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DEVELOPMENT, OPTIMIZATION AND ENHANCEMENT OF TRANSCORNEAL PERMEATION OF TIMOLOL MALEATE FROM A NOVEL *IN SITU* GEL

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ABSTRACT

Glaucoma is a chronic disease that causes irreversible blindness. Timolol Maleate is used as first line drug in treatment of glaucoma. Poor ocular bioavailability and therapeutic response shown by accustomed ophthalmic system can be overcome by use of *in situ* gelling system which undergoes reversible sol to gel phase transition in cul-de-sac by physical stimulation. Present work describes formulation and evaluation of novel pH sensitive *in situ* gel system of Timolol Maleate. Carbopol 974P was used as pH sensitive polymer with HPMC K15M as viscosity modifier. 3^2 factorial design was used to study the effect of independent variables viz. concentrations of Carbopol 974P and HPMC K15M on dependent variables like *in vitro* drug diffusion and viscosity. Optimized batch showed 88.48% drug diffusion upto 8h. Optimized formulation was evaluated for various parameters such as drug release study, isotonicity, texture analysis, preservative efficacy studies, sterility testing as per IP 2010, accelerated stability studies. *Ex vivo* transcorneal permeability study was carried out on goat eye cornea which showed that EDTA (0.5%) increases drug penetration by 1.90 fold and showed no corneal damage after histological study. In conclusion, prepared formulation is stable, non-irritant and breakthrough in treatment of glaucoma.

KEYWORDS: Glaucoma; *in situ* gel; transcorneal permeation; polymeric system; optimization and validation.

1. INTRODUCTION

Glaucoma is second most leading cause of world's blindness characterized by irreversible damage to the ganglionic cells and the optic nerve.[1,2,3] Scientist referred glaucoma as silent thief of sight because it considerably affect to patient without any prior indication. It is broadly divided in to two classes as open angle and closed angle glaucoma. This classification is based on mechanism of outflow of aqueous humor.[4,5] Survey report of WHO stated that it may affect around 80 million up to year 2020.[6] Presently, treatment of glaucoma focuses mainly on lowering of IOP. On the basis of which several classes of topical IOP lowering drugs have been developed. It includes beta blocker, prostaglandin analogue (PGA), alpha-adrenoceptor agonist (AA) and topical carbonic anhydrase inhibitors (CAI's).[7]

Timolol Maleate (TM) is used as first line drug in management of open angle glaucoma and occasionally in secondary glaucoma.[8] It is non-selective beta-adrenergic receptor blocker causes suppression of aqueous humour formation by blockage of the beta receptors in ciliary body.[9,10]

Conventionally it is available in the form of solution which gets immediately eliminated from the precorneal area. Further, shorter contact time with poor corneal permeability results into poor ocular bioavailability (10%) and decreased patient compliance.[11,12,13] Several novel drug delivery systems (NDDS's) have been developed which includes inserts, ointment, nanosuspension etc. However these systems suffer from several drawbacks such as blurred vision associated with ointment, low patient compliance from inserts and high cost of nano suspension. These problems can be overcome by using *in situ* gel forming systems.[14]

In situ drug delivery systems consist of polymers that exhibit sol to gel phase transition in cul-de-sac by several physicochemical parameters.[15] Depending upon method used for sol to gel phase transition three types of *in situ* gels are widely accepted as pH triggered system, ion activated system and temperature dependent system.[16] Pharmaceutically significant gels can be prepared by using various materials. Carbopol 974P is pH sensitive polymer which shows sol to gel transition in aqueous solution when pH is raised above 5.5. It is polyacrylic acid (PAA) which is required in high concentration to form stiff gel. At higher concentration it forms highly acidic solution which is not easily neutralized by buffer action of tear fluid. Reduction in its concentration without affecting the gelling capacity and viscosity was achieved by addition of viscosity increasing polymers such as HPMC.[12,17,18,]

The objective of present research was to develop pH sensitive *in situ* gelling system of Timolol Maleate which increases ocular residence time of drug, reduces dose and dosing frequency, decreases cost of treatment with increased patient compliance. A combination of Carbopol 974P and HPMC K15M was used as polymeric vehicle for formulation of Timolol Maleate (TM) *in situ* formulation that would gel when instilled into eye.

2. MATERIAL AND METHODS

2.1 Material

Timolol Maleate and Carbopol 974P were gifted by FDC Limited, Mumbai, India and Lubrizol advanced material India Pvt. Ltd., Mumbai, India respectively. HPMC K15M was purchased from S. D. Fine, Mumbai. HPLC grade methanol was purchased from Qualigens Fine chemicals, Mumbai. All other ingredients were of analytical grade. IOTIM (Timolol Maleate eye drops 0.5% by FDC) was brought from local medical shop.

2.2 Analytical method development

To quantitate the content of Timolol Maleate in samples reversed phase (RP)-HPLC method was developed and validated as per ICH guidelines Q2(R1). Shimadzu RP-HPLC instrument (CFR-21) equipped with photodiode array detector (PDA) and C_{18} column of Kromasil (250 mm × 4.6 mm, 5µm particle size) was used. Mobile phase consisted of phosphate buffer: methanol (60:40 v/v) and pH 3.5 was maintained by O-phosphoric acid. Elution was measured at 295 nm with flow rate of 1.0 ml/min.

2.3 Stability of Drug

Timolol Maleate was subjected for forced degradation study to check stability under various stressed conditions of acid, base, light, and oxidation as per ICH guidelines Q1A (R2). Elution was measured by validated (RP)-HPLC method.

2.4 Preparation of *in situ* gelling systems

Timolol Maleate *in situ* gel was formulated using HPMC K15M and Carbopol 974P which were allowed to soak overnight. Solution of drug, sodium chloride and benzalkonium chloride was prepared in purified water. Drug solution was added to the polymeric solution under constant stirring to get uniform solution. Final volume was adjusted with purified water. The developed formulation was filled in glass vials of 10 ml capacity, closed with gray butyl rubber closure and sealed with aluminium cap. Prepared formulation was then subjected for terminal sterilization by autoclaving at 121^{o}C, 15 p.s.i. for 20 minutes. This sterilized *in*

situ gel of Timolol Maleate was then subjected for evaluation.[18] Composition of different batches of Timolol Maleate *in situ* gel is shown in Table 1.

2.5 Full factorial experimental design

For optimization of Timolol Maleate *in situ* gel, 3^2 randomized full factorial design was selected. The design was applied to study the effect of concentration of Carbopol 974P and HPMC K15M on formulation. The amount (%) of pH sensitive polymer, Carbopol 974P (X_1) and the amount (%) of viscosity modifier, HPMC K15M (X_2) were selected as independent variables, in this study. These two factors were evaluated at 3 levels as higher, middle and lower levels with coding +1, 0 and −1 respectively. Levels of X_1 were selected as 0.15%, 0.3% and 0.45% and for X_2 0.25%, 0.5% and 0.75%. The dependent or response variables included viscosity at 20rpm (Y_1), cumulative % drug diffused at 8h (Y_2).

2.6 Evaluation of formulation

2.6.1 Physicochemical Characterization

Prepared formulations were evaluated for clarity, pH and gelling capacity. The clarity of formulation before and after gelling was determined by visual inspection under black and white background. The pH of all formulations were determined immediately after preparation as well as after 24h using digital pH meter (Equip-Tronics, EQ 610) [19]. Gelling capacity was determined by placing 100µl of sample in a vial containing 2ml artificial tear fluid (ATF) and gelation time was recorded.[18]

2.6.2 Effect of formulation variable on viscosity

The developed formulation was poured into the small sample adaptor of Brookfield viscometer (Oswal's Scintefic PES / Mcop Pharmaceutics) and angular velocity was gradually increased from 5 to 200 rpm. Average of thee reading was used to calculate the viscosity. Formulation was then poured into vials and pH raised to 7.4 by adding 0.5 M NaOH. Viscosity of resultant gel was then measured.[18]

2.6.3 Effect of formulation variable on *in vitro* drug diffusion

In vitro drug diffusion study was carried out by using Franz diffusion cell in triplicate. Freshly prepared ATF was placed in receptor compartment. In between receptor and donor compartments dialysis membrane of pore size 0.22 µm was placed. Whole assembly was kept on the thermostatically controlled magnetic stirrer to simulate *in vivo* conditions and temperature of medium was maintained at $37^{o}C \pm 0.5^{o}C$. Medium was continuously stirred at

20 rpm. In donor compartment, 1ml of formulation was placed. 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 analysed by using developed and validated RP-HPLC. Percentage cumulative drug diffusion was calculated.[19]

2.6.4 *Ex vivo* transcorneal permeability study

Mudgil and Pawar[20] suggest that goat cornea is ideal model to check transcorneal permeability of drug. The fresh whole eyeballs of goat were obtained from local butcher's shop and transported in laboratory in normal saline solution (4°C). Cornea was then carefully excised along with 2-4 mm of surrounding sclera tissue and washed with saline solution. Excised cornea was placed in between donor and receptors compartment of Franz diffusion cell in such a way that epithelial surface faced the donor compartment. Receptor compartment contained freshly prepared ATF. Whole assembly was placed on thermostatically controlled magnetic stirrer and temperature (37°C ± 0.5°C) as well as stirring rate (20 rpm) was maintained. In donor compartment, 1ml of prepared formulation was placed. Simultaneously, 1ml solution of IOTIM (marketed formulation) was placed in another donor compartment. Samples (0.5ml) were withdrawn at predetermined time interval of 1h to 5h and same volume was replaced by ATF. Samples were then diluted up to 10ml and analysed on RP-HPLC.[20] Study was performed in triplicate and data was further evaluated. Depending upon amount of drug permeated, different ocular penetration enhancers like EDTA (0.5% w/w) and tween-20 (1% v/v) were used.[21,22,23]

2.6.5 Histological study of goat eye cornea

To evaluate effect of *in situ* formulation on 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 formulation. 0.1% (w/w) sodium dodecylsulfate (SDS) solution in phosphate buffer saline (PBS) was used as the 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). Cross sections were observed microscopically for any modifications.[24]

2.6.6 *In vitro* drug release study

As per Song et al.[14] and Pund et al.[25], *in vitro* release test was carried out by using USP dissolution test apparatus Type II. A 1 ml volume of the formulation and IOTIM solution was

accurately filled into dialysis bag (Himedia, India) separately. Test was carried out in 500 ml freshly prepared ATF, which was used as release medium. The temperature and rotating rate were maintained at 34 ± 1°C and 50 rpm, respectively. Aliquots (5 ml) were withdrawn from the release medium at each sampling time and replaced by an equal volume of the release medium. The samples were subjected to HPLC analysis to determine the Timolol Maleate concentration. Percentage cumulative drug release was calculated. The data obtained of optimised Timolol Maleate *in situ* gel was further subjected to check drug release kinetics.

2.6.7 Determination of isotonicity

Isotonicity is important characteristics of ophthalmic formulation which has to be maintained to prevent any tissue damage or irritation to the eye. Tonicity is refers to the osmotic pressure exerted by salts in aqueous solution. Ophthalmic formulation must possess osmotic pressure within the range of 290-310 mOsmol/kg.[26] Tonicity of optimized *in situ* gel was determined by using digital osmometer (Osmomat 030/050 Terminal).

2.6.8 Texture Analysis

The consistency, firmness and cohesiveness of *in situ* gel is assessed by using texture profile analyzer. This mainly indicates gel strength and easiness in administration. Texture analysis provides information on hardness, compressibility and adhesiveness which can be correlated with various parameters like ease of removal from container, good spreadability on corneal surface and adherence to mucous layer in order to sustain ocular residence time.[16]

The optimized batch was subjected for texture analysis by using Brookfield CT3 Analyzer. Experiment was done by placing the gel in standard beaker below the probe. Then analytical probe was immersed into sample. Texture analyzer was set to the gelling strength test mode with test speed 0.5 mm/s. Trigger force of 3g was selected. Formulation was analyzed at pH 4 and then on pH 7 i.e. before and after gelling.

2.6.9 Sterility Testing

Ocular *in situ* gel was sterilized by autoclaving at 121^{o}C at 15lb pressure for 21 minutes and evaluated as per Indian Pharmacopoeia (2010) for 14 days sterility testing.[10]

2.6.10 Preservative Efficacy Study

Preservative efficacy study of *in situ* gel was performed as per Indian Pharmacopoeia (2010) by challenging the formulation with *Staphylococcus aureous* and *Pseudomonas aeroginosa*.[10]

2.6.11 Accelerated stability studies

Stability studies for the optimized batch F7 was carried out to determine the effect of added formulation additives on the stability of drug and also to determine physical stability of formulation under accelerated storage conditions. The optimized batch F7 was subjected to elevated temperature and humidity conditions of 25±1°C/ 60%RH, 30±1°C/ 65% RH and 40 ± 2°C/ 75 ± 5 % RH. Samples were withdrawn at the end of 0, 30, 60 and 90 days and evaluated for physical appearance, pH, active drug content and drug release.

3. RESULTS AND DISCUSSION

3.1 Analytical method development and stability of Timolol Maleate

HPLC method was developed and validated with parameter like linearity, precision, accuracy, robustness and recovery study. Percent RSD for all parameter was found to be less than 2. Forced degradation study was conducted as per ICH guidelines and Timolol Maleate was found to be stable in all condition except basic environment. Chomatogram of Timolol Maleate is shown in Figure 1.

3.2 Full factorial experimental design

To study the effect of independent variables on responses Design Expert 8.0 software was used. Experimental design layout developed for 9 possible batches of Timolol Maleate *in situ* gel is shown in Table 2. Out of the various models such as Linear, 2FI, Quadratic and Cubic which fit well was suggested by software and was tested for analysis of variance (ANOVA). Regression polynomials were calculated for the individual dependent variables and then contour plots and 3D surface graphs were obtained for each individual dependent variable. Mathematical models were generated for each individual dependent variable or response (R) and expressed as equation 1-2. X_1 and X_2 are the main effects which represent the average result of changing one factor at a time from its low to high value and X_1 X_2 are interaction terms show how the response changes when two factors are simultaneously changed. Nonlinearity is investigated by polynomial terms X_1^2 and X_2^2.

3.3 Evaluation of formulation

3.3.1 Physicochemical characterization

All formulations were found to be transparent above pH 7. The pH of formulations was found in the range of 3-5. This is pH of formulation in container, which get change towards neutral side by buffer action of tear fluid in cul-de-sac. Gelling capacity of formulations was observed in tear fluid. Respective results are shown in Table 3.

3.4.2 Effect of formulation variables on viscosity

Viscosity of solution after instillation into the eye is desired feature for sustained action of drug by increasing ocular residence time. Formulations at pH 4 showed free flowing property, as pH of formulations was raised to 7.4 by 0.5 N NaOH it showed drastic change in viscosity. Formulations were checked at 5, 10, 20, 30, 40, 50, 80, 100, 150, 200 rpm. Comparison of all batches at pH 4 and 7.4, at different rpm is shown in Figure 2. As the angular velocity increases formulations showed decreased viscosity. Hence; formulations have shear thinning characteristics. Viscosity of formulations at 20 rpm is shown in Table 2. Formulation F9 containing highest level of Carbopol 974P and HPMC K15M showed highest level of viscosity.

On applying factorial design, the two factorial model was suggested by software and found to be significant with model F value of 93.38, p value <0.0001 and R^2 value of 0.986 which implied that model was significant. There was only 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.05 for each term was obtained which indicated that every model term was significant. In this case X_1, X_2, were significant model terms. The model for response Y_1 (viscosity) is as follows:

$$Y_1 = +20010.11 + 8794.83(X_1) + 4721.50(X_2) + 2257.75\,(X_1X_2) \qquad (1).$$

Above equation (eqn.1) indicates that X_1 (concentration of Carbopol) and X_2 (concentration of HPMC) has positive effect on viscosity. However effect of X_1 is more significant than X_2 . X_1 and X_2 have positive combined effect on viscosity. Effect of X_1 and X_2 can be further explained by contour plot and response surface plot (Figure 3).

3.4.3 Effect of formulation variables on *in vitro* drug diffusion

For optimization, *in vitro* drug diffusion studies were conducted. The cumulative percent of Timolol Maleate diffused as function of time is shown in Figure 4. *In vitro* conditions may vary from those likely to be encountered in the eye. However, results clearly showed that the gels have ability to sustain the release of drug. Batch F9 contains highest level of polymer with least drug diffusion. Batch F7 showed better sustaining effect with 88.48% drug diffusion upto 8h.

On applying factorial design, the quadratic model was suggested by software and found to be significant with model F value of 85.23, p value <0.002 and R^2 value of 0.993 which implied that model was significant. And there was only a 0.01% chance that a "Model F-Value" this

large could occur due to noise. Values of "Prob > F" less than 0.05 for each term was obtained which indicated that every model term was significant. In this case X_1, X_2, X_1^2, X_2^2 were significant model terms. The model for response Y_2 (percentage drug release) is as follows:

$$Y_2 = +91.46 - 5.66\,(X_1) - 5.20\,(X_2) - 1.54(X_1\,X_2) - 4.44\,(X_1^2) - 0.48(X_2^2) \qquad (2)$$

Above equation (eqn.2) indicates that X_1 (concentration of Carbopol) and X_2 (concentration of HPMC) have negative effect on diffusion of drug. Both have significant effect. X_1 and X_2 have negative combined effect on drug diffusion. Effect of X_1 and X_2 can be further explained by contour plot and response surface plot (Figure 5).

3.4.4 Optimization of formulations

The 3^2 factorial experimental design was applied to optimize response variable Y_1 and Y_2. It is reported that if viscosity of formulation after gelling is very high it retards the drug release and at the same time it may result in discomfort to patient. On the other hand, less viscosity results in poor corneal residence time.[18] Hence formulation which shows optimum viscosity with desired drug release is to be selected as optimized formulation. Design expert 8.0 software was used for optimization. After inserting the responses in software, it suggests number of possible solutions. A solution with desirability (D) 1 or near to 1 can be chosen as optimized formulation.[27] Batch F7 showed desirability value 1 for viscosity and drug diffusion (Figure 6.) was considered optimized batch and used for further evaluations. Suggested model was subjected to validation.

3.4.4.1 Validation of model

Software suggested all possible combination of independent variable other than those used for optimization and their effect on dependent variable as shown in Table 4.

Out of all suggestions, any combinations of independent variable i.e. concentration of Carbopol 974P and HPMC K15M can be selected randomly and used for validation of suggested model.[28] Experiments were carried out by using batch 1 and 7. Data obtained by experiment was used for % prediction error calculation. Result obtained for viscosity and drug diffusion is shown in Table 5. Percent prediction error was calculated by using formula as given below;

$$\%\ prediction\ error = \frac{Experimental\ value - predicted\ value}{Experimental\ value} \times 100$$

As % prediction error was found to be less in all cases, model suggested by software was found to be valid. Similar conclusion was drawn by Joshi et al.[29]

3.5 *Ex vivo* transcorneal permeation study

Drug candidate must possess adequate permeability to be delivered successfully through ophthalmic route. Physicochemical properties of drug plays key role in permeability. Goat cornea was used for *ex vivo* transcorneal permeability study of TM *in situ* gel. Cumulative percent drug permeated for Timolol Maleate *in situ* gel (batch F7) with and without permeation enhancer along with marketed formulation as function of time is shown in Figure 7.

Transcorneal permeability of Timolol Maleate was calculated by using equation 3 and 4 given by Pund et al.[25]

$$P_{app} = J_{ss}/C_d \qquad (3)$$

Where; P_{app} is apparent permeability coefficient, *Jss* is steady state flux and C_d is initial donor chamber concentration of drug.

J_{ss} was calculated by plotting cumulative amount of Timolol Maleate (µg) permeated per unit area against time.

$$D = P_{app} \times L/K \qquad (4)$$

Where; *D* is steady state diffusion coefficient, *L* is diffusion path length, *K* is partition coefficient of drug.

The permeation characteristics of F7 formulation, with penetration enhancers and without penetration enhancer with that of marketed formulation (IOTIM) is shown in Table 6.

Formulation F7 containing no penetration enhancer showed total 7.61% drug release as compared to marketed formulation which showed total 9.13% drug release. Maximum increase in permeability was obtained from formulation containing EDTA as penetration enhancer. Formulation containing 0.5% EDTA enhanced transcorneal permeability by 1.90 fold and transported 14.50% of drug after 5 h. While 1.0% tween-20 showed less significant effect which transported 8.55% of drug upto 5 h with 1.12 fold increased in corneal permeation. Majumdar et al.[22] used 0.01% EDTA as penetration enhancer to enhance the penetration of Acyclovir. However; Plazonnet and Gurny[21] showed that, EDTA can be used safely upto 1% in ophthalmic formulations.

Therefore batch F7 containing 0.5% EDTA as penetration enhancer, 0.45% Carbopol 974P, 0.25% HPMC K15M was considered as final optimized batch and subjected for further evaluation.

3.6 Histological study of goat eye cornea

It is essential to establish safety profile of ophthalmic preparation used in chonic treatment. The ocular irritation and tissue damage by use of Timolol Maleate *in situ* gel was evaluated by histological study of goat eye cornea. Paraffin section of cornea stained with haematoxylin and eosin was observed under motic microscope. Histological section of formulation treated and untreated cornea were found to be unaffected. Structure of cornea was preserved in both cases. On the other hand, section of 0.1% SDS treated cornea (positive control) showed marked alteration on the corneal epithelium and stroma layer. No haemorrhage and necrosis effect was observed on formulation treated cornea. Results of histological studies are shown in Figure 8. These results are in accordance with those observed by Jain et al.[24], where they evaluated PLGA-chitosan nanoplexes on goat eye cornea. From histological study it can be conclude that prepared *in situ* formulation is safe for clinical application.

3.7 *In vitro* drug release study

Optimised batch F7 was subjected for *in vitro* drug release study by using USP Type II dissolution apparatus. The *in vitro* release of drug depends on two simultaneous processes, water migration into *in situ* gelling system and diffusion of drug though system. I*n vitro* drug release study result was compared with IOTIM (marketed formulation). Prepared *in situ* gel showed 91.08% drug release upto 8 h as compared to IOTIM which showed 100.01% drug release at the end of 3 h. Study clearly revealed that optimised *in situ* gel exerts sustain drug release profile for more than 8 h as compared to conventional marketed formulation IOTIM. This prolonged release of drug may be due to the formation of hydrogen bonds between drug and polymers, which have helped in rate control release of drug.[14] Comparative plot of cumulative percent drug release by F7 formulation and IOTIM is shown in Figure 9.

3.7.1 *In vitro* release kinetics

The drug release data obtained from *in vitro* release experiments (Batch F7) was subjected to various kinetic equations to evaluate the drug release kinetics. The kinetic models used were viz. zero order, first order, Higuchi model, Hixon-Crowel cube root and Korsemeyer-Peppas model. The regression coefficient (r^2) obtained from all models are listed in Table 7.

Korsemeyer-Peppas model was found to be suitable to explain release kinetic of drug (r^2= 0.991 with value of n=0.226). Similar model was observed by Pund et. al.[20] for Venlafaxine intranasal *in situ* gel. When n is less than 0.5, release of drug takes place according to Quasi Fickian diffusion mechanism. If n=0.5, drug release is solely diffusion controlled i.e. Fickian diffusion while if 0.5<n<1 indicates anomalous transport (non-fickian diffusion). Results of *in vitro* drug release study revealed the Quasi Fickian diffusion behaviour of release of Timolol Maleate from *in situ* gel. Similar model was observed by Pund et. al.[25]

3.8 Isotonicity

Tonicity of optimized batch was found to be 305 mOsmol/kg indicating the ease and comfort to eye after administration of prepared formulation.

3.9 Texture analysis

Gelling strength of *in situ* gel was measured in terms of hardness. At pH 4, hardness was found to be 17 g (Figure 10.) while after gelling i.e at pH 7.4 it was found to be 25g (Figure 11). It clearly indicates that gelling strength of prepared formulation at physiological pH was more than at pH 4. Hence, formulation can be easily placed in to eye and after gelation it can remain for longer time in ocular cavity.

3.10 Sterility Testing

Optimized formulation was subjected for sterility testing. There was no turbidity observed after 14 days of incubation at specified condition. However considerable turbidity was observed in all the medias incubated as positive control. Thus prepared formulation was considered as sterile. Similar results were observed by Geethalakshmi et al.[30] for Betaxolol *in situ* gelling system.

3.11 Preservative Efficacy Study

To check effectiveness of added preservative, preservative efficacy study was conducted. No microbial growth was observed at the end of 28 days of incubation. Results clearly show that added concentration of benzalkonium chloride (0.02% w/v) was significant to inhibit the growth of microorganism.

3.12 Accelerated stability studies

Optimized *in situ* formulation was subjected for stability studies. At the end of three month drug content was found to be 99.10% with no significant change in pH, appearance and drug release. From results prepared formulation was found to be chemically stable at all storage conditions.

Table 1: Composition Of Different Batches Of Timolol Maleate *In Situ* Gel

Name of excipients	Different batches of Timolol Maleate *in situ* gel (%w/v)								
	F1	F2	F3	F4	F5	F6	F7	F8	F9
Timolol Maleate	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17
Sodium Chloride	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80
Carbopol 974P	0.15	0.15	0.15	0.30	0.30	0.30	0.45	0.45	0.45
HPMC K15M	0.25	0.50	0.75	0.25	0.50	0.75	0.25	0.50	0.75
Benzalkonium chloride	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Water q.s.	100	100	100	100	100	100	100	100	100

Table 2: Experimental Design Layout Of Timolol Maleate *In Situ* Gel

Run	FC	Coded levels of variables		Viscosity (cps) (Y_1)	% drug release (Y_2)
		Factor X_1 (Carbapol 974P)	Factor X_2 (HPMC K15M)		
1	F1	-1	-1	9220	97.06
2	F2	-1	0	11400	91.85
3	F3	-1	1	13200	90.09
4	F4	0	-1	14122	96.98
5	F5	0	0	20100	92.44
6	F6	0	1	25460	85.92
7	F7	1	-1	21189	88.48
8	F8	1	0	31200	81.215
9	F9	1	1	34200	75.34

Table 3: Physicochemical Evaluation Of Timolol Maleate *In Situ* Gel

Batch	Clarity		pH		Gelling capacity
	At normal pH	At pH above 7	0 hr	24 hr	
F1	Clear	Clear	4.26	4.29	+
F2	Clear	Clear	4.20	4.20	+
F3	Clear	Clear	4.05	4.03	+
F4	Slight milky white	Clear	3.87	3.88	+ +
F5	Slight milky white	Clear	3.85	3.86	+ +
F6	Slight milky white	Clear	3.84	3.84	+ + +
F7	Milky white	Clear	3.77	3.79	+ + +
F8	Milky white	Clear	3.75	3.77	+ + +
F9	Milky white	Clear	3.74	3.75	+ + +

Footnote: +, gels after a few minutes, rapidly dissolves; ++, gels immediately, dissolves after few hours; +++, gelation immediately and remains gel for extended period.

Table 4: Optimized Formulas Suggested By Software

Batch	Carbopol 974P	HPMC K15M	Predicted value	
			Viscosity	% drug diffusion
1.	0.441	0.428	26322.995	84.147
2.	0.430	0.413	25342.765	85.502
3.	0.361	0.529	24216.593	87.782
4.	0.373	0.545	25372.890	86.563
5.	0.433	0.676	32542.332	78.560
6.	0.280	0.535	19450.534	91.452
7.	0.297	0.494	19706.079	91.706
8.	0.238	0.642	18526.880	90.607
9.	0.282	0.448	18005.197	93.156
10.	0.371	0.635	27308.146	84.717
11.	0.287	0.460	18544.254	92.727

Table 5: Validation Of Model

Variables		Predicted value		Experimental value		% prediction error	
X_1	X_2	Y_1	Y_2	Y_1	Y_2	Y_1	Y_2
0.441	0.428	26322.995	84.147	26901	82.91	2.14	-1.49
0.297	0.494	19706.079	91.706	20225	92.12	2.56	0.449

TABLE 6: *EX VIVO* PERMEATION CHARACTERISTICS

Formulation (F7)	J_{ss} ($\mu g\ cm^{-2}h^{-1}$)	P_{app} ($cm\ h^{-1}$) $\times 10^{-2}$	D (cm^2h^{-1}) $\times 10^{-4}$	% total drug permeated
Without penetration enhancer	200.9	13.39	74.38	7.61
Marketed formulation	803.6	16.07	89.20	9.13
With 0.5% EDTA	382.9	14.50	80.50	14.50
With 1.0% tween-20	225.6	15.04	83.58	8.55

Table 7: *In Vitro* Release Kinetics Of Drug

Kinetic model	r^2
Zero-order	0.739
First-order	0.957
Higuchi	0.941
Korsemeyer-Peppas	0.991
Hixson-Crowell cube root	0.890

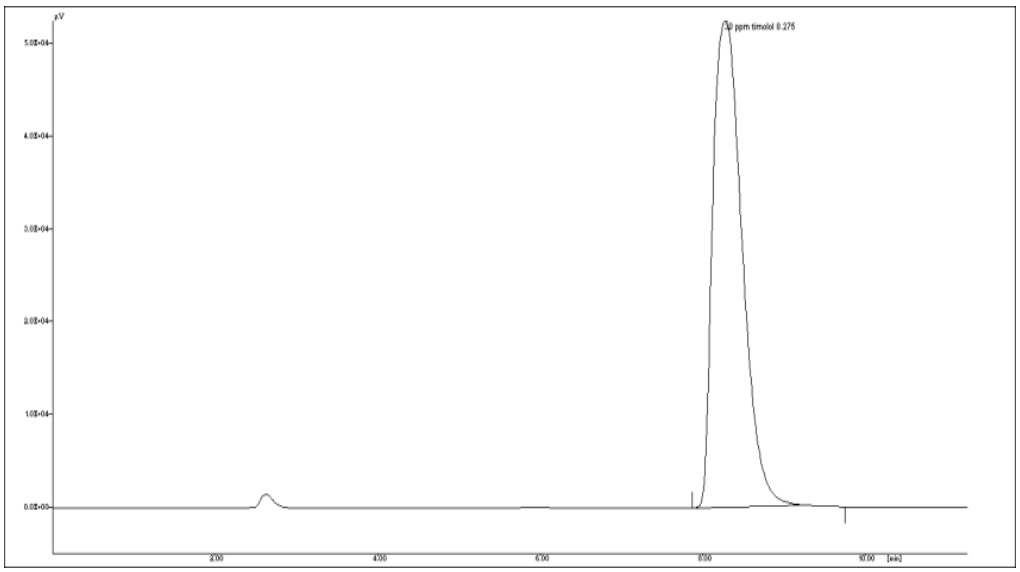
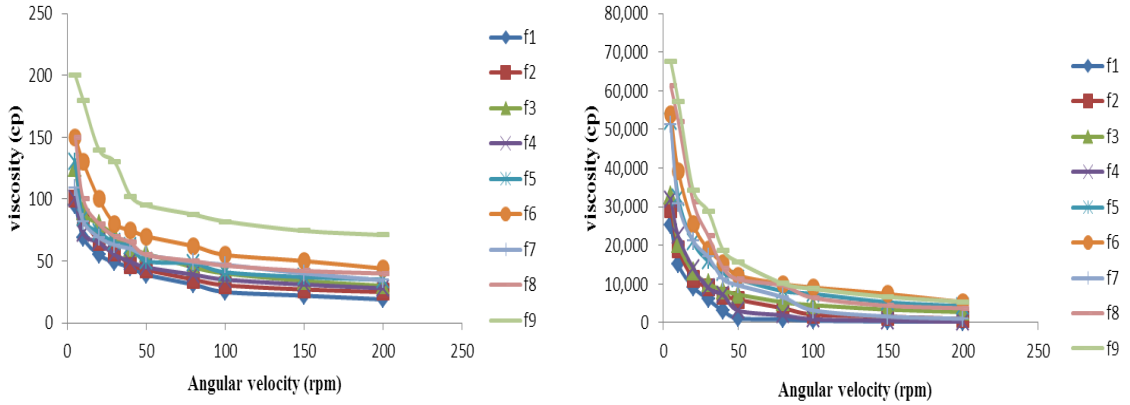
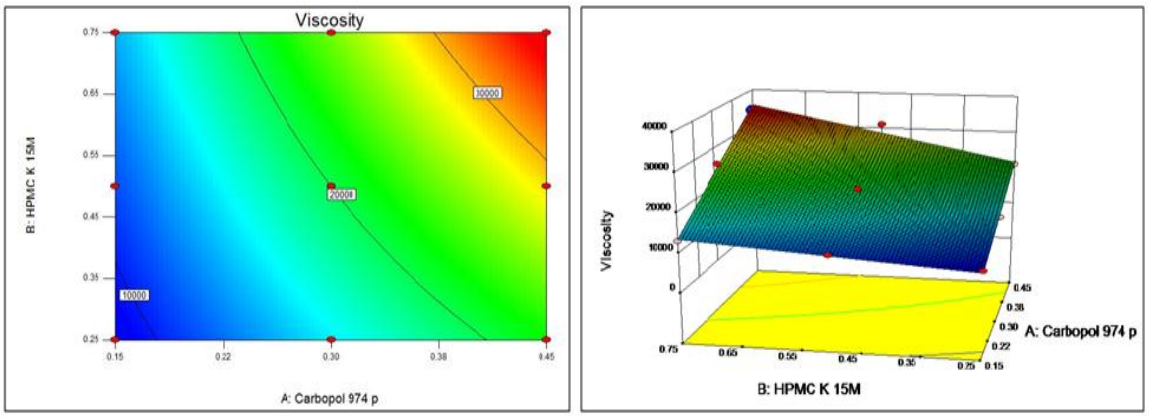


Fig. 1: Chromatogram of Timolol Maleate



(a) **(b)**

Fig. 2: Rheological profile of *in situ* gelling systems at pH 4.0 (a) and pH 7.4 (b)



(a) **(b)**

Fig. 3: Two dimensional contour plot (a), three dimensional (3D) response surface plots (b) for response Y_1

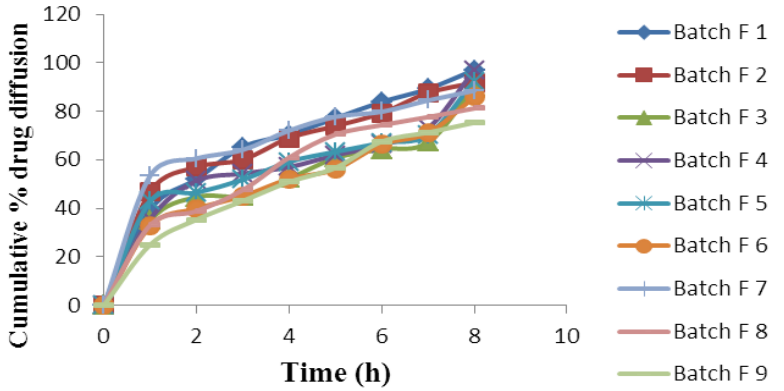
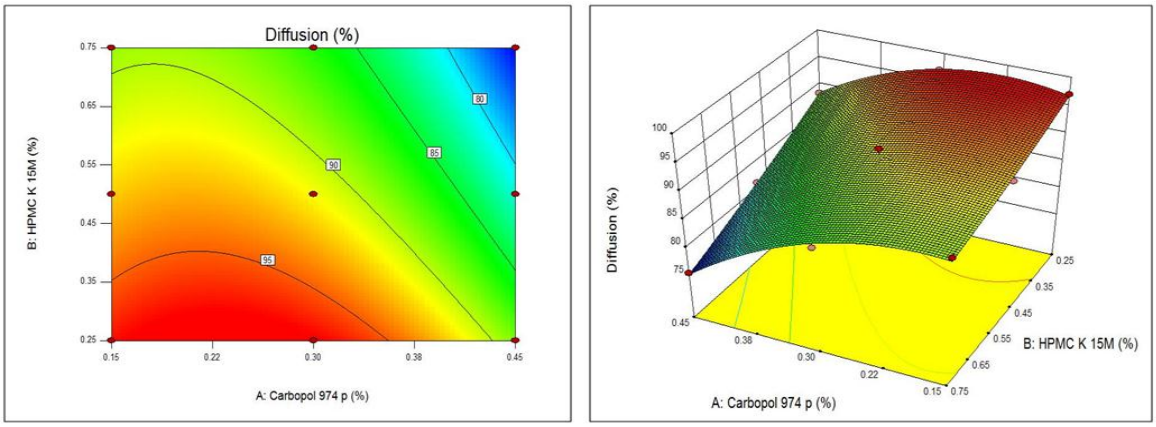
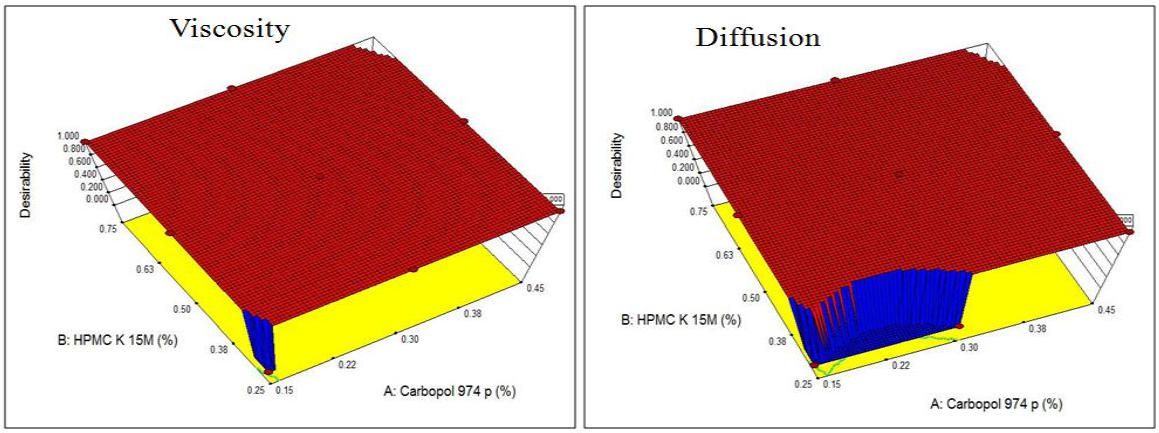


Fig. 4: Comparative cumulative % drug diffused from various batches of Timolol Maleate *in situ* gel



(a) **(b)**

Fig. 5: Two dimensional contour plot (a), three dimensional (3D) response surface plots (b) for response Y_2



(a) **(b)**

Fig. 6: Three dimensional (3D) response surface plots for desirability function of viscosity (a) and drug diffusion (b).

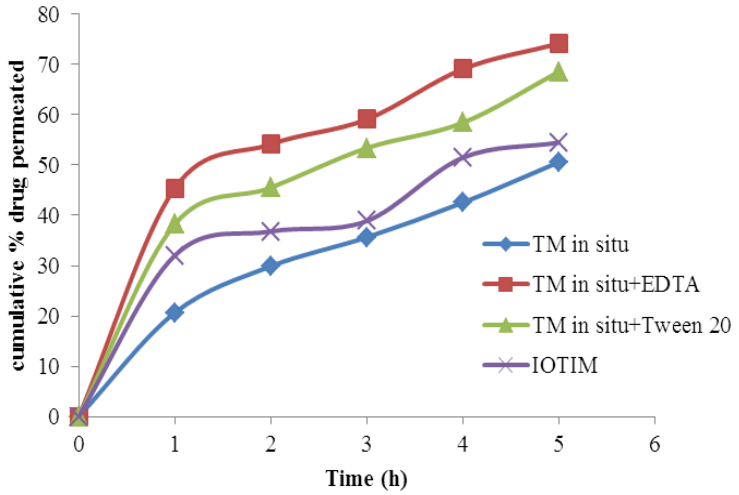
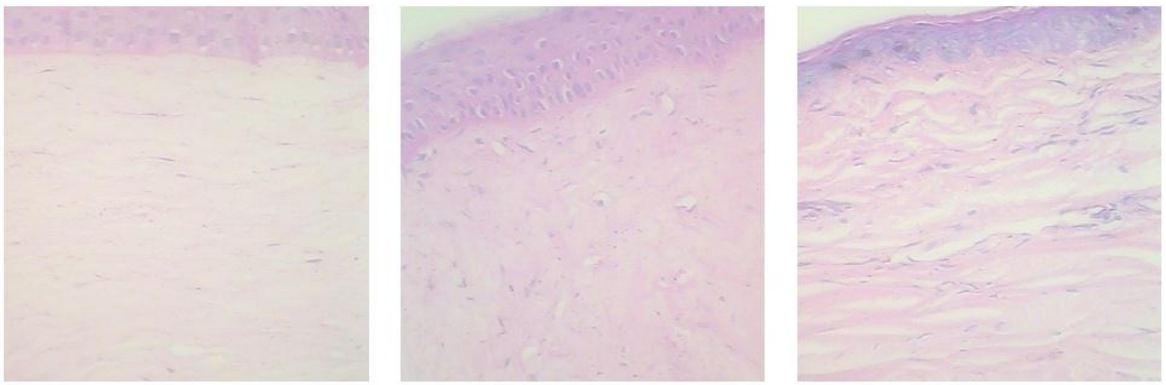


Fig. 7: Comparative *ex vivo* transcorneal cumulative % drug release



(a) (b) (c)

Fig. 8: Histological section of goat eye cornea (magnification 40X) a) negative control: untreated cornea, b) test specimen: formulation treated cornea, c) positive control: SDS treated cornea for 5h

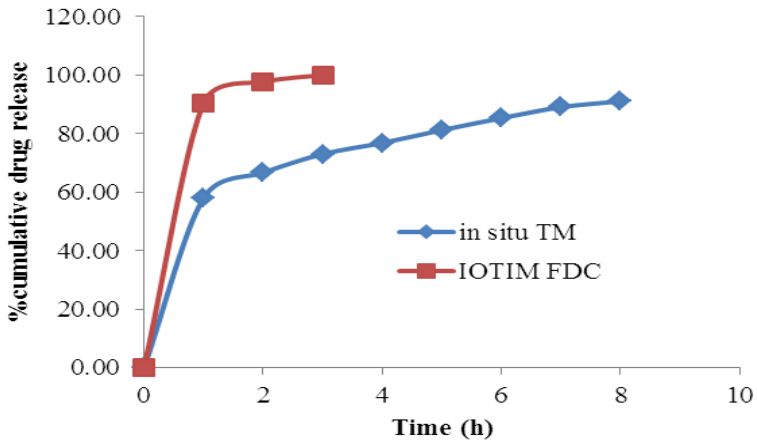


Fig. 9: Comparative *in vitro* drug release study

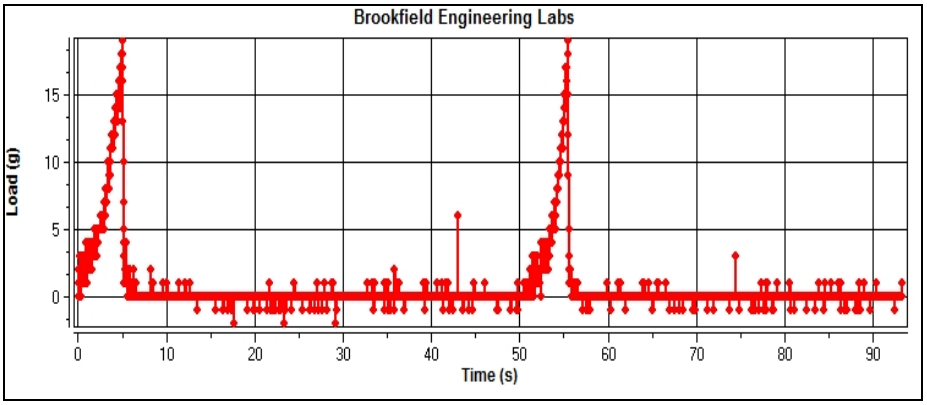


Fig. 10: Hardness of *in situ* gel at pH 4

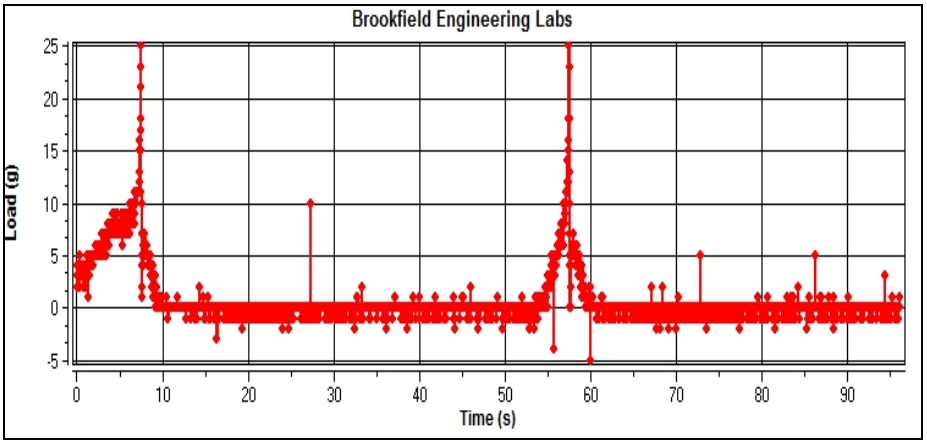


Fig. 11: Hardness of *in situ* gel at pH 7.4

4.0 CONCLUSION

The pH triggered Timolol Maleate *in situ* gel was successfully formulated by using Carbopol 974P and HPMC K15M. Formulation was optimized by 3^2 randomized full factorial design for two responses viz. viscosity at 20 rpm and cumulative percent drug diffused at the end of 8 h. Optimized formulation (Batch F7) was liquid at pH 4 and gel above pH 7 indicating *in situ* transition at physiological pH. Optimized *in situ* gel passes all safety tests used for evaluation of ophthalmic formulation. *In vitro* drug release study showed sustained release of drug from *in situ* gel over period of 8h as compared to marketed formulation IOTIM (FDC). EDTA (0.5% w/v) was found to be suitable penetration enhancer which showed 1.90 fold increased transcorneal permeability of drug. Formulation showed less eye irritation as compared to SDS (positive control) during histological study on goat eye cornea. Stability study showed that optimized formulation is stable over a period of 3 month. Hence; prepared formulation have great advantage over conventional marketed formulation in regard sustain release profile which can be further evaluated in clinical study.

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