RESEARCH PAPER

Development and Evaluation of pH triggered *In-situ* gelling systems for ocular delivery of Levofloxacin hemihydrates: *In-vitro* and *In-vivo* studies

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ABSTRACT

Eyes are delicate and most vital organs of the body whose defence mechanism restricts entry of exogenous substance. Conventional drug delivery systems get washed off within a short period of time that usually cause poor bioavailability and therapeutic responses because high tear fluid turnover and dynamics cause rapid elimination of the drug from the eye. In-situ gelling ophthalmic drug delivery system is one of the new methods that are developed to overcome these bioavailability problems. In-situ gelling systems are viscous polymer based liquids that exhibit sol-to-gel phase transition on the ocular surface due to a change in a specific physicochemical parameter like temperature, ionic strength, or pH triggered in-situ systems. Using this formulation of pH triggered in-situ gel systems, the release of drug can be sustained for longer periods of time, therapeutically more efficacious, non-irritant and stable than conventional eye drops.

Keywords: In-situ gel, ophthalmic drug delivery, pH triggered, sol-to-gel phase transition.

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INTRODUCTION

Eyes are important sensory organs in the human body, which convert light to an electric signal that later will be interpreted by brain[1]. It can restrict the entry of any exogenous substance because of its anatomical physiological structure and defence mechanisms[2]. But, as eyes are unique organs, they also can be infected by various diseases like conjunctivitis, dry eye syndrome, glaucoma, keratitis, trachoma and so on[3]. Therefore, to target the drug at a required ocular site in therapeutic dose has been one of the most challenging tasks until now[4]. Various factors like nasolacrimal drainage of drug, binding of drug to lachrymal protein, induced lachrymation, availability of limited corneal area create a barrier for absorption of drug through ocular routes[3,5]. There are two types of ophthalmic drug delivery systems, classified as conventional and newer drug delivery systems. The conventional ophthalmic drug delivery system in the form of eye drops has a dynamic effect and high tear fluid turnover that causes rapid pre-corneal elimination of the drug and also only 1-10% of topically applied drug get absorbed that often results in poor bioavailability and therapeutic response[6]. Consequently, to achieve the desired therapeutic effect, frequent instillation of concentrated solutions is needed. Due to tear drainage, more than 75% of the administered dose of the drug goes through the nasolacrimal duct and goes into the gastrointestinal tract, leading to systemic side effects[2, 7]. In order to enhance the ophthalmic bioavailability and lengthen the residence time of instilled dose, many ophthalmic vehicles have been developed, such as aqueous gels, inserts, ointments and suspensions. However, because of low patient compliance in using the inserts and the side effect of using an ointment such as blurred vision, these ocular drug delivery systems have not been used extensively until now.

For the past few years, these new drug delivery systems that have been developed received significant interest by ophthalmologists is in-situ gel systems. In-situ gel forming system has showed their potential in increasing the residential time because of bio-adhesiveness of formed gel that has been produced. Additionally, polymers used to achieve in-

situ gelling may result in sustained release of drug molecules[8, 9, 10]

In-situ gelling systems are described as low viscosity solutions that phase transition in cul-de-sac to form viscoelastic gel. This sol-to-gel phase transition happens due to conformational changes of polymer in response to a physiological environment. In-situ formulations are more acceptable for patient because they are administered as solution or suspension which immediately undergoes to gelation as coming in contact with the eye[11]. Depending on the method chosen to cause sol-to-gel phase transition on the surface of the eyes, three types of in situ gelling systems are widely accepted namely ion activated systems, pH triggered systems and temperature sensitive systems[4]. The ideal properties for in-situ gel formulation can be divided into three categories involving a physical state – the formulation should be free flowing liquid which allows ease of administration with reproducible dose delivery to the eyes: Phase transition – as drug has been instilled, it should undergo sol-to-gel formation by phase transition[12]. Strength of gel – to withstand the sheer force in cul-de-sac phase so it can prolong residence time of the drug, and the gel formed should be strong enough[5].

Use of In-situ gel forming polymeric formulations may increase patient compliance by a decrease in frequency of administration and overall cost of treatment[13,14]. In this article, a summarized concept of approaches used in stimuli responsive systems, which is pH by triggered in situ gelling systems specifically, along with information on different polymers that can be used in this approach, an example list of FDA approved-based on the concept of ocular pH triggered in-situ gel, review in the field of pH triggered in-situ gelling system, and a basic method of preparation for pH triggered in-situ gelling system.

Levofloxacin hemihydrates are 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 pH triggered in-situ ophthalmic gel system for Levofloxacin hemihydrates by using Carbopol 934p as gelling agents.

Carbopol 934p is a synthetic polymer composed of 62% of carboxyl groups with approximate molecular weight (3×10^6). 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. 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.

EXPERIMENTAL

Materials and methods:

Levofloxacin hemihydrates were kindly supplied as a gift sample from Orgochem Ltd, Gujarat. Carbopol 934p was 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 the formulations

Table (1) shows the composition of in situ-gelling systems based on Carbopol 934p as a pH activated polymer. 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 carbopol solution under constant stirring to obtain a uniform solution. Distilled water was then added to make the volume up to 100 ml.

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 membraneusing a modified Franz diffusion cell. The dissolution medium used was artificial tear fluid freshly prepared (pH 7.4). Cellophanemembrane, previously soaked overnight in the dissolution medium, was tied to one end of a specificallydesigned 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 thereceptor medium surface. The shaft was rotated at 100 rpm. Aliquots, each of 1-ml volume, were withdrawnat hourly intervals and replaced by an equal volumeof the

Receptor medium. The aliquots were dilutedwith the receptor medium and analyzed by UVspectrophotometer at 288 nm.

In vivo drug release studies

In-vivo release studies of Levofloxacin hemihydrates 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 humor was sampled from 6 eyes for each formulation using a 28-gauge needle. Aqueous humor 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 hemihydrates content by HPLC.

HPLC analysis:

Quantitative estimation of Levofloxacinwas done by HPLC. A filtered and degassed mixtureof phosphate buffer pH 3.0 and acetonitrile (80:20) wasused as the mobile phase. The mobilephase was delivered at a flow rate of 1.0 ml/min, theinjection volume was 20 µl and the effluent was monitoredat 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 incubatingformulations 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 soya bean-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 hemihydrates 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 twohours, 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 bydiscoloration 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.

Selection of excipients:

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 various pH based formulations are shown in Table (1). The drug content was found to be satisfactory.

Table (1): 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 hemihydrates.The drug contentfor pH triggered in-situ gels drug content is within 98.76 to 101.65% as shown in Table (1).

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.FormulationsC1, C2, C6 and C9 showed better gelling capacity as shown in Table (1)

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 (2).

Table (2): 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°C and 37°C					
	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:

Figure. 1 show the percentage of Levofloxacin hemihydrates released as a function of time for C1-C9 respectively. As per figures C9 showed better release of 90.21 % 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 hemihydrates 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 C9 with marketed eye drop is shown in Figure.2.

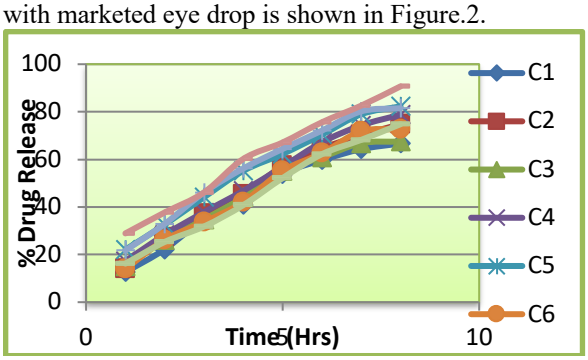


Figure 1: In-vitro release kinetics of C1-C9 batches

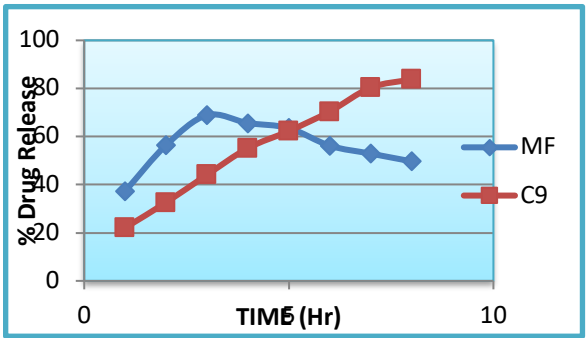


Figure 2: Comparison of In-vitro release kinetics of C9 with marketed formulation (MF)

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. 3.

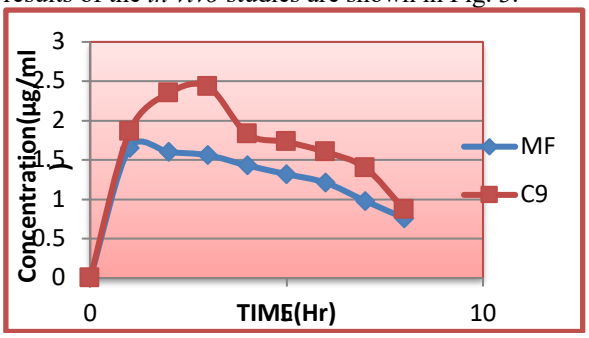


Figure 3:Tear fluid concentration profile of marketed eye drop & pH activated in-situ gel (C9)

Table (3): Pharmacokinetic parameters of Levofloxacin in aqueous humor after instillation C9

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

The level of Levofloxacin in aqueous humor after topical instillation of C9 was shown in Fig.3.The aqueous humor content of Levofloxacin was significantly higher at all time points 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 **C9** was fairly high for up to 8 hr in contrast to its other formulations.

Sterility test

The optimized formulation 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 at 30-35 ^{0}C in case of fluid thioglycolate medium and at 20-25^{0}C in the case of soya bean 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 4. In infectedcondition 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 5.This indicates that Levofloxacin shows antimicrobial activity even in acidic condition of eye (Infectious condition of eye) .

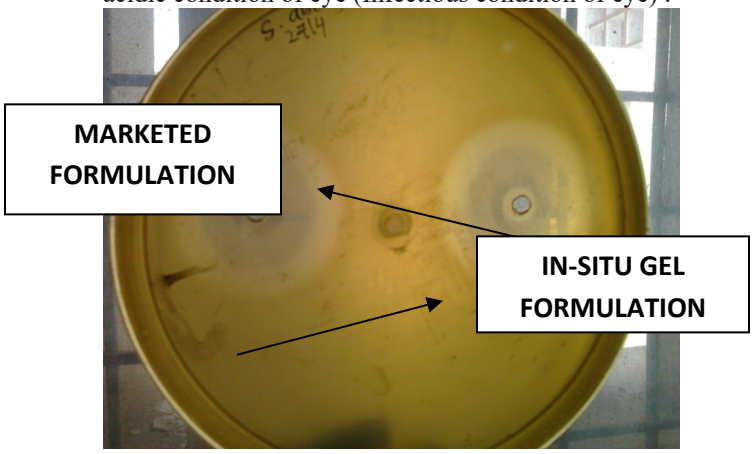
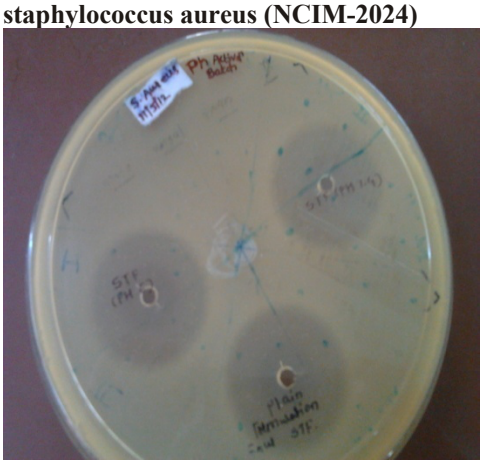


Figure 4: Zone of inhibition shown by marketed formulation with In-situ gelling system against staphylococcus aureus (NCIM-2024)



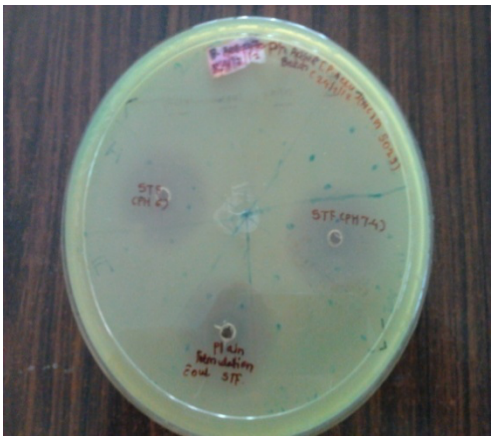
(a) Staphylococcusaureus
(b) Pseudomonas aeruginosa

Figure 5: Zone of inhibition shown by C9 against (a) Staphylococcus aureus (NCIM-2024) (b) Pseudomonas aeruginosa(NCIM-5029)

CONCLUSION:

Many advantages from using this method which is stimuli responsive polymers as a modified release pharmaceutical dosage forms, including the systemic side effects that caused from the conventional ophthalmic formulation which is drained out from the eye and then goes to systemic circulation through esophageal route can be avoid using stimuli-responsive polymers because this method will release and distribute the drug in a specific target, and also pH sensitive gels that has excellent job in transforming from sol-to-gel form instantly when coming in contact to ocular fluid and exhibiting better therapeutic level, thus reducing the side effects or adverse systemic reactions. Furthermore, patient compliance and reduction in therapeutic doses have also been shown by this selectivity type of pharmaceutical drug delivery systems. There are several types of sensitive-polymers; however, pH sensitive polymers are those that have provided promising results.

pH sensitive polymer is a smart polymer that contains acidic or alkaline functional groups in its structure and can be triggered to undergo sol-to-gel transformation by a change in the pH of the surrounding area. Many problems that occur because of the formulation using conventional drug delivery systems can be solved by using in situ gelling system as new ocular drug delivery systems, including low response in therapeutic effects, poor bioavailability because of the rapid elimination of the drug from the eyes that happen because of high tear fluids turn over the dynamics, that leads to systemic side effects or adverse effects and also poor patient adherence to the therapy given. This newer technology in ophthalmic drug delivery systems is directed towards an amalgamation process, which includes build-up systems which can not only extend the contact time of the vehicle at the ocular surface but also slow down the removal of the drug from the eyes.



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