Micro spect in vivo bio-distribution study of azithromycin ophthalmic in situ gel

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ABSTRACT
Azithromycin (AZT) is a broad-spectrum antibiotic and is found in ocular tissues when administered systemically. AZT inhibits RNA-dependent protein synthesis and hence has effective bactericidal capability against Staphylococcus aureus, Pseudomonas aeruginosa, which are the primary causative organisms for bacterial infection. In situ ophthalmic gels are systems which undergo a sol-to-gel transformation when instilled in eyes. In situ gels overcome the shortcoming of ophthalmic drops as they get washed out and diluted due to tear fluid. The aim and objective of present study was to formulate in situ ophthalmic gelling systems of Azt and determine in vivo ocular residence time in rat eyes of Tc\textsuperscript{99} labelled Azt by Micro SPECT. The in situ gel was formulated using Poloxamer 407, which is a temperature-induced gelling agent and HPMC K4M, which is known to increases mucosal adhesivity and enhance viscosity to facilitate sustained release of drug. The formulations developed were evaluated for pH, clarity, viscosity, gelling capacity and % drug release. The selected formulation was subjected to isotonicity and In Vivo Bio-distribution studies. Experimental studies on compatibility showed no interaction between polymers and AZT. AZT was found soluble in PB6.8. All formulations were found clear immediately after preparation and after sterilization & pH after gelation was satisfactorily in the range of 6 to 7. Viscosity and Gelation capacity of in situ gel increased with increase in polymer concentration. Formulations F2 showed desired results w.r.t viscosity, gelation capacity, drug release. In Vivo Biodistribution studies of Tc\textsuperscript{99} labelled AZT by Micro SPECT showed there was a significant increase in ocular residence time of in situ gel when compared with Tc\textsuperscript{99} labelled marketed solutions.

INTRODUCTION
The eye is considered a unique sensory organ which is having intricate design and is anatomically and physiologically different from other organs. There are many ophthalmic dosage forms available, but there is a substantial barrier which hinders ocular drug absorption and penetration. Hence desired drug concentrations is mostly not achieved mainly into the posterior part of the eyes (Balasingam et al., 2017). Eye infection, such as conjunctivitis, infectious keratitis, and Infectious endophthalmitis, is the most common problem (Watson et al., 2018). Azithromycin (Azt) is a broad-spectrum antibiotic which is effective against Gram-negative, Gram-
positive bacteria including Staphylococcus aureus, CDC coryneform group G, Streptococcus pneumoniae, Streptococcus mitis group and Hemophilus influenza. Increased tissue concentrations of Azt are found in the ocular tissues such as the eyelid, ciliary body and conjunctiva when administered. Azt is very effective for the treatment of Chlamydia trachomatis ocular infection (Opitz and Harthan, 2012). Mechanism of action of Azt is due to its binding to bacterial ribosome. This ribosomal binding inhibits RNA-dependent protein synthesis, thus resulting in bactericidal action (Sevillano et al., 2006; Retsema and Fu, 2001).

Various studies have demonstrated that Azt has powerful bactericidal capability against Staphylococcus aureus (Meyer et al., 1993), Pseudomonas aeruginosa (Imamura et al., 2005) the causative organisms for bacterial infection. Many conventional and newer ophthalmic drug delivery systems are available, to incorporate Azt, out of which patients very well accept eye drops. Still, it faces a significant problem, i.e. rapid precorneal drug loss (Sampath et al., 2012) due to increase production of tear fluid, blinking of the eyelid, and ocular barriers pose a challenge for ocular permeation of drug. Also when applied topically not more than 5% of drug reaches to targeted tissues (Patel et al., 2013).

To improve ocular drug bioavailability, in recent years, work is focused on the formulation of “in situ gel” (Sampath et al., 2012). These systems are solution during instillation and get transformed to gels in response to pH, temperature and specific ions. Acceptability of these in situ gels is high as it has the advantage of comfort and ease of application as solution and retaining as a gel when it enters the eye. The pre-corneal residence time of in situ gelling systems is for several hours (Acharya et al., 2019). Moreover, in situ gels overcome drawbacks like blurred vision shown by other newer ocular drug delivery systems like inserts, ointment, suspensions, and aqueous gels (Rathore et al., 2010). The aim and objective was to formulate in situ ophthalmic gelling systems of Azt and perform in vivo ocular residence time in rat eyes of Tc$^{99}$ labelled Azt by Micro SPECT.

MATERIALS AND METHODS

Azt was obtained from Yarrow chemicals, Pune. Poloxamer 407 from Sigma Aldrich, Mumbai, HPMC K4M from Eisen Pharmaceuticals, Pune. Benzalkonium chloride, Magnesium chloride, Calcium chloride dehydrate, Sodium bicarbonate, Potassium chloride, Sodium chloride, Stannous Chloride was received from Poona Chemical Lab. Reagent, Poona, and Nutrient agar from Hi-Media labs, Mumbai.

Drug Identification Studies

AZT was checked for its purity by checking its melting point, FTIR, DSC and $\lambda$ max (Kondalkar and Dev, 2019). The melting point was determined by the capillary method. The FTIR spectrum of Azt was compared with standard FTIR spectra. DSC thermograms were recorded by placing AZT in aluminium
### Table 1: Formulation and Development of Azt in situ gel

| Batch Code | Poloxamer 407 (%w/v) | AZT (%w/v) | HPMC K4M (%w/v) | Benzalkonium Chloride (%w/v) | Sodium Chloride (% w/v) |
|------------|-----------------------|------------|-----------------|-------------------------------|------------------------|
| F1         | 18.5                  | 1.5        | 1.00            | 0.01                          | 0.9                    |
| F2         | 18.5                  | 1.5        | 1.25            | 0.01                          | 0.9                    |
| F3         | 18.5                  | 1.5        | 1.50            | 0.01                          | 0.9                    |
| F4         | 19                    | 1.5        | 1.25            | 0.01                          | 0.9                    |
| F5         | 19                    | 1.5        | 1.00            | 0.01                          | 0.9                    |
| F6         | 19                    | 1.5        | 1.50            | 0.01                          | 0.9                    |
| F7         | 19.5                  | 1.5        | 1.00            | 0.01                          | 0.9                    |
| F8         | 19.5                  | 1.5        | 1.25            | 0.01                          | 0.9                    |
| F9         | 19.5                  | 1.5        | 1.50            | 0.01                          | 0.9                    |

### Table 2: Animal details used during Micro SPECT/CT studies

| Lab           | Particulars               |
|---------------|---------------------------|
| IAEC No.      | 12/2015                   |
| Bred used to study | Male Sprague Dawley rat |
| Size Weight   | 10.5 cm, 230gm - 250gm   |
| Site of sample injection | Right Eye |
| Dose instilled| 20µl                      |

### Table 3: Ex-vivo % cumulative drug release, Viscosity and Gelling time (F1-F9)

| Formulation | Coded Values A | B | Factor 1 A:Poloxamer 407 % | Factor 2 B:HPMC K4M % | Response 1 Cumulative Drug Release % | Response 2 Viscosity cps | Response 3 Gelling Capacity sec |
|-------------|----------------|---|---------------------------|-----------------------|-------------------------------------|--------------------------|-------------------------------|
| F1          | -1             | -1 | 18.5                      | 1                     | 98.12                               | 987.5                    | 21                            |
| F2          | -1             | 0  | 18.5                      | 1.25                  | 98.54                               | 1584.2                   | 20                            |
| F3          | -1             | +1 | 18.5                      | 1.5                   | 97.71                               | 1994.6                   | 20                            |
| F4          | 0              | -1 | 19                        | 1                     | 96.25                               | 1962.8                   | 17                            |
| F5          | 0              | 0  | 19                        | 1.25                  | 96.21                               | 3005.8                   | 16                            |
| F6          | 0              | +1 | 19                        | 1.5                   | 94.87                               | 3104.4                   | 16                            |
| F7          | +1             | -1 | 19.5                      | 1                     | 92.11                               | 3154.7                   | 16                            |
| F8          | +1             | 0  | 19.5                      | 1.25                  | 92.01                               | 3788.2                   | 15                            |
| F9          | +1             | +1 | 19.5                      | 1.5                   | 91.58                               | 3874.4                   | 15                            |

### Table 4: Counts of AZT plus -99Tc retained at the end of 30, 60 and 120 mins

| S No | Particular                          | Counts at the end of 30 min (kBq/CC) | Counts at the end of 1 hour (kBq/CC) | Counts at the end of 2 hours (kBq/CC) |
|------|-------------------------------------|-------------------------------------|--------------------------------------|--------------------------------------|
| 1    | Marketed Azt eye drops              | 17                                  | 12                                   | 8                                    |
| 2    | Formulation F2 -Azt in situ gel drops | 49                                  | 41                                   | 28                                   |
Figure 4: DSC Thermo gram of Azt

Figure 5: IR spectra of Azithromycin, Poloxamer 407, HPMC K4M and physical mixture

Figure 6: Graphical representation of Calibration Curve for AZT

Figure 8: Response Surface Plot for Viscosity

Figure 9: Response Surface Plot for Gelling Capacity

Figure 7: Response Surface Plot for Ex Vivo % Cumulative Drug Release

Figure 10: Normal Red blood cells without ophthamlic in situ gel
crucibles and heating in a range from 0°C to 300°C using DSC 6220 (SEIKO), Japan. The rate at which air was purged was 10 ml/min. Determination of λ max of Azt was carried out by preparing a solution with concentration 100 µg/ml in phosphate buffer pH 6.8 (PB6.8). PB6.8 was used as blank. The UV spectrum was obtained in a wavelength range of 200-400 nm.

**Compatibility studies**

Individual FTIR spectra of the Azt was compared with combination spectra of Azt with Poloxamer 407 and HPMC K4M. Differential Scanning Calorimetric (DSC) studies were carried out to analyze the thermal behaviour of the physical mixture of Azt in the presence of polymer.

**Solubility Studies**

The solubility of Azt was determined in various solvents, Acetate buffer IP (pH 6.8 & 7.4) and Phosphate buffer (pH 6.8 & 7.4) for selecting a suitable vehicle.

**Calibration Curve**

Calibration curve of the Azt was plotted by recording the absorbance of solutions (Azt in PB6.8) in different concentrations (2-16 µg/ml) at λ max 247 nm using Shimadzu UV-1800 spectrophotometer.

**Formulation and Development**

To study the effect of concentration of poloxamer 407 and HPMC-K4M on the formulation of gel, 3² randomized full factorial design of Azt in situ gel was used. The amount of poloxamer 407 (%) and the amount of HPMC K4M (%) were selected as independent variables. These two factors were evaluated each at three levels. The coding was -1, 0 and +1 respectively for lower, middle and higher levels of each element. The % cumulative drug release at 8h (Y1), viscosity at RT (Y2) and gelling capacity (Y3) are dependent variable or response. Poloxamer 407 is a temperature-induced gelling agent, while HPMC K4M is known to increases mucosal adhesivity ([Patel et al., 2016](#)). In situ formulations (F1 to F9) as shown in Table 1 were prepared by varying concentration of these polymers. The response surface graphs were plotted. The actual and coded values are as given in Table 3.

The data obtained were analyzed by the observed results from the multiple regression analysis using Design Expert Version 12. The equation obtained was $Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_{11}X_1X_1 + \beta_{22}X_2X_2 + \beta_{12}X_1X_2$ where $Y$ is the measured response, $X$ is the levels of independent factors, and $\beta$ is the regression coefficient. $X_1$, $X_2$ indicate the amount of Poloxamer 407 & HPMC K4M. These polymeric solutions were prepared by dissolving polymers into cold distilled water and then keeping them
overnight in the refrigerator. Drug AZT, Preservative Benzalkonium Chloride and tonicity modifying agent Sodium Chloride were mixed with the polymeric solution, and distilled water was used to make up the volume. These in situ gels were further evaluated for appearance, viscosity, pH, the capacity of gelling and % drug release.

**Appearance and pH**

Formulations clarity (F1 to F9) was determined by visual examination, immediately after preparation in solution form and after sterilization & gelation under light alternatively against black and white backdrop while pH of sol was determined by pH meter.

**In Vitro gelation study**

Gelling capacity was determined by depositing a drop of formulation in 2 ml of freshly prepared artificial tear fluid having pH 7.4 and maintained at 37°C in a vial. Time taken for gel formation and for the gel to dissolve recorded visually.

**Viscosity Studies**

Viscosity of the formulations was evaluated after gelation. Gelation was done by keeping formulation in a warm environment. Viscosity was recorded by taking the formulations into small sample adaptor and increasing angular velocity from 0.5rpm to 50rpm by using Brookfield viscometer (Saxena and Singh, 2015).

**Ex vivo drug release studies**

Ex vivo diffusion were done using Franz Diffusion cell. PB6.8 was used as dissolution medium, and Goat cornea was used as a semi-permeable membrane. The goat cornea was carefully removed and washed with cold saline (Paliwal et al., 2009). The corneas were then kept in a freshly prepared cold solution of tear fluid having pH 7.4. Care was taken so that the corneal side remains in continuous contact with a formulation in the donor compartment. The receptor compartment was filled with PB6.8 at 34°C ± 0.5°C. The receptor medium was stirred with a magnetic stirrer. The samples were withdrawn at different time intervals for analysis of AZT content. Receptor phase is reloaded with an equal volume of PB6.8 at each time interval. Formulations F2 showed desired results w.r.t viscosity, gelation capacity, drug release, and was considered for further studies.

**Isotonicity**

Isotonicity if not maintained, can lead to irritation of eye and tissue damage. 0.1ml of optimum formulation F2 was added in a few drops of blood. Observations were done under a MOTIC microscope at 45X magnification & compared with marketed Azt eye drops (Venkatesh et al., 2014).

**Animal Study: In Vivo Bio-distribution of Tc⁹⁹ labelled AZT by Micro SPECT (single-photon emission computed tomography)**

Percentage of AZT that binds to the Tc⁹⁹ was determined by TLC method and calculated as per Equation (1). The procedure followed for AZT & Tc⁹⁹ binding is laid down in Figure 1. Animal details used during in vivo studies are tabulated in Table 2. The micro SPECT images were acquired using the tri-modality gamma imaging system (Suma et al., 2017) after placing AZT in situ gel (formulation F2) - Tc⁹⁹ and AZT eye drops-Tc⁹⁹ in the rat eye. Whole-body SPECT was performed after 30min, 1h and 2h of installation of Tc⁹⁹ labelled AZT and AZT eye drops-Tc⁹⁹. Rats were placed on a stationary bed having a gannya movement from 0° to 360°. The radius of rotation (ROR) was 35° and field of view (FOV) was 120. Images were taken every 20 min at 64 projections. After SPECT imaging, whole-body CT images (magnification 1.3, FOV 91.08, energy 75 KeV, 300ms and 512 projections) were subsequently acquired without disturbing the position of rats (Prabhu et al., 2017). Gaseous isoflurane anaesthesia was used to restrain rat at a stationary position over the detector. SPECT / CT images were analyzed using PMOD software, version 3.2. The region of interest (ROI) was obtained. Gamma emittance was expressed in % ID/cc (Prabhu et al., 2017).

**Stability Studies**

Formulation F2 was stored for 180 days at 4°C, 25°C and 40°C (Matthews, 1999). Samples were evaluated after 30, 60 and 180 days for appearance and AZT content (Saxena and Singh, 2015).

**RESULTS AND DISCUSSION**

**Drug Identification Studies**

Melting point of drug under consideration was in range 113-115°C as reported in the literature for AZT. The FTIR of drug under consideration Figure 2 was found to match with standard FTIR of AZT. The recorded UV spectra of a drug under consideration showed absorbance maxima (λmax) at 247 nm in PB6.8 Figure 3. The DSC thermogram of the physical mixture showed a characteristic endothermic peak corresponding to Azt around 123°C Figure 4. From above obtained results it can be confirmed that drug under consideration was AZT and pure.
Compatibility studies

Combination IR spectra of AZT with Poloxamer 407 and HPMC was obtained as shown in Figure 5. When compared with AZT, no peak distortions were found. Thus, it can be concluded that AZT is compatible with Poloxamer 407 and HPMC. The DSC thermogram of the physical mixture showed a characteristic endothermic peak corresponding to AZT around 123°C, and no interaction between AZT and polymers were observed.

Solubility Studies

AZT was freely soluble in ethyl alcohol, water, 2-propanol acetone and PB6.8. As AZT was soluble in PB6.8, hence it was used as a solvent for further studies as a vehicle.

Calibration Curve for AZT

Absorbance recorded at various concentrations (0-16 µg/ml) showed that Beers Lambert’s law was obeyed, coefficient of correlation and slope was 0.9984 and 0.1033 respectively (Figure 6).

Evaluation of AZT in situ gel

pH and Appearance

Formulations F1 to F9 was found clear immediately after preparation. Sterilization and gelation did not affect the clarity of formulations while pH after gelation of the formulations was found satisfactorily in the range of 6-7.

Optimization of Independent Variables of in situ Gel Formulation

The concentration of AZT, Benzalkonium chloride and sodium chloride were kept constant. The effects of Poloxamer 407 and HPMC K4M on the % Cumulative Drug Release, Viscosity, and Gelling Capacity were analyzed.

Ex Vivo % Cumulative Drug Release

% cumulative drug release from in situ gels is depicted in (Table 3). The drug release decrease with increase in polymer concentration. It indicates that amongst all formulations, F2 shows desired sustained release up to 8h due to proper concentration of poloxamer 407 and HPMC K4. The relation between influence of viscosity on drug diffusion follows the Stokes-Einstein equation, with increased thickness of the formulation there is decreased distribution of the drug across the gel. The cumulative drug release ranges from 91.58 to 98.54%, which has been shown in Table 3. Multiple regression analysis for the drug release as per the factorial design revealed the right fit R² = 0.99 with the following equation:

\[ \% CR = 96.10 - 3.11X_1 - 0.3867X_2 - 0.03X_1X_2 - 0.7650X_1X_3 - 0.480X_2X_3 \]  

(2)

As per Equation (2) and Figure 7, values of coefficient indicate a good fit. The negative sign of X₁ indicates the retarding nature of the ophthalmic in situ gel. Although the HPMC K4 M with weak negative coefficient value delays the drug release comparatively stable coefficient value of Poloxamer 407 accelerates it. Poloxamer 407 is used alone as the gelling agent. It explains the temperature sensitivity in situ hydrogel for prolonged drug delivery due to its suitable reverse thermo-sensitive property. Poloxamer based gels contain large aqueous channels. A higher concentration of Poloxamer 407 causes a more significant tortuosity in the aqueous phase and also results in a shorter inter-micellar distance. This all results in contributing to the drug release rate reduction due to a high number of cross-links between neighbouring micelles and a higher number of micelles per unit volume. The negative sign of X₁X₃ explains the same.

Effect of Viscosity

The viscosity ranges from 3874.4 to 987.5 cps which has been shown in Table 3. Multiple regression analysis for the drug release as per the factorial design revealed the right fit R² = 0.99 with the following equation:

\[ Viscosity = 2877.4 + 1041.8X_1 + 478.0X_2 - 71.8X_1X_2 - 127.0X_1X_3 - 279.6X_2X_3 \]  

(3)

At R.T., the formulations were in a liquid state, thus exhibited low viscosity. Solutions were subject to a warm condition to mimic condition in the eye showed increased in viscosity. The viscosity of gel increases with increase in the concentration of poloxamer 407 & HPMC K4 (Table 3). F1 to F3 Batches shows very low viscosity while F6 to F9 was highly viscous at room temperature. This high viscosity may be irritant to the eye and may cause discomfort to the patient on application further leading to noncompliance.

As per Equation (3) and Figure 8, values of coefficient indicate an excellent fit response. Poloxamer gels at room temperature exist as low viscosity solutions, but at body temperature converts to as gel, making them ideal thermosensitive polymers. If we remove PPO and PEO diblock copolymers from poloxamer 407, we could increase the interactions between poloxamer 407 polymer chains which can lead to an increase in viscosity. As reflected by the positive coefficients of X₁ and X₂, the thickness of poloxamer gel was increased by using cellulose.
derivatives as compared with poloxamer gel alone. Viscosity and polymeric content are found directly proportional. Viscosity increased with the increase in the concentration of polymer. The same is also supported by Figure 8.

Effect of Gelling Capacity

The gel formulation was visually evaluated, and it is observed that all batches showed gelation on contact with ATF and retained gel structure for more than 8h. The gelling capacity ranges from 15 to 21 seconds (Table 3). F8 and F9 these two batches are highly viscous solution even before subjecting to gelation and hence can't be easily administered into the eye. Individual polymers had shown negative effect whereas combination had a positive impact on gelling capacity which can be seen with multiple regression analysis for the drug release as per the factorial design revealed the right fit $R^2 = 0.99$ with the following equation:

$$Gelling Capacity = 16.3 - 2.50X_1 - 0.50X_2 + 1.50X_1X_2$$

As concentration increases poloxamer 407 is transformed from a low viscosity solution at low temp to a gel under the ambient temperature. At low temperatures molecules of poloxamer 407 are surrounded by a hydration layer, an increase in temperature causes breakage of the hydrogen bond. At low poloxamer 407 concentration solution tends to lose the gelation ability because of dilution by lacrimal fluid. Hence, the influence of change in concentration of poloxamer 407 was observed for its in-vitro, reducing gelling capacity. The same is depicted in Equation (4) and Figure 9.

Isotonicity

RBC’s were found to be intact, and no RBC was ruptured after addition of AZT in situ solutions as seen in Figure 10 and Figure 11.

Sterility Test

There was no turbidity observed at the end of 14 days. Formulations (F2) passed the test for sterility as per IP 2007.

Stability Studies

Stability studies established that in situ formulations of Azt (F2) was stable under given conditions.

Animal Study: In Vivo Bio-distribution of Tc$^{99m}$ labelled AZT by Micro SPECT (single-photon emission computed tomography)

Drug Tc$^{99m}$ binding was found to be 98.13%. Figure 12 shows the actual images of Micro SPECT/CT for Azt branded eye drops, while Figure 13 shows the real images obtained by Micro SPECT/CT for AZT in situ gel. Counts of AZT plus - Tc$^{99m}$ retained at the end of 30, 60 and 120 mins is tabulated in Table 4. The red zone in Figure 13, Micro SPECT/CT, which determines the Drug-radioactive zone is more significant and retained in a larger amount than in Figure 12. Also the Radioactivity concentration counts of in situ gel at the end of 30, 60 and 120min is higher than eye drops, both indicating that the gel was retained in the eye for a more extended period.

CONCLUSION

Azt in situ gel was successfully formulated using gelling agent poloxamer 407 in combination with mucoadhesive and viscosity-enhancing agent HPMC K4M. The formed in situ gel was assessed for appearance, pH, gelling capacity, clarity, viscosity and isotonicity. Formulated in situ gel indicated prolonged ex vivo drug release for 8h. Formulation F2 was found optimum and was considered for further studies. In-vivo Micro SPECT/CT images showed that on instillation into the eye as sol, the formula converts to gel in the cul-de-sac and retained in the eye for a more extended time than marketed eye drop. Finally, we can conclude formula (F2) represents a promising alternative to the conventional eye drops as it has prolonged drug release and longer precorneal residence time leading to higher availability. This system has ease of administration than usual gels, and the reduced frequency of administration will result in better patient compliance.

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Conflict of interest

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