Acta Scientific Pharmaceutical Sciences (ISSN: 2581-5423)

Volume 3 Issue 2 February 2019

Research Article

Preparation of Gellan Gum and Chitosan based *In-Situ* Gel of Timolol Maleate for Ophthalmic Drug Delivery and Evaluation of Physicochemical Properties and Drug Release Profile

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Received: December 12, 2018; **Published:** January 25, 2019

Abstract

The aim of the present study was to develop ion activated and temperature dependent *in-situ* gels of Timolol maleate (TM) by cold method. Gellan gum was used as ion activated system whereas chitosan was used as temperature dependent system. Among the different batches IGG5 and IGC4 were considered as optimum gelling system because in these system sol-gel transition took place within short period of time i.e. 84.19 sec and 95.32 sec respectively. Further optimum batches were utilized for preparation of 0.5%w/v Timolol maleate *in-situ* gel. Prepared *in-situ* gels were isotonic and sterile in nature as it passes the test for sterility. The results indicated a large increase in viscosity at 37°C which provided sustained release of the drug over a 10 hour period. *In-vitro* corneal permeation study showed that *in-situ* gel showed almost 2-folds increase in permeability compared to marketed formulation. From *in-vitro* release studies, it could be concluded that the developed *in-situ* gelling systems were thus a better alternative to conventional eye drops.

Keywords: Timolol Maleate; Gellan Gum; Chitosan; Cold Method; *In-Situ* Gel

Introduction

Glaucoma is one of the serious eye disorders. In glaucoma intra ocular pressure (IOP) of eye increases and causes damage to the optic nerve, which gradually leads to loss of vision. Development of glaucoma in an individual is due to imbalance between aqueous humour secretion and drainage processes within the ocular chamber [1]. There are several classes of drugs and their dosage forms available to treat glaucoma. The conventional eye drops occupied more than 90% of marketed formulations. High acceptance range of conventional eye drops is due to simple instillation into the eye with accuracy of doses, ease of production, economical cost, patient compatibility and tolerability [2]. However, those eye drops has various disadvantages like short residence time, poor bioavailability, poor permeability, lacrimal fluid dilution and rapid precorneal drainage [3].

To overcome such problems various formulations like viscous solutions, ointments, gels or polymeric inserts have been developed with the view to extend the ocular residence time of drug for topical application to the eye [4,5].

In recent years, a major progress in development of ophthalmic formulations has been made by the ophthalmic gel technology called as "*in-situ* gel". These *in-situ* forming systems are liquid upon instillation and converted into form viscoelastic gels in response to environmental changes such as pH [6], temperature [7] and specific ions [8]. *In-situ* gelling systems are more acceptable for the patients, since they are instilled into the eye as a solution and immediately converted into a gel when it contact with the eye. Several studies showed that the pre-corneal residence time of such *in-situ* gelling systems are for several hours [7,8].

Timolol maleate is a non-selective beta blocker used in the treatment of open-angle glaucoma. It decreases the IOP by reducing the aqueous humor production through blocking of the beta receptors in the ciliary body [9]. Efficacy of the marketed ocular timolol

maleate products is limited by extremely low bioavailability, frequent instillation, and concomitant patient compliance. Also there is an excessive loss of drug due to its systemic absorption and causes respiratory, cardiovascular and central nervous system side effects [10]. To minimize such problems, delivery systems based on the concept of *in-situ* gel formation can be developed. Such delivery systems consist of phase transition systems that are instilled in a liquid form and shift to the gel or solid phase once in the cul-de-sac of the eye.

Objective of the Study

The objective of this study is to formulate *in-situ* gelling system of Timolol maleate that sustained the drug release for prolonged period of time.

Materials and Methods

Pure drug Timolol maleate was purchase from Yarrow Chem products, Mumbai, India. Carbopol 940p, gellan gum and chitosan were procured from Himedia, Mumbai. All other reagents used were analytical grade.

Preparation *in-situ* gelling system

For the preparation of *in-situ* gel, gellan gum and chitosan were selected as polymer and carbopol 940p as co-polymer. A polymer solution was prepared according to the cold method. Briefly, gellan gum or chitosan alone or in combination with carbopol 940p was accurately weighed and solubilized in required volume of distilled water by continuous stirring (Remi instruments, India) for 10 minutes. The concentration of gellan gum and chitosan was kept constant and only the concentration of carbopol 940p was varied [11]. Based on the *in-vitro* gelation time and gelation capacity, optimum batch was selected for the preparation of Timolol maleate loaded *in-situ* gel. During this study, 0.1% w/v of methyl paraben was used as preservative along with 0.45% w/v NaCl for tonicity adjustment. The composition of different gelling systems is shown in table 1 and Timolol maleate loaded *in-situ* gel is shown in table 2.

Gelling system	Gellan gum (%w/v)	Chitosan (%w/v)	Carbopol 940p (%w/v)	Distilled water (%w/v)
IGG1	0.5	--	--	q.s.*
IGG2	0.5	--	0.2	q.s.*
IGG3	0.5	--	0.3	q.s.*
IGG4	0.5	--	0.4	q.s.*
IGG5	0.5	--	0.5	q.s.*
IGC1	--	0.5	--	q.s.*
IGC2	--	0.5	0.2	q.s.*
IGC3	--	0.5	0.3	q.s.*
IGC4	--	0.5	0.4	q.s.*
IGC5	--	0.5	0.5	q.s.*

Table 1: Composition of *in-situ* gelling systems.

q.s.*: Indicates quantity sufficient to make 100 ml.

Characterization of *in-situ* gel preparations

Clarity test and pH

The clarity of the formulations after and before gelling was determined by visual examination of the formulations under light alternatively against white and black backgrounds, with the contents set in motion with a swirling action. It was also observed for formation of turbidity or any unwanted particles dispersed in the prepared solution [12]. The pH of the *in-situ* gels was evaluated using digital pH metre (Anamatrix instruments, Mumbai).

Determination of gelation time and gelation temperature

The gelation time was determined by gradually increasing the temperature of the formulations, and the time required by the formulations (containing different concentrations of the polymers) to form a stiff gel was recorded using a digital stopwatch [13].

In-vitro gelling capacity

The *in-vitro* gelling capacity was determined by placing freshly prepared solution (0.5 ml) of *in-situ* gel in a vial containing freshly prepared simulated tear fluid (pH 7.4) and equilibrated at 37ºC. The visual assessment of gel formation was carried out. Time required for gelation as well as time taken for the formed gel to dissolve were also noted. Different grades were allotted with the gel integrity, and rate of formation of gel with respect to time. The grades were given as no gelation (-), gelation after few minutes and remains for 1-2 hours (+), gelation immediate and remains for up to

Gelling system	Timolol maleate (%)	Gellan gum (%w/v)	Chitosan (%w/v)	Carbopol 940p (%w/v)	Methyl paraben (%w/v)	NaCl (%w/v)	Distilled water (%w/v)
IG1	0.5	0.5	--	0.5	0.1	0.45	q.s.*
IG2	0.5	--	0.5	0.4	0.1	0.45	q.s.*

Table 2: Composition of Timolol maleate *in-situ* gel.

8 hours (++), gelation immediate and remain extended time (+++), and very stiff gel (++++) [14].

In-vitro gel erosion study

Transfer 1 gram of prepared solution in vials (inner diameter = 12.0 mm) and placed the vials in a water bath, maintained at a constant temperature of 37°C. After the formulations had transformed into gels, 2 ml of STF (pH 7.4) was pre-warmed to 37°C and was carefully layered over the gel surface. At predetermined intervals, the entire release medium was removed, and the weight of the vial and the remaining gel was recorded. *In-vitro* gel erosion study was conducted for 72 hours. The percentage weight loss of the gel was calculated by dividing the decrease in the weight of the gel by the initial gel weight [12].

Viscosity and rheological studies

Brookfield digital viscometer (model RV-DV2T) was used for the determination of viscosity and rheological properties using spindle no 4. The viscosity of gel was measured at different angular velocities at a temperature of 25°C. A typical run comprised changing of the angular velocity from 5 to 25 rpm. The viscosity measurements were done before (at pH 6.0) and after gelling (at STF pH 7.4) [13].

Drug content estimation

Drug content was determined by suitably diluting *in-situ* gel formulation with STF and analyzed by UV spectroscopy at 294 nm for timolol maleate [15].

Isotonicity evaluation

Isotonicity is important characteristic of the ophthalmic preparations. Isotonicity has to be maintained to prevent tissue damage or irritation to cornea. Opthalmic formulations were subjected to isotonicity testing to evaluate their isotonic (osmotic pressure same as body fluid), hypotonic (osmotic pressure greater than body fluid) and hypertonic (osmotic pressure less than body fluid). The tonicity of Timolol maleate cubogel was determined by hemolytic method. In this method prepared formulations were mixed with few drop of blood and observe under microscope at 45× and observe the effect of formulation on red blood cells (RBCs) such as, swelling, bursting, and cremation [16]. Finally, compare the shape of formulation mixed blood cell with isotonic (0.9% NaCl), hypotonic (0.45% NaCl) and hypertonic (3%) solution.

Test for sterility

Sterility is one of the most vital requirements for an ophthalmic preparation. The tests for sterility are intended for detecting the presence of viable forms of microorganisms in prepared ophthalmic formulations. In this study, sterility test were performed for aerobic bacteria (*Staphylococcus aureus*, ATCC 6538) and anaerobic bacteria *Bacteroides vulgatus,* ATCC NO. 8482) using fluid thioglycollate medium (FTGM) and for fungi (*Candida albicans*, ATCC 10231) using soyabean casein digest medium (SCDM) [17].

The entire study was performed in aseptic conditions in a laminar air flow hood. Glassware was autoclaved prior to use and then placed under the hood. All non-autoclavable materials were thoroughly wiped with isopropyl alcohol to make them free from microorganisms.

In-vitro release studies

The release of TM from the in-situ gel was evaluated by using Franz diffusion cell, to measure the diffusion of the drug across a cellophane membrane. The cell consisted of two compartments i.e. the donor compartment and the receptor compartment. A previously socked cellophane membrane was placed between these two compartments. Briefly, 1 ml of formulation was placed in donor compartment above the membrane. The receptor compartment contained 25 ml of stimulated tear fluid (STF) as receptor medium. The composition of STF was NaCl 6.8g, $NaHCO_3$ 2.2g, $CaCl_2 \cdot 2H_2O$ 0.08g, KCl 1.4g, and water up to 100 ml. The diffusion cell was placed over a magnetic stirrer maintained at 50 rpm at 37 ± 0.5°C [13]. At predetermined time intervals, aliquots of the release medium were withdrawn and were diluted with the receptor medium and receptor compartment was compensated with an equal volume of fresh receptor medium. The drug concentrations in the release medium at various time intervals were analyzed spectrophotometrically at 294 nm (Shimadzu 1800 UV-Visible spectrophotometer).

In-vitro corneal permeation studies

The procedure followed for corneal permeation studies was same as *in-vitro* drug release studies. Only difference is cellophane membrane used in *in-vitro* release study was replaced with goat cornea in *in-vitro* corneal permeation studies.

Data, obtained from the *in-vitro* corneal permeability study was used to calculate cumulative percentage of drug permeated, flux

(J), and apparent permeability coefficient (Papp). Apparent permeability coefficient was also calculated using the following equation:

$$\text{Papp} = \frac{dQ}{dt} \times \frac{1}{A \times Co \times 3600}$$

Where, dQ/dt ($\mu g/cm^2.h$) is the flux across the corneal tissue. A is the area of diffusion (cm^2), Co is the initial concentration of drug in donor compartment (µg/ml) and 3600 is taken as the factor to convert hour into second. The flux across the cornea was obtained from the slope of the regression line obtained from the linear part of the curve between the amount permeated (Q) versus time (t) plot and lag time (T_{lag}) was extrapolation of the linear portion to the x-axis [18,19].

In-vitro release kinetics studies

In order to understand the kinetics of drug release from *in-situ* gel formulation, data obtained from *in-vitro* drug release study were fitted into a zero-Order (cumulative percentage of drug released vs time), first Order (log cumulative percentage of drug remaining vs time), Higuchi's model (cumulative percentage of drug released vs square root of time, SQRT) and Korsmeyer-Peppas model (log percentage cumulative drug release Vs log time). By comparing the r^2 values obtained, the best fit model was selected [20].

Statistical analysis

All experiments were performed in triplicate and data were reported as a mean ± SD. Student's t-test was performed on the data sets using SPSS 16.0 for Windows®. Differences were considered significant for P values < 0.05.

Result and Discussion

For the preparation of *in-situ* gelling system, gellan gum and chitosan were used as polymers and carbopol 940p was used as co-polymer. The concentration of polymer was kept constant i.e. 0.5% w/v, whereas concentration of co-polymer was varied (0.2% - 0.5%). Formulated *in-situ* gels were evaluated for clarity, pH, gelation time, gelation temperature, gelling capacity, *in-vitro* gel erosion study, viscosity and rheological studies.

Clarity test and pH

Clarity test was carried out immediately after the preparation of *in-situ* gel formulation under white and black background. *In-situ* gel formulations were prepared by using gellan gum and chitosan alone or in combination with co-polymer i.e. carbopol 940p in different ratios. All the prepared formulations were clear or transparent in appearance without any turbidity and suspended particles or impurities. Hence, all the batches of in-situ gel passed the clarity test. pH is one of the most important parameter involved in the ophthalmic formulation. The two areas of critical importance are the effect of pH on solubility and stability. Ophthalmic formulations should have pH range in between 5 to 7.4 [21]. The observed pH was lies in the ranges between 6.1 to 6.7. Results are showed in table 3.

Formulation	Clarity	pH	Gelation temperature (°C)	Gelation time (sec)*	Gelling capacity
IGG1	Clear	6.3	No gelation up to 45°C	--	-
IGG2	Clear	6.7	38°C	190.45 ± 2.28	+
IGG3	Clear	6.2	37°C	150.22 ± 2.43	++
IGG4	Clear	6.7	37°C	128.36 ± 3.47	+++
IGG5	Clear	6.3	36°C	84.19 ± 2.36	+++
IGC1	Clear	6.5	No gelation up to 45°C	--	-
IGC2	Clear	6.2	37°C	178.31 ± 3.65	+
IGC3	Clear	6.6	37°C	139.54 ± 2.38	+++
IGC4	Clear	6.1	36°C	95.32 ± 2.46	+++
IGC5	Stiff	6.6	36°C	46.20 ± 2.32	++++

Table 3: Results of gelation temperature and gelation time.

*Average of six determinations (n = 6, ± SD).

Gelation time and gelation temperature

Gelation time and gelation temperature of prepared *in-situ* gel formulations was determined by the tilting method [12] (Figure 1), where sol-to-gel transition end-point of prepared formulation was determined by tilting the test tube at 90°. The temperature was increased from 35° to 45°C at the rate of 1°C/0.5 min. The gelation temperature of the prepared *in-situ* gelling systems was found to be in the range of 35° - 38°C except for formulation IGG1 and IGC1. Formulation batches IGG1 and IGC1 did not show any sign of gelation up to 45°C, which might be due to the presence of less concentration of polymer in those batches. Specifically, the gelation temperature for the formulation containing combination of polymers (gellan gum or chitosan) and co-polymer (carbopol 940p) was found to be in the range of 35° - 38°C, which is considered appropriate for the *in-situ* gelling of the system [12].

Figure 1: Sol-to-gel transition of the in-situ gelling system. (A) Dispersion at 25°C and (B) dispersion at the gelation temperature (36 - 38°C).

Gelation time of the prepared *in-situ* gel formulations was co-polymer concentration dependent i.e. when the concentration of carbopol 940p was increased the conversion time of sol-to-gel time of the formulation was decreased. *In-situ* gel IGG5 containing gellan gum in combination with carbopol 940p in 1:1 ratio showed shortest gelation time i.e. 84.19 seconds. Whereas, formulation IGC5 containing chitosan in combination with carbopol 940p in 1:1 ratio showed shortest gelation time i.e. 46.20 seconds. The *in-situ* gel formulation showed a rapid sol-to-gel transition at temperatures close to physiological temperature i.e. 37 ± 1.0°C. Results are showed in table 3.

In-vitro gelation capacity

The two essential requirements for phase transition system such as in-situ gel are viscosity and gelling capacity. Gelation capacity is the rate and extent of conversion of sol-to-gel. Moreover, the flow behavior of any liquid formulation is an important parameter involved in handling and in-vivo performance, because too viscous leads to difficulty in instillation; on the contrary, and too low viscosity leads to increase drainage of applied formulation. From visual and manual inspection it was found that except formulation IGG1 and IGC1 containing 0.5% of gellan gum and chitosan alone respectively, all other formulations underwent transition into gel phase upon contact with STF (pH 7.4). Formulation IGG1 and IGC1 did not showed any signs of sol-gel transition up to 45°C temperature, so these batches are rejected from the study. However, formulations containing combination of polymer (gellan gum or chitosan) and co-polymer (carbopol 940p) showed rapid sol-to-gel transition. Gelling capacity of in-situ formulations IGG4 and IGG5 was found to be good as these formulations under goes rapid gelation and remains for period of extended time, whereas in case of chitosan based in-situ gels, formulation batches IGC3, IGC4 and IGC5 showed immediate gelation. Even though formulation IGC5 showed rapid gelation, gel formed was very stiff in nature, hence rejected from the study (Table 3). However, it was clear that the nature of the gel formed depended upon the ratio of polymer and co-polymer. Based up on the results of gelation time and gelling capacity, formulation batch IGG5 and IGC4 was selected for the evaluation of *in-vitro* release studies.

Viscosity and rheological studies

The variation in viscosity under different conditions is an important rheological parameter to be considered in the utilization and *in-vivo* performance of pH as well as ion activated *in-situ* gels. Formulations having excessively high viscosity are difficult to administer, whereas with low viscous formulation drainage of the formulation is a challenge. As such, the optimum preparation should possess lower viscosities under storage conditions and higher viscosities in physiological environments.

The viscosity of *in-situ* gel was evaluated using Brookfield viscometer under increased shear stress. Viscosity was evaluated at formulation pH (pH 6.0 i.e. before gelation and at ocular pH (STF pH 7.4) i.e. after gelation. It was observed that formulations containing combination of polymer and co-polymer imparted viscosity to the formulation without affecting its clarity (except IGC5, containing chitosan: carbopol 940p 1:1 ratio formed turbid solution). Formulation batches IGG1 and IGC1 containing 0.5%w/v of gellan gum and chitosan alone respectively, were low viscous solutions and there was no much difference in viscosity at formulation pH (pH 6.0) and at ocular pH (pH 7.4) and there was no sign of gelation up 45°C, hence rejected from study. Formulations prepared with combination of polymer and co-polymer showed concentration dependent increase in viscosity. On increase in the concentration

of carbopol 940p (co-polymer) viscosity of the formulation was also increased gradually. At pH 6.0 (25°C), all formulations were in a liquid state and exhibited low viscosity (Figure 2). When the pH and temperature was increased to 7.4 and 37°C respectively, solution state was transformed into the gel of high viscosity (Figure 3). The viscosity was also evaluated under different shear stress. Viscosity of the prepared formulations was decreased with increase in shear stress and exhibited more pseudo-plasticity.

Figure 2: Comparative rheological profile of in-situ gel formulations before gelation.

Figure 3: Comparative rheological profile of in-situ gel formulations after gelation.

Drug content estimation

Drug content was determined using UV method. The drug content was found to be 97.78 ± 0.34% and 98.06 ± 0.371% respectively for IG1 and IG2 formulation.

In-vitro gel erosion study

The *in-vitro* erosion test was conducted for 72 hours to evaluate the gel strength of the prepared formulation. Gel erosion was calculated by determining the gel loss (% w/w) at various time intervals, after the gel-containing vials were allowed to incubate at 37°C. The formulations batches IGG2, IGG3, IGG4, IGG5, IGC2, IGC3, IGC4, and IGC5 showed 49.0%, 38.15%, 33.36%, 30.54%, 43.62%, 36.97%, 32.94% and 24.57% respectively, after 72 hours of the study. Formulation batches IGG1 and IGC1 containing gellan gum and chitosan alone respectively does not showed any sign of gelation up to 72 hours of study, hence not included in the study.

The *in-vitro* gel erosion study showed that the formulation containing chitosan: carbopol 940p in 1:1 ratio had relatively higher gel strength (only 24.57% of gel erosion after 72 hours). Formulation containing polymer and co-polymer combination showed that the gel strength of the system was increased when the concentration of carbopol 940p was increased. This might be the fact that the carbopol 940p act as a gelling agent and it tends to improve the gel strength. The optimum batch was selected by studying the effect of various concentrations of carbopol 940p (0.2 - 0.5%) on the viscosity, gel erosion, gelation time and gelling capacity. When the con-

centration of carbopol 940p was increased from 0.2 to 0.5%, the gel strength was also found to be increased. However, the gel formulation containing 1:1 ratio of chitosan: carbopol 940p resulted in a turbid formulation. Thus, a concentration of 0.5% carbopol 940p in case of gellan gum based formulation and concentration of 0.4% carbopol 940p in case of chitosan based formulations were found to be optimal combination for the preparation of ion activated and pH responsive in-situ gel formulations; this concentration resulted in a gel with sufficient gel strength without any noticeable turbidity. Results are shown in table 4.

***In-situ* gel formulation**	**Percentage gel loss (%w/w)**				
	6 hours	**12 hours**	**24 hours**	**48 hours**	**72 hours**
IGG1	No gel formation	No gel formation	No gel formation	No gel formation	No gel formation
IGG2	4.4 ± 0.06	7.8 ± 0.95	16.4 ± 1.63	26.7 ± 2.02	49.0 ± 2.72
IGG3	3.8 ± 0.17	7.5 ± 1.05	15.9 ± 2.12	23.4 ± 2.60	38.15 ± 3.03
IGG4	3.4 ± 0.72	7.1 ± 1.14	13.4 ± 2.18	20.6 ± 1.98	33.36 ± 2.72
IGG5	2.8 ± 0.09	6.8 ± 1.71	10.2 ± 2.08	18.7 ± 2.52	30.54 ± 2.42
IGC1	No gel formation	No gel formation	No gel formation	No gel formation	No gel formation
IGC2	4.1 ± 0.16	7.5 ± 1.06	14.9 ± 1.96	24.88 ± 2.26	43.62 ± 2.16
IGC3	3.5 ± 0.09	7.2 ± 1.16	12.7 ± 1.21	20.84 ± 2.72	36.97 ± 3.07
IGC4	3.0 ± 0.14	6.9 ± 1.11	10.6 ± 1.61	18.5 ± 3.04	32.94 ± 3.22
IGC5	2.5 ± 0.17	4.7 ± 1.09	7.9 ± 1.55	12.8 ± 1.86	24.57 ± 2.88

Table 4: Results of *in-vitro* gel erosion studies as a function of time.

In-vitro drug release studies

The *in-situ* gel formulations of TM were solutions at room temperature and turned into stiff gels at body temperature and pH. Franz diffusion cell was used to determine *in-vitro* drug release studies. Marketed formulation released 98.74% of drug within 2 hours, whereas *in-situ* gel formulations sustained the drug release over a period of 10 hours. Drug release from the *in-situ* gel was significantly higher than that of marketed formulation ($P > 0.05$). Presence of gellan gum in IG1 may form gel network after being influenced by cations from tears, and sustained the drug release. In IG2 formulation, chitosan was used as gelling agent. Chitosan is a cationic polymer and it electro-statically interacts with negatively charged mucus, giving way to the mucoadhesion property to prepared formulation. The optimized batch of TM *in-situ* gel formulation showed sustained release behaviour over a period of 10 hour. At the end of 10 hour, more than 95% of TM was released from both formulations. The results of release studies showed there was no significant difference ($P < 0.05$) in drug release from both batches. Thus, it conforms sustain release behavior of the *in-situ* gel formulations. It was also observed that *in-situ* gel showed burst release (initial quick release) of the drug, it would be beneficial for achieving the therapeutic concentration of drug in a short period of time. This burst release might be due to initial migration of the drug toward the surface of the matrix of the gel [21]. Later on it showed sustained release behaviour of the drug. These sustain release behaviour would be beneficial for reducing number of application of drugs per day. The results of *in-vitro* drug-release study of marketed formulation and TM-loaded *in-situ* gel are shown in figure 4.

Figure 4: Comparative drug release profile of TM from in-situ gel and marketed formulation.

In-vitro release kinetics studies

Mathematical models like zero-order, first-order, Higuchi model, Hixson-Crowell and Korsmeyer-Peppas models are important tool to understand the drug release kinetics of prepared formulations. The *in-vitro* release data were fitted to those kinetics models to predict the drug release mechanism from the prepared *in-situ* gel formulations. The results showed that all the formulations were best explained by Higuchi release kinetics model, as the plots shows high linearity (r^2 = 0.996 - 0.998) in comparison to zero order (r^2 = 0.931 - 0.941), first order (r^2 = 0.922 - 0.968) and Hixson-Crowell model (r^2 = 0.588 - 0.613). Other researchers also reported Higuchi square root model for *in-situ* gel formulation [22]. Hence, it can be concluded that prepared *in-situ* gel formulations followed Higuchi's release pattern, where the drug diffuses at a slower rate as the distance for diffusion increases, referred to the square root kinetics.

To confirm the drug release mechanism, the drug release data were further analyzed using Korsmeyer-Peppas release kinetics model. For the *in-situ* gel formulations Ig1 and IG2, the values of release exponent (n) were found in the range of 0.90 - 0.93, which indicate that prepared *in-situ* gel formulation followed Super-case II transport mechanism as their 'n' values are higher than 0.89 [23].

Sterility testing

Sterility is one of the pre-requisite criteria for ophthalmic preparations. The presence of microbes in the preparation may cause irritation, inflammation or may infect the corneal surface. Sterility of prepared formulations was ensured during the preparation process. In-situ gel formulations of Timolol maleate were sterilized by autoclaving at 15lbs pressure (121°C) for 15 minutes [13]. Formulations are tested for sterility using direct inoculation technique in a FTGM (for aerobic and anaerobic bacteria) and SCDM (for fungi) as per IP procedure. The formulations incubated with media suitable for the growth and proliferation of aerobic/anaerobic bacteria and fungi showed no growth (no turbidity) at the end of 14 days. The negative control and test samples remained clear when compared with positive control (showed turbidity). Results of sterility testing (Figure 5-7), showed there was no evidence of microbial growth in the 'test' and 'negative control' tubes, whereas 'positive control' tube showed macroscopic evidence of microbial growth (turbidity). The results suggested that the cubogel and in-situ gel tested for aerobic/anaerobic bacteria and fungi passed the test for sterility and also it proved the effectiveness of autoclave sterilization.

Figure 5: Sterility test for aerobic bacteria in fluid thioglycollate media.
-ve control: Sterile water
+ve control: *S. aureus*
Test: In-situ gel formulation

Figure 6: Sterility test for Anaerobic bacteria in fluid thioglycollate media.
-ve control: Sterile water
+ve control: *B. vulgatus*
Test: In-situ gel formulation

Figure 7: Sterility test for fungi in soybean casein digest media.
-ve control: Sterile water
+ve control: Candida albicans
Test: In-situ gel formulation

Isotonic evaluation

Isotonicity means that the tone of the cell will not be affected, either by the ingress of water from the instilled solution (in case of hypotonic solution) or egress of water from the cell (in case of hypertonic solution). If the formulation is hypo/ hypertonic in nature, it tends to irritate sensitive tissues like eyes and also cause cellular damage and ocular pain [24].

Prepared formulations were mixed with blood and were observed under microscope at 45×. Similarly few drops of isotonic (0.9% NaCl), hypotonic (0.45% NaCl) and hypertonic (3% NaCl) solutions were also mixed with blood and observed under microscope at 45×. The size of RBCs was not altered (no bulging or shrinkage) when exposed to test formulation and was found to be very much

similar as when treated to normal saline solution (Figure 8-11). The results obtained from the above study revealed that optimized formulation was isotonic with lachrymal fluid and/or blood [25]. Blood cells treated with hypotonic solution appears to be swollen (Figure 12), whereas hypertonic solution treated blood cell were shrunk (Figure 13).

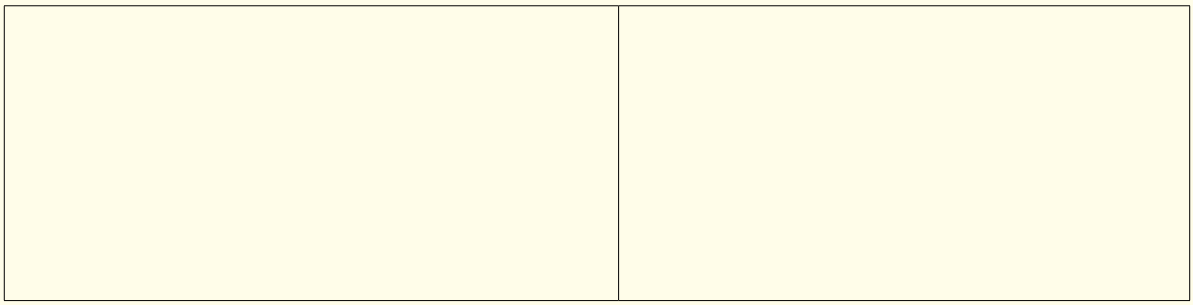

Figure 8: Red blood cell alone.

Figure 9: RBCs with normal saline (0.9% NaCl).

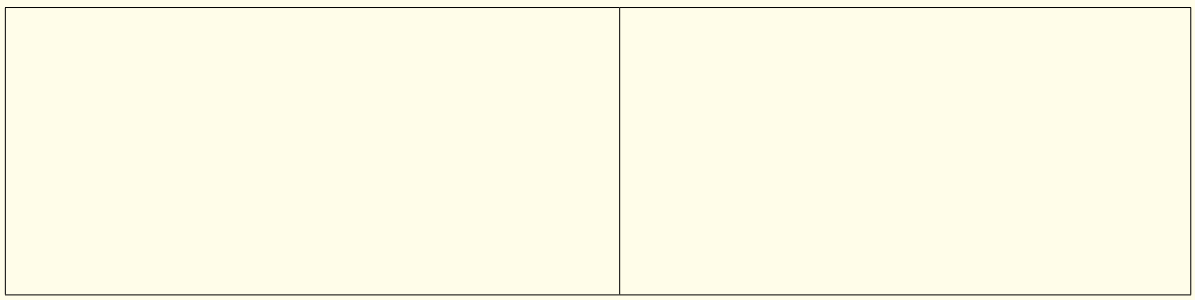

Figure 10: RBCs with in-situ gel (IG1).

Figure11: RBCs with in-situ gel (IG2).

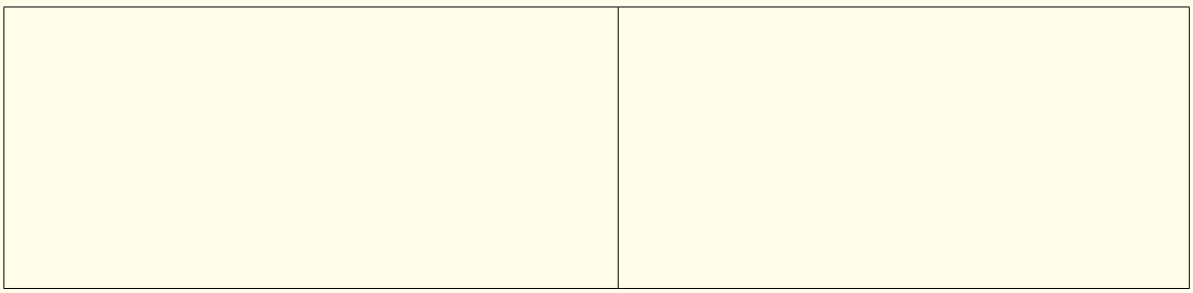

Figure 12: RBCs with hypertonic solution.

Figure 13: RBCs with hypotonic solution.

In-vitro corneal permeation studies

Ability of the substance to permeate the corneal barrier depends on various factors i.e. chemical nature of substance, size and conformation, lipid/water partition co-efficient, and degree of ionization etc. Epithelium of cornea is lipidic in nature and it is main barrier for hydrophilic drug whereas the aqueous stroma of cornea is the prime barrier for hydrophobic agent [17]. Transcorneal permeation of Timolol maleate *in-situ* gel was compared with marketed formulation (Figure 14). The drug permeated from marketed formulation was 19.48% whereas *in-situ* gel formulations IG1 and IG2 released 39.04% and 37.31% in 10 hours. *In-situ* gel formulation showed a significantly higher permeation ability as compared to marketed formulation ($P > 0.05$). As showed in table 5, apparent permeability coefficient (Papp) of Timolol maleate in *in-situ* gel formulation IG1, IG2 and marketed formulation was 1.56×10^{-5}, 1.41×10^{-5} and 0.749×10^{-5} cm/s, respectively. Compared with marketed formulation, Timolol maleate *in-situ* gel IG1 and IG2 exhibited a 2.08 and 1.88-fold increase in Papp, suggesting that higher amount of Timolol maleate *in-situ* gel were taken up by the goat cornea than that of marketed eye drops. The higher corneal permeation ability

of *in-situ* gel might be due to higher mucoadhesive nature of the polymer used in the *in-situ* gel formulation.

Figure 14: Comparative %CDP of prepared TM in-situ gel and marketed formulations.

Formulation code	Flux (J) (µg/cm^2·h)	Papp × 10^{-5} (cm/s)
MF	10.12	0.749
IG1	21.08	1.56
IG2	19.24	1.41

Table 5: Steady state fluxes (J) and apparent permeability coefficient (Papp), of various formulations.

Conclusion

In order to minimize the drawbacks of conventional eye drops formulations *in-situ* gel formulations were prepared. *In-situ* gel formulation of Timolol maleate was prepared by cold method. Two different types of gelling systems i.e. ion activated system and temperature dependent system were used. Gellan gum acts as ion activated system and chitosan acts as temperature dependent gelling system. *In-situ* gel was isotonic and sterile in nature as it passes sterility test. *In-situ* gel sustained the drug release upto 10 hours which was comparatively longer period than marketed eye drops. *In-vitro* corneal permeation efficacy of *in-situ* gel was significantly higher than marketed formulation. In conclusion *in-situ* gel might be a good alternative for conventional eye drops as it sustains the drug release for prolonged period of time and may also reduces the number of application of the drug. Further animal studies will be performed to understand the *in-vivo* efficacy of the drug.

Acknowledgement

We are thankful to Principal, Sri Adichunchanagiri College of Pharmacy, Bangalore, and Rajiv Gandhi University of health sciences, Bangalore, for providing all necessary facilities to carry out this PhD research work.

Conflict of Interests

The authors declare no conflict of interests regarding the publication of this article.

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