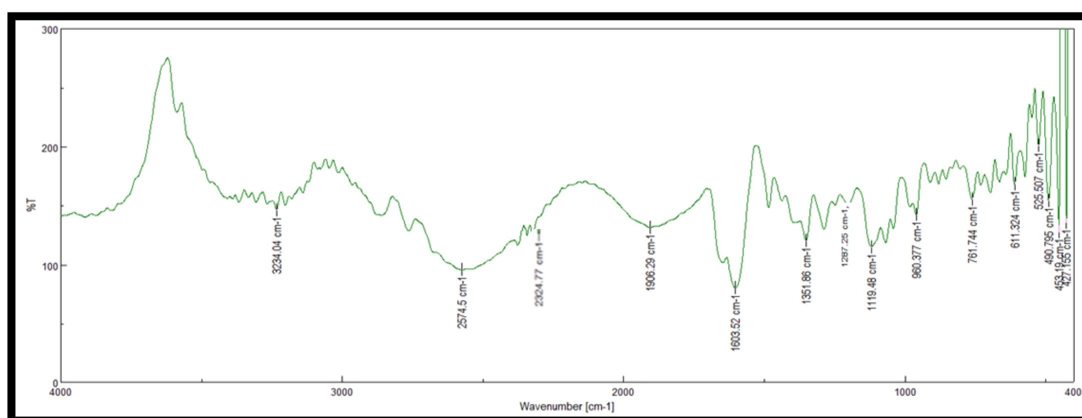


Fig. 1. FT-IR spectra of brimonidine tartrate

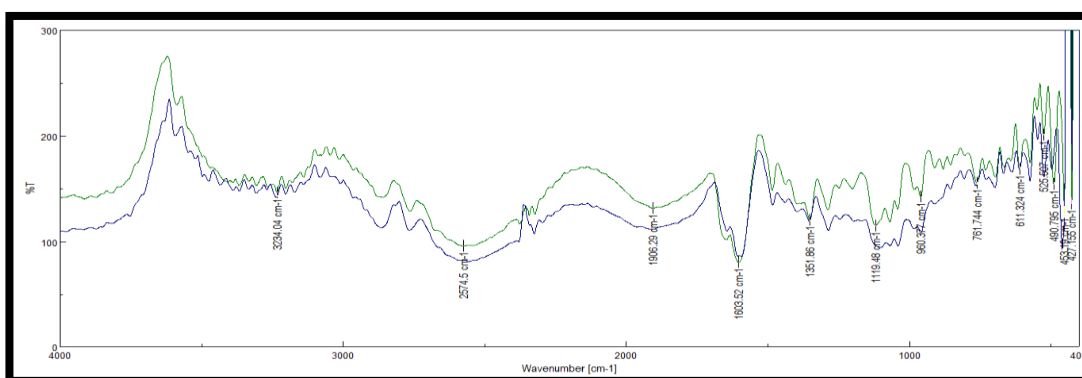


Fig. 2. FT-IR spectra of brimonidine tartarate and excipients mixture

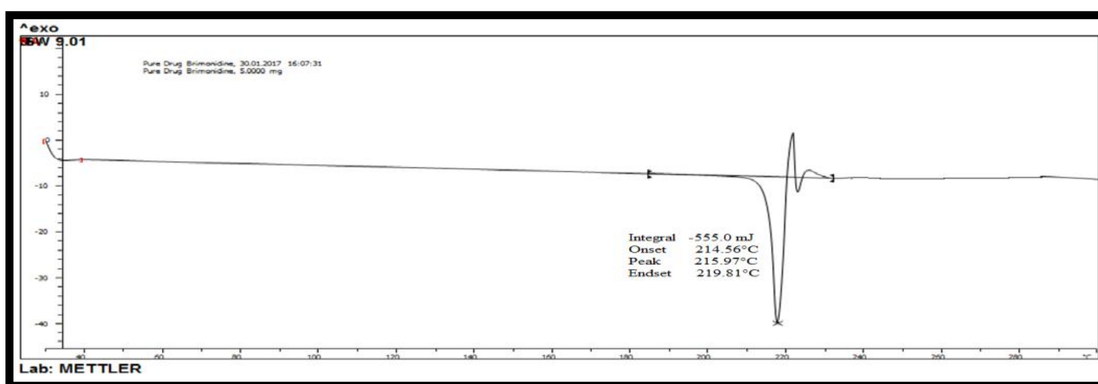


Fig. 3. DSC thermogram of brimonidine tartrate

Table 2. The optimized data of HPLC analysis

Mobile Phase	0.01 Mol/L Citric acid monohydrate: Methanol : Water (30:20:50) pH3 maintained by using triethylamine
Column	Kromasil – C 18 Column (250 mm × 6.5 mm × 5 um)
Flow rate	1 ml/min
Detection wavelength	253 nm
Injection volume	20 µl
Run time	10 minutes
Retention time	6 mins

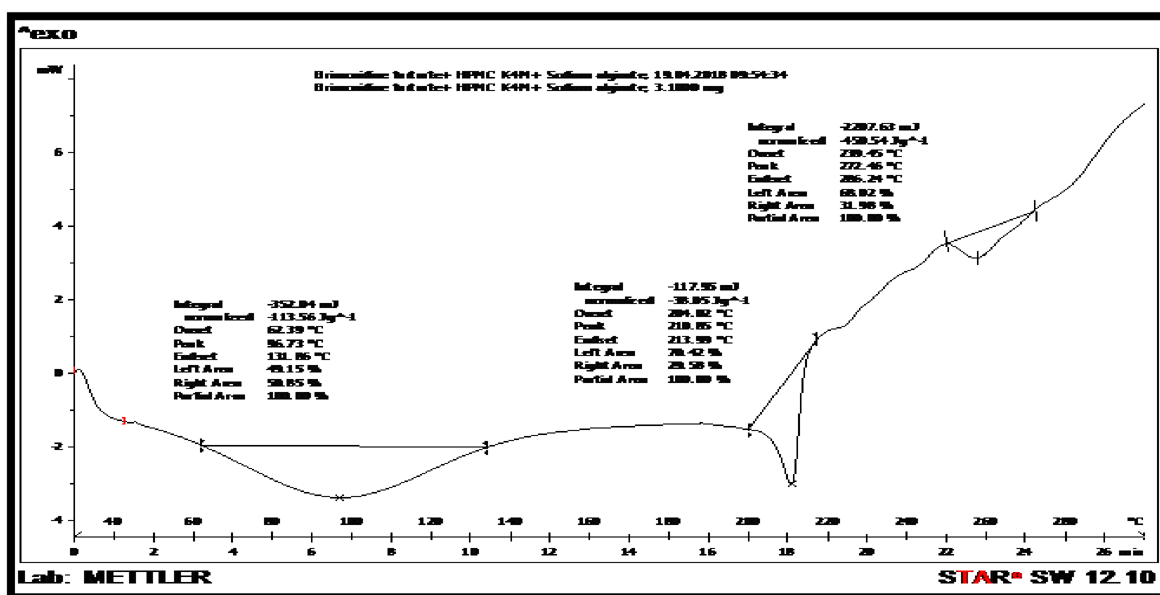


Fig. 4. DSC thermogram of brimonidine tartrate and excipients mixture

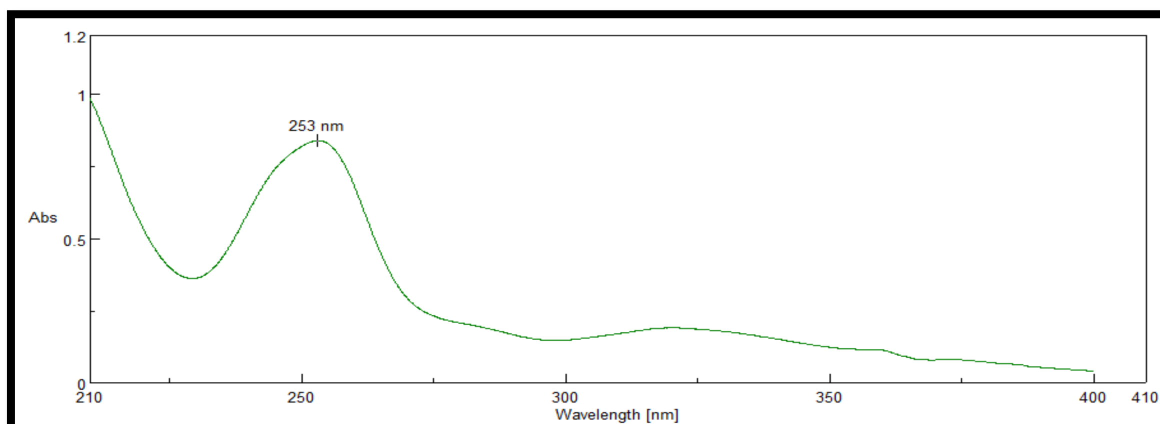


Fig. 5. UV spectra of brimonidine tartrate in distilled water

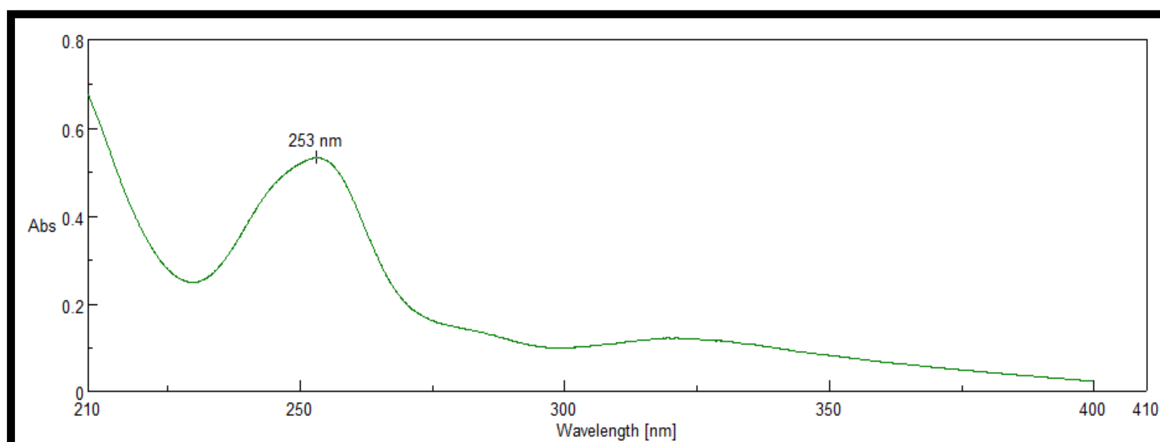


Fig. 6. UV spectra of brimonidine tartrate in tear fluid

8.3 Formulation and Optimization of Brimonidine Tartrate *in Situ* Gel

Design expert (Version 10.0, Stat-Ease Inc., Minneapolis, MN) software was used for studying the effect of independent variables on responses. Various models such as Linear, 2 FI and Quadratic were fitted to the data and the model which fit well was suggested by software and tested for (ANOVA). Regression polynomials were calculated for the individual independent variables and then contour plots and 3D surface plots were obtained for each individual dependent variable. Mathematical models were generated for studying the effect of independent variable (X1 and X2) on dependent variable (Y1, Y2 and Y3) or response (R) and expressed equations (1) - (3). The 3^2 factorial design selected for studying effect of independent variables Sodium Alginate (X1) and HPMC K4M concentration (X2) on dependent variables the percent cumulative drug release, viscosity and gelling capacity. The Experimental design layout developed for nine possible combinations of brimonidine tartrate *in situ* gel formulations is shown in Table 3. Explained quadratic equations obtained from software were used to explain the effect of independent variables on responses. High R^2 value indicated best fitted quadratic model. The conclusion can be drawn with the help of polynomial equations by considering mathematical signs (positive or negative) and the magnitude of the coefficient [18].

8.3.1 Effect of formulation variable on the percent cumulative drug release

In *vitro* drug release study: The release of the drug is faster from the solution as compared to the polymeric matrix system. Effect of viscosity on the drug diffusion was described by the Stokes-Einstein equation which says that by increasing the viscosity of formulation, decreases the rate of diffusion. Through gel matrix into receptor compartment. The *in vitro* release data of various formulations are shown in Fig. 7.

A model "F value" could occur due to noise. Values of "Prob> F" less than 0.0500 indicate that model terms were significant. In this case X1, X2, X12and X22 were significant model terms.

The model for response Y 1 is as follow:

$$Y1 = 95.07 - 0.8250X_1 - 1.76X_2 + 1.66X_1X_2 - 1.64X^2_1 - 1.68X^2_2$$

From eq. (1) it is clear that drug release rate appeared to decrease with an increasing amount of factor X1 (concentration of sodium alginate) and factor X2 (concentration of HPMC K4M). The release of the drug was found to be dependent on concentration of gelling agent and drug release modifier. The combined effect of factor X1 and factor X2 can be further interpreted with the help of a cuntour plot and 3D response surface plots Fig. 8 showing the effect of concentration of sodium alginate and HPMC K4M on the percent of cumulative drug release at 8 h.

8.3.2 Effect of formulation variables on viscosity (viscosity study)

At R.T the formulations were in a liquid state and exhibited low viscosity. Solutions were administered into eye and were converted into a gel with high viscosity Fig.9. Viscosity of the formulation decreases by increasing the angular viscosity.

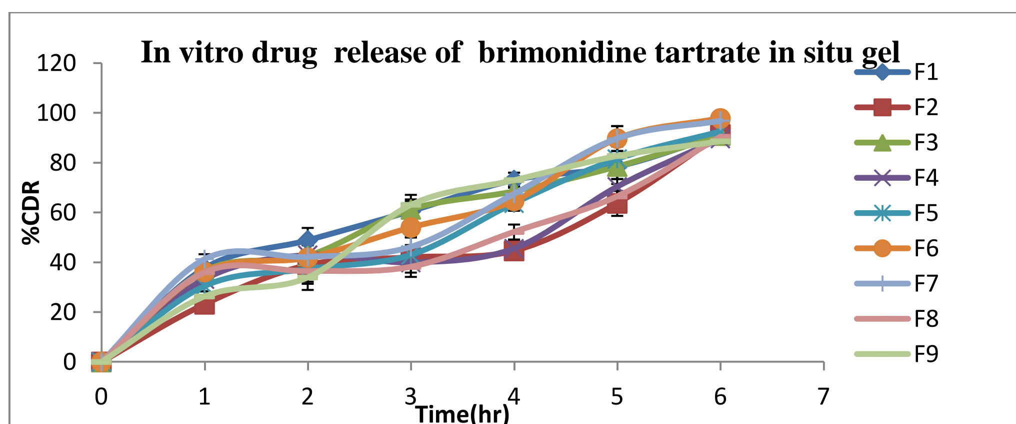
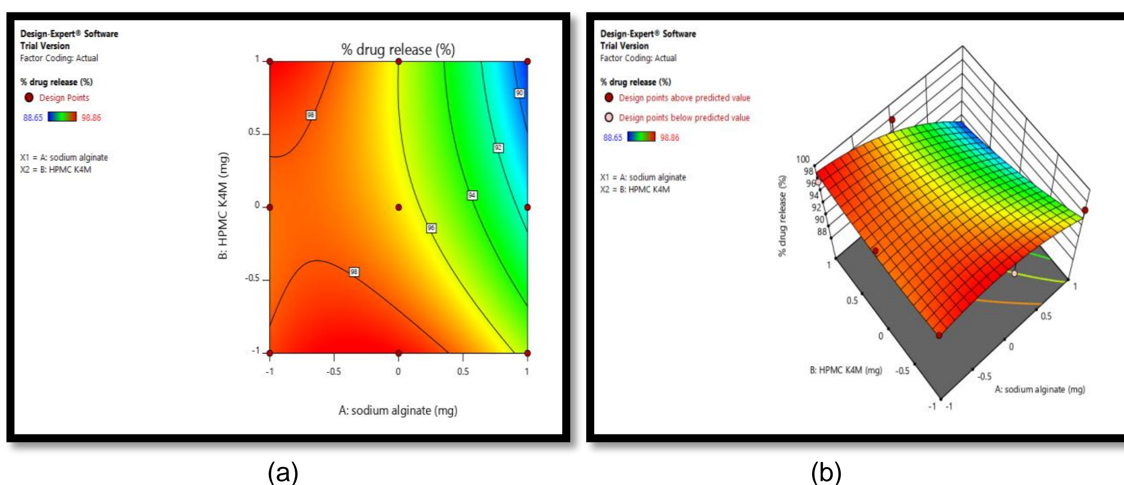


Fig. 7. *In vitro* drug release of brimonidine tartrate *in situ* gel

Table 3. Experimental design layout of brimonidine tartrate *in situ* gel Formulations

Batch Code	Factor X1(Sodium Alginate)	Factor X2 (HPMC K4M)	Response 1(Y1)	Response 2(Y2)	Response 3(Y3)
	Coded levels of variables		**Gelling time (S)**	**Viscosity (cps)**	**% Cumulative drug release**
F1	-1	-1	30	76.85	98.76
F2	-1	0	26	136.00	98.23
F3	-1	+1	18	182.00	97.56
F4	0	-1	20	196.60	97.14
F5	0	0	22	205.10	96.45
F6	0	+1	21	218.20	97.82
F7	+1	-1	20	232.30	96.85
F8	+1	0	15	278.15	89.61
F9	+1	+1	12	287.65	88.65



(a) (b)

Fig. 8. (a) Two dimensional counter plot, (b) dimensional (3 D) response surface plot for response Y3 (The percent of cumulative drug release at 8 h)

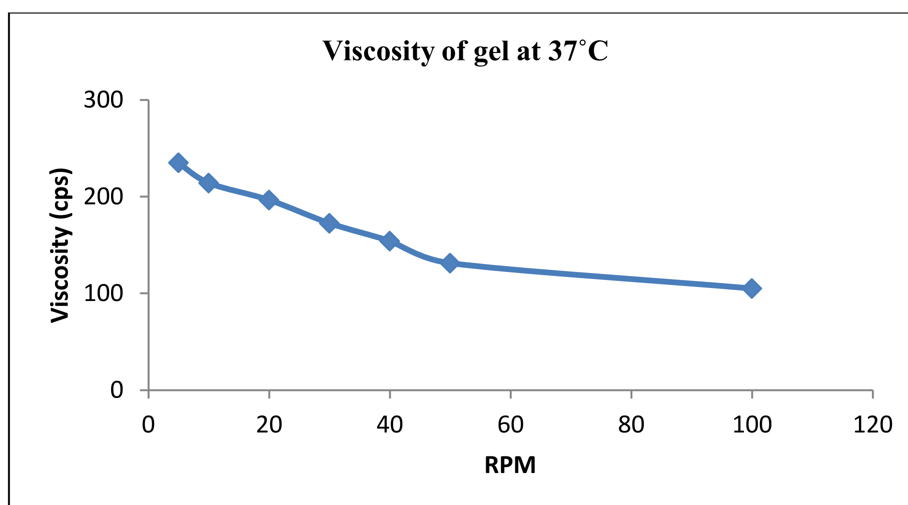


Fig. 9. Viscosity of brimonidine tartrate *in situ* gel in ATF

by applying factorial design, quadratic model was suggested by software for response Y2 and found to be significant with model F value 9.66, p value of 0.0455 and R^2 value of 0.9752 which implied that model was significant,and there was only a 4.55% chance that a "model F- value" this could occur due to noise. Values of "Prob> F" less than 0.0500 indicated that the model terms were significant. In this case X1, X2, X12and X22 were significant model terms [19]

The model for response Y2 is as follows:

$Y2 = 169.78 + 54.00X_1 + 63.67X_2 +48.00X_1X_2 - 53.33X^2_1 +0.66X^2_{2\ eq}$ (2)

From eq. (2) it is clear that the viscosity rate appeared to increase with an increasing amount of factor X 1 (concentration of Sodium alginate) and factor X2 (concentration of HPMC K4M).

The combined effect of factor X1 and factor X2 can be further interpreted with the help of contour plot and 3D response surface plots Fig. 10. showing the effect of concentration of sodium alginate and HPMC K4M on gel viscosity at R.T.

8.3.3 Effect of Formulation Variables on Gelling Time (Viscosity Rate)

By applying factorial design, quadratic model was suggested by software for response Y3 and found to be significant with model F value of 13.15, p value of 0.0297 and R^2 value of 0.9910. which implied that the model was significant,and there was only a 2.97 % chance that a "model F-

value” this could occur due to noise. Values of “Prob> F” less than 0.0500 indicate that the model terms were significant. In this case X1, X2, X12and X22 were significant model terms.

The model for response Y3 is as follows:

$$Y3 = 17.33 + 3.00X_1 - 3.67X_2 + 3.75X_1X_2 + 1.00X^2_1 - 5.00X^2_2 \quad \text{eq (3)}$$

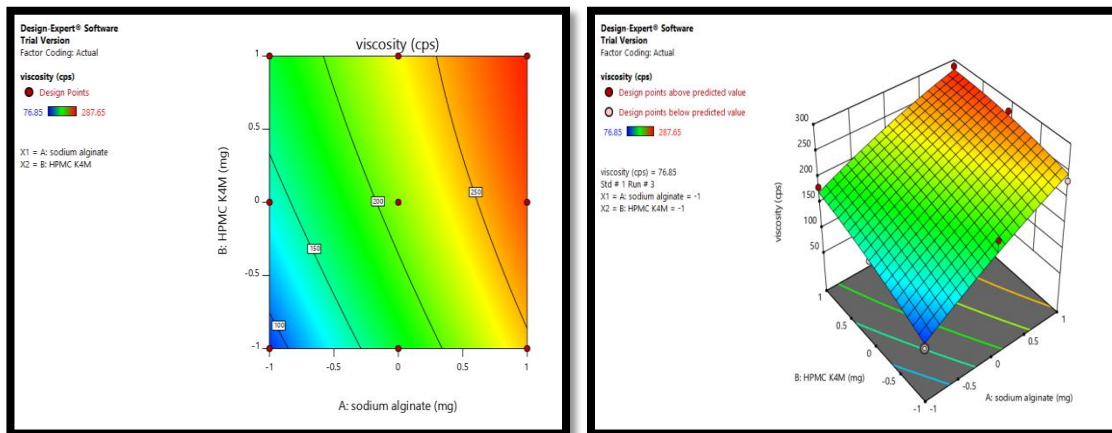
From eq. (3) it is clear that the appeared to increase with an increasing amount of factor X 1 (concentration of sodium alginate) and factor X2 (concentration of HPMC K4M) [20].

The combined effect of factor X1 and factor X2 can be further interpreted with the help of contour plot and 3D response surface plots Fig.11. showing the effect of concentration of Sodium alginate and HPMC K4M on gelling capacity.

8.3.4 Validation of model

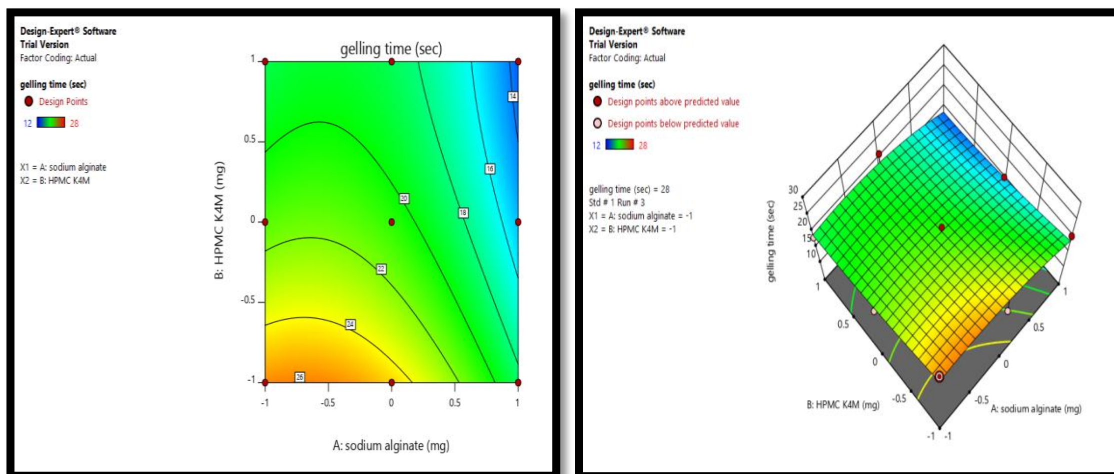
The optimized compositions of *in situ* gel were required to obtain desired responses and they are shown in Table 4. Any composition with desirability near to 1 can be chosen as optimized formulation The percent prediction error was found to be < 2%, it indicates that model used is reliable. The optimized batch is shown in Table 4 was selected based upon the desirability, which should be near to one.

The reliability of the developed model was evaluated by experimentally determining the



(a) (b)

Fig. 10. (a) Two dimensional counter plot, (b) (3 D) response surface plot for response Y2 (Viscosity)



(a) (b)

Fig. 11. (a) Two dimensional counter plot, (b) three dimensional (3 D), response surface plot for response Y3 (Gelling Capacity)

responses for the optimized trials Table 4 along with several random trials covering the entire range of experimental domain. The variables of check points, predicted and experimental values of all response variables and the percent of prediction error in prognosis are shown in Table 5.

8.3.5 Optimization of formulation

The optimized batch of *in situ* formulation is given in Table 6.

8.4 Characterization of Brimonidinet Artrate *in Situ* Gel

8.4.1 Physicochemical evaluation

i) Clarity: Clarity is one of the most important parameter of ophthalmic formulations. All prepared formulations were evaluated for clarity by visual observation against a black and white background. The clarity of all formulations was found to be satisfactory before and after the terminal sterilization Table 7.

ii) pH value: The developed formulations were evaluated for pH value by using calibrated Equip-Tronics digital pH meter model EQ- 610. All the sample solutions showed different pH value, as shown in Table 7.The pH values of all the prepared formulations ranged from 6.32 to 6.86, 6-7 pH considered acceptable to avoid the risk of irritation upon application to the eye [21].

iii) *In vitro* gelation study: All prepared formulations were evaluated for gelling capacity to check *in situ* gelation. Gelling capacity was determined by placing a drop of formulation in a vial containing 2 ml of freshly prepared ATF. The gel formulation was visually evaluated. It was observed that F3- F7 batches showed gelation on contact with ATF and retained the gel structure for more than 8 h. Formulations F1 and F2 showed gelation after administration of solution within 25-30 S and retaine for a longer time. Formulations F8 and F9 has high viscosity which is a characteristic that does not allow an easy easily administration to eye. A high concentration of sodium alginate and HPMC K4M shows gelation within 20-22 S and remain up to 8h.

B. Viscosity study: Viscoelastic fluids with viscosity that is high at low shear rates and low at high shear are preferred. I*n situ* gelling system exhibited pseudoplastic rheology, as the viscosity decreased by increasing angular velocity as shown in Fig. 12. If viscosity is high at high shear rate it results in ocular irritation. The viscosity of gel increases as the concentration of sodium alginate and HPMC K4M is increased in the formulation. Formulation F1- F7 showed very low viscosity, and formulation F8 and F9 were highly viscous at RT, which may be irritant to the eye. High viscosity may cause patient discomfort on the application of *in situ* gel, thus leading to patient noncompliance. F6 showed increase in viscosity after mixing the formulation into ATF. It was indicated that gel had high retention time in eye compared to solution. *In situ* gel system shows pseudo plastic behavior which is helped maintain sustained release of the drug from the conjunctival sac of the eye without difficulty in blinking [22].

Table 4. *In situ* gel formulation and their overall desirability

Batch	Variable		Response			Desirability
	X1(sodium alginate)	X2 (HPMC K4M)	Y1Gelling time	Y2Viscosity	Y3% Drug release	
1	1.50	0.6	9.302	299.17	85.193	1.000
2	1.45	0.6	9.778	297.473	85.663	0.976
3	1.44	0.6	9.926	296.928	85.809	0.969
4	1.39	0.6	10.435	295.00	86.314	0.942

Table 5. Comparative levels of predicted and observed responses for the optimized formulation

Variable		Predicted value			Experimental value			% Prediction error		
X1	X2	Y1	Y2	Y3	Y1	Y2	Y3	Y1	Y2	Y3
1.50	0.6	15	292.15	89.90	12	287.65	88.65	-20.0	-1.56	-1.41
1.45	0.6	16	291.40	89.70	15	289.32	89.65	-6.6	-0.72	-0.05
1.44	0.6	16	287.31	89.30	14	286.99	88.99	-14.2	-0.11	-0.35
1.39	0.6	17	281.44	91.02	15	279.89	91.16	-13.3	-0.55	0.04

% Prediction error = [(Experimental value-Predicted value) /Experimental value] ×100

Table 6. Optimized composition for *in situ* gel

Ingredient's	Quantity(%w/v)
Sodium Alginate(Protanal CR 8133)	1
HPMC K4M	0.6
Water	100

Table 7. Clarity, pH value and gelling time of brimonidine tartrate *in situ* gel

Formulation	Clarity	pH value	Gelling time(S)
F1	++++	6.32+0.02	30+0.05
F2	++++	6.70+0.03	26+0.03
F3	++++	6.86+0.03	18+0.08
F4	++++	6.52+0.04	20+0.06
F5	++++	6.45+0.01	22+0.03
F6	++++	6.63+0.02	21+0.06
F7	++++	6.64+0.01	20+0.01
F8	++++	6.65+0.02	15+0.04
F9	++++	6.85+0.01	12+0.09

(Clarity: +++ clear, ++ slightly clear, + translucent)

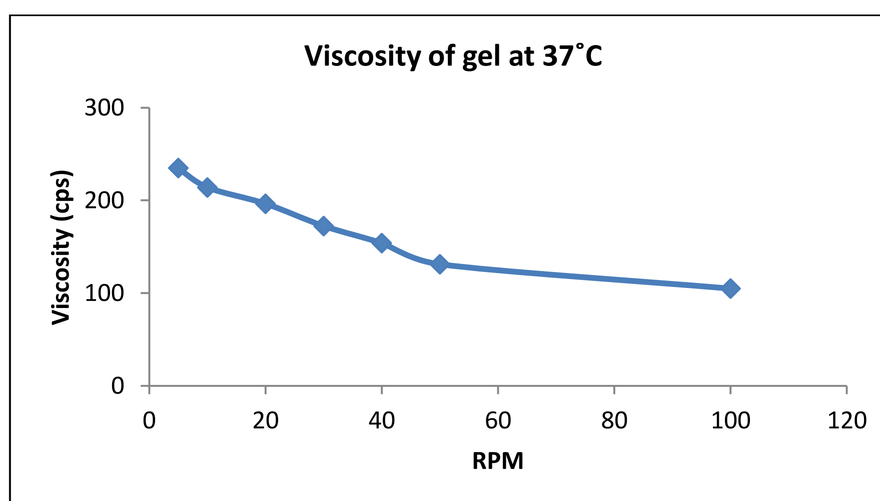


Fig. 12. Viscosity of brimonidine tartrate *in situ* gel in ATF

C. *In vitro* drug release: The decrease in the drug release was observed with an increase in polymer concentration. It indicates that amongst all formulations, F6 showed desired sustained release of up to 8h due to proper concentration of sodium alginate and HPMC K4M. The relation between the viscosity and the drug diffusion described by the Stokes–Einstein equation which demonstrates that, increased viscosity of the formulation resulted in slower diffusion of the drug across the gel matrix and into the receptor medium [23].

D. Release kinetic model: Drug release data for all batches were fitted into various kinetic models like zero order, first order, Higuchi, Hixson Crowell and Korsmeyer Peppas equations in order to determine the release mechanism and regression coefficients (r^2 value). The kinetic release model for F6 is shown in Table 8. The formulation F6 was selected based on desirability (1.00). Fig. 12 shows viscosity of brimonidine tartarate *in situ* gel in ATF After comparing the all kinetic models it is observed that the zero order kinetic model was best fitted model for the drug release from *in situ* gelling system, with the r^2 value of 0.9720 and 'n' value of 0.8196 it indicated a non Fickian transport, an anomalous drug diffusion.

E. Content uniformity: The drug content of brimonidine tartrate *in situ* gel was determined by validated HPLC method and the percent of the drug content of all formulations was found to be

in the range of 97.76-99.73% (n= 3 , mean ± S.D given in Table 9

F. Sterility testing as per I.P 2014: It is essential that ophthalmic formulation free from microorganism. Optimized formulation was subjected to sterility testing. There was no turbidity observed after 14 days of incubation at specified condition. However considerable turbidity was observed in all media incubated as positive control Fig.13.

G. Preservative efficacy study as per I.P 2014: To check the effectiveness of added antimicrobial a preservative, preservative efficacy study was conducted. Observations are shown in Table 10 and Fig. 14. No microbial growth was observed at the end of 28 days of inoculation. Results indicated that added concentration of benzalkonium chloride is significant to inhibit microbial growth for 28 days [24].

H. *Ex vivo* permeation study: A drug candidate must possess adequate permeability to deliver successfully through ophthalmic route. Physicochemical properties of drug plays key role in permeability. Goat cornea was used for *ex vivo* transcorneal permeability study of brimonidine tartrate *in situ* gel as shown in Fig.15. The percent of cumulative drug release through goat cornea was found to be 94.25 ± 1.84% Table 11

Table 8. Kinetic parameter of brimonidine tartrate *in situ* gel

Sr. No.	Kinetic models	r^2 Brimonidine Tartrate *in situ* gel	n
1.	Zero order	0.9720	0.8196
2.	First order	0.880	0.8315
3.	Higuchi	0.815	0.7815
4.	Hixon Crowel cube root	0.294	0.7513
5.	Korsemeyer-Peppas	0.342	0.6218

(a)

(b)

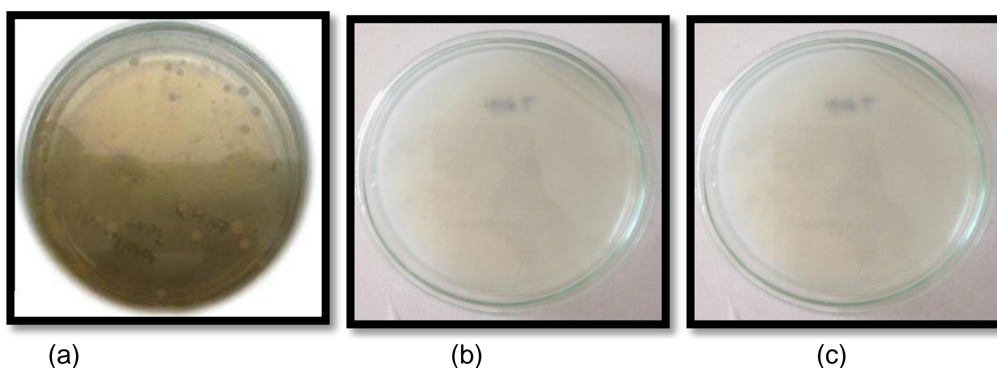
Fig. 13. Sterility test as per IP 2014 SCDM Sample a)After incubation and FTM Sample b) after incubation

Table 9. Determination of drug content

Batch	Absorbance	Concentration(µg/ml)	Actual concentration (µg/ml)	% Drug content
F1	0.1062	1.82	2	98.18±0.04
F2	0.1058	1.81	2	98.16±0.06
F3	0.1037	1.78	2	98.96±0.03
F4	0.1183	1.97	2	99.28±0.07
F5	0.1190	1.99	2	99.56±0.04
F6	0.1189	1.98	2	99.73±0.06
F7	0.1067	1.80	2	98.75±0.02
F8	0.1185	1.97	2	99.25±0.04
F9	0.1063	1.83	2	98.61±0.03

Table 10. Microbial count during preservative efficacy study

Microorganism	0 h.	24 h.	7 days	14 days	28 days
Staphylococcus aureus	50	0	0	0	0



(a) (b) (c)

Fig. 14. Preservative efficacy study as per IP 2014 (a) 7days (b) 14days (c) 28days

Fig. 15. Isolated goat eye ball

I. Histopathology study: The optimized formulation was subjected to a histopathology study. Results are shown in Fig.17. It was found that goat cornea showed no damage in case of the test sample [25].

J. Stability study: Optimized formulation was subjected to stability testing. Results are shown in Table 11. It was found that the formulation remained stable at various conditions of temperature and relative humidity used per ICH guidelines [26].

Table 11. Accelerated stability studies of *in situ* gel formulation

Time (Months)	Temperature/% RH	Appearance	Parameters			
			% Drug content	pH	Gelation Time (S)	% Drug release
1		No change	98.80±0.08	6.6±0.045	20±0.47	97.79±0.23
2	40 ± 2°C/ 75 ± 5%	No change	98.78±0.01	6.5±0.047	21±0.42	97.81± 0.19
3		No change	98.80±0.05	6.5±0.046	21±0.43	97.80±0.20

**n=3mean ± S.D.*

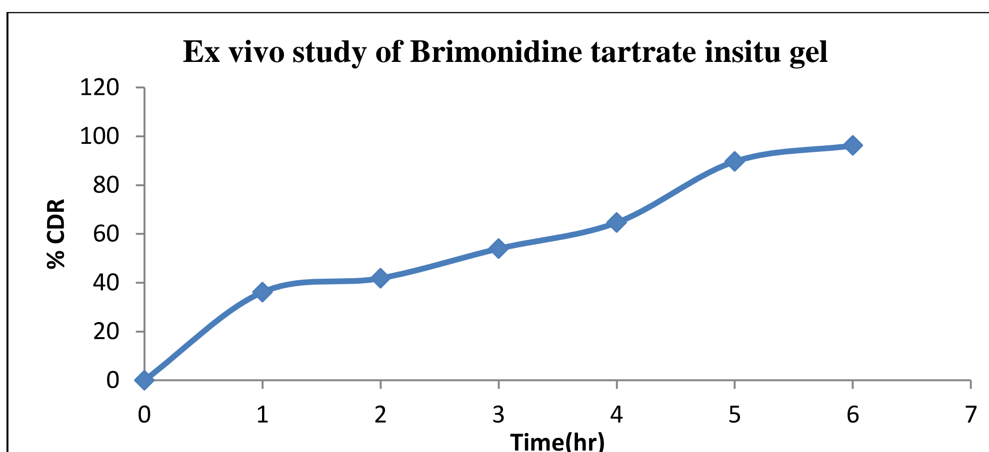
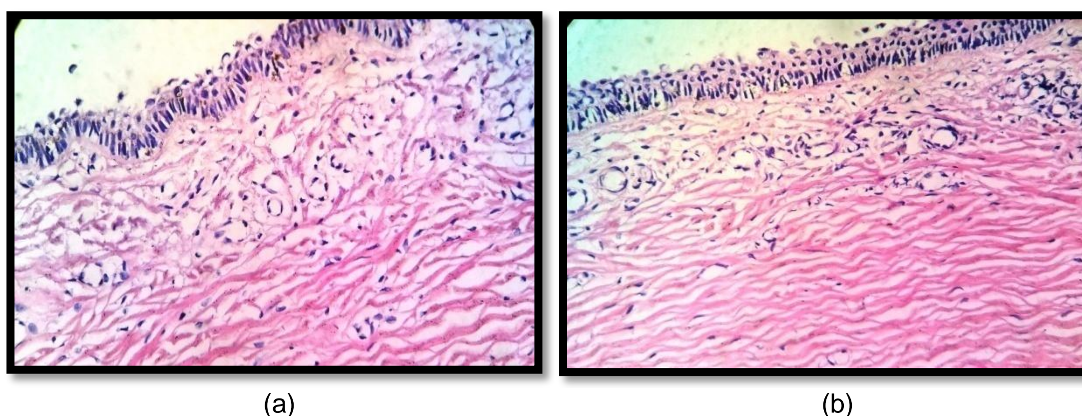


Fig. 16. ***Ex vivo*** **transcorneal percent of cumulative drug release**



(a) (b)

Fig. 17. Cross sections of goat cornea (a) control (b) test

9. CONCLUSION

The prepared *in situ* gel is evaluated for all the desired parameter like efficacy and safety studies. The *ex vivo* studies showed the promising result. Accelerated stability studies show no change in the drug content. [27] Developed formulation successfully overcome drawbacks of the conventional eye drops with safe, non-irritant approach to improve bioavailability and therapeutic activity [28].

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

ACKNOWLEDGEMENTS

Authors are thankful to FDC Mumbai for providing gift sample of Brimonidine tartrate. Authors are grateful to Principal Dr. K. G. Bothara and Dr.Dnynesh Limaye for providing excellent facility for research work.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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