PREPARATION AND CHARACTERIZATION OF pH BASED CARBOPOL 934P IN-SITU HYDROGELS FOR THE TREATMENT OF HARMFUL BACTERIAL INFECTIONS

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Carbopol 934P, HPMC, Topical route, HET-CAM study, Precorneal

ABSTRACT: The purpose of the present study describes the preparation of pH based in-situ gel formulations were applied for the harmful bacterial infections by using carbopol 934P and HPMC derivatives. The simple procedure was adopted for preparing these formulations. To eliminate or reduced adverse systemic effects produce from bacterial conjunctivitis, topical route of administration, such as eye drops was preferred for this type of infections and it should be installed into the eye’s cornea. These eye drops are more effective to provide therapeutic index and also to achieve good bioavailability in the eye. Forthcoming, the developed in-situ gel formulations were characterized by gelling capacity, pH, clarity, appearance, viscosity determination, surface tension, drug content, in-vitro drug release, HET-CAM study, permeation study, and in-vivo ocular irritation study. The optimized formulation (F2) was set up to be non-irritant and well tolerable from the results both in-vitro HET-CAM study and ocular irritation study. The developments in-situ gel formulation forms good gelling capacity, clear/translucent in appearance, after administration in the eye. The developed in-situ gel formulations control the precorneal drainage, increased resistance time on the eye, improved patient compliance by increasing the bioavailability of drug concentrations to the eye.

INTRODUCTION: Globally, the human eyes are regarded by some bacteria and fungi which cause dangerous infections such as endophthalmitis, fungal endophthalmitis, and orbital cellulitis. Among the different infections, fungal endophthalmitis is the dangerous infection, which is either exogenous or endogenous 1, 2, 3, 4, 5, 6. The onset of activity of fungal infection is gradually delayed than bacterial infections. The fungi species such as Fusarium is mainly involved in causing different infections such as keratitis, scleritis, and intraocular infections. Aspergillus keratitis is a type of fungi that cause industrial trauma or surgery. Bipolaris, Curvularia, Phialophora, and Lecythophora are mainly concerned in keratitis and intraocular infections. Purpureocillium lilacinum is mainly responsible for the infection in contact lens and intraocular lens implants. The fungal species such as Candida species is mainly involved in ocular infections 7, 8, 9, 10. The severe infection of the cornea is mainly due to microbial keratitis. The acute and chronic effects of keratitis are chronic superficial eye disease, trauma, use of contact lenses and immune deficiency.
In the USA, it is reported that the patients having microbial keratitis infections owing to the usage of the soft contact lens are in the range of 40 to 210 per 100,000 per year. In most countries, the species like Fusarium keratitis is mainly responsible for causing the infection due to contamination in the contact lens solution. About 80% of healthy people have an eye infection due to Acanthamoeba keratitis. Panophthalmitis is a type of eye infection occurs, especially in ocular tissue layers including episclera. One of the specific characteristics of panophthalmitis is the severe pain during eye movement. Uveitis is one of the eye infections and is found in 11.1% of instances which is caused primarily due to different clinical conditions caused by various usual fungi, bacteria, viruses, and fastidious bacteria. The bacterium, Bartonella henselae causes uveitis in 6.1% cases during the year 2001-2007.

The pharmaceutical scientist, facing more struggles in the area of ophthalmic drug delivery system to treat a disease in constant and conventional attacks. In human, an eye plays a vital role to see colorful things, and it is highly resistant to unknown (foreign) substances. The drug concentration at a specific site of action and their ensuing pharmacological effect are correlated. The therapeutically effective concentration is administered through topical application and is successful in certain infections such as keratitis and bacterial conjunctivitis. During the usage of eye drops, bacteria and their antigens are washed away, and the adverse effects of drugs are either decreased or eliminated. The effective therapeutic drug concentration is achieved through frequent drug administration, varying drug concentration, the duration of the drug in the cornea, the lack of an intact corneal epithelium and the study of the lipophilic nature of the drug where the epithelium is intact.

During the topical administration of ocular drugs, merely 5% of the total drug is penetrating the cornea and contact the internal anterior eyes tissue. The efficient and prompt drainage by the relative impermeability of cornea to hydrophobic and hydrophilic molecules, non-corneal absorption, nasolacrimal apparatus are resulting in deprived ocular bioavailability. During drug administration, the eye barrier must be protected without any tissue damage. One of the specific ways to improve drug bioavailability is to increase drug residence time inside the eye. An ample number of in-situ gelling systems has been developed to lengthen the pre-corneal drug residence time, better patient compliance and therefore improve ocular bioavailability.

Most of the available in-situ gelling systems are mainly as formulations in the form of eye solution, which could be dropped in the eye that further undergo transition into a gel. The transition of the sol-to-gel phase is mainly due to a change in pH, ions, and temperature in the cul-de-sac and hence lengthen the precorneal residence time. These types of in-situ gelling systems have been widely used to consider the precorneal drainage of artificial tear products, thermosetting gels, liposomal formulation of tropicamide, ophthalmic ointments, w/o microemulsions, an ion and pH-activated in-situ gelling system based on gellan gum and chitosan and alginate and HPMC based an ion activated in-situ gelling system. Past 2 decades an ample number of literature has reported on the development of in-situ gel preparation in an aspect of the conversion of sol to stiff gel when it contacts with the eye. Due to this, the vision is blurred, and it could get normal after extended minutes. Whereas in this study, the prepared in-situ formulation is specifically in the form of intermediate gel when it contacts.

This is specifically prepared in such a way to avoid the blurred vision. The current study is mainly on the development of pH-based in-situ gelling systems of ofloxacin to treat severe bacterial eye infections. The formulation is prepared and optimized using various tests such as ex-vivo permeation study, HET-CAM membrane test, and in-vivo corneal irritation study. Carbopol 934P and HPMC derivatives were used to develop the pH-based in-situ gelling system. Among fluoroquinolones, ofloxacin is preferred due to its pH 6 which is quietly bearable by eyes, maximum inherent solubility and utmost diffusion rate into ocular tissues. This drug is specifically considered due to their elevated diffusion rate into the tissues.
Fourier Transforms Infra-Red Spectrum (FTIR) Studies: To characterize the functional groups, FT-IR spectra of pure drug, polymers and prepared formulation were compared to each other. The samples were measured from 4000 to 400 cm⁻¹ using Perkin Elmer FTIR spectrophotometer.

Differential Scanning Calorimeter (DSC): DSC analysis was performed for the prepared formulation using thermal analysis system (DSC-60, Shimadzu). About 5 mg of the sample was heated at 10 °C/min at a constant rate in an aluminium pan under a nitrogen atmosphere.

Characterization of Prepared in-situ Gel: The presence of any particulate matter was observed by visual appearance. The determination of formulation clarity before and after gelling formations was done through visual examination under light against white or dark backgrounds. pH of the prepared formulation was measured using pH meter. In-vitro gelling capacity was ascertained using the visual method. The colored formulation of different concentrations in the range of 20µl - 50µl was added dropwise with the use of a pipette. The stiff gel like structure had been formed during the reactivity between the colored formulation and STF fluid, pH 7.4. The gelling capacity of the prepared formulation was assessed based on the stiffness of a stiff formed gel.

Drug content of the prepared formulation was done by diluting about 1 ml of prepared formulation in 0.1N HCl. Further, the sample was subjected to UV-Visible spectrophotometry at 294 nm. The prepared in-situ gel formulations were taken in a sampling tube, and their viscosities were measured at 25 °C using a Brookfield Viscometer DV2T model. The samples were measured at a rate of 15 rpm for 5 min. The surface tension of prepared formulations was analyzed using a drop weight method. The surface tension was calculated based on Eq. (1).

\[ \gamma_L = \frac{W_L}{W_w} \times \gamma_w \]  

Where, \( \gamma_L \) - surface tension of liquid sample, \( W_L \)-Weight of 1 drop of liquid sample, \( W_w \)-Weight of 1 drop of water, \( \gamma_w \) - surface tension of water.

In-vitro release study of the prepared formulations was done using dialysis bag membrane method.
About 3 ml of sample was withdrawn on 1 h interval for about 8 h and substituted with fresh STF fluid. Further, the samples withdrawn for every hour were subjected to UV visible spectrophotometry analysis to measure the cumulative % drug release at 294 nm.

Bio-adhesion Measurement: The method was based on the measurements of the shear stress or tensile strength necessary to break the adhesive bond between test formulation and the model membrane. The prepared formulation was squashed in between two model membranes and set on the flexible support in the assemblies for 10 sec after the formation of the adhesive bond, the force required to separate the formed bond was measured and calculated as a bio-adhesive force.

Evaluation of in-vitro Release Kinetics: To evaluate in-vitro release kinetics, the data obtained were fitted using various kinetic models such as Zero-order reaction, first-order reaction, Higuchi's Kinetics model, Korsmeyer Peppas reaction and Hixson Crowell erosion equation methods.

In-vitro Permeation Study: The freshly obtained goat cornea was obtained from the slaughtered house and mounted on the modified Franz diffusion cell (FDC) apparatus. In the receptor chamber, STF fluid was maintained at 37 ± 1 ºC and on continuous stirring. About 2 ml of test samples were taken at regular time intervals and replaced with fresh STF fluid. Each prepared formulation was allowed to permeate nearly for 0.5-8 h. Further, the samples were subjected to UV-Visible spectrophotometer at 294 nm to analyze the concentration of ofloxacin. The permeated drug concentration at different time intervals was resolutely using the standard curve.

\[
\% \text{ Permeation} = \frac{\text{Amount of drug permeated in receptor}}{\text{Initial amount of drug in donor}} \times 100
\]

Apparent Permeability Coefficient: The freshly obtained goat cornea was employed to assess apparent permeability coefficient using Eq. (2).

\[
P_{\text{app}} = \frac{\Delta Q}{\Delta t \times A} \quad \text{(2)}
\]

Where, \(\Delta Q / \Delta t\) - is the flux across corneal tissue \(\mu g/\mu L\), \(A\) - is the diffusion area (cm²); \(CO\) - is the initial concentration of drug in donor compartment \(\mu g/cm^3\) and 60 is taken as a factor to convert min into sec. The graph was plotted between the amount of permeated liquid and time.

In-vitro HET CAM Test: HET-CAM test was done to assay severe ocular irritants and corrosives potential. The test substance was administered on embryonated hen’s egg membrane, and their immediate effects were studied. The white Leghorn hen’s fertile eggs were stored at a temperature and relative humidity of 37 ± 0.2 ºC and 58 ± 2% respectively for 8 days. The test substances and control were dissolved in 2.5% (w/v) agarose to achieve the final concentration of 25 µg/µL. After incubation, the air cell on the eggs was marked. The marked area on the eggs was sliced off without disturbing the chorioallantoic membrane in eggs. The generated liquids were placed directly on CAM, and the eggs were observed for 300 sec for the indication of any lysis reactions or hemorrhage on CAM. The negative and positive controls used in this study were dissolving 0.9% (w/v) sodium chloride (NaCl) and 1% (w/v) sodium hydroxide (NaOH) in distilled water respectively. Once test substance is applied on CAM, the capillaries and chorioallantoic blood vessels were observed for any irritants/corrosive effects such as hemorrhage, hyperemia, and clotting. Each prepared formulation was tested for any irritant effects at different time intervals.

Acute Eye Irritation / Corrosion Study: The optimized formulation was evaluated in-vivo in an animal model, Male New Zealand white rabbits. The protocol used in this study was according to Draize technique and also approved by the Animal Ethical Committee (IAEC/51/SRU/550/2017). The rabbits were housed in cages at 27 ± 0.5 ºC and fed with standard diet and water. The left and right eyes of white rabbits were marked as control and test respectively. The control eye has no sample mentioned as blank whereas the test eye was injected with 0.1 ml of formulation. Further, the eyes were observed at different time intervals such as 1, 24, 48, 72 h and 1 week after having an exposure with the optimized formulation. The ocular changes were graded using a scoring system which incorporates score rating and any alteration on conjunctiva, eyelids, cornea, and iris. Besides, the eyes of white rabbits were also observed regularly for redness, swelling, and watering of the eye.

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Stability Studies: To study the stability of different prepared formulations, the guidelines offered by International Conference on Harmonization guidelines were adopted for this study. The stored in-situ gel was maintained at a temperature, relative humidity and refrigerator condition of 25 ± 2 °C, 60 ± 5% and 5 ± 2 °C respectively 39, 40. The stored vials were analyzed for every 30 days to study their gelling effect, appearance, drug content and pH.

Antibacterial Activity: Activity was determined by agar diffusion test employing a cup plate technique. The drug was allowed to diffuse through a solid agar medium. The standard minimum inhibitory concentration (MIC 2 μg/mL) of control and developed formulations containing in-situ gel formed was prepared. A total of 100 ml of nutrient agar media was prepared and sterilized at 15 lb/sq-inch pressure for 20 min in an autoclave; 0.5 ml of microorganism suspension was poured into the above medium which is maintained at a temperature of 50 °C to 58 °C. This will be done in an aseptic condition. Immediately 20 ml of the microbial agar suspension was poured into each petri plate. After solidification of the media, sterile solutions of ofloxacin (standard solutions) and the developed formulations diluted suitably with sterile distilled water (test solutions) were poured into the cup of sterile nutrient agar Petri plates.

**TABLE 1: OPTIMIZATION OF FORMULATIONS (F1-F5)**

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Ofloxacin conc. (g)</th>
<th>Carbopol 934P (g)</th>
<th>HPMC (g)</th>
<th>pH</th>
<th>Clarity</th>
<th>Gelling time</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>0.3</td>
<td>0.5</td>
<td>0.1</td>
<td>6.18</td>
<td>C</td>
<td>++</td>
</tr>
<tr>
<td>F2</td>
<td>0.3</td>
<td>0.5</td>
<td>0.1</td>
<td>6.3</td>
<td>C</td>
<td>+++</td>
</tr>
<tr>
<td>F3</td>
<td>0.3</td>
<td>0.5</td>
<td>0.1</td>
<td>6.27</td>
<td>C</td>
<td>+++</td>
</tr>
<tr>
<td>F4</td>
<td>0.3</td>
<td>0.5</td>
<td>0.4</td>
<td>6.28</td>
<td>C</td>
<td>+++</td>
</tr>
<tr>
<td>F5</td>
<td>0.3</td>
<td>0.5</td>
<td>0.5</td>
<td>6.24</td>
<td>C</td>
<td>+++</td>
</tr>
</tbody>
</table>

XRD Spectrum Analysis: XRD spectral peak positions of the drug, excipient and the mixture of excipients were obtained. XRD spectrum of ofloxacin indicated peak position (2θ) at 20.77º, 22.10º, 24.05º, 26.24º, 26.97º, 27.68º and 29.75º. XRD spectrum of carbopol 934P showed peak position (2θ) at 27. 21º, 36.21º, 53.54º and 70.93º. XRD spectrum of the physical mixture of the drug and polymer showed only two sharp peaks at 20.53º and 27.16º.

The remaining peak positions in the physical mixture were not sharp which is mainly due to the peak positions present in drug and polymer were merged and is indicated in Fig. 1. XRD peak signals indicated that the prepared formulation was in amorphous state than the pure drug.

FT-IR Spectra: The presence of functional groups was confirmed by using FT IR (Instrument-JASCO 4100). The readings were obtained between 400 cm⁻¹ to 4000 cm⁻¹ using a KBr pellet technique. FT-IR study was carried out for drug and various excipients. The results showed that various stretching, bending and rocking vibration based on the groups present.

Sterility Test: The aerobic, anaerobic bacteria and fungi of the optimized best formulations underwent sterility test using thioglycolate and soya bean casein that was made by dissolving 500 mg of peptic digest of animal tissue. To obtain 100 ml so, as to adjust the pH 7.1 ± 0.2. Then was a drained, centrifuged, and disseminated into flask 7 of 10 ml quantities and was allowed to sterilize at 121 °C for 20 min. The positive and negative control tests for growth promotion and sterility were performed respectively. The microorganisms like Bacillus subtilis (aerobic), Bacteroides vulgaris (anaerobic) and Candida Albicans (fungi) respectively were used as test organisms. The incubation was done, and growth was observed 41.

RESULTS: Characterization of the Optimized Formulation: The developed in-situ gel formulations were optimized and adopted in the simple procedure and composition was given in Table 1.
TABLE 2: INTERPRETATION OF FT-IR SPECTRUM OF DRUG, POLYMER AND PHYSICAL MIXTURES

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Fig. 3</th>
<th>Wave number (cm⁻¹)</th>
<th>Vibrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>(a)</td>
<td>3042, 2787, 1709, 1620, 1520, 1454, 1287, 198, 1137, 1048, 1099, 954, 876, 798 and 704</td>
<td>N-H, C=H, C=O and C=N are stretching, N-H, C-H, C-N, C-H &amp; C-C bending and N-H rocking</td>
</tr>
<tr>
<td>2.</td>
<td>(b)</td>
<td>2936, 2361, 1703, 1451, 1415, 801 and 618</td>
<td>C-H, C-H, C=O and C-N are stretching, C-C, C-H, O-H are bending, and C-H rocking</td>
</tr>
<tr>
<td>3.</td>
<td>(c)</td>
<td>3407, 2927, 2386, 1710, 1619, 1521, 1399, 1050, 949 and 774</td>
<td>O-H, C-H, C=O-H, C=O, C=O-C and C=O are stretching, N-H is bending, and C-H rocking</td>
</tr>
<tr>
<td>4.</td>
<td>(d)</td>
<td>3434, 2925, 2362, 1709, 1619, 1242, 1197, 1050, 961 and 708</td>
<td>O-H, C=O-H, C=O, C=O-C and C=O are stretching, N-H and O-H are bending and N-H rocking</td>
</tr>
<tr>
<td>5.</td>
<td>(e)</td>
<td>3429, 2914, 1709, 1620, 1401, 1197, 105, 949 and 707</td>
<td>O-H, C=O-H, C=O-C, C=O, C=O, C=O are stretching, N-H and O-H are bending, N-H and C-H are rocking</td>
</tr>
</tbody>
</table>

**Fig. 3** showed FT-IR spectra of pure drug, polymer and their physical mixture. The interpretations of FT-IR spectra for the respective compounds were given in Table 2.

**DSC Studies:** The physical state of the drug in the formulation was examined through differential scanning calorimetry (DSC).

The endothermic melting transition of pure ofloxacin was observed at 275 °C and 278 °C which was very close to its reported melting point. In the thermogram of formulation, the melting endothermic peak appeared near the melting temperature of ofloxacin at 275 °C and it’s slightly weak which was shown in Fig. 4. Hence, the thermogram studies revealed the physical nature of drug was found to be stable. DSC studies of the pure drug, polymer and the mixture of formulations showed that they form thermally good stability Fig. 4.

**Gelling Capacity:** The pH, visual appearance, gelation studies of the formulations F1-F5 were
shown in Fig. 5. All the developed formulations were found to be slightly pale white dispersion in the pH range of 6.20 to 6.30, and after adding STF fluid pH 7.4 immediate stiff gelation were found for formulations, F1-F5 were represented in Fig. 5a, b, c, and d.

Fig. 5 showed the optimized formulations F1-F5. After the addition of 20-50 µl formulation to STF fluid, the transition of sol to gel formation occurs. The formed gel remained nearly for 8-10 h. The indications of gelling time are as follows. (a) (+) Gelation occurred within few minutes, and the formed gel-like structure dissolved rapidly, (b) (++) Gelation remains for 6-8 h and (c) (+++) Gelation will be retained for more than 8-10 h. Based on the gelation time, the gelling capacities of the prepared formulations were analyzed and used for further analysis. The combination of ofloxacin, carbopol 934P, and both high and low viscous HPMC have maximum gelling capacity than other formulations.

**Surface Tension:** The surface tension of the prepared formulations F1-F5 was analyzed through drop weight method of analysis using Stalagrometer. The surface tension showed trivial differences between different prepared formulations.

The results revealed that the surface tension of the prepared formulations F1-F5 was in the range of 36.5-54.12 dynes/cm. Among all the formulations, formulation F5 showed the maximum surface tension of 54.12 dynes/cm. Next, to F5, F1 showed the highest value of 44.05 dynes/cm Fig. 6.

**Evaluation of Mucoadhesive Strength:** During in-situ preparation, long retention and bio-adhesion are important characteristics to evaluate mucoadhesive strength. Both carbopol and HPMC function as a permeation enhancer and mucoadhesive agent and the study of adhesion force proves the mucoadhesive nature of carbopol and HPMC. Due to abundant hydrogen bond forming groups in carbopol, it is rich in hydrogen property and thus has specific bio-adhesive property. When carbopol is mixed with widely used
bio-adhesive material like HPMC, the synergic effect of bio-adhesive property is more predictable. To improve the physical property of the formulation such as mucoadhesive strength, the mucoadhesive polymer is added and is vital for the formulation to remain at the site of action for the effective amount of time. The results were found to be around (F1 - 3.421 ± 104 dynes/cm², F2 - 12.157 ± 127 dynes/cm², F3 - 13.019 ± 075 dynes/cm², F4 - 16.947 ± 023 dynes/cm², F5 - 19.142 ± 035 dynes/cm²)

**Drug Content:** The drug content present in the prepared formulations F1-F5 was analyzed using UV visible spectrophotometer at 294 nm. The drug content was in the range of 96.87% to 98.67% for all the developed formulations F1-F5 Fig. 7.

**Viscosity:** The viscosities of all the prepared formulations F1-F5 were measured using Brookfield Viscometer DV2T model and are reported in Fig. 8. The results showed that when there are increases in the concentration of polymer, viscosities of the respective formulations were found to be increased. Viscosities of the prepared formulations F1-F5 were in the range of 94.6-102.12cPs. Among all the formulations, formulation F5 showed the maximum viscosity of 102.12cPs Fig. 8.

**In-vitro Release Study:** The result of in-vitro drug release of in-situ gel formation of all the formulations was shown in Fig. 9.

**In-vitro Transcorneal Approach - Permeation Experiment:** Ex-vivo study was done for the optimized formulation, F2 by plotting cumulative percent drug release concerning time and is shown in Fig. 10.
The formulation F2 showed the maximum percent drug release of 95.01% at the end of 7 h Fig. 10. The permeability coefficient of the developed formulation F2 was found to be Kp = 0.0065 cm/h.

**Hen’s Egg Test on Chorioallantoic Membrane (HET-CAM):** In substituting to animal experimentation, HET-CAM test is an alternative method to assay corrosives/severe ocular irritants on CAM of the fertilized eggs. This test is helpful to analyze any damages caused by test substance on CAM.

HET-CAM (Hen’s Egg Test - Chorioallantoic Membrane) study was carried out for F2 with suitable positive and negative controls and is shown in Fig. 11. The result showed that there is no significant damage to CAM membrane during the application of formulation F2 on CAM and no irritations were observed in the conjunctiva Fig.11 and in Table 3.

![Fig. 11: (a) Fertilized eggs, (b) HET-CAM study using the optimized formulation F2, (c) negative control using sodium chloride, (d) positive control using sodium hydroxide and (e) the CAM membrane shows that they have no damage or redness](image)

**TABLE 3: SCORE OBTAINED IN HET – CAM TEST**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Time (in Min)</th>
<th>Sodium hydroxide and normal saline as control (positive and negative)</th>
<th>Developed in-situ gel formulations (F2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>Egg 1</td>
<td>0</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>Egg 2</td>
<td>0</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>Egg 3</td>
<td>0</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>Average mean (n=3)</td>
<td>0</td>
<td>0.5</td>
<td>0</td>
</tr>
</tbody>
</table>

Score test – 0-3 is normal, 3-5 is a mild irritant, 5-9 is a moderate irritant, and 9-11 is a severe irritant.

**Acute Eye Irritation / Corrosion Study:** This study was carried out to observe any irritations in the conjunctiva of the eyes and the score was tabulated in Table 2. While applying the test substance on the eyes of white rabbits, the eyes were observed regularly for redness, swelling and watering of the eye were examined by the use of light source from a standard ophthalmoscope. From Table 4, it is inferred that there is no ocular irritation on white rabbit eyes and hence it is confirmed that the optimized formulation, F2 was found to be non-irritant.

According to Draize score test, the results of the ocular irritation studies indicated that the formulation has no average score, Zero. The excellent ocular tolerance was noted. There was no ocular damage or abnormal clinical signs in cornea, iris, conjunctiva, and chemosis. During initial and confirmatory tests, the animals showed no microscopic lesions in both eyes Table 4.

**TABLE 4: MEAN SCORE FOR EYE IRRITATION**

<table>
<thead>
<tr>
<th>A. no</th>
<th>Initial and Confirmatory Tests</th>
<th>Mean Score for 24, 48 and 72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ocular Region</td>
<td>Control (Left eye)</td>
</tr>
<tr>
<td></td>
<td>Cornea</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Iris</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Conjunctivae</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Chemosis</td>
<td>0</td>
</tr>
</tbody>
</table>

**Ocular Region**

- Cornea
- Iris
- Conjunctivae
- Chemosis

**Mean Score for 24, 48 and 72 h**

- A. no. – Animal Number, Cornea (0 – no ulceration or opacity), Iris (0 - normal), Conjunctivae (0 - normal), Chemosis (0 - normal)
Based on the above observations, the prepared in-situ gel formulation was found to be non-irritant to the rabbit white eyes.

**Antibacterial Activity:** Antimicrobial efficacy study was performed on F2 formulation using gram +ve *Bacillus subtilis* and gram -ve *E. coli* organism. The zone of inhibition of F2 ophthalmic formulation found to be 30.33 and 29.33 mm, respectively, for gram +ve *S. aureus* and gram –ve *E. coli* organism. The results of antimicrobial activity are as shown in Fig. 12 and Table 5. The study indicated ofloxacin retained its antimicrobial activity when formulated as gel-forming confirming that they are sterile and free from micro-organisms as shown in Table 6.

**Sterility Test:** To obtain or for best formulation seven days of sterility test was done which brought out that the negative group showed no signs of precipitation which in turn indicated that there were no microorganisms.

**TABLE 5: ANTI-BACTERIAL ACTIVITY OF FORMULATION F2**

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Microorganisms</th>
<th>1000 µg</th>
<th>500 µg</th>
<th>250 µg</th>
<th>Distilled water</th>
<th>Ofloxacin 10 µg</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td><em>Escherichia coli</em></td>
<td>31</td>
<td>30</td>
<td>27</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td><em>Bacillus subtilis</em></td>
<td>30</td>
<td>30</td>
<td>31</td>
<td>-</td>
<td>20</td>
</tr>
</tbody>
</table>

**TABLE 6: STERILITY TEST FOR IN-SITU GEL FORMULATION**

<table>
<thead>
<tr>
<th>Sterility test</th>
<th>Results obtained</th>
<th>Negative control</th>
<th>Test sample</th>
<th>Positive control</th>
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<tbody>
<tr>
<td>Test for Aerobic bacteria (<em>Bacillus subtilis</em>)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Test for Anaerobic bacteria (<em>Bacteroides vulgaris</em>)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Test for Fungi (<em>Candida albicans</em>)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

(-) The absence of microorganisms  (+) Presence of microorganisms

In the positive control, the microorganisms were introduced in the growth medium showing a milk-white precipitate which was significant. The tubes with F2 formulation in-situ gel was clear

**TABLE 7: STABILITY STUDIES OF THE FORMULATIONS F1-F5 AS PER ICH GUIDELINES FOR 0-6 MONTHS**

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Drug content (%) at 0 month (n=3) ± S.D</th>
<th>Drug content (%) at 3 month (n=3) ± S.D</th>
<th>Drug content (%) at 6 month (n=3) ± S.D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25ºC</td>
<td>25ºC</td>
<td>40ºC ± 75% RH</td>
</tr>
<tr>
<td>F1</td>
<td>96.87 ± 0.25</td>
<td>96.51 ± 0.17</td>
<td>95.88 ± 0.18</td>
</tr>
<tr>
<td>F2</td>
<td>98.67 ± 0.11</td>
<td>97.59 ± 0.15</td>
<td>96.65 ± 0.43</td>
</tr>
<tr>
<td>F3</td>
<td>97.28 ± 0.09</td>
<td>97.09 ± 0.28</td>
<td>96.24 ± 0.16</td>
</tr>
<tr>
<td>F4</td>
<td>97.54 ± 0.41</td>
<td>97.23 ± 0.21</td>
<td>96.15 ± 0.08</td>
</tr>
<tr>
<td>F5</td>
<td>96.49 ± 0.26</td>
<td>96.15 ± 0.30</td>
<td>95.79 ± 0.29</td>
</tr>
</tbody>
</table>

Further, the formulation kept for stability studies were undergone the parameters of the test like the appearance, the pH, gelling capacity and drug content were shown good results after 6 months as shown in Table 8.
DISCUSSION: The optimization of formulation indicated that the combination of carbopol 934P, HPMC (both low and high viscosity) showed the maximum period of gelling capacity when compared to other formulations as shown in Table 1. XRD signals indicated that the formulation was amorphous in condition when compared with the pure individual drug as shown in Table 2.

DSC studies of the pure drug, polymer (carbopol 934P) and the mixture of formulations showed that they form thermally good stability. Fig. 4 the gelling capacity of the formulations were carried out and shown in Table 3. The surface tension of the formulations F1-F5 was calculated by the drop weight method by using Stalagmometer. The results revealed that the surface tension of the prepared formulations F1-F5 was found to be in the range of 36.5 - 57.14 dynes/cm as shown in Fig. 5.

Drug content of the formulations F1-F5 was calculated and analyzed by using UV visible spectrophotometer at 294 nm. The drug content was found to be in the range of 96.87% to 98.67% for all developed formulations F1-F5 as mentioned in Fig. 6. Brookfield viscometer DV2T model analyzed the viscosity of all formulations (F1-F5) by using S18 spindle at 15 rpm for the duration of 5 min. Results depicted that as the concentration increased the viscosities of the formulations were also found to be increased. The viscosities of all formulations F1-F5 were 94.6-102.12 cPs plotted in Fig. 8.

In-vitro release studies of the developed formulations F1-F5 showed the % drug release of F1- 89.38%, F2-95.28%, F3-89.85%, F4-90.03% and F5-94.32% as in increased order as shown in Table 4. The pharmacokinetic studies revealed that the optimized formulation F2 obeyed the Higuchi kinetic model (R² value = 0.9988) and followed the diffusion mechanism. Ex-vivo studies were performed for the optimized formulations (F2) which showed the maximum percent of drug release 95.01% at the end of 7 h as shown in Table 5. HET-CAM studies were performed for the optimized formulations F2, and it doesn’t show any significance redness or damage to this CAM membrane, and also no irritations were observed as shown in Table 6 and scoring test as mentioned in Table 3.

The ocular irritation studies revealed that no irritation in the rabbit’s eye. Hence the developed formulation in-situ gel was found to be non-irritant. The results of the ocular irritation studies indicated that the formulation has no average score, i.e. (Zero) according to the Draize score test. Excellent ocular tolerance was noted; no ocular damage or abnormal clinical signs in the cornea, iris, conjunctiva, and chemosis were visible. In initial and confirmatory test animals showed no microscopic lesions were observed in both eyes from control (left) and treated (right) as shown in Table 7. Based on the above observations under tested conclusion, the in-situ gel formulation was found to be non-irritant to the eye.

The antibacterial activity shows the zone of inhibition for the formulation F2 good inhibition activity as shown in Table 8 and Table 9. The tubes with F2 formulation in-situ gel was clear confirming that they are sterile and free from micro-organisms as shown in Table 10.

Stability studies were carried out for the developed formulations (F1-F5) as per the ICH guidelines for 0-6 months. The drug content of the formulations F1-F5 was calculated by UV-visible spectrophotometer at 294 nm for 0 months, 3rd month and 6th month at room temperature (25°C) and 40 ºC ± 75% relative humidity as shown in Table 7 and 8.

| TABLE 8: AFTER STABILITY DATA STUDIES TEST PARAMETERS WERE CARRIED OUT |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| **Months**      | **Formulation F2 at 25 ± 2°C/5% RH** | **Formulation F2 at 40°C ± 2°C/75% RH** |
| 0               | Translucent    | Translucent    | Translucent    | Translucent    |
| 3               | 6.05           | 6.01           | 6.04           | 6.09           |
| 6               | 96.65 ± 0.43   | Translucent    | 97.57 ± 0.21   | Translucent    |
|                 |                |                | 96.89 ± 0.19   | 95.51 ± 0.27   |
| **Gelling studies** | +++            | +++            | +++            | +++            |

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The formulations were found to be stable in both the temperature (25°C ± 2°C and 40°C ± 75% Rh) and were above T90% as per the ICH guidelines.

CONCLUSION: From the above study work, it was accomplished that the developed in-situ gel formulations system is a feasible choice when compared to conventional drops by its ability to enhance bioavailability through its long precorneal residence time, ability to sustain drug release.

Also, it is important in case of administration affords, decreased frequency of administration and resulting in better patient acceptance. Our wish is that this research will improve the quality of life likely, there by developing professional functioning by way of fall in the frequency of administration of dosage form, biodegradable polymers provide prolong action and enhanced bioavailability could be achieved by a single instillation of eye drops. Hence, to conclude it is one of the future novel delivery system in the field of ocular therapeutics for the effective treatment of bacterial infections.

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