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Original Article

Development and *in vitro*/*in vivo* evaluation of thermo-sensitive *in situ* gelling systems for ocular allergy

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Ocular allergy is one of the most common disorders of the eye surface. Following diagnosis this condition is typically treated with preparations containing antihistamines. However, anatomy of the eye and its natural protective mechanisms create challenges for ocular drug delivery. Rapid elimination of antihistamine substances due to short residency times following application can lead to insufficient treatment of ocular allergies. With this in mind, the aim of this study was to prepare a controlled ocular delivery system to extend the retention time of olopatadine hydrochloride (OLO) and in doing so to reduce the need for frequent application. We developed extended-release ocular *in situ* gelling systems for which *in vivo* retention times were determined in sheep following *in vitro* characterization and cytotoxicity studies. *In vivo* results were then compared to commercially available Patanol eye drops. the transparent gels formulated using appropriate amounts of polymers and having longer ocular retention times appear to be a viable alternative to commercially available eye drops.

Keywords: Olopatadine hydrochloride. Ocular allergy. *In situ* gelling system.

INTRODUCTION

Generally, there are two main purposes of ocular drug applications: treating ocular diseases emerging on the surface (conjunctivitis, keratitis, etc.) and treating those emerging in deeper layers (glaucoma, uveitis, etc.). The most common commercial dosage form for ocular administration is the eye drop which makes up approximately 90% of the formulations on the market (Bourlais *et al*., 1998; Bain *et al*., 2009; Vadlapudi *et al*., 2015; Mehanna, El-Kader, Samaha, 2017).

Ocular allergies include seasonal allergic conjunctivitis, hay fever, vernal keratoconjunctivitis, chronic allergic conjunctivitis, and atopic conjunctivitis. Furthermore, contact lens-induced papillary conjunctivitis and papillary conjunctivitis which develops after surgery are also included within the scope of ocular allergies. Seasonal allergic conjunctivitis, which is the most common form of ocular allergy, is a condition caused by a sudden reaction to substances like pollen, animal hair, dust, or chemicals. (Palmer, 2007). The most important symptom of ocular allergy is itchiness. Swelling on the conjunctiva can also occur. Palpebrae may become tumid and a change can occur within the structure of tender collagen fibers around the eye depending on the degree of swelling (Barney, Cook, Stahl, 2014).

Olopatadine hydrochloride (11-[(Z)-3-(dimethylamino)propylidene]-6,11-dihydrodibenz [b, e] oxepin-2-acetic acid hydrochloride, OLO) is a white, crystalline, water-soluble compound with a molecular weight of 373.88 (Ohmori *et al*., 2002; Leonardi, Quintieri, 2010). OLO is an anti-allergic *active* pharmaceutical ingredient with histamine H_1 receptor antagonistic action which also has an effect on human conjunctival mast cells. The compound is capable of suppressing the mast cell concentration at least ten times more than its clinically effective concentration (Brockman *et al*., 2003; Tamura, Komai, 2008). Due to its antihistaminic feature and effect on mast cells, treatment begins rapidly and long-term protection is provided (Abelson, Spitalny, 1998).

Although topical application offers many advantages for the treatment of ocular surface disorders, it can result

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in severe bioavailability issues due to various biological factors involved in the process of eye protection (Kaur *et al*., 2004). Many commercial drug companies suggest 50 µL as the maximum volume for eye drop application; however, only about 30 µL remains on the eye surface. Therefore, active pharmaceutical ingredient is wasted because of the excessive application volume required (Kaur *et al*., 2004; Rawas-Qalaji, Williams, 2012).

Following ocular application, pre-corneal factors and anatomic hindrance have a negative effect on the bioavailability of topical formulations. Pre-corneal factors include solution drainage, loss due to blinking, formation of tear film, and increase in tear secretion. Tears secreted from the lacrimal glands at the corner of the eye orbit are a mixture of antimicrobial enzymes, lysozyme, salts, and mucus. Formation of a tear film indicating the presence of a healthy eye surface is the first step in providing high resistance. This film cleans the pathogens on the ocular surface and plays a protective role by forming a hydrophilic surface. Considering all of the pre-corneal factors, actual contact time of an applied ocular dose was determined to be low and < 5% actually was instilled into the intraocular tissues (Başaran *et al*., 2010; Perrie *et al*., 2012; Morrison, Khutoryanskly, 2014). Based on these facts as well as other issues, new drug delivery systems providing continuous and controlled release such as *in situ* gel, nanoparticles, microemulsions, liposomes, nanosuspensions and ocular inserts have emerged in recent years (Tangri, Khurana, 2011).

There has been a growing interest in ocular *in situ* gelling systems which undergo physical or chemical changes in their structure and transform into a gel form. This phenomenon has been used by many researchers to increase the retention time of drugs on the ocular surface to provide sustained release, to increase stability of protein-based drugs, and to formulate active drug delivery systems when required (Thrimawithana *et al*., 2012; Patil, Kumar, 2015).

In situ gel is generally expressed as a polymer solution in liquid form when applied which is converted into a semi-solid gel phase when exposed to physiologic conditions. Gelling can be achieved by ultraviolet application or with solvent exchange depending on temperature, pH, and ion change (Rathore, 2010).

Triblock copolymers of polyethylene glycol-*b*-polypropylene and glycol-*b*-polyethylene glycol, commercially available as Pluronic, are non-ionic, water-soluble materials of great interest as pharmaceutical excipients. These polymers have an amphiphilic character, surfactant property and may interact with hydrophobic surfaces and biological membranes. Pluronic is a polymer which undergoes a gelation process depending on elevation of temperature. It can be used frequently in ophthalmology due to its transparent structure and the fact that it causes no disturbance in vision after application (Batrakova, Kabanov, 2008; Khateba *et al*., 2016).

Poloxamer, belonging to the same group as Pluronic, is a biocompatible polymer commonly used for medicinal and pharmaceutical purposes. Various potential uses for Poloxamer in oral preparations are being examined in addition to ocular, nasal, topical, rectal, vaginal, and intrauterine applications.

To overcome the short residence time and to reduce the application frequency of conventional ocular delivery systems containing an antihistaminic substance, an OLO sustained-release drug delivery system was designed to treat ocular allergies (Paavola, Bernards, Rosenberg, 2016).

MATERIAL AND METHODS

Material

Poloxamer (Sigma, code: 16758), Pluronic F 127 (Sigma, code: P2443) and hydroxypropyl methylcellulose (HPMC, Sigma code: H7509) used as polymers for *in situ* gel formulations were purchased from Sigma-Aldrich (Germany), sodium chloride (NaCl) used for isotonic adjustment from Merck (Germany), benzalkonium chloride used as preservative from Fluka, and distilled water from Millipore.

Simulated tear fluid (STF) consisted of 0.680 g NaCl (Merck), 0.220 g $NaHCO_3$ (Sigma-Aldrich, code: S6014), 0.008 g $CaCl_2 \cdot 2H_2O$ (Sigma-Aldrich, code: 449709), 0.14 g KCl (Merck), and distilled water to 100 mL.

Preparation of *in situ* gel systems

Two methods, namely the 'hot method' and 'cold method', were used to prepare the gel systems. Polymeric solutions were prepared using the cold method (Li *et al*., 2015). Briefly, the required quantity of polymer was dispersed in cold water with continuous magnetic stirring at 4 ºC for at least 24 hours. Formulation studies were started by determining constituent ratios. For polymers in different percentages, proportions were selected in parallel with that reported in the literature (Rawat, Warade, Lahoti, 2010). The amount of NaCl for formulations was calculated according to Raoult's law to obtain isotonic aqueous solutions. The concentration of the preservative was 0.01% benzalkonium chloride in accordance with the

TABLE I - Composition percentage of *in situ* gel formulations

Code	Polymers			Olopatadine Hydrochloride	NaCl	Benzalkonium Chloride	water
	Poloxamer®	Pluronic® F 127	HPMC				
P10	10	-	-	0.1	0.884	0.01	qs 100
P12	12	-	-	0.1	0.884	0.01	qs 100
P14	14	-	-	0.1	0.884	0.01	qs 100
P16	16	-	-	0.1	0.884	0.01	qs 100
P18	18	-	-	0.1	0.884	0.01	qs 100
P20	20	-	-	0.1	0.884	0.01	qs 100
F10	-	10	-	0.1	0.884	0.01	qs 100
F12	-	12	-	0.1	0.884	0.01	qs 100
F14	-	14	-	0.1	0.884	0.01	qs 100
F16	-	16	-	0.1	0.884	0.01	qs 100
F18	-	18	-	0.1	0.884	0.01	qs 100
F20	-	20	-	0.1	0.884	0.01	qs 100
FH1205	-	12	0.5	0.1	0.884	0.01	qs 100
FH121	-	12	1	0.1	0.884	0.01	qs 100
FH1405	-	14	0.5	0.1	0.884	0.01	qs 100
FH141	-	14	1	0.1	0.884	0.01	qs 100
FH1605	-	16	0.5	0.1	0.884	0.01	qs 100
FH161	-	16	1	0.1	0.884	0.01	qs 100

most commonly used ophthalmic ingredient on the market. Compositions of the *in situ* gel formulations prepared are detailed in Table I.

Measurement of sol-gel transition (gelation temperature)

The most ideal ophthalmic *in situ* gel formulation is one that transitions at 32°C which is the temperature of the ocular surface. Tsol-gel of formulations vary depending on the amount of polymer. Therefore, to determining the gelation temperature of each formulation prepared, the *Test Tube Tilting Method* from 0 °C to 50 °C was employed (Bain *et al*., 2009). The study proceeded with those gelling systems undergoing transition at appropriate temperatures. Briefly, a 2 mL aliquot of prepared solution was transferred to a 5 mL test tube in a water bath maintained at 0 °C. The temperature of the bath was incrementally increased to 50 °C. At every temperature point the solution was permitted to equilibrate for 1 min. The samples were checked for gelation by tilting the test tube at 90°. The solutions were said to gel when no mobility was observed. Measurements were made in triplicate and the mean value calculated.

U-HPLC analysis of OLO

Samples were analyzed using a U-HPLC system (Agilent Technology 1290 Infinity, USA) installed with Zorbax Eclipse Plus C18 (2.1x50 mm 1.8 Micron). The column compartment was temperature-controlled and a PDA detector was employed throughout the analysis.

A new U-HPLC method was developed for OLO. Methanol-distilled water-sodium acetate buffer (40:50:10 v/v/v) was determined to be the mobile phase after testing different ratios and phases at a flow rate of 1 mL/min. The column oven was set at 40°C. Sodium acetate buffer 0.1 M prepared using acetic acid was adjusted to pH 4.5. The U-HPLC method was validated with respect to system suitability, linearity, precision, accuracy/specificity, selectivity, limit of detection (LOD), quantitation (LOQ), and stability (ICH, 2005).

Characterization of *in situ* gelling system

Physical appearance and clarity

In situ gel formulations prepared containing the active ingredient were visually examined for physical appearance. The clarity of gelling systems was assessed

using white and black backgrounds to avoid overlooking particulate matter.

pH analysis

The pH values of freshly prepared dispersions were determined at 25±1 °C by WTW Profi Lab (pH 597, Weilheim, Germany). All analyses were repeated in triplicate.

Gelling capacity

A drop of the formulation was placed into 2 mL of freshly prepared STF and the gelling time was visually recorded to determine gelling capacity of the formulations prepared using active substances. The code system specified in Table II was used to rate the gelling capacities (Rathore, 2010; Morsi *et al*., 2016).

TABLE II - Grading of gelling capacities

-	No gelation occurred.
+	The gel formed after a few minutes and dissolved rapidly.
++	The gel formed immediately and remained for a few hours.
+++	The gel formed immediately and remained for an extended period of time.

Swelling study

STF was used at a temperature of 32±1 °C for formulation swelling studies. A volume of 1 mL of the formulations was placed on a dialysis membrane and sealed to prevent leakage. Prior to testing (t_0), gel weight was measured and recorded, after which the gel was kept in STF for specified times (0.5, 1, 2, 3, 6, 12, 24 hours). Swelling rates were calculated using the following formula: (Bhowmik *et al*., 2013).

$$\text{Swelling ratio (t) \%} = \{(\text{gel weight (t)} - \text{gel weight } (t_0)) / \text{gel weight } (t_0)\} \times 100$$

Examination of rheological behavior and viscosity

Rheological properties were determined using a cone-and-plate geometry rheometer with a diameter of 40 mm (Brookfield, USA). Measurements and viscosity changes were repeated at two different temperatures, 25±1 °C and 32±1 °C. Shear rates against shear stress were calculated to provide further information regarding flow properties.

In vitro drug release study

STF was used as the release medium for obtaining *in vitro* release profiles of freshly prepared formulations (Başaran *et al*., 2010). Release studies were performed using the dialysis membrane method with magnetic stirring at a speed of 100 rpm and at an ocular surface temperature of 32±1 °C (Sniegowski *et al*., 2015). Release tests were repeated 3 times for each formulation. Quantification of the active ingredient, OLO, was achieved using the pre-validated U-HPLC method. Release medium with a total volume of 40 mL was adjusted to obtain sink conditions. The 1 mL of STF sample withdrawn each time was replaced with a fresh sample.

Cytotoxic evaluation

The MTT method was used to investigate the cytotoxic effect of various OLO-containing formulations on cells in comparison to Patanol, the only commercial product containing OLO. Mouse NIH 3T3 fibroblasts were cultured. Formulations containing 2% OLO were dispersed in the medium and applied onto the cells at concentrations of 5, 10, 30, 90, 120, 150, and 200 μg/100 μL. At the end of 12- and 24-hour incubation periods, absorbance was measured at 572 nm using a BioTek Cytation 5 (BioTek Instruments, Germany) multiple plate reader. For the cytotoxicity tests, 3 plates were used for each formulation and 8 wells were used for each concentration. Intact cells incubated in the culture medium were used as the control group. Results were expressed as the percentage of absorbance of control cells. Since no cytotoxicity was observed during the first 12 hours of incubation, IC50 (inhibitor concentration causing 50% decrease in cell proliferation) values were determined at the end of the 24-hour incubation period.

In vivo retention time study

The *in vivo* experimental protocol was approved by the Local Animal Ethical Committee of Osmangazi University (Approval No. 518-1). *In vivo* studies were performed at the MD Center Livestock Farm and accompanied by a veterinary surgeon. A total of 6 healthy female/male Anatolian Merino sheep, 1-2 years old, weighing 25-40 kg were used. Within the scope of the study, each of the 5 formulations was applied to one eye of the 6 sheep once a day without disturbing the animals. Following the application, tear samples were collected and measured using Schirmer's tear test at the 30th minute and 1st, 2nd, 3rd, 6th, 24th and 48th hours (Figure 1). The test membrane was contacted with the eye for 30 seconds at each sampling time point. A total of 14 tear samples were collected from the sheep, that is, 7 samples from each eye.

Sex-related changes were not taken into consideration since both female and male sheep were used in each group. Samples taken from the test membrane were stored at -20°C until analyzed. The amount of OLO in tear samples was determined by the U-HPLC method. Data obtained indicated retention time of formulations in the eye.

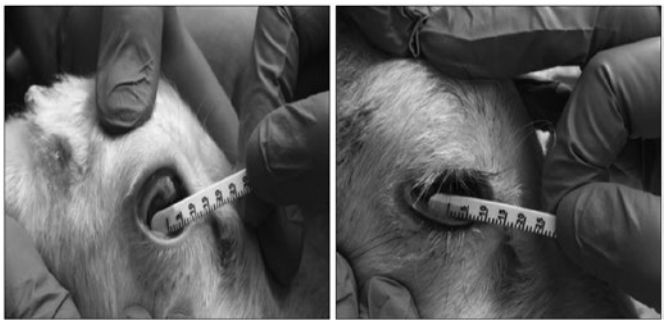

FIGURE 1 - Sampling with Schirmer tear test membrane.

Statistical data analysis

The difference between the test animals and the control group was evaluated statistically by applying a one-way ANOVA Tukey test. The GraphPad Prism version 5.0 statistical program was used for the statistical analysis of the complete data obtained. The significance level was evaluated as 95% ($p<0.05$).

RESULTS AND DISCUSSION

Preparation of *in situ* gel systems

The widespread ocular use and non-toxic nature of Poloxamer and Pluronic F were advantageous in this study. Furthermore, the water-soluble character of HPMC, transparency, and proper viscosity of its solution and widespread use in ophthalmology supported the preference of gel formulations in this study (Khangtragool, 2014).

Aqueous solutions of different concentrations of Poloxamer and Pluronic F were prepared and evaluated for gelation temperature to identify the compositions suitable for use as *in situ* gelling systems.

Measurement of Tsol-gel (gelation temperature)

A perfect thermosensitive ophthalmic gel should have a Tsol-gel higher than room temperature, ideally 30°C, and form a gel at precorneal temperature even when diluted by a small volume of tear liquid. If transition occurs at temperatures below 30°C, the *in situ* gel would already be gelled when instilled to the eye and if gelation happens at higher temperatures, the formulation would be drained by lachrymal secretions without filling the need (Venkatesh *et al.*, 2011).

Gelation temperature differs depending on the concentration of ingredients in the formulation, particularly the polymer. Therefore, formulations to be applied ophthalmically should gel at the surface temperature of the eye. When the polymer concentration is too low, gelation at 32°C will not be achieved and use of polymer at very high concentrations increases both the cost and the toxic effects. Therefore, determining the minimum concentration at which gelation occurs at 32°C is the goal (Jeong, Kim, Bae, 2002). In the *Test Tube Tilting Method* used in this study, the temperature is gradually increased over time so that the phase transition can be visually recorded and the gelation temperature determined. The gelation temperatures of the formulations are presented in Table III. The Tsol-gel obtained for various Poloxamer concentrations (14%-20% w/w) is in accordance with the results described in the literature and proves that the Tsol-gel is dependent on polymer concentration (Gratieri *et al*., 2010). Formulations demonstrating ratios of polymer (P16, F16, F1405) which could gel at 32°C were selected for *in situ* preparations.

U-HPLC analysis of OLO

The U-HPLC method used to determine OLO in both *in vitro* and *in vivo* studies was preferred because of its many advantages. This method is very sensitive and the detection limits (LOD and LOQ) are rather low. Due to the much smaller particle size in U-HPLC columns, it is more resistant to pressure and the signals are clearer due to the low inter-particle spaces within the column. In addition, the analysis period is short enough to be expressed in seconds and the volume of the mobile phase is much lower (Novakova, Matysova, Solich, 2006).

As a result of the validation study, the determination coefficient (r^2) was found to be 0.9997 and linearity, accuracy, and precision in the operating range were observed to be within acceptable limits. The limit of quantification (LOQ) was determined as 2.3188 µg/mL and the limit of detection (LOD) was found to be 0.7652 µg/mL.

Characterization of *in situ* gelling system

Physical appearance and clarity

Physical appearance and transparency are the first issues investigated when examining physicochemical properties of gel systems. In most studies when the existence of particles was visually observed under light on

a black-and-white background no changes in transparency and uniform appearance were observed in the formulations prepared (Gupta *et al*., 2007).

pH analysis

The pH value should be 7.2±0.2 to ensure maximum comfort after application of an ideal ophthalmic preparation. However, preparations with different pH values can be tolerated due to the buffering capacity of tears. Therefore, an ophthalmic preparation with a pH value between 4.0 and 8.0 can be applied (Ammar *et al*., 2009; Fialho, Silva-Cunda, 2004).

Average pH value of the *in situ* gel formulations we prepared was between 6.50 and 7.00, which was appropriate for ophthalmic application (Table III). Fortunately, there was no need for pH adjustment using any additional chemicals because substances added to the formulation could be detrimental in terms of toxicity or stability. In a similar study, it was reported that the average pH value of the ophthalmic *in situ* gel system developed was approximately 6.5 and this value was tolerated by the eyes (Mandal *et al*., 2012).

TABLE III - pH, gelling capacity and gelation temperature of *in situ* gelling (n=6)

Code	pH (Mean ± SE)	Gelling capacity	Gelation temperature (°C)
P16	6.61±0.00	++	32
F16	6.60±0.00	++	32
FH1405	6.70±0.00	+++	32

Gelling capacity

Viscosity and gelling capacity are the two essential features of *in situ* gel systems. Formulations with appropriate viscosity and gelling can easily be applied as eye drops leading to increased patient compliance. Gelling capacities of the formulations determined visually according to the coding system reported in the literature are presented in Table II (Gupta *et al*., 2007; Rathore, 2010).

As shown in Table II, formulations containing 20% polymer had a grade of "+++" as the gel formed at room temperature (< 25ºC) and did not exhibit free-flowing properties at 4ºC. However, P16 and F16 achieved a "++" grade for gelling capacity as it formed immediately and remained for a few hours. The HPMC added formulation, FH1405, had a grade of "+++" as the gel formed at 32ºC. Formulations containing the lowest polymer content did not exhibit gelling capacity as they remained in a solution state at both non-physiological (4ºC) and physiological (32ºC and over) conditions showing no gelation.

From these outcomes, it was discovered that the thermosensitivity of gel is profoundly concentration dependent, with higher concentrations leading to faster gelation at lower temperatures (Almeida *et al*., 2013).

Swelling study

One of the tests used in considering an *in situ* gel system is the swelling capacity. Transformation of a gel system depending on time and temperature are examined with a swelling study (Ju *et al*., 2013). This test also gives some ideas as to the cross-linking ratio of gel, diffusion of active substance, and its interaction with the tissue (Diniz *et al*., 2015).

When swelling of *in situ* gel formulations calculated using STF were compared, it was found that the swelling of F16 was higher than P16 and FH1405 (Fıgure 2). Weight of the gel increased in a rapid pattern until the 6th hour followed by an increase in a slower pattern. Considering the duration of ophthalmic retention, it was decided to continue the test up to 24 hours.

The perfect formulation in solution form ought to have an ideal viscosity that will allow for easy installation into the eye, and a fast Tsol-gel. In this case, P16 and F16 gave reliable results and were selected as the optimized batches.

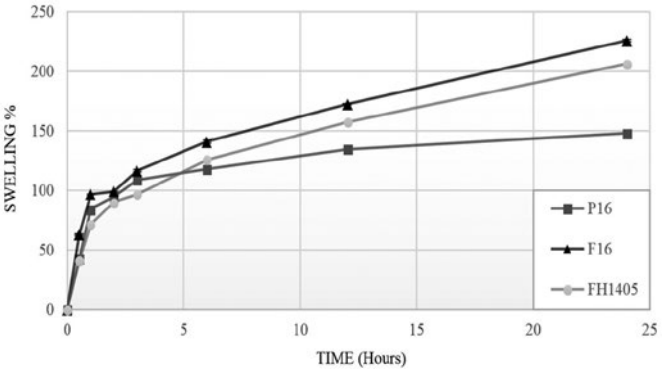

FIGURE 2 - Swelling profiles of *in situ* gel formulations (n=3).

Examination of rheological behavior and viscosity

Viscosity of all formulations prepared presented linear behavior until 30ºC (Figure 3). Just after 30ºC an increase in viscosity was seen which remained constant above 35ºC. Constant viscosity above 35°C helps maintains longer ophthalmic retention time of formulations. When the formulations were tested rheologically at a shear rate range of 0-800 sec^{-1} at 25°C, linear correlation between shear rate and shear stress was determined, indicating a Newtonian flow type (Figure 4). When solution transforms

to a gel at 32°C, shear stress and shear rate conform to the pseudoplastic flow model. Thus, temperature dependent rheological behavior of all formulations was confirmed by the Newtonian flow model at 25ºC and the pseudoplastic model at 32ºC (Lin, Sung, Vong, 2004; Chang *et al*., 2002).

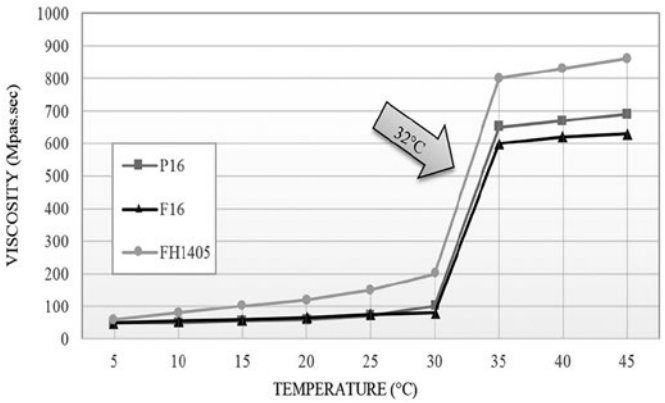

FIGURE 3 - Effect of temperature on viscosity of formulations (n=6).

In vitro OLO release study

An *in vitro* release study was performed for P16, F16, and FH1405 in comparison to Patanol. From Figure 5, it is obvious that OLO in a structured *in situ* gel system resulted in a significant ($p < 0.05$) slower release of OLO. Three different *in situ* gel system preparations exhibited similar release profiles (Figure 5).

Formulations P16, F16, and FH1405 showed 60.33%, 61.21%, and 53.73% drug release, respectively at the end of 24 h. Initially, release of drug from these formulations was higher due to the bursting effect and as the time period increased the gelation effect occurred and release rate was finally retarded. P16 and F16 formulations demonstrated higher release compared to FH1405. This difference was attributed to the higher gelation capacity and viscosity of the FH1405 formulation compared to the other two formulations. The results show that the percentage of OLO released from P16 and F16 were nonsignificantly ($p < 0.05$) lower than that released from FH1405. This outcome might be due to HPMC, which may have played a significant role in retarding the release of the drug, probably by slowing down polymer disintegration (Morsi *et al*., 2016).

OLO in Patanol eye drops was released rapidly during the first minute and its concentration exceeded 75% at the 6th hour. All the formulations prepared demonstrated extended and retarded release characteristics when compared to Patanol.

Cytotoxic evaluation

It is important that the formulations developed have low body cell toxicity. The MTT test, involving water-soluble yellow tetrazolium dye reduction by living cells to a purple formazan salt insoluble in aqueous solutions, was used in this study to determine the cytotoxic potentials of all formulations. The amount of formazan produced is directly proportional to the amount of living cells which can be measured spectrophotometrically (Arranja *et al*., 2014; Şenel, Büyükköroğlu, Yazan, 2015).

In this study, concentration dependent cell depletion was observed in the first 12 hours with respect to formulations prepared using HPMC, with cell viability not going below 50% even at the highest dose (Figure 6).

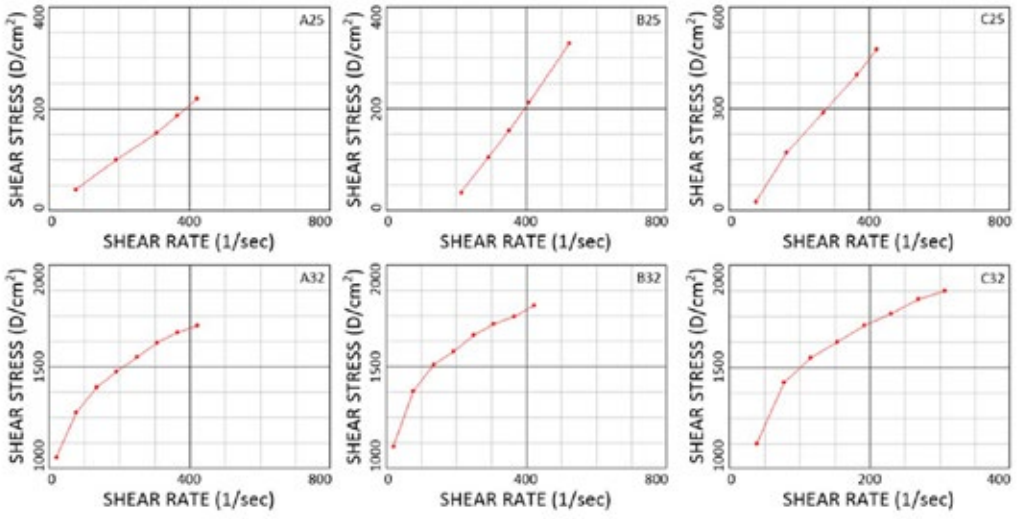

FIGURE 4 - Effect of temperature on rheological behavior of formulations. (A25 F16, 25 °C; B25 P16, 25 °C; C25 FH1405, 25 °C; A32 F16, 32 °C; B32 P16, 32 °C; C32 FH1405, 32 °C).

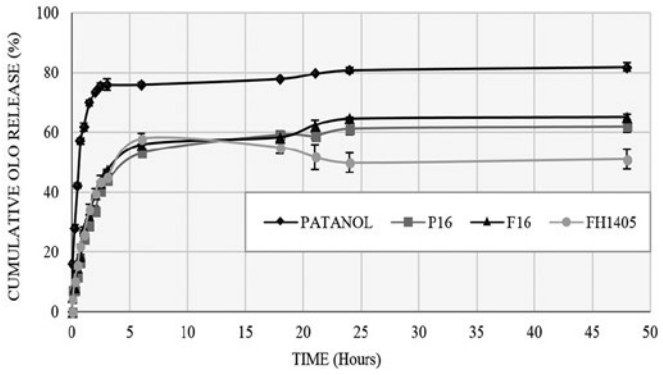

FIGURE 5 - Cumulative release percentage from gel formulations in simulated tear fluid (n=3).

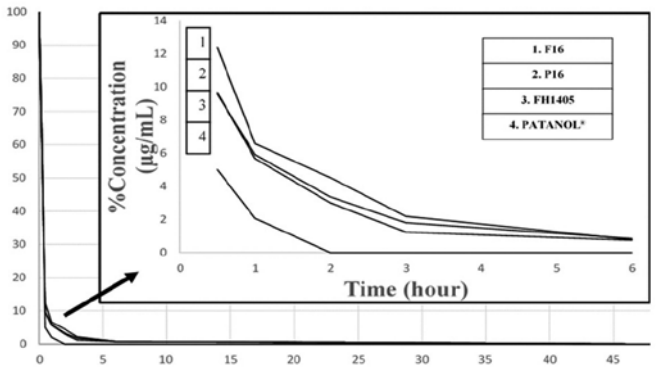

FIGURE 7 - Concentration-time profiles of OLO after administration of in situ gel formulations and Patanol (n=6).

However, cell viability was close to 50% at approximately 30 µg/100 µL at 24 hours. Higher cytotoxicity was observed in Pluronic F 127 formulations, whereas Poloxamer 407 formulations showed 50% cell viability only at the highest dose of 200 µg/100 µL. When we evaluated cytotoxicity for Patanol, the cell viability decreased to 50% only at the highest dose (200 µg/100 µL) during the 12 hour incubation period, while cell viability decreased to < 50% at approximately 90 µg/100 µL after 24 hours of incubation.

It was determined that the formulations prepared in this study showed cytotoxicity only at high doses depending on concentration and time. While some of the results obtained are consistent with those in the literature, some are not. These discrepancies may be attributed to the sparse number of previous studies and differences in concentrations and time values used in those studies.

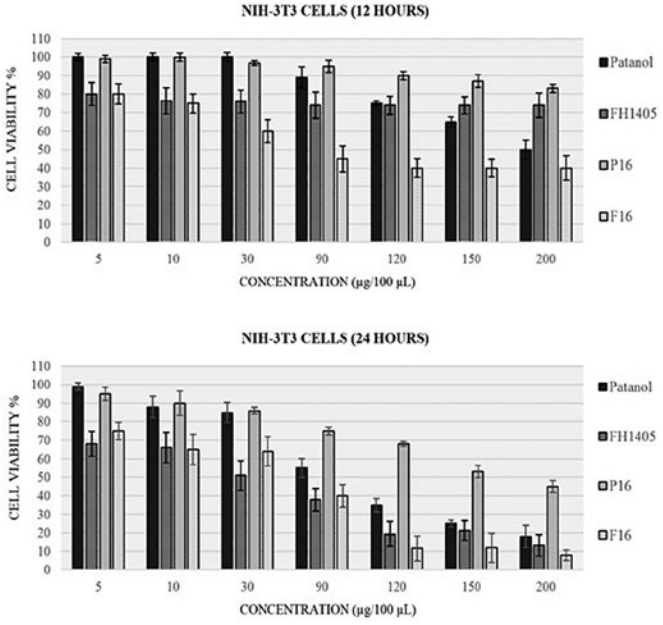

FIGURE 6 - Cell viability of formulations on human 3T3 cells.

In vivo retention time studies

The amount of OLO in tear samples taken from the sheep was successfully measured using the validated U-HPLC analytical method. Samples collected by soaking them on 'Schirmer's tear test' membranes appeared to result in enhancement of bioavailability due to the prolonged ophthalmic retention time (Byrro *et al*., 2012; Bhatta *et al.*, 2012).

Redness must be controlled for in eyes that receive formulations. From application to final tear sampling there was no redness or irritation in the eyes of the sheep used in this study. According to our findings, OLO in all formulations was determined to be at significant ophthalmic concentrations until 6 hours, while no OLO was found in the Patanol group in the 2 hour samples. OLO detected between 6 and 24 hours was found to be under analytically acceptable concentration limits (LOD and LOQ); therefore, the concentration could not be calculated at 24 hours.

A statistically significant difference was observed between the Patanol group and all formulations ($p<0.05$). It was proved that ophthalmic retention time was much longer for *in situ* gel preparations than solution application. Therefore, the frequency of application can be decreased and patient compliance increased with the added benefit of a reduction in the risk of toxic effects resulting from frequent dose repetition.

CONCLUSION

The present study showed the development and evaluation of OLO-loaded *in situ* gelling systems. The most important parameter for the success of treating allergic conjunctivitis, which is an ocular superficial disorder, is to ensure that the active substance remains on the eye surface for an extended period of time. Rapid

excretion resulting from drainage following eye drop application causes frequent repetition of dosing, which may lead to irritation and eye toxicity long-term. The *in situ* gel systems developed in this study provided sustained release over an extended period with once-daily application. In this study, a thermosensitive *in situ* gel of OLO was prepared which exhibited appropriate gelation temperature, quick transition, sufficient viscosity, extended drug release, and negligible ocular toxicity. *In vivo* retention time studies showed that commercially available Patanol ophthalmic solution was effective for only 2 hours while the gel systems developed and tested were still at significant ophthalmic OLO concentrations at 6 hours after administration. Use of *in situ* gel systems appears to be promising as an alternative to eye drops. Benefits of such a system include increased patient compliance as a result of the need for less frequent application sufficient to treat pharmacologic allergy symptoms.

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