Formulation and Optimization by Factorial Design of *In-situ* Ophthalmic gelling systems of Levofloxacin hemihydrates

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Abstract:
Many topically instilled ocular drugs undergo rapid elimination due to tear turnover thereby showing poor absorption. A prolonged precorneal residence time may result in higher absorption and prolonged duration of their therapeutic effect. *In-situ* gels are retained better in the eye than the conventional ocular dosage forms like eye drops as solutions and suspensions thereby enhancing bioavailability and duration of therapeutic activity. These are viscous polymer-based liquids that exhibit sol-to-gel phase transition on the ocular surface due to change in specific physico-chemical parameters like temperature, ionic strength, or pH. The present work describes the formulation and evaluation of two in-situ ocular delivery systems of antibacterial agent Levofloxacin hemihydrates based on Ion activation and pH activation approach. Nine formulations (C1-C9) based on the concept of pH triggered in situ gelation using Carbopol 934p and nine formulations (S1-S9) based on Ion activated in situ gelation using Sodium alginate were prepared with HPMC K4M as a common viscolyzer. The formulations were evaluated for clarity, pH measurement, gelling capacity, drug content estimation, rheological study, in-vitro drug release study and in-vivo studies on rabbit eye and Antimicrobial efficacy study. Studies showed that the formulations release drug in a sustained manner up to 8 hrs. They are non-irritating with no ocular damage.

Key words: Levofloxacin, In-situ gelling systems, Carbopol 934p, Sodium alginate.

Introduction:

Topical administration is generally considered the preferred route for the administration of ocular drugs due to its convenience. Drugs applied in this manner can be packaged in multiple forms, including solutions, ointments, and suspensions. Drug absorption occurs through corneal and non-corneal pathways. Most non-corneal absorption occurs via the nasolacrimal duct and leads to non-productive systemic uptake, while most of the drugs transported through the cornea is taken up by the targeted intraocular tissue. Unfortunately, corneal absorption is limited by drainage of the instilled solutions, lacrimation, tear turnover, metabolism, tear evaporation, non-productive absorption/adsorption, and limited corneal area, poor corneal permeability, binding by the lachrymal proteins, enzymatic degradation, and the corneal epithelium itself. These limitations confine the absorption window to a few minutes after administration and reduce corneal absorption to < 5%[1]. Consequently; multiple daily dosing is often required, leading to decreased patient compliance. To increase both retention time on the surface of the eye and corneal absorption, novel topical systems have been

developed, including ones that utilize in situ gelling polymers, mucoadhesive polymers, inserts, and ocular penetration enhancers[2]. In situ-forming hydrogels are liquid upon instillation and undergo phase transition in the ocular cul-de-sac to form viscoelastic gel and this provides a response to environmental changes. Three methods have been employed to cause phase transition on the surface: change in temperature, pH, and electrolyte composition[3].Sodium alginate, the sodium salt of alginic acid, is a natural hydrophilic polysaccharide containing two types of monomers, β-D-mannuronic acid (M) and α-L-gucuronic acid (G) which forms three-dimensional hydrogel matrices .Sodium Alginate was chosen as a vehicle for ophthalmic formulations since it exhibits a prolonged precorneal residence of formulations containing alginic acid was looked for, not only based on its ability to gel in the eye but also because of its mucoadhesive properties. Alginate transforms into stable gel upon exposure to divalent cations, which is not easily eroded by tear fluid[4].

Carbopol 934p is a synthetic polymer composed of 62% of carboxyl groups with approximate molecular weight (3×10^6)[5]. Carbopol shows a sol-to-gel phase transition in aqueous solution when the pH is raised above its pKa of i.e. about 5.5 and also it has mucoadhesive properties[6]. In order to reduce the polymer concentration and improve the gelling properties, viscosity enhancing agent has been used which is hydroxypropyl methylcellulose (HPMC K4M). Also it strengthens the gel formed even after the dilution with the tear fluid. Levofloxacin hemihydrate is a fluoroquinolone derivative used to treat external infections of eye such as acute and sub acute bacterial conjunctivitis, keratitis, keratoconjuctivitis and corneal ulcers. It is available in market as 0.5%w/v eye drops. The objective of present study was to develop and evaluate an Ion activated and pH triggered in-situ ophthalmic gel system for Levofloxacin hemihydrates by using Sodium alginate and Carbopol 934p respectively as gelling agents.

Materials and methods:

Levofloxacin hemihydrate was kindly supplied as a gift sample from Orgochem Ltd, Gujarat. Carbopol 934p and Sodium alginate supplied by Signet chemicals. Hydroxyl propyl methyl cellulose (HPMC K4M) was obtained from Colorcon Asia Pvt. Ltd. All chemicals were used of analytical grade.

Preparation of formulations:

Table (1) and (2) shows the composition of in situ-gelling systems based on Sodium alginate and Carbopol 934p respectively. Ion activated in-situ solutions were prepared by dispersing the required amount of Sodium alginate and HPMC K4M in distilled water with continuous stirring. Levofloxacin hemihydrates (0.5% w/v) were dissolved in distilled water. Solution of benzalkonium chloride and mannitol were then added to the above solution. The resultant solution was then added to alginate solution under constant stirring to obtain a uniform solution. Distilled water was then added to make the volume up to 100 ml[7].

The pH triggered in-situ gels were prepared by dispersing HPMC K4M in 75 ml of Citrophosphate buffer pH 6.0 and allowed to hydrate. Carbopol 934p was sprinkled over this solution and allowed to hydrate overnight. The solution was stirred continuously. Levofloxacin hemihydrates were dissolved in 0.01 N sodium hydroxide solutions and pH was adjusted. Benzalkonium chloride (BKC) and Sodium chloride was then added to the above solution with continuous stirring. The drug solution was added to the Carbopol–HPMC solution under constant stirring until a uniform solution was obtained. Buffer was then added to make up the volume to 100 ml[8]. The formulations, in their final pack, were subjected to terminal sterilization by autoclaving at 121^0C at 15 lb or 20 min.

Evaluation of formulations:

Test for appearance/ clarity

All the formulations were checked for general appearance i.e. colour, odour, any suspended particulate matter etc. The clarity was checked using wooden board with black and white background. The vials were held horizontally and gently rotated immediately under the lamp and then inverted once or twice to detect foreign particles.

Determination of pH

The pH of each formulation was recorded using a calibrated pH meter. The pH of all formulations was recorded immediately after preparation as well as after 24 hours of storage at room temperature.

Gelation Studies

The gelling capacity was determined by placing 2 drops of prepared system in a vial containing 2 ml of artificial tear fluid freshly prepared and equilibrated at 37^0C.The gel formation was visually evaluated; time for gelation and the time taken for the gel to dissolve were noted. The composition of artificial tear fluid was sodium chloride (0.670 g), sodium bicarbonate (0.200 g), calcium chloride·2H2O (0.008 g), and purified water q.s. (100 g). To note gelation observations the lowest scores (+) were assigned to those products in which the phase transition occurred only after 60-90 sec. and the formed gels collapsed within 1-2 hrs. The highest scores (+++) were assigned to those products for which the phase transition commenced within 60-90 sec. and the gels so formed were stable for about 7-8 hrs. The moderate scores (++) were assigned to the products, which could form the gel in 60-90 sec.but failed to maintain gel structure for more than 3hrs.

Drug content

The drug content was determined by suitably diluting solution with phosphate buffer pH 7.4. Aliquot of 1ml was withdrawn and further diluted to 10 ml with phosphate buffer pH 7.4. Levofloxacin concentration was determined at 288 nm by using UV-Visible spectrophotometer.

Rheological studies

Viscosity of the instilled formulation is an important factor in determining residence time of drug in the eye. The prepared solutions were allowed to gel in the simulated tear fluid and then the viscosity determination were carried out by using Brookfield viscometer with angular velocity run from 10 to 100 rpm.

In-vitro release study:

The in vitro release of Levofloxacin from the formulations was studied through cellophane membrane using a modified Franz diffusion cell. The dissolution medium used was artificial tear fluid freshly prepared (pH 7.4). Cellophane membrane, previously soaked overnight in the dissolution medium, was tied to one end of a specifically designed glass cylinder. A 1-ml volume of the formulation was accurately pipetted into this assembly. The cylinder was attached to the metallic driveshaft and suspended in 50 ml of dissolution medium maintained at 37^0C so that the membrane just touched the receptor medium surface. The shaft was

rotated at 100 rpm. Aliquots, each of 1-ml volume, were withdrawn at hourly intervals and replaced by an equal volume of the receptor medium. The aliquots were diluted with the receptor medium and analyzed by UV spectrophotometer at 288 nm.

In vivo drug release studies:

In vivo release studies of Levofloxacin hemihydrate from the prepared *in situ* gelling formulations were carried out using six male New Zealand albino rabbits each weighing 2.5–3.0 kg and with no signs of ocular inflammation or gross abnormalities.

Method:

50-µl of Levofloxacin hemihydrates optimized *in situ* gelling formulations (0.5%w/v) and marketed eye drop (0.5% w/v; marketed preparation) were instilled in the lower cul-de-sac of each eye, and the upper and lower eyelids were gently held closed for 2 min to maximize drug-cornea contact. At 0.5, 1, 2, 4, 6, and 8 h post dosing, the eyes were anesthetized using 4% topical xylocaine solution and the aqueous humour was sampled from 6 eyes for each formulation using a 28-gauge needle. Aqueous humour samples (100 µl) were mixed with acetonitrile and kept in a refrigerator for 1 h. The mixture was then centrifuged at 3000 r/min for 15 min and 20 µl of the supernatant obtained was used to determine the Levofloxacin hemihydrate content by HPLC.

HPLC analysis:

Quantitative estimation of Levofloxacin was done by HPLC. A filtered and degassed mixture of phosphate buffer pH 3.0 and acetonitrile (80:20) was used as the mobile phase. The mobile phase was delivered at a flow rate of 1.0 ml/min, the injection volume was 20 µl and the effluent was monitored at 235 nm using Gatifloxacin as internal standard.

Pharmacokinetic analysis:

The area under the curve, $AUC_{(0-8)}$ (µg.h/mL) for Levofloxacin concentration in the aqueous humor was calculated using the trapezoidal rule. The maximum Levofloxacin concentration Cmax (µg/ml) in the aqueous humor and the time at which Cmax is achieved i.e. Tmax (hr) were determined from actual data point.

Sterility testing:

IP method (1996) was followed for the sterility testing of eye drops. Sterility testing was carried out by incubating formulations for not less than 14 days at 30 to 35^0 C in the fluid thioglycolate medium to find the growth of anaerobic bacteria and at 20 to 25^0 C in the soyabean-casein digest medium to find the growth of aerobic bacteria and fungi in the formulation.

Antimicrobial efficacy study:

Antimicrobial efficiency studies were carried out to ascertain the biological activity of sol-to-gel systems against microorganisms (Staphylococcus aureus and Pseudomonas aeruginosa) This was determined in the agar diffusion medium employing “cup plate technique”. Sterile solution of marketed Levofloxacin hemihydrate eye drops was used as a standard. The standard solution and the developed formulations (test solution) were taken into separate cups bored into sterile Muller Hinton Agar (MHA) previously seeded with organisms (*Staphylococcus aureus and Pseudomonas aeruginosa*). After allowing diffusion of solutions for two hours, the plates were incubated for 24 h at 37^0C. The zone of inhibition (ZOI) was compared with that of the standard. The activity of optimized formulations were also checked in infectious condition of eye i.e. STF pH 6.0 and compared with that of normal condition of eye i.e. STF pH 7.4

Results and Discussion:

Selection of vehicle:

Buffers contribute significantly to chemical stability and clinical response and also influence the comfort and safety of the product; hence it is important to select a suitable buffer which ensures product stability and desired drug solubility. The studies in various buffer solutions indicated that the drug was soluble in acetate buffer IP of pH 4.6, 4.8 and 5.0 and in citrophosphate buffer of pH 6.0 at the dosage level of 0.5%, w/v. The solutions were stable to elevated temperatures and autoclaving. However, their instability to light as evidenced by discoloration of the exposed solutions necessitated their packing in amber vials. It has been reported that the ocular penetration of Levofloxacin, is maximum at pH of about 6.5. Citrophosphate buffer, pH 6.0, was therefore selected as a vehicle for the pH activated in-situ gel preparation. Sodium alginate was found to be more stable in deionised water as compared to buffer that's why it was selected as vehicle for Ion activated in-situ gel preparation.

Selection of excipients:

Sodium alginate and Carbopol 934p were selected as gelling agent because of its property to transform into gel in contact with tear fluid. These agents alone could not be used for ocular therapeutics due to its low mucoadhesive property, hence HPMC K4M was used along with these gelling agents to impart mucoadhesive properties. Benzalkonium chloride is mostly used in ophthalmic as preservative and also it is stable over wide temperature and pH conditions. Sodium chloride and Mannitol were used as tonicity adjusting agents to maintain preparation isotonic.

The composition, drug content, gelling capacity of the various Ion activated and pH based formulations are shown in Table (1) and (2), respectively. The drug content was found to be satisfactory.

Table (1): Characterization of sodium alginate In-situ gelling system of Levofloxacin

Code	Sodium alginate	Hpmc k4m	Gelling capacity	pH	Drug content	Clarity	Appearance
S1	1.0	0.4	+++	6.0	99.98	Clear	Light yellow
S2	0.5	0.3	+	6.0	102.02	Clear	Light yellow
S3	1.5	0.5	+++	6.4	99.76	Clear	Light yellow
S4	0.5	0.5	+	6.2	98.75	Clear	Light yellow
S5	0.5	0.4	++	6.2	100.03	Clear	Light yellow
S6	1.5	0.3	+++	6.3	100.48	Clear	Light yellow
S7	1.5	0.4	+++	6.0	101.20	Clear	Light yellow
S8	1.0	0.5	+++	6.2	99.34	Clear	Light yellow
S9	1.0	0.3	++	6.3	100.36	Clear	Light yellow

Table (2): Characterization of Carbopol In-situ gelling system of Levofloxacin

Code	Carbopol 934p	Hpmc k4m	Gelling capacity	pH	Drug content	Clarity	Appearance
C1	0.4	0.4	+++	6.2	99.21	Clear	Light yellow
C2	0.4	0.5	+++	6.4	98.76	Clear	Light yellow
C3	0.5	0.6	++	6.0	101.65	Clear	Light yellow
C4	0.3	0.4	+	6.2	100.87	Clear	Light yellow
C5	0.3	0.5	++	6.4	99.98	Clear	Light yellow
C6	0.5	0.5	+++	6.3	99.89	Clear	Light yellow
C7	0.4	0.6	++	6.1	99.12	Clear	Light yellow
C8	0.3	0.6	++	6.2	100.24	Clear	Light yellow
C9	0.5	0.4	+++	6.1	100.23	Clear	Light yellow

Appearance, clarity, pH and drug content:

The appearances of all formulations were light yellow in colour and were clear. Terminal sterilization by autoclaving had no effect on the formulations. The haziness observed during autoclaving due to precipitation of HPMC at elevated temperature was found to disappear and the clarity was regained after overnight standing. The pH of all the formulations was found to be within the range of 6.0 to 6.5, which is desirable for absorption of Levofloxacin hemihydrate. The drug content of ion activated in-situ formulations was within the range of 98.75% to102.02%, showed the uniform distribution of drug in the ophthalmic formulations and the results are shown in Table (1) while for pH triggered in-situ gels drug content is within 98.76 to 101.65% as shown in Table (2).

Gelling capacity:

The viscosity and gelling capacity plays important role for in situ gelling system. The formulation should have an optimum viscosity for easy instillation into the eye as a liquid which undergo sol-to-gel transition. Formulations S1, S3, S6, S7, S8, C1, C2, C6 and C9 showed better gelling capacity as shown in Table (1) and (2).

Rheological studies:

The formulations were shear thinning and an increase in shear stress was observed with increase in angular velocity (pseudo plastic rheology). The administration of ophthalmic preparations should influence as little as possible the pseudo plastic character of the precorneal tear film. Since the ocular shear rate is very large ranging from 0.03 s^{-1} during interblinking periods to 4250–28,500 s^{-1} during blinking, viscoelastic fluids with a viscosity that is high under conditions of low shear rate and low under conditions of high shear are preferred[9]. In the case of pH triggered in-situ gelling system, formulations were in a liquid state at pH 6.0 and exhibited low viscosity. An increase in pH to 7.4 (the pH of the tear fluid) caused the solutions to transform into gels with high viscosity as shown in Table (4) same in case of ion activated in-situ gelling system shown in Table(3).

Table (3): Viscosity of ophthalmic formulations of sodium alginate with HPMC K4M at 10, 50 & 100 rpm.

Code	Viscosity of formulations at 10, 50 and 100 rpm both at 25ºC and 37ºC					
	25^0c	37^0c	25^0c	37^0c	25^0c	37^0c
	10	10	50	50	100	100
S1	72	38242	63	11220	58	9729
S2	60	18400	56	7840	52	6289
S3	78	42212	72	12200	69	9810
S4	62	21432	58	7920	55	6123
S5	67	20121	53	6850	49	4024
S6	71	39273	61	12121	57	9932
S7	76	40280	64	12010	54	9813
S8	79	38276	63	11389	53	9623
S9	68	36209	48	11254	41	9523

Table (4): Viscosity of ophthalmic formulations of carbopol 934p with HPMC K4M at 10, 50 & 100 rpm.

Code	Viscosity of formulations at 10, 50 and 100 rpm both at 25 oC and 37 oC					
	25^0c	37^0c	25^0c	37^0c	25^0c	37^0c
	10	10	50	50	100	100
C1	62	37400	55	6523	49	3056
C2	67	38900	63	7840	53	3210
C3	72	42500	69	11360	62	9921
C4	79	19280	74	8533	69	5209
C5	82	20212	79	8976	73	6289
C6	85	43243	83	11270	76	9729
C7	89	32176	95	9890	888	8827
C8	93	22726	101	10210	96	9120
C9	97	39300	107	11230	110	9603S

In-vitro release:

Fig. 1 and Fig. 2 show the percentage of Levofloxacin hemihydrate released as a function of time for S1-S9 and C1-C9 respectively. As per figures S3 and C9 showed better release of 86.2 and 90.21 % respectively up to 8 hrs. The *in vitro* drug release conditions may be very different from those likely to be encountered in the eye. However, the results clearly showed that the gels have the ability to retain Levofloxacin hemihydrate for a prolonged period of time (8 hr) and premature drug release does not occur. From the results it is concluded that the high viscosity plays important role in controlling the release of drug from the formulations, when the polymer concentration increases drug release decreases, and when polymer concentration decreases drug release from the formulation increases. Comparison of in-vitro release of formulations S3 and C9 with marketed eye drop is shown in Fig.3 and Fig.4.

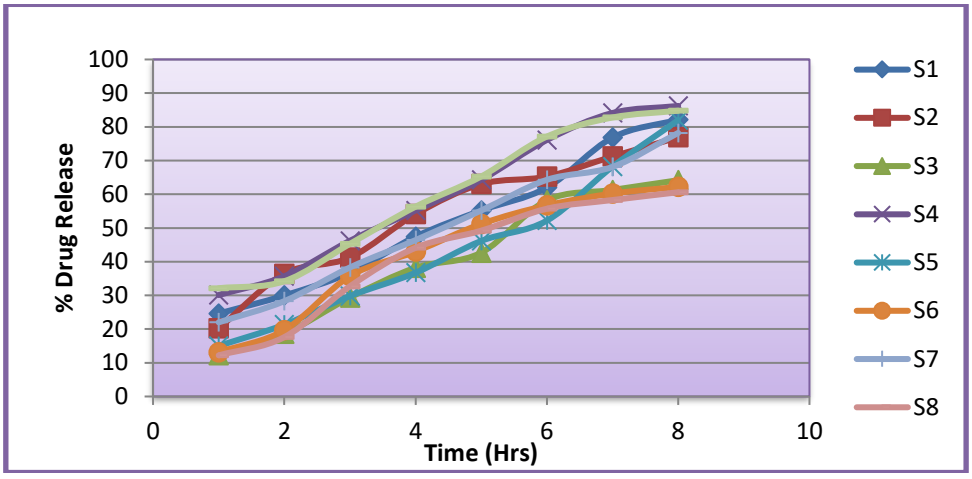


Figure 1: In-vitro release kinetics of S1-S9 batches

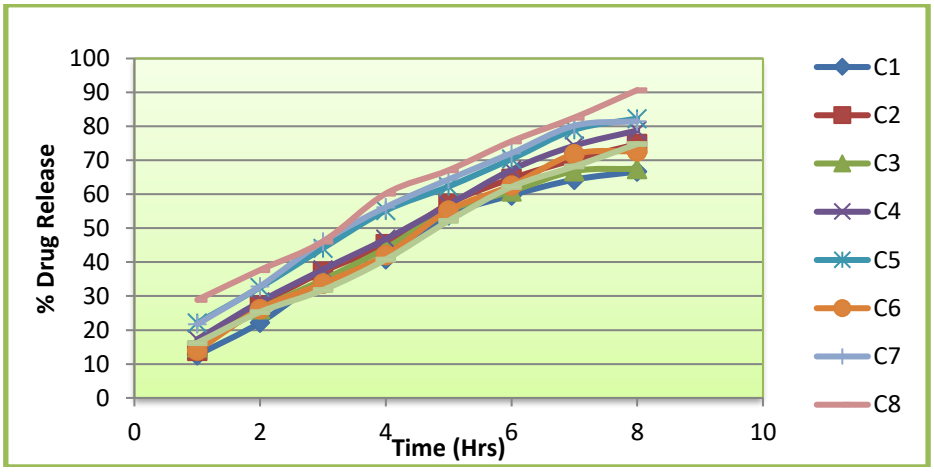


Figure 2: In-vitro release kinetics of C1-C9 batches

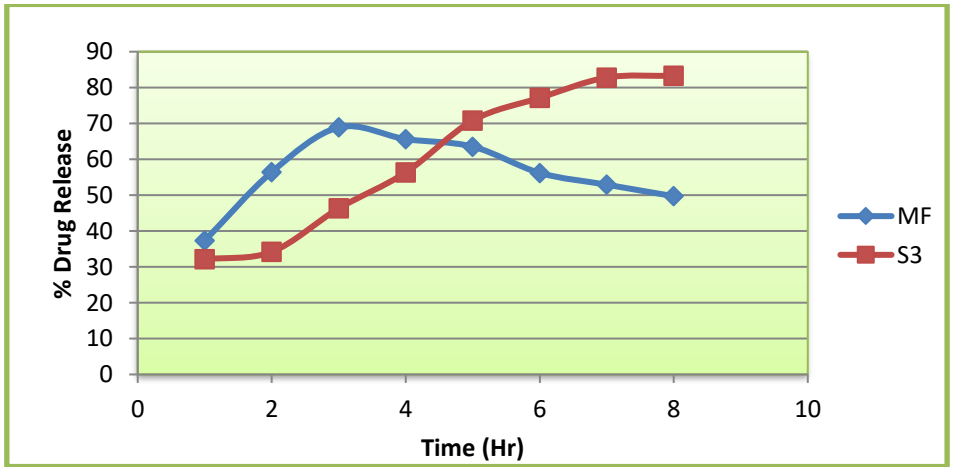


Figure 3: comparison of In-vitro release kinetics of S3 with marketed formulation (MF)

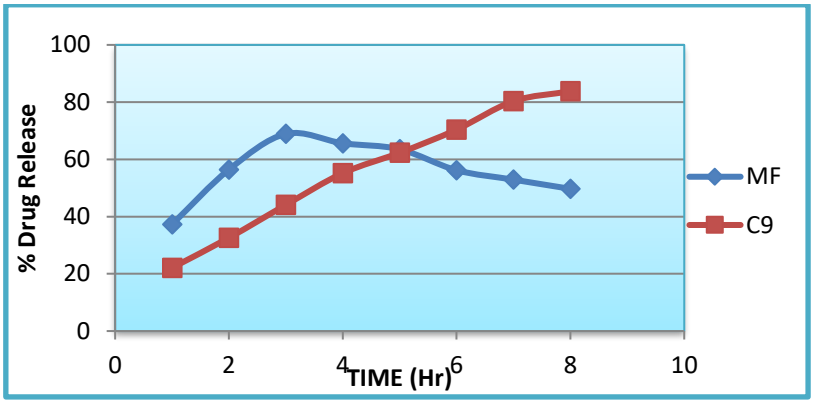


Figure 4: comparison of In-vitro release kinetics of C9 with marketed formulation (MF)

In-vivo release:

The *in vitro* release studies were completely free from any complications due to variability in precorneal factors, such as blinking, lachrymation, tear turnover, and drug washout. The *in vitro* studies provided the relative permeation characteristics of Levofloxacin from different formulations, but they could not simulate real *in vivo* conditions. It is, therefore, necessary to study the *in vivo* ocular absorption of the drug from the formulations. The results of the *in vivo* studies are shown in Fig. 5 and Fig 6

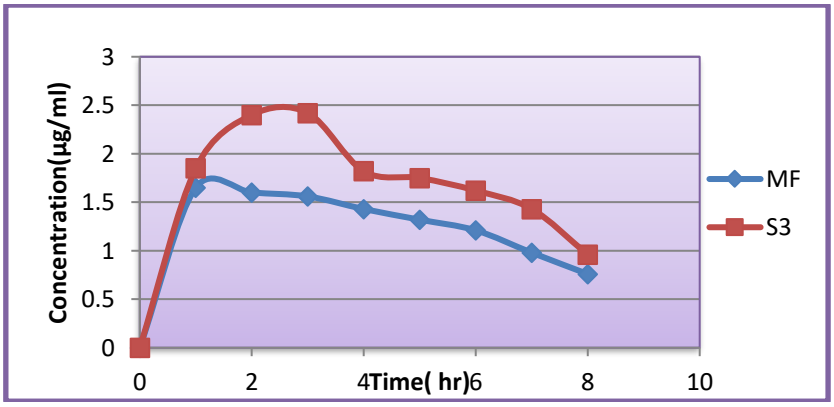


Fig.5 Tear fluid concentration profile of marketed eye drop & ion activated in-situ gel (S3)

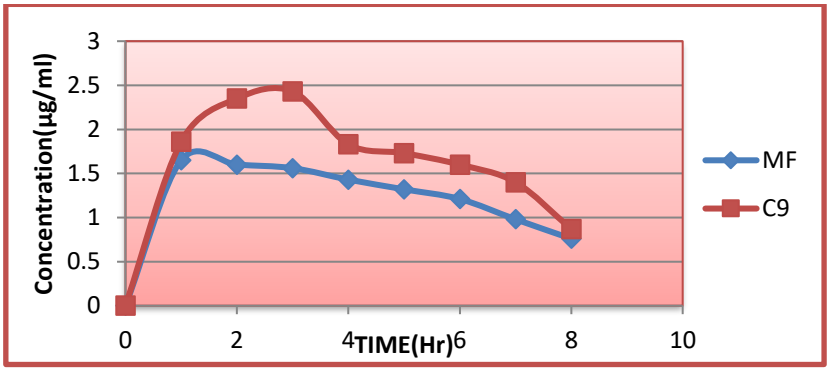


Fig.6 Tear fluid concentration profile of marketed eye drop & pH activated in-situ gel (C9

Table (5): Pharmacokinetic parameters of Levofloxacin in aqueous humor after instillation of S3 and C9

Ocular delivery system	Cmax (µg/ml)	Tmax (h)	$AUC_{(0-8)}$ (µg.h/ml)*
MF	1.65	1	12.69
S3	2.40	3	18.30
C9	2.35	3	17.62

The level of Levofloxacin in aqueous humor after topical instillation of S3 and C9 was shown in (Fig.5 and Fig.6). The aqueous humor content of Levofloxacin was significantly higher at all time points after administration of S3 than that obtained after instillation of C9. It was interesting to note that the aqueous humor level showed a maximum at 3 hr post administration which decreased gradually afterwards since the higher concentration of the antibiotic is always desirable at early time of infection. More specifically, the intraocular Levofloxacin level attained in the aqueous humor following administration of S3 was fairly high for up to 8 hr in contrast to its intraocular level from C9.

Sterility test:

The optimized formulation S3 and C9 passed the test for sterility as there was no appearance of turbidity and hence no evidence of microbial growth when incubated for not less than 14 days a 30-35 ^{0}C in case of fluid thioglycolate medium and at 20-25^{0}C in the case of soyabean casein digest medium.

Antimicrobial efficacy study:

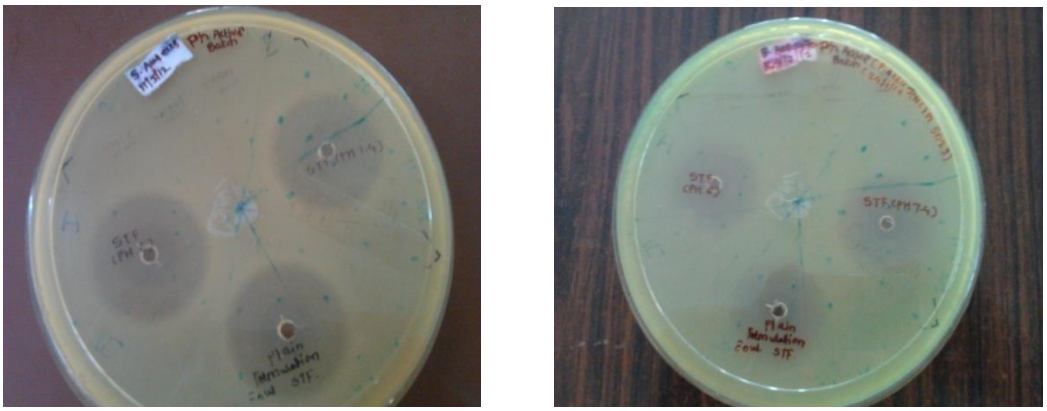
The study indicated that the Levofloxacin retained its antimicrobial efficacy even after formulated as an *in situ gelling system*, this was indicated by same zone of inhibition (3.5 cm) observed in case of both marketed formulation and in-situ gel system against staphylococcus aureus as shown in figure 7. In infected condition of eye, pH of eye becomes acidic i.e pH 5-6. Antimicrobial efficacy of optimized in-situ formulations were tested in acidic condition i.e.

STF(pH 6) and compared its zone of inhibition in STF (pH 7.4) against Staphylococcus aureus (2024) and Pseudomonas aeruginosa (NCIM-5029) which was same as shown in figures 8 and 9 .This indicates that Levofloxacin shows antimicrobial activity even in acidic condition of eye (Infectious condition of eye) .

FIGURE 7: Zone of inhibition shown by marketed formulation with In-situ gelling system against staphylococcus aureus (NCIM-2024)

(a) Staphylococcus aureus **(b) Pseudomonas aeruginosa**

Figure 8: Zone of inhibition shown by S3 against (a) Staphylococcus aureus (NCIM-2024) (b) Pseudomonas aeruginosa(NCIM-5029)



(a) Staphylococcus aureus **(b) Pseudomonas aeruginosa**

Figure 9: Zone of inhibition shown by C9 against (a) Staphylococcus aureus (NCIM-2024) (b) Pseudomonas aeruginosa (NCIM-5029)

Conclusion:

In this study we investigated the potential of In-situ gelling systems triggered by Ion and pH for Levofloxacin hemihydrate delivery to ocular tissue. Sodium alginate system compared to carbopol exhibited higher Levofloxacin level and prolonged residence time in the eye. Therefore, sodium alginate In situ gel system could improve ocular bioavailability more as compared to carbopol system.

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