



Formulation and Evaluation of Topically Retained Hydrogels Loaded with Timolol Maleate Microspheres for Extended Release in Treating Glaucoma

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Article History	Abstract
Received: 06 June 2023 Revised: 05 Sept 2023 Accepted: 11 Oct 2023	Background: Conventional aqueous eye drop formulations have a shorter retention period and wash out of the precorneal region more easily. In this study, biodegradable microsphere-loaded hydrogels were developed to extend the time of residence and improve the drug bioavailability. The prepared microspheres were fabricated using PLGA as the biodegradable polymer and PVA as the surfactant. Hydrogels were prepared using Carbopol 940 and Gellan gum as gelling agents. Methods: The microspheres were created through a technique known as double emulsion solvent evaporation method. Evaluation included for its percentage yield, entrapment efficiency, particle size and surface morphology of the prepared microspheres. Timolol Maleate microsphere loaded hydrogel underwent assessments for pH, spreadability, rheological behaviour, sterility, antimicrobial efficacy, in vitro release studies, drug release kinetics and ocular irritation. Results: FTIR analysis confirmed compatibility between the drug and polymer. SEM observations indicated spherical microspheres with sizes ranging from 1 to 12 μm, suitable for ocular application. All formulations exhibited pH values between 4.2 and 5.0, with gelation occurring at 35°C to 38°C. Formulation F3 demonstrated optimal viscosity (1486 – 1850 cps) and good spreadability. Sterility tests were successful, and F3 displayed a drug release of 82.6% after 24 hours. Antimicrobial studies exhibited inhibition zones, and ocular tests on rabbits revealed no irritation. Stability analysis indicated no significant changes in pH or clarity. Conclusion: Based on physiological, rheological, and in vitro evaluations F3 was identified as the most suitable formulation for extended Timolol Maleate release via hydrogel, holding promise for effective glaucoma treatment.
CC License CC-BY-NC-SA 4.0	Keywords: Biodegradable, Polymeric microspheres, Hydrogel, Double emulsion solvent evaporation technique, Ocular drug delivery, Glaucoma.

1. Introduction

Advancing ophthalmic drug delivery is a significant challenge within the pharmaceutical field. The distinctive arrangement and operation of the human eye render it resilient to foreign agents¹. Historical approaches to ocular drug delivery included topical application, systemic administration, or direct injections. However, conventional forms like solutions, suspensions, and ointments are associated with drawbacks such as quick pre-corneal elimination and vision blurring². To enhance ocular bioavailability and overcome these issues, the focus has shifted towards controlled and sustained drug delivery³.

Ophthalmic hydrogel microspheres are a promising avenue to improve drug delivery. These microspheres offer advantages over traditional forms, including extended ocular residence, controlled release, accurate dosing, and prolonged shelf life⁴.

Glaucoma, a condition characterized by increased intraocular pressure, can lead to vision loss if left untreated. Microspheres present a favorable strategy for glaucoma treatment, as they enhance ocular bioavailability compared to standard eye drops. Smaller particle sizes are generally better tolerated, making microspheres preferable for long-acting ocular drug delivery⁵.

Hydrogels hold immense potential as ocular drug carriers⁶. These three-dimensional, hydrophilic polymer networks can absorb substantial amounts of water, swelling in aqueous environments to form a gel that can release solvents in a controlled manner. By controlling permeation and diffusion, hydrogels can retain both hydrophobic and hydrophilic agents⁷. Combining hydrogels with microspheres can further augment drug delivery. The aqueous nature of hydrogels also offers protection for cells and pharmacological agents. Their structure can be designed as nondegradable or degradable based on application requirements.

Primary treatment for glaucoma involves self-administered topical eye drop medications. Regular use is crucial to prevent vision deterioration and blindness. However, adherence rates are often below 70%, primarily due to the frequency and complexity of administration⁸.

Biodegradable polymer microspheres find common use in surface application. The prepared microspheres can be incorporated into traditional formulations like creams, lotions, gels, ointments, tablets, and powders, providing versatility in formulation^{9,10}. Ophthalmic hydrogel microspheres, in particular, provide advantages such as prolonged residence, controlled release, accurate dosing, and extended shelf life. These gels combine the benefits of solutions (accurate dosing) and gels (prolonged residence) for improved ocular bioavailability. pH-sensitive hydrogels with thermoresponsive properties, loaded with biodegradable polymer microspheres, present a novel ocular drug delivery system capable of prolonged glaucoma treatment¹¹.

2. Materials And Methods

Materials: Timolol Maleate was procured from Chetana Pharmaceuticals Private Limited, situated in Perinthalmanna., India. PLGA was provided as a gift sample by HLL Lifecare Ltd, Thiruvananthapuram, India. All additional chemicals utilized were of analytical quality and procured from local providers.

Timolol Maleate Microspheres Formulation^{12,13}: Microspheres were produced by using a double emulsion solvent evaporation method with a water-in-oil-in-water (w/o/w) approach. Timolol Maleate (500 mg) was first dissolved in 5 mL of deionized water, while PLGA (500 mg) was dissolved in 15 mL of dichloromethane solvent. The two phases were emulsified, and the emulsion produced was the resulting emulsion was added dropwise to a 1% w/v PVA aqueous solution. After being stirred for a duration of 3 hours, the microspheres were harvested through centrifugation and subjected to triple washing with deionized water.

Table 1. Formulation of Timolol Maleate Microspheres Loaded Hydrogel

Formulation Ingredients	F1	F2	F3	F4	F5	F6
Timolol Maleate (%)	0.5	0.5	0.5	0.5	0.5	0.5
Gellan gum (%)	0.2	0.4	0.6	0.2	0.4	0.6
Carbopol (%)	0.2	0.4	0.6	-	-	-
Sodium alginate (%)	-	-	-	0.2	0.4	0.6
Benzalkonium Chloride (%)	0.01	0.01	0.01	0.01	0.01	0.01
Sodium chloride (%)	0.9	0.9	0.9	0.9	0.9	0.9

Microsphere Characterization¹⁴: Microspheres fabricated through the double emulsion solvent evaporation technique were subjected to analysis for percentage yield, particle size studies, entrapment efficiency, and surface morphology.

Percentage Yield¹⁵

Microspheres produced at the end were weighed and its percentage yield was calculated by the below mentioned equation.

$$\% \text{Yield} = \frac{\text{PRACTICAL YIELD}}{\text{THEORETICAL YIELD}} \times 100$$

Entrapment Efficiency¹⁶

Entrapment efficiency was calculated by dissolving a specified amount of the collected microspheres in dichloromethane and was then diluted to a final volume of 10 mL using distilled water. Prepared microsphere solution was centrifuged at 1000 rpm and the resultant supernatant was and then its absorbance was calculated by UV Spectrophotometer at the λ_{max} of 292 nm¹⁷. The percentage of encapsulation efficiency was determined using the subsequent equation:

$$\% \text{ Entrapment Efficiency} = \frac{\text{Mtotal} - \text{Msupernatant}}{\text{Mtotal}} \times 100$$

Where, Mtotal = Mass of total drug and Msupernatant= Mass of drug in supernatant solution

Particle Size Studies¹⁸

Particle size studies was analysed by using optical microscope and this method is suitable for counting of particles of 1 μm and greater size. The optical microscopic technique entails calibrating the eyepiece micrometer against the stage micrometer. The stage micrometer divides one millimeter into 100 equal units, making each division equivalent to 10 μm . The particles were measured, and the mean diameter was computed using the subsequent equation;

$$\text{Average diameter} = \Sigma d/n \times CF$$

Where, n = number of microspheres, d = diameter of microspheres, CF = calibration factor

Surface Morphology¹⁹

Determined through Scanning Electron Microscopy (SEM) to assess surface topography and morphology. So, the surface morphology of Timolol Maleate microspheres will be obtained by the SEM analysis.

Preparation of Timolol Maleate Microspheres Loaded Hydrogel^{20,21}

Hydrogel formulations were created using different polymer soaked in deionized water overnight for swelling. Benzalkonium chloride solution was added, followed by drug-loaded microspheres. Viscolizers were added with slow stirring to prevent foam formation. The mixture was stirred until a clear dispersion formed.

Evaluation of Timolol Maleate Microspheres Loaded Hydrogel^{22,23}

Clarity Test and pH²⁴

These characteristics are especially important in sterile ophthalmic preparations. Variations often indicate poor mixing of ingredients or contamination. Variations in colour can often be attributed to the growth of microorganisms. The formulations were examined for their transparency both prior to and after gelling using visual inspection under alternating light conditions in white and dark backgrounds. The formulation under study was also evaluated for turbidity and the presence of undesired particles within it. The pH of the formulation was required to remain consistent and not cause irritation to the patient upon administration. The pH was evaluated using digital pH metre²⁵.

Determination of Gelation Temperature

Determined using the tilting method to identify the temperature at which the solution gels. The temperature at which the solution becomes gel was noted by gradually increasing the temperature²⁶.

Rheological studies

Viscosity was determined using a Brookfield viscometer after allowing the solutions to gel in simulated tear fluid. Viscosity of eye preparations is the prime important factor in determining the residence time of the active ingredient in our eye. The hydrogel formulated were allowed to form a gel in the STF and its viscosity was noted in Brookfield Viscometer by taking spindle no.62 and the angular viscosity was gradually increased from 1 to 4 rpm. The shear rate was reversed and the best average of the two readings was taken to calculate the viscosity²⁷.

Spreadability

Evaluated by placing an excessive sample between two glass slides and observing the duration it took for the upper slide to traverse the lower surface²⁸. A weight of 1000 g was applied for 5 minutes, followed by the addition of 50 g to the pan. The time was recorded, and the spreadability was computed using:

$$S = \frac{ML}{T}$$

Where, M = weight tied to upper slide (g), L = length moved on the glass slide (cm), T = time taken (sec)

Sterility Testing^{29,30}

Followed the guidelines outlined in the Indian Pharmacopoeia (IP), in which the testing was performed using Fluid Thioglycolate Medium and Soybean Casein Digest Medium. Three batches of the medium were prepared, each contained within a set of test tubes. The initial set functioned as a negative control to assess the sterility of the media. The second set served as a positive control, with sterilized media inoculated with *Staphylococcus aureus*. The third set was dedicated to testing the formulated product. Fluid thioglycolate medium and Soyabean casein digest medium were separately prepared by suspending the required ingredients in 1000 mL distilled water and boiled until it dissolves completely. Then it was subjected to autoclave at 15 lbs pressure, 121°C for 15 minutes and after cooling 25 mL of both medium were transferred into test tubes by sterile pipette and sterile syringe. Subsequently, a defined volume of the formulated product was aseptically transferred to both media and then incubated for a duration of 7 days³¹. The incubation temperatures were maintained at 30 °C to 35°C in Fluid Thioglycolate Medium and 20 to 25°C in Soybean Casein Digest Medium. The culture tubes were periodically observed to check any growth of microorganisms.

In Vitro Release Studies³²

The prepared formulations were studied using Simple Franz Diffusion Cell which has a donor-receiver compartments partitioned with sigma dialysis membrane soaked in the receptor medium, simulated tear fluid pH 7.4. The diffusion medium was stirred at 500 rpm maintained at $37 \pm 0.5^\circ\text{C}$. The formulated products were applied onto the membrane, with the surface facing the donor compartment, and the subsequent drug release was observed for a duration of 24 hours. Samples were collected at specific time intervals and subjected to analysis using a UV spectrophotometer at 292 nm³³. The information derived from the in vitro diffusion assessments was then matched with kinetic equations to determine the underlying mechanism of drug release from the in-situ hydrogel formulation

Comparative Evaluation of Marketed Formulation of Timolol Maleate Eye Drops with the Prepared In-Situ Gels

In vitro release studies for the prepared marketed formulation of Timolol Maleate, TIMOZ eye drops is performed using Simple Franz-Diffusion Cell tied with a sigma dialysis membrane soaked in the simulated tear fluid pH 7.4³⁴. 100 mL of diffusion medium was stirred at 50 rpm maintaining $37^\circ\text{C} \pm 0.5^\circ\text{C}$. The one end of the diffusion tube was covered by a membrane and placed such that it touches the diffusion medium present in receptor compartment. Followed by the withdrawn of drug samples at the interval of one hour for a period of 6 hours an analysed under UV at 292 nm using the simulated tear fluid as blank³⁵.

Drug Release Kinetics

The *in vitro* data for drug release was employed for assessment using kinetic models including Zero order, First order, Higuchi, and Korsmeyer-Peppas. The most appropriate model was selected based on goodness of fit and high regression coefficient value. The regression coefficient (r^2) value closer to 1 indicates that the model fits to the required release mechanism³⁶.

Ocular Irritation Studies^{37,38}

Novel ophthalmic delivery system was evaluated by considering an injury to the eye. The injuries were measured according to Draize test, considering that the eye is an incredibly sensitive, delicate, and invaluable sensory organ, the Timolol Maleate-loaded microspheres hydrogel, which had been developed, underwent *in vivo* investigations. These investigations were carried out after getting the approval from the Institutional Animal Ethical Committee under the registration number IAEC 051/18. The main objective of the study was to find out the potential for irritation caused by the test substance after a solitary application to the eye of rabbits³⁹.

The study was conducted in accordance with the recommendations provided in the OECD Guideline for the Testing of the Chemicals No. 405 titled "Acute Eye Irritation/Corrosion" (Adopted: 24th April 2002). The study involved a thorough assessment of ocular irritation and defects in rabbits' eyes using an ophthalmoscope. Only rabbits with intact ocular structures were included in the study. In the initial phase, a solitary rabbit was subjected to the test formulation. Specifically, 0.1 mL of the substance to be tested was carefully introduced into the conjunctival sac of the eye of rabbit by gently retracting the lower eyelid. Subsequently, the eyelids were briefly held together to prevent substance loss before being released. The untreated left eye was used as a control.

After the administration of the formulation, the rabbit's eyes were examined for indications of ocular harm and irritation at specific time intervals: 1 hour, 24 hours, 48 hours, and 72 hours. Recorded numerical values were linked to each animal, ocular tissue, and observation time, forming the basis for result interpretation. Indicators were designated as A (redness), B (chemosis), and C (discharge), while iris-related observations were marked as D, and those regarding the cornea were identified as E (degree of opacity) and F (area of cornea affected).

The scoring system for each tissue was as follows:

- Score for conjunctivae = $(A + B + C) \times 2$
- Score for iris = $D \times 5$
- Score for cornea = $(E \times F) \times 5$

The cumulative scores for each time point were utilized to determine the ocular irritancy potential, which was then categorized using a modified version of the approach proposed by Kay JH and Calandra JC. This classification involved the summation of all scores obtained at different time points to assess the overall ocular irritancy potential of the test substance⁴⁰.

Accelerated Stability Studies⁴¹

To evaluate the physical and chemical stability of the product, we conducted stability assessments. Stability involves the ability of a pharmaceutical product to maintain its intended physical, chemical, and microbiological characteristics throughout its designated shelf life. The purified sterile formulation underwent an assessment of its stability by being packaged in glass vials sealed with grey butyl rubber closures. These vials were then placed within a stability chamber, where the conditions were maintained at $40 \pm 2^\circ\text{C}$ and $75 \pm 5\%$ relative humidity, for a duration of 6 months. Periodic sample withdrawals enabled analysis of drug content, pH, visual presentation, and transparency.

3. Results and Discussion

The aim of this investigation was to prolong the release of Timolol Maleate using an innovative formulation, with the goal of reducing the frequency of administration. Preformulation studies adhered to Timolol Maleate formulation development requirements. FTIR analysis indicated no drug-excipients interaction. The drug exhibited an absorption maximum at 292 nm. Timolol Maleate microspheres, fashioned via double emulsion solvent evaporation using PLGA as biodegradable polymer, PVA as

surfactant, and Dichloromethane as solvent, yielded a 95% w/w and 93.25% entrapment efficiency. Microsphere diameter averaged $6.15 \pm 2.86 \mu\text{m}$, visualized through optical microscopy, and SEM imagery revealed sizes between 1 – 12 μm .

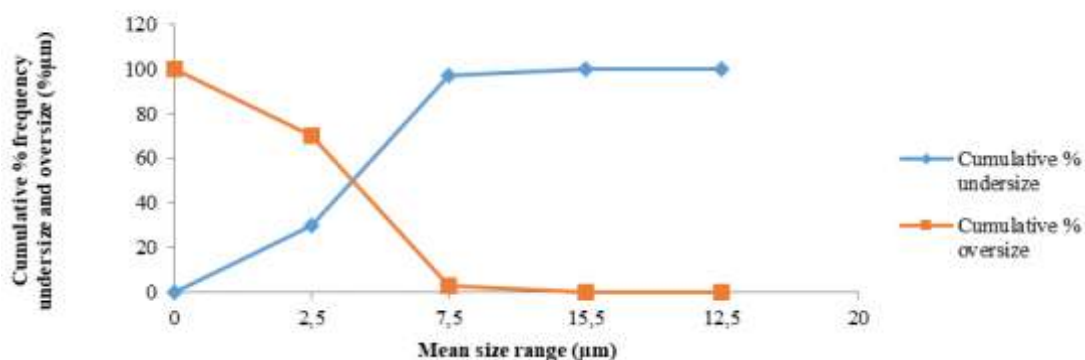


Figure 1. Cumulative Percentage Frequency Curve for Particle Size Analysis

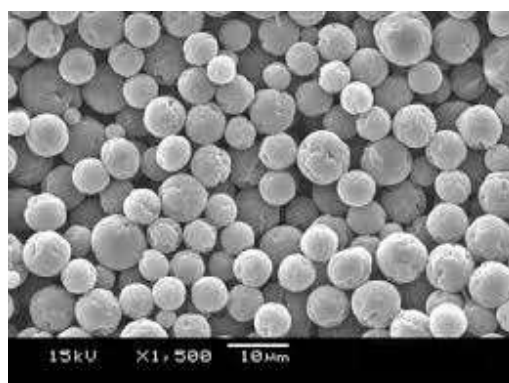


Figure 2. Scanning Electron Photograph of Timolol Microspheres

Hydrogel integration involved loading Timolol Maleate microspheres into Carbopol 940-based gels. All hydrogel formulations displayed clarity, transparency, and pH values (4.2 - 5.0) fitting ophthalmic requirements. Gelation temperatures ranged between 35 – 38 °C. Viscosity was optimized for ocular formulations, with F3 demonstrating appropriate viscosity both before (1000-1280 Cps) and after (1486-1850 Cps) gelation. Spreadability was measured at 13.5-19.2 g.cm/sec, with F3 showcasing favourable spreadability (19.2g.cm/sec).

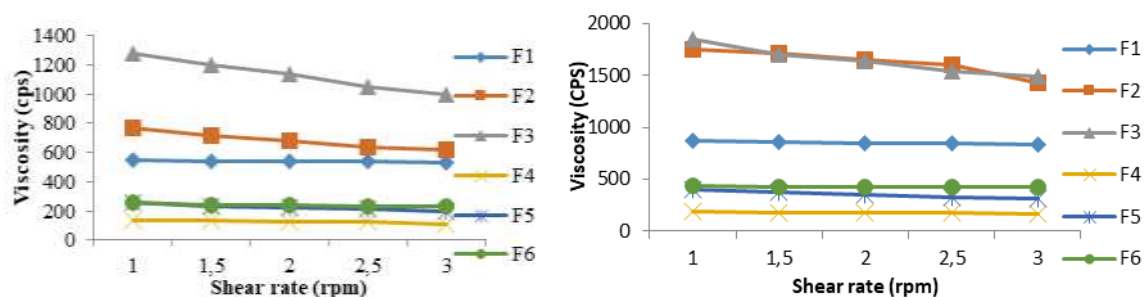


Figure 3. Viscosity of Timolol Maleate Microspheres Loaded Hydrogel (a) Before and (b) After Gelation

Sterility tests affirmed the absence of microbial growth in all formulations after 7 days' incubation at specified temperatures. In vitro diffusion studies revealed drug release percentages between 70-82.6%, with F3 achieving an optimal 82.6% release after 24 hours. F3's in vitro release data fit Zero order kinetics, suggesting Higuchi model applicability and fickian diffusion mechanism.

Comparative *in vitro* release testing of the marketed eye drops, TIMOZ, in a cellophane membrane diffusion cell resulted in a 3-hour release period. Test samples exhibited efficacy against gram-positive and gram-negative strains. Ocular irritation assessment of the optimized F3 formulation displayed with no irritation, with an excellent ocular tolerance and with no observable corneal, iridial, or conjunctival effects. Stability studies spanning 3 months confirmed F3's stability, as physical appearance, clarity, and pH remained unchanged, aligning with ICH guidelines. This affirms its suitability for long-term usage.

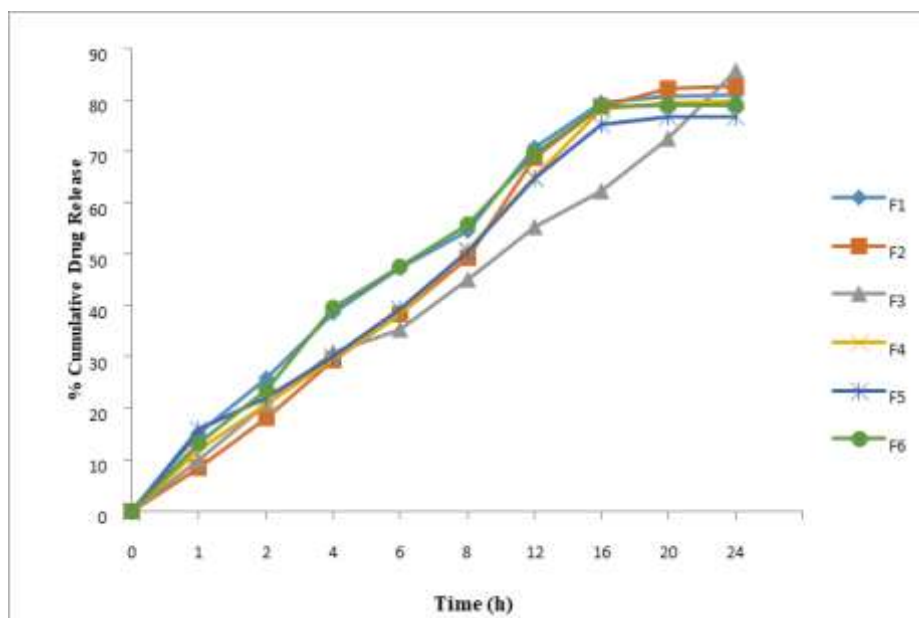


Figure 4. Percentage Cumulative Release of Timolol Maleate from Hydrogel

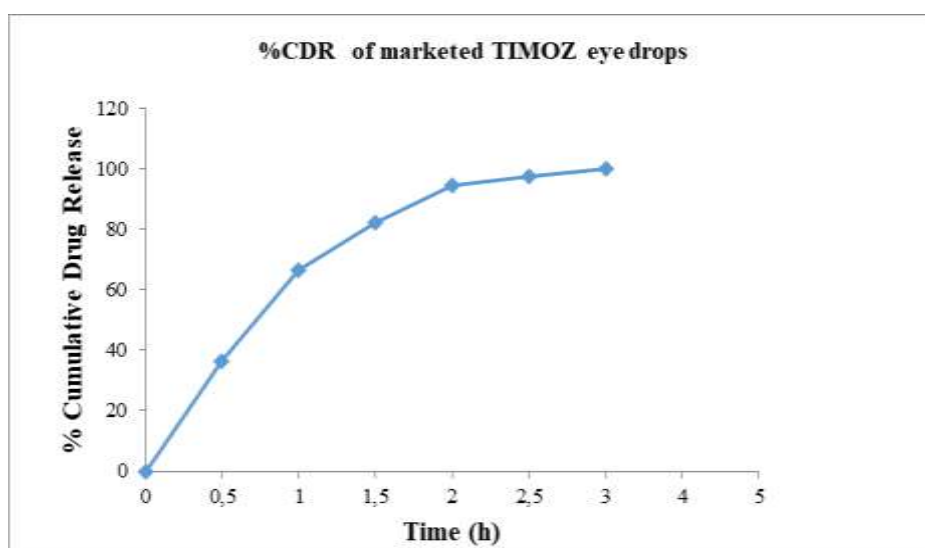


Figure 4. Percentage Cumulative Release of Timolol Maleate from Conventional Marketed Eye Drops

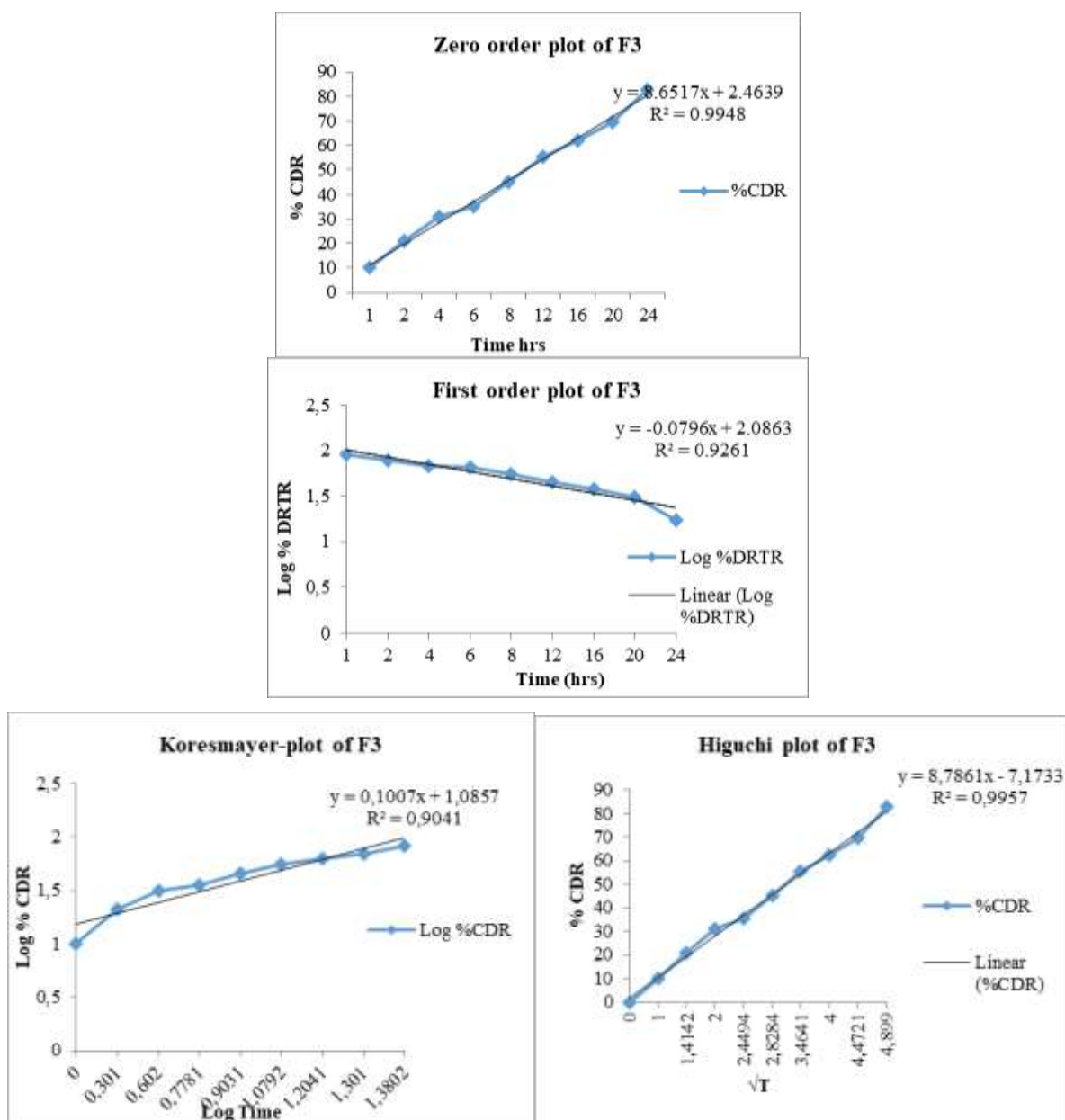


Figure 5. Kinetic Modelling of Timolol Maleate Microspheres Loaded Hydrogel

Preformulation studies ensured drug-excipient compatibility, microsphere preparation, and subsequent hydrogel integration. The developed formulation F3 exhibited optimal characteristics in terms of viscosity, spreadability, and release kinetics, along with excellent ocular tolerance. Stability tests bolstered its long-term utility. This novel formulation holds potential in reducing dosing frequency and improving patient adherence.

4. Conclusion

The project aimed to develop a topically retained Timolol microsphere-loaded hydrogel for extended release in glaucoma treatment. The formulation successfully achieved the objective of diminishing the need for frequent drug dosing by prolonging the release of Timolol. The Timolol Maleate loaded hydrogel, prepared using Carbopol 940 as the gelling agent, exhibited suitable pH and gelation temperature for ophthalmic use. Its viscosity remained within the desired range before and after gelation. The hydrogel formulations underwent a 7-day sterility test, and no microbial growth was observed in any of the samples. In conclusion, the developed topically retained Timolol microsphere-loaded hydrogel (formulation F3) has the potential to improve glaucoma treatment by providing an extended release of the drug, reducing dosing frequency, and maintaining ocular tolerability while ensuring sterility. Further investigations and longer-term stability studies are warranted to assess its

long-term efficacy and safety. This project centered on the creation of a Timolol microsphere-loaded hydrogel with sustained release capabilities for glaucoma therapy. The formulation effectively achieved its goal of reducing dosing frequency by extending Timolol release. Incorporating Carbopol 940 as the gelling agent, the hydrogel loaded with Timolol Maleate exhibited a pH and gelation temperature that were conducive to ophthalmic applications. The hydrogel's viscosity maintained optimal levels before and after gelation. Through a 7-day sterility test, all hydrogel formulations exhibited absence of microbial growth. In summation, the developed Timolol microsphere-loaded hydrogel (designated as F3) demonstrates promise in advancing glaucoma treatment through prolonged drug release, diminished dosing frequency, upheld ocular tolerance, and ensured sterility. To fully ascertain its prolonged efficacy and safety, additional investigations and extended stability assessments are warranted.

References:

1. Willoughby CE, Ponzin D, Ferrari S, Lobo A, Landau K, Omid Y. Anatomy and physiology of the human eye: Effects of mucopolysaccharidoses disease on structure and function — A review. *Clinical and Experimental Ophthalmology*. 2010;38:2-11.
2. Cholkar K, Dasari SR, Pal D, Mitra AK. Eye: Anatomy, physiology, and barriers to drug delivery. In: Mitra AK, Editor. *Ocular Transporters and Receptors*. Woodhead Publishing Limited; Cambridge, UK: 2013. pp. 1-36.
3. Gaudana R, Ananthula HK, Parenky A, Mitra AK. Ocular drug delivery. *The American Association of Pharmaceutical Scientists Journal*. 2010;12:348-60.
4. Osswald CR, Kang-Mieler JJ. Controlled and extended release of a model protein from a microsphere-hydrogel drug delivery system. *Annals of Biomedical Engineering*. 2015;43:2609-2617.
5. Lavik E, Kuehn MH, Kwon YH. Novel drug delivery systems for glaucoma. *Eye*. 2011;25:578.
6. Agrawal AK, Das M, Jain S. *In situ* systems as “smart” carriers for sustained ocular drug delivery. *Expert Opinion on Drug Delivery*. 2010;9:383-402.
7. Chang D, Park K, Famili A. Hydrogels for sustained delivery of biologics to the back of the eye. *Drug Discovery Today*. 2019;24:1470-82.
8. Quigley HA, Broman AT. The number of people with glaucoma worldwide in 2010 and 2020. *British Journal of Ophthalmology*. 2006;90(3):262-7.
9. Ulbrich K, Pechar M, Strohalm J, Subr V, Rihova B. Synthesis of biodegradable polymers for controlled drug release. *Annals of the New York Academy of Sciences*. 1997;831:47-56.
10. Jonas JB, Aung T, Bourne RR, Bron AM, Ritch R, Panda-Jonas S. Glaucoma. *Lancet*. 2017;390:2183-93.
11. Lavik E, Kuehn MH, Shoffstall AJ, Atkins K, Dumitrescu AV, Kwon YH. Sustained Delivery of Timolol Maleate for Over 90 Days by Subconjunctival Injection. *Journal of Ocular Pharmacology and Therapeutics*. 2016;32(10):642-9.
12. Liu W, Lee BS, Mieler WF, Kang-Mieler JJ. Biodegradable Microsphere - Hydrogel Ocular Drug Delivery System for Controlled and Extended Release of Bioactive Aflibercept *In Vitro*. *Current Eye Research*. 2019;44(3):264-74.
13. Bodomeier R, McGinity JW. Solvent selection in the preparation of Poly(D,L-lactide) microspheres prepared by solvent evaporation method. *International Journal of Pharmaceutics*. 1988;43:179-86.
14. Hincal AA, Calis S. Microsphere preparation by solvent evaporation method. In: Wise DL, editor. *Handbook of pharmaceutical controlled release technology*. 2nd ed. New York: Marcel Dekker, Inc; 2000. pp. 329-43.
15. Yang YY, Chung TS, Ng NP. Morphology, drug distribution, and in vitro release profiles of biodegradable polymeric microspheres containing protein fabricated by double-emulsion solvent extraction/evaporation method. *Biomaterials*. 2001;22:231-41.
16. ODonell PB, McGinity JW. Preparation of microspheres by the solvent evaporation technique. *Advanced Drug Delivery Reviews*. 1997;28:25-42.
17. Makwana SB, Patel VA, Parmar SJ. Development and characterization of *in-situ* gel for ophthalmic formulation containing ciprofloxacin hydrochloride. *Results in Pharma Sciences*. 2016;6:1-6.
18. Gan L, Gan Y, Zhu C, Zhang X, Zhu J. Novel microemulsion *in situ* electrolyte-triggered gelling system for ophthalmic delivery of lipophilic cyclosporine A: *In vitro* and *in vivo* results. *International Journal of Pharmacy*. 2009;365:143-9.
19. An injectable thermosensitive polymeric hydrogel for sustained release of Avastin® to treat posterior segment disease. *International Journal of Pharmacy*. 2015;490:375-83.
20. Osswald CR, Kang Mieler JJ. Controlled and extended release of a model protein from a microsphere-hydrogel drug delivery system. *Annals of Biomedical Engineering*. 2015;43:2609-17.
21. Bertram JP, Saluja SS, McKain J, Lavik EB. Sustained delivery of timolol maleate from poly(lactic-co-glycolic acid)/poly(lactic acid) microspheres for over 3 months. *Journal of Microencapsulation*. 2009;26:18-26.

22. Nasir F, Iqbal Z, Khan A, Ahmad L, Shah Y, Khan AZ, Khan JA and Khan S. Simultaneous determination of timolol maleate, rosuvastatin calcium and diclofenac sodium in pharmaceuticals and physiological fluids using HPLC-UV. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*. 2011;879:3434-43.
23. El Kamel AH. *In vitro* and *in vivo* evaluation of pluronic f127-based ocular delivery system for timolol maleate. *International Journal of Pharmacy*. 2002;241:47-55.
24. Jung HJ, Chauhan A. Extended release of timolol from nanoparticle-loaded fornix insert for glaucoma therapy. *Journal of Ocular Pharmacology and Therapeutics*. 2013;29:229-35.
25. Zhang HH, Luo QH, Yang ZJ, Pan WS, Nie SF. Novel ophthalmic timolol maleate liposomal-hydrogel and its improved local glaucomatous therapeutic effect *in vivo*. *Drug Delivery*. 2011;18:502-10.
26. Huang SF, Chen JL, Yeh MK, Chiang CH. Physicochemical properties and *in vivo* assessment of timolol-loaded poly(d,l-lactide-co-glycolide) films for long-term intraocular pressure lowering effects. *Journal of Ocular Pharmacology and Therapeutics*. 2005;21:445-53.
27. Dubey A, Prabhu P. Formulation and evaluation of stimuli-sensitive hydrogels of timolol maleate and brimonidine tartrate for the treatment of glaucoma. *International Journal of Pharmaceutical Investigation*. 2014;4:112-8.
28. Johnson TV, Fan S, Zhan G, Camras CB, Toris CB. Efficacy and mechanisms of intraocular pressure reduction with latanoprost and timolol in participants with ocular hypertension: a comparison of 1 and 6 weeks of treatment. *Journal of Glaucoma*. 2010;19:356-64.
29. Fatouros DG, Bouwstra JA. Iontophoretic enhancement of timolol across human dermatomed skin *in vitro*. *Journal of Drug Targeting*. 2004;12:19-24.
30. El Kamel AH. *In vitro* and *in vivo* evaluation of pluronic F127-based ocular delivery system for timolol maleate. *International Journal of Pharmacy*. 2002;241:47-55.
31. Jung HJ, Chauhan A. Extended release of timolol from nanoparticle-loaded fornix insert for glaucoma therapy. *Journal of Ocular Pharmacology and Therapeutics*. 2013;29:229-35.
32. Higashiyama M, Inada K, Ohtori A, Tojo K. Improvement of the ocular bioavailability of timolol by sorbic acid. *International Journal of Pharmacy*. 2004;272:91-8.
33. Syed MF, Loucks EK. Update and optimal use of a brinzolamide-timolol fixed combination in open-angle glaucoma and ocular hypertension. *Clinical Ophthalmology*. 2011;5:1291-6.
34. Hsiue GH, Hsu SH, Yang CC, Lee SH, Yang IK. Preparation of controlled release ophthalmic drops, for glaucoma therapy using thermosensitive poly-N-isopropylacrylamide. *Biomaterials*. 2002;23:457-62.
35. Nanjawade BK, Manvi FV, Manjappa AS. *In situ*-forming hydrogels for sustained ophthalmic drug delivery. *Journal of Controlled Release*. 2007;122:119-34.
36. Chiang CH, Ho JJ, Chen JL. Pharmacokinetics and intraocular pressure lowering effect of timolol preparations in rabbit eyes. *Journal of Ocular Pharmacology and Therapeutics*. 1996;12:471-80.
37. Watanabe K, Chiou GC. Action mechanism of timolol to lower the intraocular pressure in rabbits. *Ophthalmic Research*. 1983;15:160-7.
38. Jacob TF, Singh V, Dixit M, Ginsburg-Shmuel T, Fonseca B, Pintor J, Youdim MBH, Major DT, Weinreb O, Fischer B. A promising drug candidate for the treatment of glaucoma based on a P2Y6-receptor agonist. *Purinergic Signal*. 2018;14(3):271-84.
39. Higginbotham EJ, Schuman JS, Goldberg I, Gross RL, VanDenburgh AM, Chen K, Whitcup SM. One-year, randomized study comparing bimatoprost and timolol in glaucoma and ocular hypertension. *Archives of Ophthalmology*. 2002;120(10):1286-93.
40. Shilakari AG, Asthana A, Singh D, Sharma PK. (2016). Etodolac containing topical niosomal gel: formulation development and evaluation. *Journal of Drug Delivery*. 2016:1-8.
41. Sawant D, Dandagi PM, Gadad AP. Formulation and evaluation of sparfloxacin emulsomes-loaded thermosensitive *in situ* gel for ophthalmic delivery. *Journal of Sol-Gel Science and Technology*. 2016;77:654-65.