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*RESEARCH ARTICLE*

# HET CAM IRRITANCY STUDY FOR DEVELOPMENT OF GATIFLOXACIN IN SITU GEL FORMULATION.

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Gatifloxacin, Gellan gum, Lacrimal fluid, in situ gel, HET CAM, Haemorrhage.

## *Abstract*

**Purpose:** Formulation and evaluation of an non irritant ion activated in situ gel of a flouroquinolone antibiotic, Gatifloxacin.
**Methods:** Ion activated in situ gel formulation was developed using Gellan gum as phase transition polymer and HPMC K 100M as release retardant which can undergo a sol-gel transition in the cul-de-sac of the eye.
**Results:** Gatifloxacin in situ gel formulation with pH 6.0-6.3 and 52 cps in solution form resulted in gel formation in simulated lacrimal fluid having 325 cps viscosity, indicating phase transition behaviour in physiological conditions of eye. It was found to be isotonic, as it exhibited no change in the size and shape of RBCs. Formulation was found to be non irritant to eyes exhibiting mean score of zero in HET-CAM (Hen's Egg Test Chorio Allantoic Membrane) test for ocular irritancy for 5 min
**Conclusion:** It can be concluded that the developed in situ gel formulation can be viewed as a better alternative to the conventional eye drops of Gatifloxacin by virtue of its ability to enhance pre corneal residence time and consequently ocular bioavailability with lesser frequency of administration.



## Introduction:-

Eye is a very sensitive and important organ of the body and is considered as window hinge. There are many eye diseases i.e., conjunctivitis, uveitis, glaucoma etc, that can lead to loss of vision. The bioavailability of ophthalmic drugs is, however, very poor due to efficient protective mechanisms of the eye. Blinking, baseline and reflex lachrymation, and drainage remove rapidly foreign substances, including drugs from the surface of the eye.[1] Topical administration of eye drops in the lower cul-de-sac is the most common method of drug delivery for the treatment of ocular diseases [2]. There are the most commonly available ophthalmic preparations such as drops and ointments about 70% of the eye dosage formulations in market [3]. However, one of the major problems encountered with the eye drops is the rapid and extensive elimination induced by tear turnover, blinking and drainage of formulation which leads to short pre-corneal residence time and poor ocular bioavailability. As a result, frequent instillation of eye drops is needed in order to achieve desired drug concentration and therapeutic effect [4]. An increase in dosing frequency or use of highly concentrated solution to compensate for short ocular residence time is undesirable because of poor patient compliance and risk of toxicity due to ophthalmic absorption via the nasolacrimal duct [5].

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To increase ocular bioavailability and duration of drug action, various ophthalmic vehicles i.e., viscous solutions [6], ointments/gels [7], and polymeric inserts [8], have been used. These ocular drug delivery systems, however, have not been used extensively owing to some drawbacks, such as blurred vision from ointments, lack of patient compliance from inserts, and, sticking of eyelids from gel. As a result, an enhanced ocular bioavailability following topical drug administration remains a challenge yet to be resolved satisfactorily.

An ideal ophthalmic dosage form is one that can sustain the drug release and remain in pre-corneal contact for an extended period of time. A significant increase in the residence time of the formulation and consequently drug bioavailability can be achieved by delivery systems based on the concept of in situ gelation [9]. These delivery systems consist of polymers that exhibit sol to gel phase transition, due to change in specific physiological conditions (pH, temperature and ionic strength) in the eye [10]. Depending upon the method employed to cause phase transition on ocular surface, three types of systems are recognized, i.e., pH triggered systems-Cellulose Acetate Hydrogen Phthalate latex [11] and Carbopol [12-14], temperature dependent systems-Pluronic [15-17] and tetronics [18] and ion activated systems gellan gum [19-20] and sodium alginate [21].

Gatifloxacin is a fourth generation flouroquinolone antibiotic used for the treatment of bacterial conjunctivitis.[22] It is commercially available in the form of an eye drops and ointment. The topical ophthalmic administration of 0.3% Gatifloxacin solution is indicated in case of severe infection.

The main objective of the present work was the development of non irritant in situ gel formulation using an ion-activated phase transition polymer to deliver the drug effectively into the eye for sustained drug release and enhanced ocular drug bioavailability. For irritancy studies alternative method to Draize eye is used i.e., HET-CAM (Hen's Egg Test or Hulner Embryogen) Test.

The HET-CAM assay was evaluated by the expert panel of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods .[23]

**Hen's egg CAM** [22]
10 days after fertilization Hen's eggs are rotated in an incubator for 9 days after which time any defective eggs are discarded. The shell around the air cell is removed and the inner membranes are extracted to reveal the chorio allantoic membrane. Test chemicals are added to the membrane and left in contact for 5 min. The membrane is examined for vascular damage and the time taken for injury to occur is recorded. Irritancy is scored according to the severity and speed at which damage occurs.

**Endpoint and Endpoint Measurement** [23]
**Haemorrhage:**
recording macroscopically the appearance time in seconds COAGULATION: recording macroscopically the appearance time in seconds

**Lysis of blood vessels:**
recording macroscopically the appearance time in seconds

The original HET-CAM test was developed by Luepke and has formed the basis for several modifications to allow testing of materials with different physicochemical properties.[24]

The HET-CAM test is already used within industry for identifying potential nonirritating or mildly irritating materials during in-house screening and safety evaluations of formulations and raw materials. However, the HET-CAM assay has been accepted already by the British, French, Dutch and German authorities for the classification of severe irritants. [25-27]

Within Europe, independent validation studies were carried out by COLIPA [28-30]. The German Ministry for Research and Technology BMFT in conjunction with the German Federal Health Agency BGA [29], the French association for the welfare of laboratory animals OPAL[31], and the inter laboratory study was carried out under the auspices of the Japanese Ministry of Health and Welfare together with the Japanese Cosmetic Industry Association[32] Additionally, the HET-CAM test was included the world-wide EC/HO validation study[33] This

test is used for the detection of ocular corrosives and irritants. The potential ocular irritancy of a test substance is measured by its ability to induce toxicity in the chorioallantoic membrane of a chicken. The effects are measured by the onset of hemorrhage, coagulation, and vessel lysis. These assessments are considered individually and then combined to derive a score; irritation score (IS) which is used to classify the irritancy level of the test substance.
The formula used to generate an IS value is:

$$\left(\left(\frac{(301-Hemorrhage\ time)}{300}\right)\times 5\right)+\left(\left(\frac{(301-Lysis\ time)}{300}\right)\times 7\right)+\left(\left(\frac{(301-Coagulation\ time)}{300}\right)\times 9\right)$$

**Hemorrhage time =**
observations in seconds of hemorrhage reactions on CAM

**Lysis time =**
observations in seconds of vessel lysis on CAM

**Coagulation time =**
observations in seconds of coagulation formation on CAM

A test is considered acceptable if the negative and positive controls each induce a response, which falls within the classification of nonirritating and severely irritating, respectively as shown in table 1.

**Table 1:-**Classification Scheme for Substances as per HET-CAM Score Range Irritation Category

| S.No. | Effect | Score | Inference |
|---|---|---|---|
| 1. | No visible haemorrhage | 0-0.9 | Non- irritant |
| 2. | Just visible membrane discoloration | 1-4.9 | Slight irritant |
| 3. | Structures are covered partially due to membrane discoloration | 5-8.9 | Moderate irritant |
| 4. | Structures are covered totally due to membrane discoloration/haemorrhage | 9-21 | Severe irritant |

## Materials And Methods:-

Gatifloxacin was used as an active pharmaceutical ingredient, Gellan gum was used as in situ gel forming polymer and HPMC K100M was used as release retardant. All the other reagents were used in the present study were of analytical grade.

**Preparation of Formulations**

Boric acid and disodium edetate were dissolved in distilled water. Gellan gum and HPMC were then dissolved in the above solution. The required quantity of Gatifloxacin to give a final drug concentration of 0.3% w/v was added to the polymeric solution and stirred until dissolved and then phenyl mercuric nitrate was added to it as preservative. The formulations were filled amber colored glass vials, closed with rubber closures and sealed with aluminum caps. The formulations, in their final pack were terminally sterilized by autoclaving at 121°C temperature, 15 psi pressure for 15 min. The sterilized formulations were stored in a refrigerator until further use. Composition of formulations is mentioned as below in table 2.

**Table 2:-**Composition of Prepared In situ gelling Formulations

| Ingredients | Amount (g) | | | | |
|---|---|---|---|---|---|
| | F 1 | F 2 | F 3 | F 4 | F 5 |
| Gatifloxacin | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| Gellan gum | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 |
| Boric acid | 1.68 | 1.68 | 1.68 | 1.68 | 1.68 |
| Phenylmercuric nitrate | 0.002 | 0.002 | 0.002 | 0.002 | 0.002 |
| Disodium edetate | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 |

| | | | | | |
|---|---|---|---|---|---|
| HPMC K100M | - | 0.3 | 0.4 | 0.5 | 0.6 |
| Distilled water | 100 | 100 | 100 | 100 | 100 |

**Evaluation Of Formulations**

**Physiochemical Characterization**

The clarity of the formulation was evaluated by visual observation against white and black back grounds. pH of the formulations was determined by pH meter and it was found to be between 6.2-6.3.

**Drug Content Uniformity**

Equivalent of 100 µl of (Gatifloxacin) the formulation was diluted to 25 ml with distilled water in sterilized volumetric flak. It was estimated spectrophotometrically using double beam UV-visible spectrophotometer. For F3 desired formulation it was found to be between 98.7 ± 0.75 % (Shimadzu 1700) at 286 nm as shown in table 3.

**Table 3:-**Drug Content Uniformity

| Formulation Code | Drug content (% ± SD) |
|---|---|
| F 1 | 99.3 ± 0.45 |
| F 2 | 97.8 ± 0.11 |
| F 3 | 98.7 ± 0.75 |
| F 4 | 96.0 ± 0.60 |
| F 5 | 97.1 ± 0.05 |

**Rheological Studies**

Viscosity of the formulation was determined by Brookfield viscometer (LVT model). To assess the gelation of formulation on instillation and mixing with lacrimal fluid, the viscosity measurements were also taken after diluting the formulation with the simulated lacrimal fluid (SLF). SLF comprised of 0.670 g sodium chloride, 0.200 g sodium bicarbonate and 0.008 g calcium chloride dihydrate and distilled water q.s to 100 g [13,15] which simulated the cation content of lacrimal fluid.

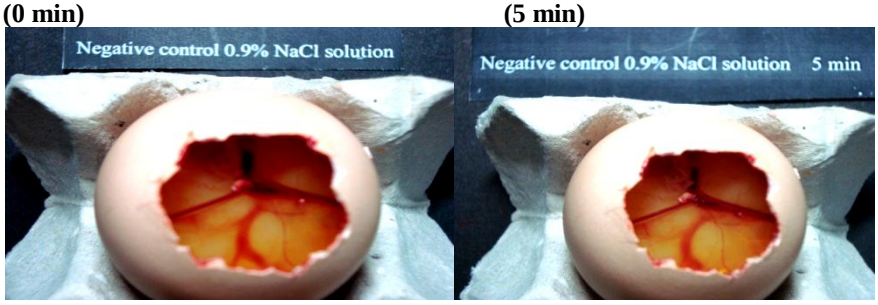
**Isotonicity Evaluation**[34,35]

Isotonicity has to be maintained to prevent tissue damage and irritation to the eye. Smear of resuspended RBCs with Gatifloxacin in situ gel formulation was prepared and observed under the polarizing microscope (Leica) at 45x magnification. Same procedure was followed for the marketed Gatifloxacin eye drops (Gatiquin$^{TM}$), isotonic solution (negative control) as well as hypertonic and hypotonic solution (positive controls). Size and shape of the RBCs with developed Gatifloxacin in situ gel formulation was compared to that with marketed Gatifloxacin eye drops (Gatiquin$^{TM}$) as well as with the positive and negative controls.

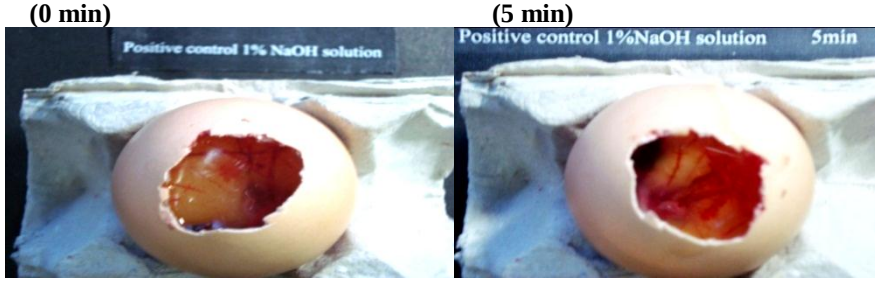
**Ocular Irritation Study (HET-CAM Test)**[36]

For the present study, HET-CAM test ( Hen's Egg Test- Chorio Allantoic Membrane) was carried out. This test is used for the detection of ocular corrosives and irritants. The potential ocular irritancy of a test substance was measured by its ability to induce toxicity in the Chorio Allantoic Membrane of a chick embryo. Fertilized hen's eggs weighing between 50-60 g were procured from poultry farm. The eggs were then candled to discard the defective ones and were then incubated in a humidified incubator at 37°C temperature and 75 ± 5% RH. The trays containing eggs were rotated manually in a gentle manner every hour. After ninth days, a window (2x2 cm) was cut on pointed end of eggs through which 0.2 ml of Gatifloxacin in situ gel formulation was instilled. A 0.9% NaCl solution was used as a negative control because it is reported to be practically non irritant being isotonic and physiologically compatible and 1% NaOH as positive control in present study. After instillation of the test samples, the chorioallantoic membrane was observed for a period of 5 min for hemorrhage, coagulation and vessel lysis as shown in Figure 1.The irritation scores are mentioned in table 1 and the observations are shown in table 4.


**Gatifloxacin in situ gel Formualtion (0 min)** **Gatifloxacin in situ gel Formulation (5 min)**




**0.9% NaCl Solution (Negative Control) (0 min)** **0.9% NaCl Solution (Negative Control) (5 min)**




**1% NaOH Solution (Positive control) (0 min)** **1% NaOH Solution (Positive control) (5 min)**

**Figure 1:-**Image representing the following in CAM at different Time Intervals

1. Developed Gatifloxacin In situ gel Formulation
2. 0.9% NaCl Solution (Negative Control)
3. 1% NaOH Solution (Positive Control)

**Table 4:-**Observations for HET-CAM Test

| Test substance | Score | Inference |
|---|---|---|
| 0.9% NaCl | 0 | Non-irritant |
| Formulation (AN/GFXGFS/07) | 0 | Non-irritant |
| 1 % NaOH | 14.27 | Severe irritant |

**In vitro Drug Release Study**

The in vitro drug release of the Gatifloxacin in situ gel formulation was estimated using modified USP dissolution apparatus-1 (Electrolab). Whatman® filter paper No 41 was placed inside the USP basket. It was then wetted by dipping in a solution of simulated lacrimal fluid for one minute to ensure the intimate contact of release medium with the formulation. Then 100 µl of the formulation was applied to it. Fifty ml of simulated lacrimal fluid was filled in a beaker and basket was rotated over its surface. A 3-3 ml aliquots of samples were withdrawn at regular time intervals and replaced with an equal volume of fresh simulated lacrimal fluid. The samples were analyzed spectrophotometrically for Gatifloxacin content using double beam UV-visible spectrophotometer (Shimadzu 1700) at 286 nm.

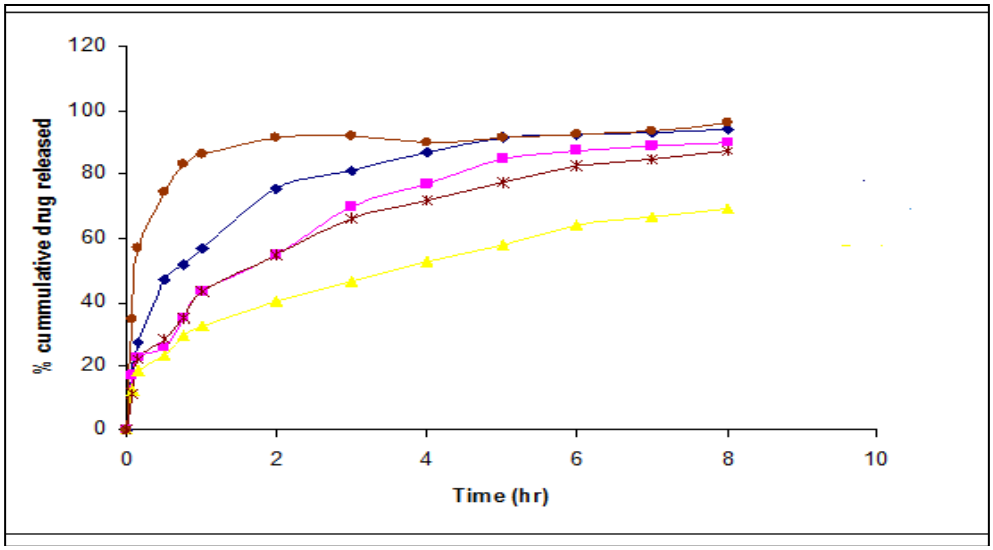



**Figure 2**:-In vitro Drug Release Study

## Results:-

The compositions of various formulations of eye drops are shown in Table 2. Different concentrations of gellan gum, i.e., 0.1-0.7% were studied for the gelling property in physiological conditions. Only 0.6% gellan gum solution exhibited desired flow characteristics and resulted in instantaneous gelation in simulated lacrimal fluid which was retained for an extended period of time. Combination of 0.6% gellan gum and 0.4% HPMC was selected, as it had satisfactory attributes of in situ gelling property, flow characteristics and prolonged in vitro release over the duration of 8 hr with 90.6% release in 8 hr. HPMC K100M was incorporated as a release retardant in the formulation. All the formulations were clear, having pH 6.0-6.3 and resulted in gel formation in SLF, clearly indicating phase transition behaviour in physiological conditions of eye. These formulations were evaluated for drug content uniformity as shown in table 3. Viscosity of the formulation was found to be 52 cps in solution form and 325 cps in gel form. Gatifloxacin in situ gel formulation was found to be isotonic, as it exhibited no change in the size and shape of RBCs. Formulation was found to be non irritant to eyes exhibiting mean score of zero in HET-CAM test for ocular irritancy for 5 min as shown in table 4. Since the immune response generated by chorioallantoic membrane of chicken simulates the ocular immune response of human eye, the developed formulation can be presumed to be non-irritant to the eyes.

## Discussion:-

Gellan gum is an anionic hetero polysaccharide which forms clear gel in the presence of monovalent and divalent ions present in cul-de-sac of the eye. Different concentrations of gellan gum, i.e., 0.1-1% were evaluated for the gelling property in physiological conditions out of which 0.6% resulted in instant gelation, and retained for an extended period of time. HPMC K100M was employed as a release retardant gave desirable results in concentrations range of 0.3-0.5%. As, isotonicity is a desirable attribute of an ophthalmic formulation, sodium chloride and boric acid were studied as an isotonicity adjusting agents. Sodium chloride imparted gelation of the formulation in vitro, hence, boric acid was selected as an isotonicity adjusting agent. Phenyl mercuric nitrate was used as a preservative in the formulation. 0.05% of disodium edetate was also added to enhance the solubility of Gatifloxacin in water and prevent its crystallization in freeze thaw conditions. Formulations were sterilized by autoclaving at 121˚C temperature, 15 psi pressure for 15 min.

Combination of 0.6% gellan gum and 0.4% HPMC K100M was selected as an optimum composition, as it exhibited desirable flow characteristics, physico-chemical properties and in vitro drug release. By the in vitro drug release study, it was found that gel has ability to retain Gatifloxacin for the entire duration of study (8 hr).

Gatifloxacin in situ gel formulation (F 3) was evaluated for the isotonicity and was also compared with the marketed eye drops, isotonic solution (negative control), hypertonic and hypotonic solution (positive controls). Hypertonic solution resulted in shrinkage of the cells and hypotonic solution caused bursting of the cells. Hence, it was confirmed that formulation is isotonic to eye. It was also compared with that of marketed Gatifloxacin eye drops (Gatiquin$^{TM}$).

Developed formulation was also found to be non-irritant to eyes. It was tested for irritation on the Chorio Allantoic Membrane of the chick embryo, which is a complete tissue including veins, arteries and capillaries and responds to injury with a complete inflammatory process, a process similar to that induced in the conjunctival tissue of rabbit eyes.

## Conclusion:-

It can be concluded on the bases of results and observations that the developed Gatifloxacin in situ gel formulation can overcome the drawbacks of the conventional ocular dosage forms. The developed formulation provided efficient therapy through prolonged drug release of the drug over an 8-hr period in vitro. It exhibited better antimicrobial efficacy when compared with the marketed eye drops. Formulation was isotonic and devoid of any irritant effect to the eyes. The ease of administration along with its ability to provide sustained release could result in decrease in frequency of administration thus enhancing the patient compliance.

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**Conflict of Interest**

No conflict of interest associated with this work

**Contribution of Authors**

I declare that this work was done by Ankita Kapoor and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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