

DEVELOPMENT AND EVALUATION OF CHITOSAN BASED THERMOSENSITIVE *IN SITU* GELS OF PILOCARPINE

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ABSTRACT

Several ophthalmic delivery systems have been developed to prolong the pre-corneal-drug contact time for enhancing diffusion and to improve ocular bioavailability. *In situ* gelling systems prolong the corneal contact time, thereby improving bioavailability and are convenient to administer. The aim of the present study was to develop a novel thermosensitive *in situ* gelling polymeric system of pilocarpine for prolonged retention in the eye. For this purpose, *in situ* gels were prepared using chitosan and sodium glycerophosphate. The prepared *in situ* gels were evaluated for sterility, content uniformity, gelation temperature and time, viscosity, isotonicity, *in vitro* drug release and stability studies. *In vivo* eye irritancy test was performed using albino rabbits to evaluate its acceptability. Polymeric systems were in solution form, but transformed into clear, translucent gels with increase in temperature. Viscosity of the gels increased with increase in the polymer concentration and temperature. Drug release was controlled for 12 h. The release followed fickian diffusion and showed first order kinetics, confirmed by Higuchi plots. The *in situ* gel was isotonic, non-irritant to rabbit eyes and was well tolerated. The *in situ* gels were stable during the stability testing period. The results demonstrated that pilocarpine *in situ* gels may prolong the drug residence time, enhance bioavailability thereby improve patient compliance. It may be concluded that thermosensitive *in situ* gels of pilocarpine can be a suitable alternate to conventional ocular drug delivery system.

Keywords: Pilocarpine, *In situ* gels, Chitosan, Sodium Glycerophosphate, Thermosensitive.

INTRODUCTION

Ocular drug delivery is one of the most interesting and challenging endeavours faced by a Pharmaceutical scientist. Physiological barriers to diffusion and productive absorption of topically applied drug exists in the pre-corneal and corneal spaces. The pre-corneal constraints responsible for the poor ocular bioavailability of conventional ophthalmic dosage forms (ointments, drops, lotions, suspensions) are solution drainage, lacrimation, tear dilution, tear turnover and conjunctival absorption. Drainage of drug solution from the pre-corneal area has been concerned to be the most significant factor in reducing the contact time of the drug with the cornea and consequently ocular bioavailability of topical dosage forms. The instilled dose leaves the pre-corneal area within two minutes of instillation in humans. In rabbits, the process of drainage, generally takes 5 to 10 min. Topical application of ophthalmic drugs is further made inefficient by tear turnover, which is about 16% in humans.

The conventional ophthalmic dosage forms are the simplest form of the drug delivery system, but they suffer certain drawbacks such as frequent medication, dilution and drainage of medication by tear fluid, reduced bioavailability etc. [1] Despite these severe limitations, significant improvements in ocular drug delivery have been made. The improvements have been with the objective of maintaining the drug in the bio phase for an extended period. It is a challenge to the formulator to circumvent the protective barriers of the eye, so that the drug reaches the biophase in sufficient concentration. Aqueous solutions have the disadvantage of being quickly removed from the front of the eye resulting in poor ocular bioavailability. A solution or suspension form of a drug delivery system is preferred, provided retention time in the eye is extended.

In order to overcome the drawbacks of the conventional dosage forms novel ocular drug delivery system have been developed to increase the contact time, by using viscosity enhancers, gels, penetration enhancers, prodrugs, liposomes, nanoparticles, inserts, *in situ* forming gels, oil-in-water emulsion, colloidal drug delivery system, micro particulates etc. [2,3]

In situ gels refer to polymer solutions which can be administered as liquid, and undergo a phase transition to semisolid gel upon exposure to physiological environments. The gelation can be

triggered by temperature change, change in pH, and presence of ions. [4] They can be conveniently instilled as a solution into the conjunctival sac of the eye. Upon contact with the eye, the system changes conformation and forms a clear, transparent gel. This type of formulation has advantage of a solution being patient convenient with the favourable residence time of a gel. Hence, there is a need for *in situ* gels which can prolong the drug-cornea contact time, enhance bioavailability, reduce the frequency of administration and be patient friendly.

Pilocarpine is a cholinergic parasympathomimetic, positively charged quaternary ammonium compound agent. It increases secretion by the exocrine glands, and produces contraction of the iris sphincter muscle and ciliary muscle (when given topically to the eyes) by stimulating muscarinic receptors. When applied topically to the eye as a single dose, it causes miosis, spasm of accommodation, and may cause a transitory rise in intraocular pressure followed by a more persistent fall. [5]

Chitosan is produced by deacetylation of chitin, which is the structural element in the exoskeleton of crustaceans (crabs, shrimp, etc.). It is a biocompatible, pH-dependent cationic polymer, which is soluble in water up to pH 6.2. Chitosan is bioadhesive and readily binds to negatively charged surfaces such as mucosal membranes. Chitosan enhances the transport of polar drugs across epithelial surfaces, and is biodegradable. [6] The potential use of chitosan based systems (gels, colloidal systems and nanoparticles) for improvement of the retention and absorption of drugs applied topically onto the eye has been explored previously. Because of its low toxicity and good ocular tolerance, favorable biological behavior, such as bioadhesion and permeability-enhancing properties, chitosan is considered unique material for design of ocular dosage forms. [7]

Sodium glycerophosphate is the sodium salt of glycerol 3-phosphate, an organophosphate. Sodium glycerophosphate is used as a source of phosphate in the treatment of imbalances of calcium and phosphate metabolism. It is used as a source of sodium and phosphates in tonics, also used as emulsifier. [8]

The focus of the present investigation is to prepare and evaluate sodium glycerophosphate-chitosan system as *in situ* gelling vehicle for delivery of pilocarpine.

MATERIALS AND METHODS

Materials

Pilocarpine Hydrochloride BP (mol. wt. 244.75 g/mole) was a gift sample from Medreich Pharmaceuticals, Bangalore, India; Chitosan (water soluble grade) (avg mol wt. 10,000-10,00,000) was sourced from Marine Chemicals, Cochin, India; Sodium Glycerophosphate (mol. wt. 216) was procured from Vaya Jayanthi Drug Private Limited, Hyderabad, India. Other solvents and chemicals were of analytical grade, used without further processing.

Methods

Spectral Analysis

A sensitive UV spectrophotometric method was used to analyze pilocarpine hydrochloride in solution and formulations. Weighed amount of pilocarpine was dissolved in simulated lachrymal fluid

(sodium bicarbonate-0.2 g, calcium chloride dehydrate-0.008 g, sodium chloride-0.67 g, distilled water to 100 ml) and scanned between 200-400 nm in UV spectrophotometer (Shimadzu UV 1800, Japan). The absorption maximum of 215.5 nm was observed, used for further studies. [9]

Preparation of *in situ* gel

Chitosan (water soluble) and sodium glycerophosphate were dissolved in distilled water separately at room temperature. Complete solvation was achieved by placing the solutions in refrigerator for 2 h. The glycerophosphate solution was then added dropwise into the chitosan solution with constant stirring. Pilocarpine hydrochloride (2% w/v) was dissolved in small volume of distilled water and then added to polymeric solution with stirring. The stirring was continued for 10 min to gain homogenous mixture. [10,11] Benzalkonium chloride was added as preservative in the preparation. The formulation chart is presented in Table 1.

Table 1: Composition of pilocarpine *in situ* gels

Ingredients	Formulation Code					
	F1	F2	F3	F4	F5	F6
Pilocarpine Hydrochloride (%w/v)	2	2	2	2	2	2
Chitosan (%w/v)	1.75	2.0	2.25	1.75	2.0	2.25
Sodium Glycerophosphate (%w/v)	25.0	25.0	25.0	27.5	27.5	27.5
Triethanolamine	q.s	q.s	q.s	q.s	q.s	q.s
Benzalkonium chloride (%w/v)	0.001	0.001	0.001	0.001	0.001	0.001

Sterilization of *in situ* gels

The sterilization was carried out by exposing *in situ* gels filled in glass vials to UV radiations in a UV chamber for a period of 60 min at room temperature (25°C) in air.

IN VITRO CHARACTERIZATION

Sterility Testing

The effectiveness of sterilization was ascertained by incubating *in situ* gel in fluid thioglycollate media [aerobic and anaerobic bacteria (in anaerobic chamber)] and soyabean casein digest media (fungal organisms) in an incubator (Thermocon Instruments, Mumbai, India) at 37±1°C for a period of 14 days. The growth of bacteria/fungus was evaluated at the end of incubation period.

Determination of Drug Content

1 ml of *in situ* gel was dissolved in simulated lachrymal fluid and the volume was made to 100 ml. Uniformity of the drug content was evaluated by measuring the absorbance at 215.5 nm in UV-spectrophotometer (Shimadzu-1601, Japan) after suitable dilution.

Gelation Temperature

Two milliliter of *in situ* gel was heated in a thin walled test tube (internal diameter-10 mm, length-76 mm, thickness-0.5 mm) placed in a thermostatically controlled water bath (INSIF, Ambala, India) with frequent shaking till it got converted to gel. The water was set to heat at a rate of 2°C/5 min constantly. Gelation was considered complete, when the gel in the test tube did not flow when overturned.

Gelation Time

It was done by tube inverting method. The temperature of water bath (INSIF, Ambala, India) was set at gelation temperature and allowed to maintain for 10 min. A thin walled test tube containing 2 ml of *in situ* gel was placed in water bath. The *in situ* gel was observed for gelation by inverting the test tube at periodic intervals. The gelation time was noted when there was no-flow when the test tube was inverted.

Determination of Viscosity

The viscosity studies of all the formulations were measured by using Brookfield viscometer (Model-DV II+ pro, USA) using spindle no. 5 at

30 rpm. Viscosity was measured (n=3) at two different temperatures viz. 8±1°C and 37±1°C.

Isotonicity test

The tonicity of the prepared *in situ* gels was evaluated. On two separate slides, few drops of *in situ* gels and marketed eye drops were placed separately. Few drops of freshly drawn blood was added to both slides, mixed and then observed under microscope at 45X magnification. The results were compared for any changes in RBCs in two slides.

In vitro drug release studies

In vitro drug release study was carried out using open (diffusion) tube apparatus. [12] The semi-permeable cellophane membrane, presoaked overnight in the freshly prepared simulated lachrymal fluid (7.4), was tied to one end of an open tube, acted as donor compartment. 1 ml of *in situ* gel was placed inside the donor compartment in contact with the cellophane membrane. The tube was vertically held by a stand and suspended in 100 ml of simulated lachrymal fluid maintained at 37±1°C touching the surface of receptor medium. The receptor medium was stirred at 100 rpm using magnetic stirrer. The aliquots of 3 ml were withdrawn at regular intervals and replaced by an equal volume of warm receptor medium every time. The amount of pilocarpine released was analyzed spectrophotometrically at 215.5 nm (Shimadzu UV-1800, Japan).

The drug release profiles obtained were fit into various mathematical models to determine the mechanism of drug release and release kinetics. The cumulative drug release data was fit into Korsmeyer-Peppas equation and Higuchi equation to ascertain the mechanism of drug release and its kinetics.

In vivo studies

Approval from institutional animal ethics committee was obtained for conducting the eye irritancy studies. Six adult albino rabbits of either sex weighing 2.0 ± 0.5 kg were used for the study. All rabbits were housed in separate restraining cages during the study. Food and water were freely accessible for the animals; rabbits were allowed to move their legs and eyes during the study.

At the start of the study, both eyes were washed with 2 ml water for injection and left for 10-15 min. The rabbits were instilled with

normal saline in the left eye (control) and pilocarpine *in situ* gel in the right eye (Test). After instillation, the eyes were observed with torch light and magnifying lens for reactions like discharge, redness and reflex tearing etc.

The eye irritancy potential testing was done as previously described by Draize (Draize test). [13] During the study, right eye (test) was observed for parameters like opacity, redness, chemosis (swelling) and discharge and compared with control (left eye). Conjunctiva and iris were observed for changes. Periodic observation on both eyes of the rabbit was done; ocular parameters were scored as previously described by Draize.

Stability studies of *in situ* gels

Optimized *in situ* gels were filled into glass vials and sealed with rubber caps with aluminium crimping. The stability studies were performed at $25 \pm 2^\circ\text{C}$ and $60 \pm 5\%$ RH and $30 \pm 2^\circ\text{C}$ and $65 \pm 5\%$ RH for 90 days by placing them in stability chambers (Thermolab, India).

RESULTS AND DISCUSSION

In situ gels of pilocarpine were formulated using sodium glycerophosphate and chitosan. Six formulations were prepared by varying the concentrations of polymer and co-polymer. The prepared *in situ* gels were colorless and semi-transparent with good appearance.

Chitosan solutions are reported to form clear, colorless transparent gels. Hence the chitosan was selected at 2%w/w concentration. Chitosan solutions can be transformed into thermosensitive gelling systems in the presence of polyols like glycerophosphates. These gelling systems in the presence of glycerophosphate possess a neutral pH, remain liquid at or below room temperature, and form monolithic gels at body temperature. [14] Hence the sodium salt of glycerophosphate was incorporated to prepare thermosensitive chitosan gels.

The concentrations of sodium glycerophosphate and chitosan in the gel were varied to study the effect of concentration on gelling and drug release properties. Chitosan by its inherent property of bioadhesion, adheres to the biomembrane and prolongs the corneal retention time. The contribution of chitosan in retarding the drug release along with glycerophosphate was also studied.

The initial pH of gels varied from 5.9 to 6.5; at this pH range, the gels would not cause irritation to ocular surface. It was observed that

increase in the concentration of glycerophosphate increased the pH of gels. The presence of glycerophosphate lowered the surface electrostatic charge of chitosan and elevated the pH of the system. Further, pH of gels was adjusted to 7.4 using phosphate buffer. The buffered gels were used for further studies.

Sterility is one of the pre-requisite criteria for ocular preparations. The presence of microbes in the preparation may cause irritation, inflammation or may infect the corneal surface. The gels incubated with media suitable for the growth and proliferation of aerobic/ anaerobic bacteria, fungi showed no growth at the end of 14 days. This indicated that *in situ* gels were free from bacteria and fungi; moreover, it proved the effectiveness of radiation sterilization.

The distribution of drug in the dosage form ensures the content uniformity of drug. This allows the patient to get the required amount of drug at each instillation, whereby the benefits from the drug can be gained. In all the formulations, pilocarpine was uniformly distributed and varied from $97.82 \pm 0.17\%$ to $99.20 \pm 0.09\%$.

Chitosan/glycerophosphate gel exhibited thermosensitive property, which was liquid (solution) at refrigerated temperature and solidified into a white semi-transparent hydrogel at body temperature. Gelation properties determine the ability of polymeric system to undergo transition from sol to gel state in response to environmental variables like temperature, pH, pressure, ions etc. The particular temperature when the system in sol converts to gel state in response to the temperature is termed as gelation temperature.

An ideal thermosensitive ophthalmic system should exhibit a sol-gel transition temperature higher than room temperature (25°C), preferably (30°C) and form gel at pre-corneal temperature (37°C) even though diluted by small volume of tear fluid. If gelation occurs at temperature below 30°C ; that would be difficult in instillation to eye as the system would already be gelled. If gelation occurs at temperature higher than 37°C , the formulation would be drained by lachrymal secretions without serving the purpose. Hence, an ideal ophthalmic preparation should possess a gelation temperature between $30\text{--}37^\circ\text{C}$. In the study, all formulations gelled at temperature below 40°C (Table 2). Formulation F3 and F4 recorded the highest and lowest gelation temperatures of 39.66°C and 31.33°C respectively. Since the gelation temperatures of formulation F3 and F6 were higher than body temperature, they were not suitable for ophthalmic use.

Table 2: Characteristics of pilocarpine *in situ* gels

Formulation Code	Initial pH	Drug Content (%)	Gelation Temperature ($^\circ\text{C}$)	Gelation Time (sec)	Viscosity (cps)	
					8°C	37°C
F1	5.93 ± 0.058	97.82 ± 0.17	33.00 ± 1.00	26.2 ± 1.23	437.33 ± 8.5	7634.00 ± 49.5
F2	6.00 ± 0.100	98.00 ± 1.00	36.33 ± 0.57	31.5 ± 0.98	682.33 ± 25.5	10132.00 ± 48.1
F3	6.07 ± 0.058	98.33 ± 0.71	39.66 ± 0.75	37.1 ± 1.06	877.67 ± 21.2	8376.67 ± 31.1
F4	6.20 ± 0.100	99.00 ± 0.91	31.33 ± 0.46	23.6 ± 1.26	388.67 ± 24.0	6818.67 ± 38.2
F5	6.23 ± 0.115	98.66 ± 0.58	34.00 ± 0.68	28.4 ± 1.14	582.33 ± 33.2	9705.67 ± 27.6
F6	6.47 ± 0.058	99.20 ± 0.09	38.33 ± 0.39	35.3 ± 1.34	771.00 ± 32.5	7963.33 ± 26.2

* Mean \pm SD, n = 3

It was noticed that the gelation temperature decreased with gels possessing higher concentration of glycerophosphate. Similar trend was observed with chitosan too. The concentration of chitosan and glycerophosphate significantly affected (decreased) the gelation temperature of the polymeric system. This may be due to the detachment of chitosan chain by poly-alcohol group of glycerophosphate, which accelerates the formation of a hydrophilic shell around the chitosan molecule, improving the chitosan chain protective hydration, preventing the associative effects at low temperatures and neutral pH. However, with an increase in temperature, hydrophilic interactions and hydrogen bonding play an important role and trigger physical cross-linking throughout the solution, starting the gelation process. [15]

The mechanism of sol-gel transition in the chitosan-glycerophosphate system includes hydrophobic interactions,

hydrogen bonding, electrostatic interactions, and molecular chain movement. At low temperature, the solubility of solute is probably due to hydration of the chitosan promoted by glycerophosphate. Upon heating, the chitosan chains lose their water of hydration, bonding between chains can occur and gelation proceeds. Three types of interactions may be involved in the process of gelation: (1) electrostatic attraction between the ammonium groups of chitosan and the phosphate group of glycerophosphate; (2) hydrogen bonding between polymer chains due to reduced electrostatic repulsion after neutralization of the chitosan solution with glycerophosphate; and (3) enhancement of chitosan-chitosan hydrophobic interactions by structuring action of glycerol. [13,15] Results of gelation studies were satisfactory. The gelation temperature of gel systems was in the range of 31.33°C – 39.66°C (Table 3).

The time required for the polymeric system to transform from sol-to-gel at its gelation temperature is considered as gelation time. Ideally, the system is expected to gel immediately or within a brief time upon exposure to its gelation temperature. All *in situ* systems recorded a quick gelation time, which varied from 23.6 ± 1.26 to 37.1 ± 1.06 sec (Table 2), this implies that system can undergo *in situ* gelation quickly upon instillation into the eye. If time taken for gelation is high, instilled gel will get diluted and removed quickly by tear fluid. The short gelation time observed may be attributed to the degree of deacetylation and concentration of chitosan and glycerophosphate. [16]

The viscosity of pilocarpine gels were evaluated at two temperatures, 8°C and 37°C representing their storage and body temperature. The gel was equilibrated by holding at respective temperature before the measurements were made (n=3). All the formulations exhibited viscosity characteristic to polymer. Viscosity was low at storage temperature (8°C), but their viscosities increased at body temperature studied (37°C) in lieu of sol-gel transition. As the concentration of chitosan increased the viscosity also increased. But the viscosity of *in situ* systems decreased with increase in concentration of glycerophosphate. This may be due to the protective hydration of chitosan chains in the system. Viscosity of formulations F3 and F6 was found to be comparatively less at 37°C when compared to other formulation because of incomplete gelation as they gelled above body temperature. A significant increase in viscosity was observed between the formulations at 8°C and 37°C. Formulation F3 and F2 exhibited the highest viscosity values of 877.67 ± 21.2 cps and 10132.00 ± 48.1 cps at 8°C and 37°C respectively.

Viscosity values play a significant role in optimizing the formulation suitability for instillation in the eye. It is an indication in predicting the corneal contact time with the gel. An ideal ophthalmic formulation should possess viscosity to prolong the contact time with cornea, thereby increasing the drug absorption and efficacy but able to be instilled into eye easily.

'Isoosmotic' nature is important in maintaining the normal functioning of the cells or tissues at the site of injection/ instillation. 'Isotonic' refer to preparations that are isosmotic with the cell contents, across specific membrane and in addition, maintains the tone of the membrane. Hypo/ hypertonic preparations tend to

irritate sensitive tissues like eyes and cause pain when applied to mucus membrane of eye, nose, ear etc. the degree of irritation is related degree of deviation from isotonicity.

Hypo/hypertonic preparations may affect the normal functioning of the eye. If a preparation is isotonic, the tone of the cell will not be disturbed either by ingress of water from the instilled solution (hypotonic) or egress of water of the cell (hypertonic). In either case, cellular damage occur causing pain, tissue irritation and damage. The test solution did not showed swelling or shrinkage of blood cells.

The pH of prepared *in situ* gels was lesser than the pH of lachrymal secretions. It was adjusted to the pH of the eye (7.4) before the test. It was observed in both cases, there was no change in the shape of blood cells (bulging or shrinkage), which indicated that prepared *in situ* gels were isotonic in nature.

In vitro release data provides information about the efficiency of a delivery system under test conditions. The proposed model needs to be similar with regard to *in vivo* conditions. The study helps in predicting the residence time, drug release, bioavailability and related parameters from the study. Open diffusion tube apparatus provides the simplicity and design similar to ocular conditions. The values obtained during the study will be helpful in predicting the *in vivo* performance of the delivery system.

From the *in vitro* release data, it was observed that the incorporated polymers retarded the drug release from *in situ* gels. Out of six formulations, three formulations sustained the drug release for 10-12 h, but other formulations released drug quickly within 6-8 h. Formulation F3 released pilocarpine quickly by the end of 6 h (95.23%), whereas F5 sustained the release of pilocarpine for 12 h (94.35%). An interesting observation was made during the study; the release rates of formulations F3 (95.23%, 6 h) and F6 (99.78%, 7 h) were faster than their preceding formulations [F2-92.17%, 10 h and F5-94.35%, 12 h). It was obvious to expect that higher concentration of polymer retards the drug release. In this case, the polymeric systems (F3 & F6) were in sol form and had not been completely gelled at the temperature studied (37°C), since their gelation temperature were above body temperature resulting in faster drug diffusion. The cumulative percent drug release from *in situ* gels is shown in Figure 1.

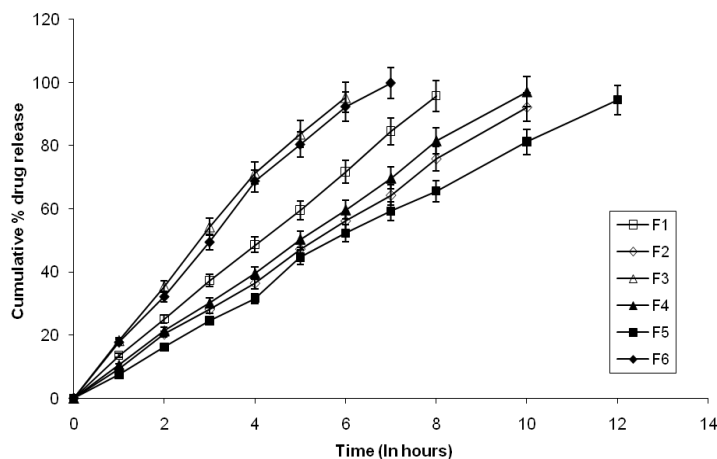


Fig.1: *In vitro* drug release profile of pilocarpine from *in situ* gels

Concentration of chitosan affected the cumulative drug release. Formulations with higher percentage of chitosan retarded the drug release. Similar trend was observed with glycerophosphate, but not to the extent of chitosan. The increment in concentration of chitosan: glycerophosphate was 0.25%: 2.5% which retarded the release of about 1.29 to 4.55% for 1 to 2 h.

In this investigation, F5 could sustain the release of pilocarpine for 12 h (94.35%). The polymer duo is ascertained to complement each

other with regard to gelling and drug release properties. These polymers have limited ability to sustain the drug for few hours in case of hydrophilic solutes. But the drug release could be prolonged if the solute is hydrophobic or if molecular weight is > 1000 g/ mole. [14]

The *in vitro* release data was quantified using PCP Disso-V2.08 software to calculate the percentage release of drug and to determine the release mechanism.

The kinetic analysis of the release profile was calculated according to the Peppas equation:

$$M_t / M_\infty = kt^n \quad (1)$$

where 'M_t' is the cumulative amount of drug released at time 't'; 'M_∞' is the total amount of drug incorporated; 'k' is the proportionality constant, the value of which depends on the structural and geometrical properties of the matrix; and 'n' is the release exponent, its value depends on the mechanism of drug release. 'R' regression coefficient was also calculated in a set of data; the model showing highest R value was taken as a best model.

If 'n' value is < 0.5, the polymer relaxation does not affect the molecular transport, hence diffusion is fickian. If n > 0.5, the solid transport will be non-fickian and will be relaxation controlled. If n =1, release follows case II transport (zero order release) and if n > 1, indicates super case II transport.

For the drug release, the best fit model was "Peppas" model. The values of "n" were calculated from the drug release data (<70%) and estimated by linear regression of log (M_t/M_∞) vs log (t), and the obtained values were between 0 and 0.5, indicating that the release of pilocarpine was by fickian diffusion. [17] The n, k and R values of each formulation are given in Table 3.

Table 3: Parameters of model fitting for *in vitro* drug release

Formulation Code	N	K	R
F1	0.3673	36.43	0.9853
F2	0.2774	52.33	0.9891
F3	0.4968	45.65	0.9816
F4	0.3719	27.54	0.9845
F5	0.2658	34.76	0.9897
F6	0.4915	49.21	0.9805

To ascertain whether the drug release from *in situ* gels followed first order kinetics, the *in vitro* data was fitted into the equation:

$$\text{Log Wt} = \text{Log Wo} + \text{Ket} / 2.303 \quad (2)$$

Where, 'Wt' is the amount of drug retained in matrix at time 't'; 'Wo' is the initial amount of drug retained in matrix; 'T' is time in hours and 'K' is the first order rate constant (hr⁻¹).

From the kinetics study, it was found that drug release from all the formulations followed first-order kinetics (concentration dependant) since a straight line was obtained when log % cumulative drug retained versus time was plotted.

To verify the drug release was diffusion controlled; release data was fitted into Higuchi equation:

$$f_t = K_H \sqrt{t} \quad (3)$$

Where, 'f_t' is amount of drug released; 'K_H' is Higuchi dissolution rate constant; '√t' is square root of time.

During the study, the amount of drug released versus square root of time was plotted. The drug release from *in situ* gels were found to be diffusion controlled, since the plots were linear and the results reinforced that drug release from the *in situ* gels was by diffusion.

Formulation F5 was considered as optimized formulation; since it was sterile, isotonic, thermosensitive, quickly gelled below body temperature, possesses optimum viscosity and sustained the drug release for 12 h. This formulation was selected for *in vivo* testing.

Ocular irritation studies were performed to know whether developed formulation cause irritation or pain when applied. When formulation F5 was instilled into eye, it transformed into gel immediately. There were no untoward reactions like redness, inflammation, reflex tearing observed after instillation of gels.

Rabbits were periodically observed for any changes in retina, iris and conjunctiva of the eye. The scoring of ocular parameters for F5 is tabulated (Table 4). Draize Test (1959) provides several criteria for evaluating irritancy potential using different parameters; it is used today in spite of considerable criticism because of its subjective evaluation which leads to inter laboratory differences. [18,19,20] There are numerous alternative methods proposed to obtain data in predicting whether a particular material will be safe for human use. These proposed methods are either non-validated or utilize the same Draize technique with a different approach viz., change in the application site, volume of drug sample, less number of animals. One approach, low volume eye test (LVET) has been proposed as an alternative to Draize test. However, the LVET has not been shown to predict the human response more closely than the Draize test for a wide array of test substances. Thus, the LVET has not yet been adopted as a reference test method by any regulatory agency. In contrast, there are no documented instances in which a substance that produced a severe irritant/corrosive response in humans was not also classified as a severe irritant/corrosive in the Draize rabbit eye test.

The results of the ocular irritation studies indicated that formulation is non irritant; an excellent ocular tolerance was noted. No ocular damage or abnormal signs to the cornea, iris and conjunctivae was observed.

Table 4: Ocular parameters and their scorings-Draize Test

Parameter	Normal scoring					Scoring for F5
	0	1	2	3	4	
Opacity	No opacity	Diffuse area, details of iris clearly visible	Easily visible translucent areas, details of iris slightly obscure	Opalescent areas, no details of iris	Opaque, iris invisible	0
Area of cornea involved	25% or less	25% to 50%	50% to 75%	> 75%	---	0
Redness	Vessels normal	Vessels definitely injected above normal	More diffuse, deeper crimson red with individual vessels not easily discernible	Diffuse beefy red	---	0
Chemosis (swelling)	No swelling	Any swelling above normal (includes nictitating membrane)	Obvious swelling with partial aversion of lids	Swelling with lids about half closed	Swelling with lids about half closed to completely closed	0
Discharge	No discharge	Any amount different from normal (does not include small amounts observed in inner canthus of normal animals)	Discharge with moistening of the lids and hairs of just adjacent to lids	Discharge with moistening of the lids and hairs and considerable are around the eye.	---	0
Iris observation	Normal	Folds above normal, congestion, swelling, iris reacts to light	No reaction to light, haemorrhage, gross destruction	---	---	0

Corneal parameters - Opacity and Area of cornea involved

Conjunctival parameters – Redness, Chemosis (Swelling), Discharge

The formulation was placed at $5\pm 3^{\circ}\text{C}$ and $25^{\circ}\text{C}/60\% \text{RH}$ representing the long term and accelerated storage conditions, since the system is meant to be stored at $5\pm 3^{\circ}\text{C}$ (refrigeration). There was no marked change in the physical property and drug content during the study period (Table 5) from *in situ* gels during stability studies, which indicated that formulation F5 exhibited good stability during investigation period.

Table 5: Results of stability studies of formulation F5

Sampling Interval	% Drug content	
	$5\pm 3^{\circ}\text{C}$	$25^{\circ}\text{C}/60\% \text{RH}$
0 Day	98.66 ± 0.58	98.66 ± 0.58
15 Days	98.60 ± 0.64	98.46 ± 0.89
45 Days	98.54 ± 0.55	98.43 ± 0.56
90 Days	97.69 ± 0.89	97.68 ± 0.98

CONCLUSION

The objective of the study was to prepare and evaluate pilocarpine *in situ* gels for sustained release. Sodium glycerophosphate-chitosan *in situ* gels of pilocarpine showed appreciable *in situ* gelling properties during the study. The prepared *in situ* gels were found to be sterile, uniform, isotonic, thermosensitive and released drug for 12 h, they were non-irritant and well tolerated during the *in vivo* eye irritancy studies in rabbits. This study revealed that *in situ* gels formulation was simple, easy to administer, comfortable, with reduced frequency of instillations and also enhance the drug activity by releasing the drug in sustained manner. The diffusion data indicated that the drug release followed fickian diffusion and first order kinetics. The formulation F5 (Chitosan-2%, Sodium Glycerophosphate-27.5% and Pilocarpine-2%) gave the best results. From the present work, it may be concluded that *in situ* gel delivery system of Pilocarpine Hydrochloride is a novel approach.

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