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Original Article

FORMULATION, OPTIMIZATION, AND EVALUATION OF *IN-SITU* GEL OF MOXIFLOXACIN HYDROCHLORIDE FOR OPHTHALMIC DRUG DELIVERY

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ABSTRACT

Objective: The present study emphasizes the synthesis, optimization, and evaluation of ocular *in-situ* gel for ophthalmic drug delivery against conjunctivitis.

Methods: Pre-formulation studies on the drug and polymers were carried out, which included the study of various physicochemical properties of the drug and drug-polymer compatibility studies. The 12 different formulations were further pre-optimised by Taguchi method for determining the number of influential factors. Furthermore, the formulation optimization was done by using 'Box–Behnken' design (BBD) (Design expert 10 software) for assessing the effect of formulation variables on product characteristics viz. viscosity, gelation temperature (GT), and mean release time (MRT). About 13 suggested runs of the experiment were carried out and formulations were optimised. Finally, three batches of the optimised formulation were prepared and evaluated for *in vitro* drug release, isotonicity of formulation, anti-microbial potential, ocular irritancy, and accelerated stability testing.

Results: Pre-formulation study confirmed the purity, solubility, and compatibility of drug measured by λ_{max}, partition coefficient, stability study, and Fourier-transform infrared spectroscopy (FTIR) analysis. Taguchi screening method suggested about 12 different formulations and 3 most prominent influential factors including viscosity, GT, and drug release. 13 different formulations designed based on 'BBD' method were further optimised by considering the most influential factors suggested by Taguchi screening. The *in vitro* evaluation of the optimised formulation gave satisfactory results in terms of drug release, and anti-microbial activity. It was found to be isotonic with no ocular irritancy. Further, the preparation immediately transformed from sol to gel upon administration into cul-de-sac region of the eye due to multi-dimensional approaches utilised for *in-situ* gel formation namely temperature change Pluronic, ion sensitivity due to Gellan-gum, pH sensitivity because of Carbopol.

Conclusion: The optimised *in-situ* gelling ocular drug formulation showed promising potency for ophthalmic drug delivery with no irritancy due to the multifactorial mechanism.

Keywords: Ocular *in-situ* gel, Pluronic *F-127*, Gellan-gum, Carbopol, Taguchi screening, Box–Behnken design

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INTRODUCTION

In-situ forming formulation is an advanced system of drug delivery in which *in-situ* phase transition occurs on the surface at the site of application. The preparations are convenient to administer and use over other drug delivery systems [1]. The phase transformation of dosage form from liquid to gel, in *in-situ* gelling formulations, takes place due to changes in physical or chemical factors, which include, changes in temperature, pH, solvent, ionic concentration and trigger one or more mechanism for transforming the liquid into gels. Since, the mechanisms are not dependent on any external factors which enhances the simplicity and reliability of formulation, encouraging self-administration and improved patient compliance [1]. Previously researchers have attempted to develop formulations to enhance the precorneal residence time by injecting the drug in cul-de-sac region of eye,. Using the polymer dependent singular approach such as pH dependence (cellulose acetophthalate), temperature dependent (polyoxyethylene polymer), in a single polymer containing formulation; the concentration of Pluronic required to achieve enough gelling property to avoid being oozed out by tears was 30% [2]. Carbopol possess self-acidic nature and may irritate the individual's eye which leads to excessive tearing and wash out of the formulation [3]. The time required to form gel is significantly large in case of Gellan-gum, which sometimes leads to oozing out of the instilled formulation due to reflex tearing before it can gel. Thus, these formulations dependent on the singular mechanism are more susceptible to failure. Also, the concentration of polymer required to achieve desirable properties is often too high, which ultimately increases the total volume and cost of manufacturing of formulation and more importantly overexposure of delicate tissues of eyes to the high concentration of polymers. Thereby, limiting the scope of the formulation [4]. Whereas, the combined use of polymers can help to develop a robust formulation that may reduce the probability of formulation failure and reduces the quantity of polymers required to develop formulation [5]. Thus, to target the limitations of the traditional formulation including rapid exclusion, insignificant ocular bioavailability and eye irritation we have proposed an innovative formulation of Moxifloxacin hydrochloride based on multifactorial approaches. We have pursued the combination of numerous mechanisms to develop a new *in-situ* gelling formulation having a relatively small concentration of each polymer. The present investigation includes the development of an optimised formulation based on pre-formulation studies and its evaluation. For this, the different sets of combined polymers in miscellaneous concentration was used to the developed formulation and optimised by design expert software (Design-Expert® 10 software) for utmost release of the drug, apposite viscosity at ocular site and optimised gelation temperature (GT). Furthermore, the ocular irritancy was observed in the rabbit's eye for 14 d.

MATERIALS AND METHODS

Materials

Moxifloxacin hydrochloride was obtained as a gift sample from INTAS pharmaceuticals, Ahmedabad, India. Pluronic F-127, Carbopol, Gellan-gum were obtained as a gift sample from Jubilant life sciences India. All other chemicals were of analytical grade. Microbial culture *Pseudomonas aueroginosa* and *Staphylococcus aureus* were obtained from microbial type culture collection (MTCC); MTCC catalog no. of both are 424 and 96 respectively, and Gene Bank, CSIR-Institute of microbial technology, India.

Pre-formulation studies

Melting point

The melting point of active pharmaceutical ingredient (API) was determined using digital melting point apparatus. A capillary tube having the diameter of 1.9 mm was broken in the length of 6 cm and one end of the tube was sealed off. Capillary tubes were filled with the sample up to

the height of 0.5 cm. The sample containing capillary tube was now placed into the sample holder of the apparatus. Finally, the melting point was determined by gradually increasing the temperature, until the sample melted, and the melting point range was observed.

Determination of λ_{max}

In distilled water and simulated tear fluid

10 µg/ml solution of Moxifloxacin hydrochloride was prepared in distilled water and simulated tear fluid (STF) and scanned for λ_{max} in 200-400 nm range. The composition of STF was sodium chloride (1.34 g), sodium bicarbonate (0.4 g), calcium chloride dihydrate (16 mg) and water up to 200 ml.

Preparation of standard curve

In distilled water

10 mg of Moxifloxacin was weighed and transferred to a volumetric flask of 10 ml capacity and diluted with distilled water. 1 ml of this stock solution was further diluted to 100 ml aliquots from the resulting solution were pipetted and diluted to make a standard solution containing 1-10 µg/ml of the drug. The absorbance of these solutions was measured at 288 nm by using UV-visible spectrophotometer. Standard curve for moxifloxacin hydrochloride was developed in double distilled water and STF pH 7.4 using UV spectrophotometry.

In STF

Similarly, distilled water dilutions of drug were prepared in STF in a concentration range of 1-10 µg/ml. Absorbance of these solutions was measured at 287 nm by using UV-visible spectrophotometer and standard curve was prepared.

Fourier-transform infrared spectroscopy analysis

IR spectrum of pure drug sample was recorded using Fourier-transform infrared spectroscopy (FTIR) alpha with ECO ZnSe ATR (bruker) and compared with reference spectra of the drug. Sample was placed on the sample platform and squeezed between the knob and sample platform.

Drug-excipient compatibility study

Prior to the formulation of Moxifloxacin hydrochloride *in-situ* gel, the drug and excipient compatibility should be determined. The physical mixture of drug (Moxifloxacin hydrochloride) and excipients (Pluronic F-127, Carbopol, Gellan-gum) in 1:1 ratio. The mixture was stored in airtight, light-resistant containers for the duration of 14 d at 37 °C. The samples were scanned by FTIR spectroscopy and the spectra were analysed [6].

Selection of dose for incorporation in *in-situ* gel

0.5% of Moxifloxacin hydrochloride was incorporated into the *in-situ* gel, which was taken as standard marketed formulation of Moxifloxacin containing maximum strength formulation recommended for the disease treatment.

Pre-optimization of *in-situ* gel

The pre-optimization of *in-situ* gel was done by using Taguchi screening design.

Screening of influential variables

We use Taguchi design for the screening of influential factors among various formulation and process variables for the development of *in-situ* gel [7]. The high and low level of various variables were screened for their influence on property of *in-situ* gel. The L8 array layout for 7 factors like rate of stirring, Pluronic *F-127*, Gellan-gum, Carbopol, pH, and stirring time, in two-level Taguchi design was adopted for the pre-optimization study. Twelve formulations of *in-situ* gel were prepared [8].

In-situ hydrogel was prepared by dispersing Pluronic F-127 into distilled water followed by continuous stirring for 1hr. Separately Gellan-gum solution was prepared by dispersing powdered Gellan-gum into distilled water at 90 °C, followed by continuous stirring until the mixture reaches room temperature and Carbopol solution was prepared by soaking the polymer in distilled water.

The partially dissolved solutions of polymers were kept in a refrigerator overnight at 4 °C [9]. Loading of Moxifloxacin hydrochloride into *in-situ* gel was done by dispersing moxifloxacin into a specific mixture of Pluronic, Gellan-gum, and Carbopol, followed by continuous stirring for 5 min. Benzalkonium chloride was also added as a preservative, the pH of the formulation was varied using 1 M hydrochloric acid or 1 M sodium hydroxide. The formulation concentration was given in table 1. Finally, the formulations were sterilised using autoclaving at 121 °C at 15 psi for 30 min. The formulations were stored at 4 °C. The variables used in the pre-optimization study of *in-situ* gel is described in table 2 below.

Table 1: Formulation of *in-situ* hydrogel

S. No.	Ingredients	Quantity (g)
1	Moxifloxacin hydrochloride	0.5
2	Pluronic*F-127*	10-20
3	Gellan-gum	0.1-0.5
4	Carbopol	0.1-0.5
5	Benzalkonium chloride	0.006
6	Distilled water	Quantity sufficient for 100 g

Table 2: L_{12} array layout as per six factor, two-level taguchi screening designs

S. No.	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6
	Rate of stirring (rpm)	Pluronic F-127	Gellan-gum	Carbopol	pH	Stirring time (min)
1	100	10	0.5	0.1	8	10
2	10	10	0.5	0.5	8	240
3	100	10	0.5	0.5	6	10
4	100	10	0.1	0.5	8	240
5	100	10	0.5	0.1	6	240
6	10	10	0.1	0.1	6	10
7	10	20	0.1	0.5	6	240
8	100	20	0.1	0.1	6	10
9	10	20	0.1	0.1	8	240
10	10	20	0.5	0.1	6	10
11	10	20	0.1	0.5	8	10
12	100	20	0.5	0.5	8	240

Evaluation of *in-situ* gelling preparation

Physical appearance

The preparation was analysed visually for physical properties such as appearance, colour, homogeneity, clarity, and consistency.

Determination of pH

10 g of the *in-situ* gel was taken, and the pH of the formulation was evaluated using digital pH meter, calibrated using standard pH buffer tablets of pH 4.0 and 7.0 at 25 °C [10].

Drug content

Uniform distribution of active ingredients is important to achieve dose uniformity. The drug content of various gels was determined by placing the sample (2 ml) of the *in-situ* gel in a 100 ml volumetric flask and diluting the same with STF of pH 7.4. The UV absorbance of the resulting sample was then measured at 287 nm and using the standard curve percentage drug content was determined [11].

***In vitro* release study**

In vitro release studies of the prepared formulation were done with the help of modified dissolution apparatus. An overnight soaked semi-permeable membrane was tied to one end of an open-ended cylindrical tube having the diameter of 3.2 cm. 2 ml of the test sample was placed into the dissolution apparatus using STF (pH 7.4) as dissolution medium. Finally, the apparatus was suspended into the beaker containing 100 ml of dissolution medium, maintained at 37±2 °C and 50 rpm (by magnetic stirrer). Periodically 1 ml of sample was withdrawn and replaced by fresh dissolution medium. The aliquots were analysed at 287 nm using UV spectrophotometry.

Mean release time-80

MRT-80 is the time required by the formulation to release 80% of its drug content. The MRT of the prepared formulations were evaluated using UV-Vis Spectrophotometer.

MRT was calculated using the equation.

$$\mathrm{MRT} = \frac{\sum_{i=1}^{n} \mathrm{tmid}\cdot\Delta C}{\sum_{i=1}^{n} \Delta C}$$

In which *i* represents the release sample number, *n* represents the number of release sample time, t_{mid} represents the time at the midpoint between *i* and *i*-1 and Δ represents the additional concentration of drug release between *i* and *i*-1 [12].

Gelation temperature

GT was measured by a magnetic bar method. In brief, 10 ml of the formulation was taken in 20 ml beaker. The formulation was stirred using the magnetic bar and gradually heated at the rate of 1 °C/min. The temperature at phase transformation of the formulation was noted when the movement of magnetic bar was hindered [13, 14].

Rheological evaluation

The rheological study of the formulation was measured by taking the sample in 150 ml beaker. Viscosity of the sample was measured using Brookfield viscometer LV-III, using appropriate spindle.

Optimization of *in-situ* gel employing Box-Behnken design

The results obtained by preoptimization study were evaluated for identification of influential factors for further optimization of the formulation. Among various factors screened Pluronic, Carbopol and, Gellan-gum were identified as most influential factors (table 3). Limits for the influential factors were also determined by factor influencing study at various factor levels [9]. Box-Behnken design (BBD) was used for the optimization study. Using center point (0,0,0) was observed in quintuplicate. Table 4 summarises the experimental design matrix of 13 experimental runs and their factor combinations.

Formulation by design validation

The predictability of Formulation by Design (FbD) optimization study was validated using several checkpoint formulations. Using numerical optimization six confirmatory run formulations were prepared based on desirability function as a check-points for validation of FbD and optimum formulation selection. The predicted and observed responses of the formulation were compared along with construction of linear correlation plots of the selected six formulations. Residual plot of predicted vs observed response was generated and linear regression was performed, and the amount of percent bias (i.e. prediction error) in prediction was analysed against observed responses. Optimised formulation of in-situ gel was used in further studies.

Table 3: Variables used in the formulation of *in-situ* gel system

S. No.	Variables	Levels		
		I	II	III
1	Pluronic *F-127*	10	15	20
2	Gellan-gum	0.1	0.3	0.5
3	Carbopol	0.1	0.3	0.5

Table 4: Combination of factors according to 3-factor box-behnken design

Run	Factors		
	Pluronic *F-127*	Gellan-gum	Carbopol
1	10	0.3	0.5
2	15	0.1	0.5
3	20	0.5	0.3
4	10	0.3	0.1
5	15	0.1	0.1
6	10	0.1	0.3
7	15	0.5	0.5
8	10	0.5	0.3
9	20	0.3	0.1
10	20	0.3	0.5
11	20	0.1	0.3
12	15	0.3	0.3
13	15	0.5	0.1

Evaluation of systematically optimised formulations

Physical appearance

The prepared formulations were inspected visually for their appearance, colour, homogeneity, clarity and consistency.

Drug content

Uniform distribution of active ingredients is important to achieve dose uniformity. The drug content of various gels was determined by placing the sample (2 ml) of the *in-situ* gel in a 100 ml volumetric flask and diluting the same with STF of pH 7.4. The UV absorbance of

the resulting sample was then measured and using the standard curve percentage drug content was determined.

Antimicrobial activity

Antimicrobial activity was performed to evaluate the efficiency of the optimised formulation as compared to the marketed formulation. For this Kirby-Bauer disk diffusion method was used where the drug-loaded disk of 10 mm diameter was incubated in nutrient agar media [15]. Nutrient media were pre-poured into the sterile petri plates and allowed to cool under laminar air flow. Microbial culture of *Pseudomonas aueroginosa* and *Staphylococcus aureus* were inoculated with the help of sterile cotton swabs and drug-loaded disks were placed in the inoculated media. After allowing an initial diffusion period of 2 hr, the Petri plates were incubated at 37±0.5 °C for 24 hr. Zone of inhibition (ZOI) was measured with the help of Vernier calliper.

Sterility testing

Sterility testing is an important parameter for ophthalmic preparations. We used direct inoculation method for evaluation of sterility of *in-situ* gel. 2 ml of preparation was taken using a sterile pipette and transferred into Thioglycolate medium and separately in Soybean-casein digest medium. The sample was incubated for minimum of 14 d at 30-35 °C and 20-25 °C respectively.

Accelerated stability testing

Optimised formulation of Moxifloxacin hydrochloride was tested for accelerated stability study (according to ICH guidelines). In brief, the optimised formulation was placed in glass vial closed by the grey butyl rubber closure and sealed with aluminum closure was stored in a stability study chamber at 40±2°C, 75±5% RH in both horizontal and vertical position for 6 mo. Periodically samples were withdrawn and evaluated for change in visual appearance, pH, gelling capacity, drug content and *in vitro* drug release.

Isotonicity evaluation

Isotonicity is an important characteristic of ophthalmic preparations. The preparation was mixed with a few drops of blood and was observed under the microscope. The integrity and shape of red blood cells were compared against 0.9% Sodium Chloride isotonic solution.

Ocular irritancy

Ocular irritancy test was done on 3 rabbit's eyes (New Zealand white, either sex), a weight of 1.5-2 kg obtained from the institutional animal house [16]. Experimental animals were acclimatised for 4 d before starting the experimental work. 0.1 ml of the optimised formulation of Moxifloxacin hydrochloride was used for administration into the cul-de-sac and observed at 1, 24, 48, and 72 hr. the procedure is repeated for the duration of 7 d. The animals were evaluated for the watering of eyes, redness, mucosal discharge and swelling [17].

RESULTS AND DISCUSSION

Pre-formulation studies

Melting point

The melting point of the sample was found to be 235-239°C, which is very close to the theoretical melting point i.e 238-242°C, indicating Moxifloxacin with high purity.

Determination of λ_{max} in distilled water and STF

The λ_{max} of the Moxifloxacin Hydrochloride was found to be 288 nm and 287 nm in distilled water and STF respectively as shown in fig. 1 and 2.

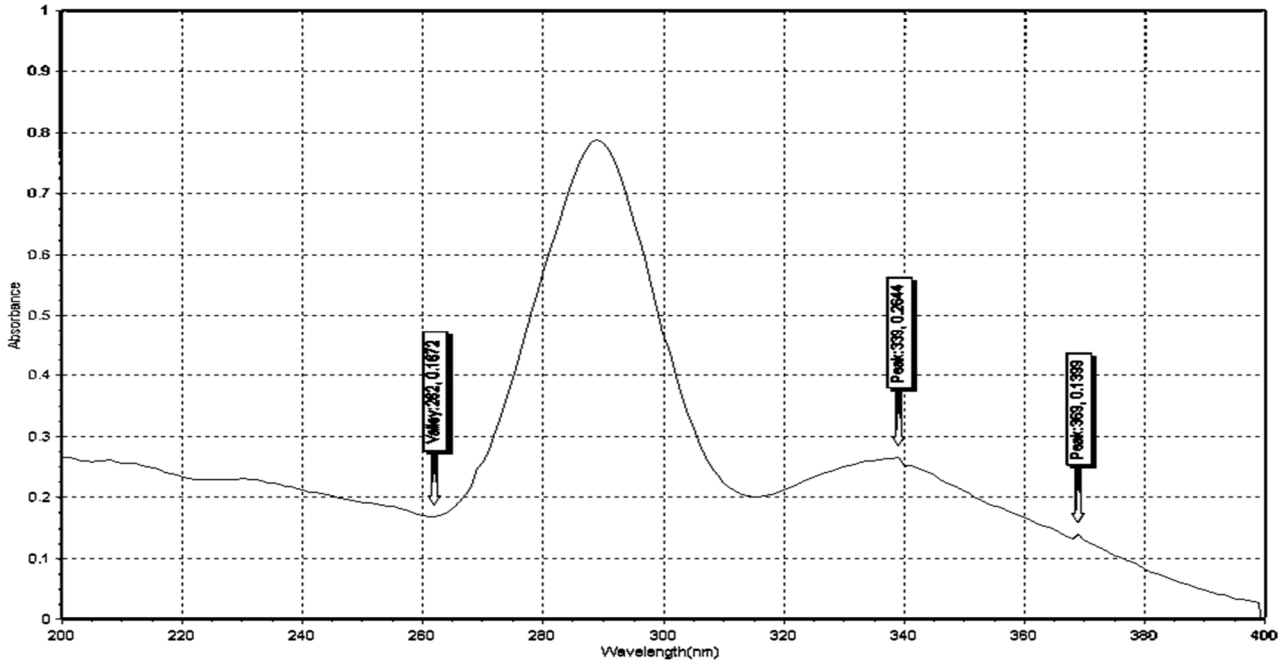

Fig. 1: Fig. shows the UV scan of mmoxifloxacin hydrochloride in distilled water

Standard calibration curve for Moxifloxacin hydrochloride in distilled water and STF

Standard calibration curve for Moxifloxacin hydrochloride in double distilled water (fig. 3) and STF (fig. 4) respectively. Equation of the line and R-square value was found to be.

Conc. = 11.3505 * A, R=0.9980 (for distilled water)

Conc. = 10.0489 * A, R=0.9995(for STF)

FTIR analysis

FTIR scan of the sample drug compared to standard confirmed the purity of sample that showed similar FTIR graph as standard Moxifloxacin hydrochloride (fig. 5). FTIR analysis compared to standard drug shows characteristic peaks in close agreement with standard, indicating Moxifloxacin hydrochloride with high purity (table 5).

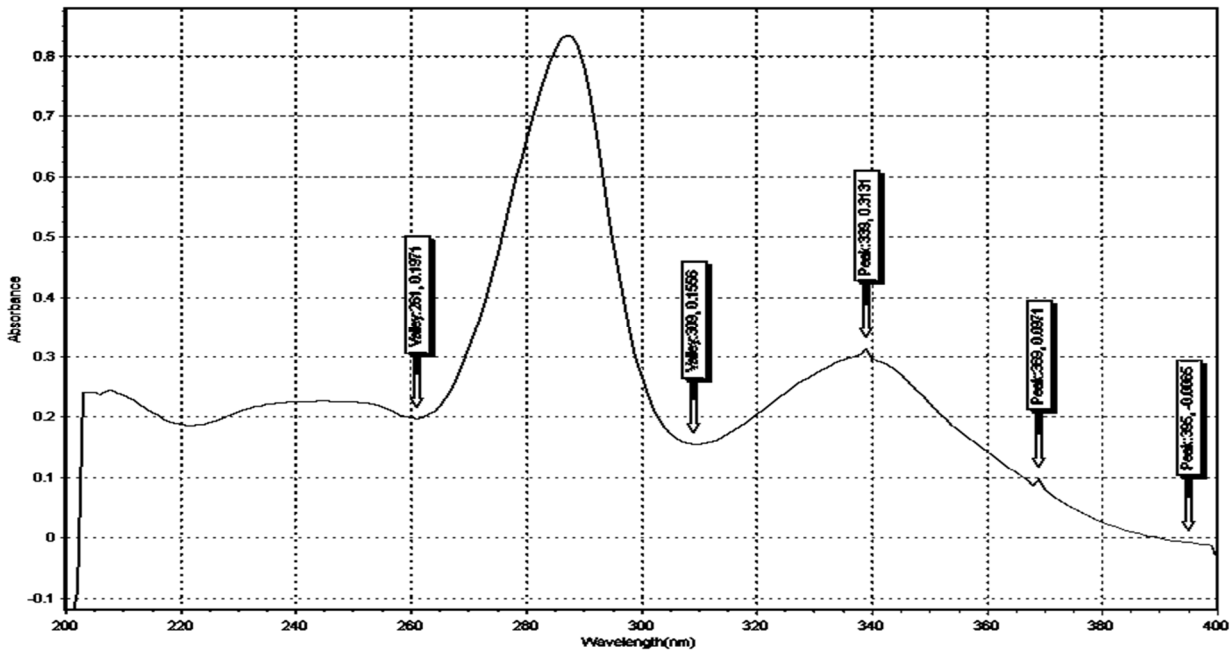

Fig. 2: Fig. shows the UV scan of Moxifloxacin hydrochloride in STF

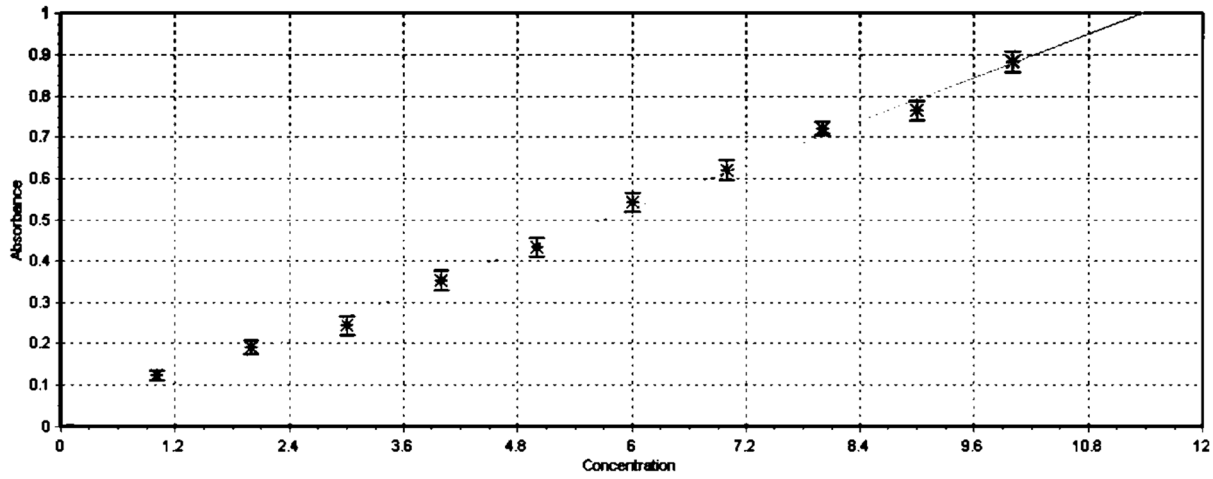

Fig. 3: Standard calibration curve for Moxifloxacin hydrochloride in double distilled water, *mean±SD (n=3)

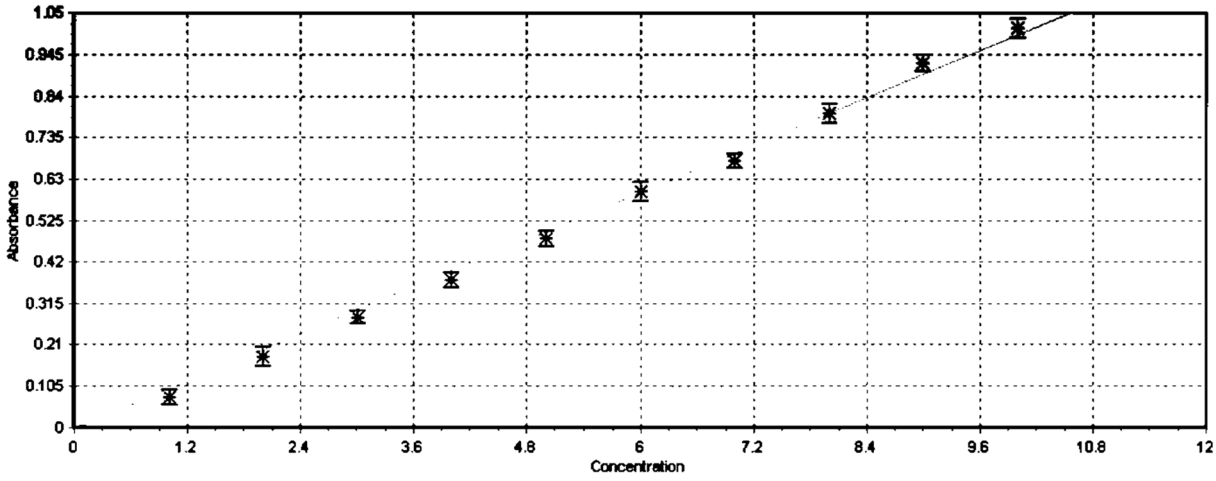

Fig. 4: Standard calibration curve for Moxifloxacin hydrochloride in STF, *mean±SD (n=3)

Table 5: Shows the characteristic peak of the standard drug compared to the test sample

Functional group	Expected frequency in pure drug (cm^{-1})	Observed frequency in the test sample (cm^{-1})
-F	1400-1000	1081
-C=O	1650-1700	1620
-NH stretching	3500-3100	3524
O-H (carboxylic group)	2700-2500	2523
Aromatic substitution	788	770

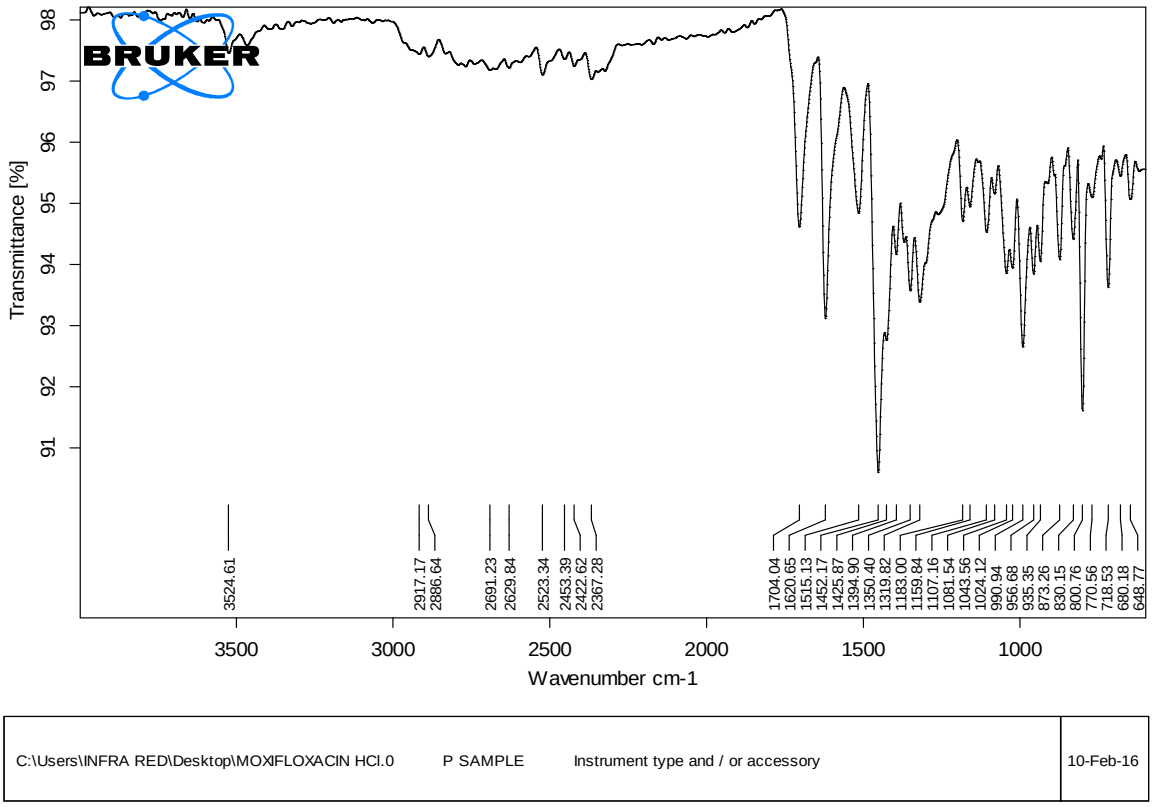

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Fig. 5: Fig. shows FTIR spectrum of sample Moxifloxacin hydrochloride

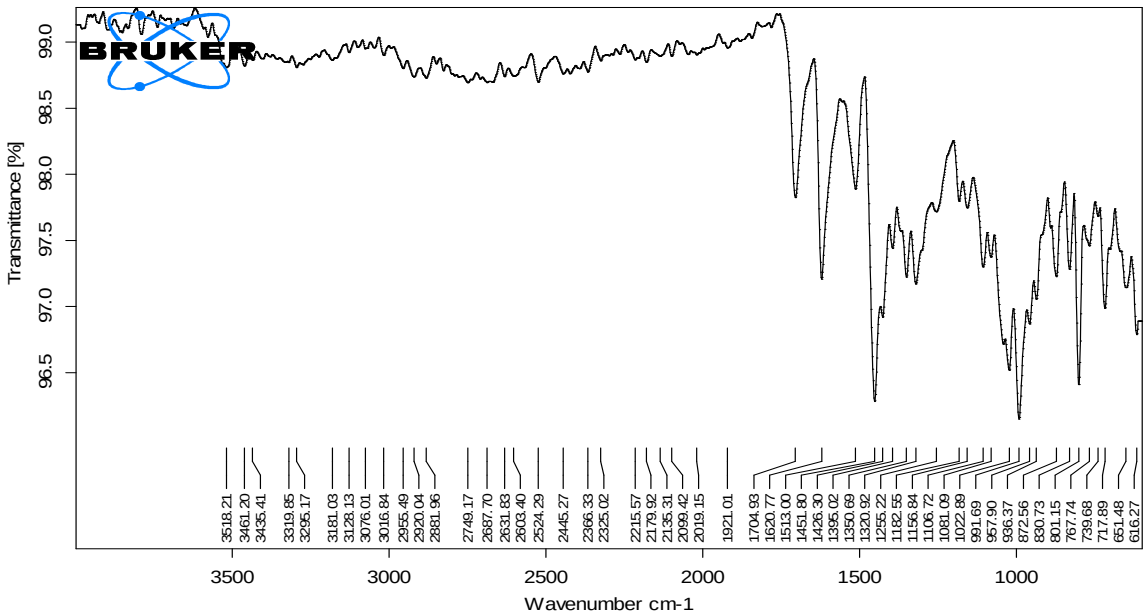

Fig. 6: Fig. shows FTIR spectrum of freshly prepared mixture of drug and excipients mixture

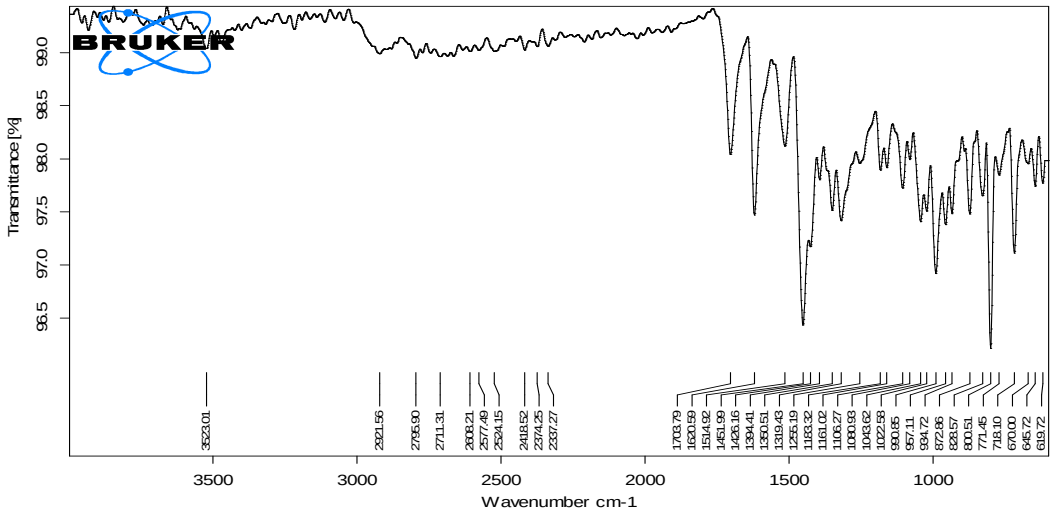

Fig. 7: Fig. shows FTIR spectrum of mixture of drug and excipients after completion of 14 d

Drug-excipient compatibility study

The FTIR graph of the formulation (fig. 7) after compatibility testing was compared with freshly prepared formulation (fig. 6) that showed no new peaks formation and indicates that there is no product degradation with time and compatibility of the ingredients of formulation with each other.

Screening of influential factors

The performance of *in-situ* gel was dependent on a number of formulation variables and process variables, which can influence each other and make it extremely difficult to study individual formulation by conventional approaches thus, requiring a greater amount of resources [18]. Hence, it is important for the individual or organization to implement FbD, to minimize the requirement.

In our study screening of various formulation and process variables significantly influencing, MRT, GT, and viscosity were done using Taguchi screening design for seven factors at two levels individually. Implementation of pre-optimization study helps in the identification of significant factors influencing response for further optimization study with maximum efficiency while utilizing minimum resources. Principally, screening relies upon the phenomenon of "sparsity effect" in which only few of the influential factors significantly affect the responses, thus, truly explaining a large proportion of experimental variation. The factors responsible for significant variation in responses are termed as influential factors, while others are termed as non-influential factors.

Model generation

The screening predicts considerable approximation of the combination of several factors and the lack of interaction. Thus, the first order Taguchi design was employed, and interaction terms were ignored. Taguchi design had an advantage of requiring the minimal number of runs (i.e. 12) for many independent variables (i.e. 6). The first order polynomial equation generated for response variables (i.e. MRT, GT, and viscosity) has been shown in Equation 0.

$y = \beta 0+\beta 1 \chi 1+\beta 2 \chi 2+\beta 3 \chi 3+\beta 4 \chi 4+\beta 5 \chi 5+\beta 6 \chi 6+€$. (0)

where,'€' represents the noise or error,

'χ' represent an independent variable,

'y' represents response and

'β' represents the coefficient.

The first-order mathematical model for each response variable has shown statistically significant ($p<0.005$), those coefficients were identified as significant through ANOVA and half normal plots were left in simplified equations. Variables having the coefficient with *p*-value were characterized as insignificant. The polynomial equations of every response variable had high values of r^2, indicating excellent fit to experimental data.

Model analysis

As shown by the fig. 8, the pluronic F-127 (B) concentration, shows a significant effect on the studied response MRT, GT, and viscosity. Gellan-gum (C) and Carbopol (D) concentration also showed the significant effect on response parameter, hence were selected as influential factor variables for the formulation of *in-situ* gel of Moxifloxacin hydrochloride.

Systemic optimization study

The objective of the pre-optimization study was to identify the significant factors influencing the formulation properties.

Calculation of coefficients

Independent variables including Pluronic F-127, Carbopol and Gellan-gum were evaluated for their final effect on MRT, GT and viscosity. Total 13 experiments were conducted, and the response data were generated in according to BBD (table 4).

Final data obtained from the experiments were well modelled by the independent variable linear function. Hence the first order polynomial equation was used for approximating the function.

$y = \beta 0+\beta 1 \chi 1+\beta 2 \chi 2+\beta 3 \chi 3+€$ (1)

'€' represents the noise or error,

'χ' represent an independent variable,

'y' represents response and

'β' represents the coefficient.

The value of responses y1 (MRT), y2 (GT) and y3 (viscosity) varies from 2.6 to 3.1 hr, 17 to 36 °C, and 210 to 485 m. Pa. S (table 6). The ratio of maximum to minimum response for response y1, y2, and y3 was 1.19, 2.11, and 2.3 respectively. Thus, power transformation was not applicable to the obtained values. The model section for response analysing was according to the sequence model sum of the square, lack of fit test and model summary statistics. The Prob>F value of $p<0.0001$, low standard deviation, high R-square value and lower predicted residual error sum of square (PRESS) value recommend selecting the linear model for analysing the responses.

Analysis of variance (ANOVA) was applicable to determine the effect of variables and their interaction. The regression model was used to develop contour plots of independent factors. The ANOVA table affirms the competence of the linear model (Model Prob>F is less

than 0.05). It also determines the powerful factors that affect the response y1, y2 and y3 of the formulation. For MRT, the concentration of Gellan-gum played a significant role, followed by other 2 factors.

Moreover, for GT, the concentration of polymer Pluronic was determined as the significant model term, whereas for viscosity concentration of Gellan-gum was found to be significant compared to a recent investigation.

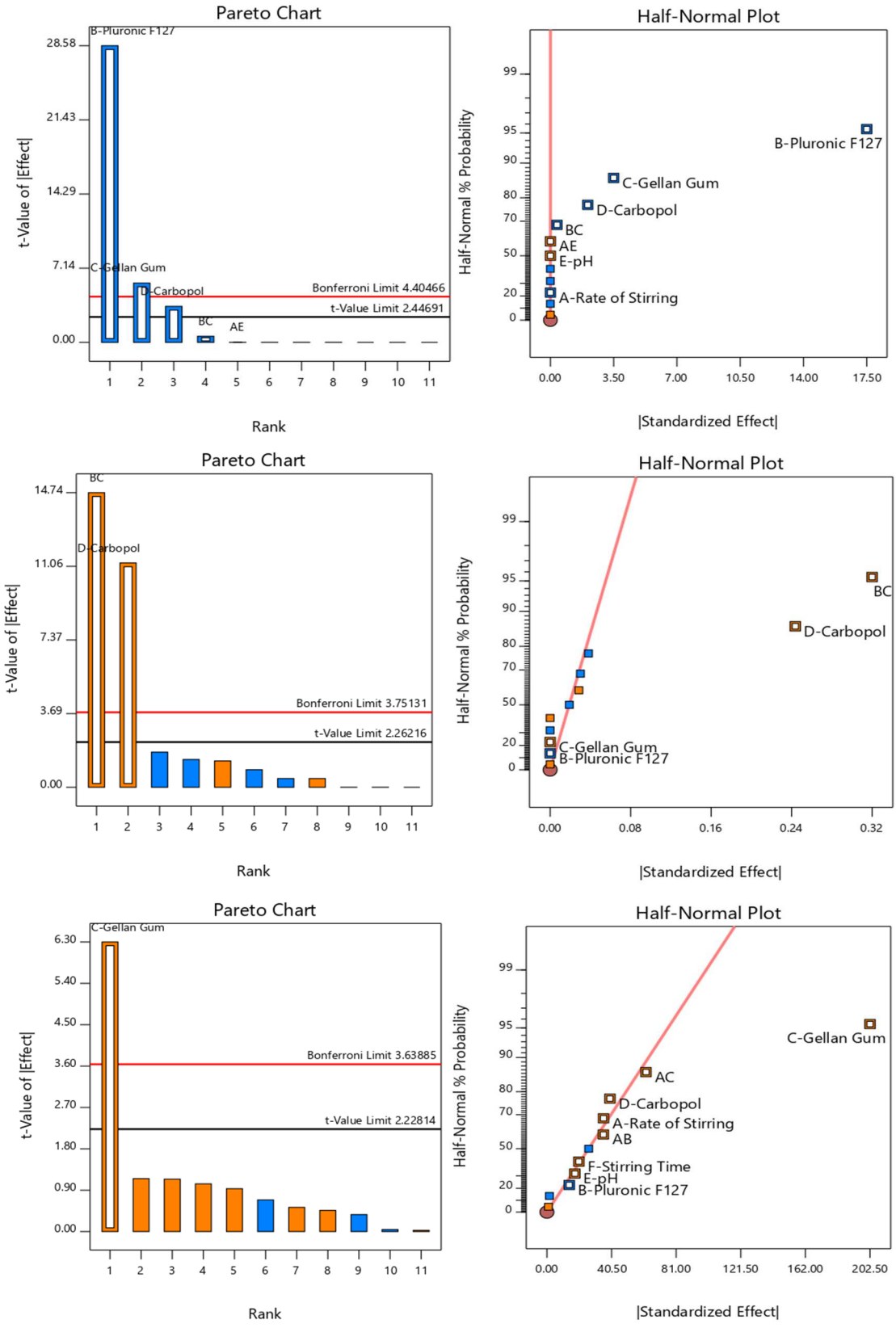

Fig. 8: Pareto and a half-normal chart showing the effect of (B) Pluronic F-127, (C) Gellan-gum, and (D) Carbopol on the response variables studied for *in-situ* gel

Table 6: Composition of various factors used in *in-situ* gel for ocular preparation of Moxifloxacin hydrochloride and responses

Run	Factors			Responses		
	Pluronic *F-127*	Gellan-gum	Carbopol	MRT (h)	GT (°C)	Viscosity (mPa. S)
1	10	0.3	0.5	2.9±0.05	34±0.12	295±0.34
2	15	0.1	0.5	2.8±0.06	27±0.14±0.14	250±0.14
3	20	0.5	0.3	3.1±0.05	17±0.16	485±0.24
4	10	0.3	0.1	2.7±0.08	36±0.06	255±0.14
5	15	0.1	0.1	2.6±0.12	29±0.14	210±0.14
6	10	0.1	0.3	2.6±0.08	36±0.08	225±0.24
7	15	0.5	0.5	3.1±0.12	24±0.19	460±0.22
8	10	0.5	0.3	2.9±0.14	33±0.22	475±0.14
9	20	0.3	0.1	2.9±0.12.12	20±0.14	265±0.14
10	20	0.3	0.5	3.1±0.13	18±0.14	307±0.24
11	20	0.1	0.3	2.8±0.08	20±0.18	235±0.14
12	15	0.3	0.3	2.9±0.12+0.12	27±0.24	280±0.14
13	15	0.5	0.1	2.9±0.22	26±0.14	460±0.14

All readings were taken in triplicate (n=3), (mean±std. deviation)

Table 7: ANOVA response for the surface model of MRT, GT and viscosity

Source	Sum of squares			df			F-value			Prob>F P-value		
	y_1	y_2	y_3	y_1	y_2	y_3	y_1	y_2	y_3	y_1	y_2	y_3
Model	0.34	538.00	1.650E+005	3	3	9	132.60	2098.20	130.63	<0.0001	<0.0001	0.0010
A	0.080	512.00	220.50	1	1	1	93.60	5990.40	1.57	<0.0001	<0.0001	0.2988
B	0.18	18.00	1.152E+005	1	1	1	210.60	210.60	820.90	<0.0001	<0.0001	<0.0001
C	0.080	8.00	34060.50	1	1	1	93.60	93.60	242.71	<0.0001	<0.0001	0.0006
Residual	7.692E-003	0.77	421.00	9	9	3	-	-	-	-	-	-
Cor total	0.35	538.77	1.654E+005	12	12	12	-	-	-	-	-	-

Optimization of formulation

Details of ANOVA of response y1, y2 and y3 as described in table 7.

The eventual mathematical model determined by software design-expert was demonstrated in equation 2, 3 and 4.

y1 (MRT) =+2.194+0.020 ∗ A+0.750 ∗ B+0.50∗ C. (2)

y2 (GT) =+54.442–1.600∗ A–7.500∗ B–5.000∗C. (3)

y3 (Viscosity) =+234.625–5.400∗ A–371.250∗ B+215.00∗ C. (4)

The positive sign represents a synergistic effect, whereas a negative sign represents an antagonistic effect.

In case of the y1 positive coefficient of A in the model refers to an increase in MRT at higher concentration of Pluronic F-127. Similarly, the positive coefficient of B and C indicated the increase in MRT with increasing other factors (Pluronic, Carbopol and Gellan-gum concentration). For y2, the negative coefficient of A, B, C referred to decrease in GT as an increase in the concentration of other factors. Whereas for the y3 negative coefficient of A and B referred to decrease in viscosity as the concentration of these factors increases, and positive coefficient represented the increase in the viscosity with an increase in the concentration of factor C.

Optimization of formulation using a numerical optimization method

Optimization of the formulation was performed to determine the levels of factors A, B, C where yield response of y1 was 2.6 to 3.1 hr (target 2.85), y2 was 17 to 36 (target 35) and y3 was 210 to 560 m. Pa. S (goal: minimize). This model predicted y1, y2 and y3 in the required range at A, B, and C values of 11.50 (g), 0.32 (g), 0.3 (g) respectively for a batch size of 200 g. Based on these values, three different batches of the *in-situ* gelling preparation were prepared and found that the obtained values were the very close agreement to the predicted values, which establishes the reliability of the optimization process. In fig. 9 the overlay plot has shown the optimised formulation as suggested by design expert software for desired range response.

Finally, the optimised concentration of variable factors was found to be, pluronic (5.75% w/v), gellan-gum (0.16% w/v), carbopol (0.15% w/v) and the final formulation is presented in table 8.

Table 8: Final optimised formulation details

S. No.	Ingredients	Quantity (g)
1	Moxifloxacin hydrochloride	0.5
2	Pluronic *F-127*	5.75
3	Gellan-gum	0.16
4	Carbopol	0.15
5	Benzalkonium chloride	0.006
6	Distilled water	Quantity sufficient for 100 g

Evaluation of systematically optimised formulation

Physical appearance

The prepared *in-situ* gel formulation was pale yellow, with no odour, homogeneous, consistent and non-gritty in nature.

Drug content

Drug content of the formulation was determined by UV method. Each 100 g of *in-situ* gel contains 0.497 g of moxifloxacin hydrochloride which indicates the percentage of drug content to be 98.29+0.43. (n=3) that complies with the official standard of 95-105%.

Drug release (*in vitro* release study)

In vitro release studies were done to evaluate the amount of drug released in STF. Periodically samples were withdrawn from the modified dissolution apparatus and analysed by UV-spectrophotometer at 287 nm. The percentage cumulative release of drug was shown in fig. 10.

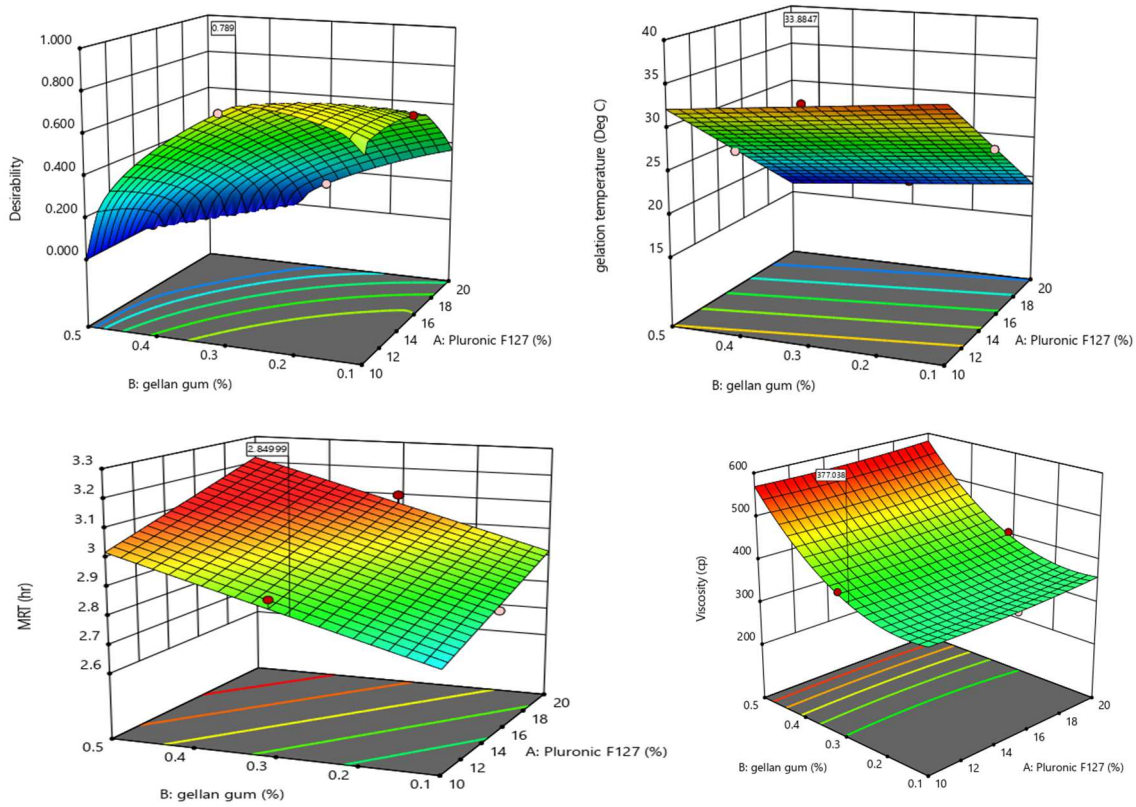

Fig. 9: 3-D surface plot for optimised parameter of *in-situ* gelling preparation. A. represent the 3-D graph of desirability, B. represents the 3-D surface plot of GT. C. represents the MRT, and D represents the viscosity 3-D surface plots. (To check concentrations of polymers on axis)

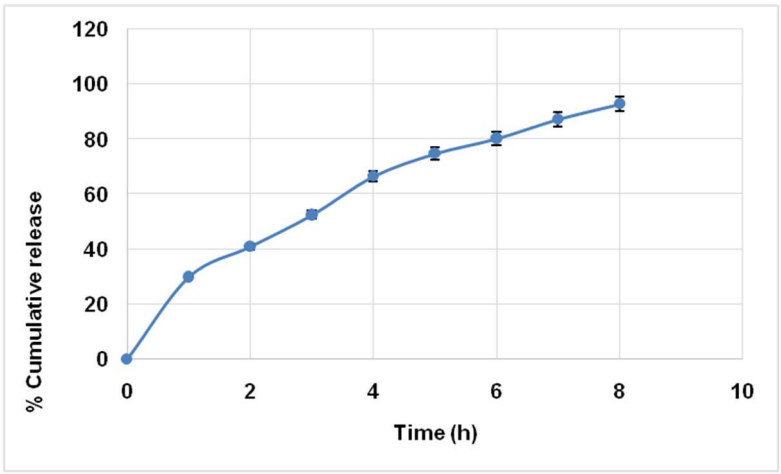

Fig. 10: *In vitro* release profile of Moxifloxacin hydrochloride from optimised formulation of *in-situ* gelling system, *mean±SD (n=3)

Release kinetics

The results of *in vitro* release profile obtained for all the formulations were plotted in kinetic models as follows:

a) Cumulative percent drug released versus time (zero order kinetic model).

b) Log cumulative percent drug remaining to be absorbed versus time (first-order model).

c) Amount of drug release or cumulative amount of drug release versus square root of time (Higuchi model).

d) Log Mt/M∞ versus log time (Korsmeyer's pepas model).

The kinetics of drug release can be estimated by comparing R2 values from graphs. R2 value is higher for in-situ gel optimised batch (R2 value: 0.9941) for higuchi model (table 9). Hence in-situ gel follows sustain release kinetics. The mechanism of drug release of the final formulation was determined by comparing the slope values of log CR v/s log time (pepas plot) with the standard values of Korsmeyer's pepas model. Slope of pepas plot was found to be 1.3567 for an in-situ gel which was greater than 1. This indicates that the mechanism of drug release to be super case-II transport [19].

Table 9: Release kinetics for optimised formulation of in-situ gel

Formulation	Zero order		First order		Higuchi model		Korsmeyer's and pepas model		Best fit model
	Slope	R^2	Slope	R^2	Slope	R^2	Slope	R^2	
Test	10.744	0.9361	0.1668	0.5456	33.752	0.9941	1.3567	0.5704	Higuchi Model

Antimicrobial activity

Antibacterial activity of optimised formulation was compared against the marketed formulation. Table 10 showed the ZOI obtained by the prepared formulation of Moxifloxacin hydrochloride as compared to the marketed formulation. The higher ZOI obtained by synthesized formulation indicate the potency of formulation that can relate the higher viscosity of formulation which results in slow and prolong the release of drug from the formulation [14].

Table 10: Anti-bacterial potency of synthesized and marketed formulation

Formulation	Zone of inhibition (diameter in mm)	
	Pseudomonas aueroginosa	***Staphylococcus aureus***
Selected formulation	31.4±0.8	31.1±0.7
Marketed formulation	31.3±1.4	30.2±1.2

*All values were represented as mean±SD (n=3).

Sterility testing

No turbidity was observed in either of the media inoculated with the sterile preparations, indicating no microbial growth in the samples incubated for more than 14 d. This confirms that the prepared formulation passed the sterility test.

Accelerated stability study

Accelerated stability studies of the optimised formulation showed no significant changes in the visual appearance, pH, gelling capacity, drug content, and *in vitro* drug release which confirm the stability of the optimised formulation for a long duration.

Isotonicity evaluation

In isotonicity evaluation, the measure of tonicity of the formulation was done by observing the structural alteration in blood cells. It is the amount of osmotic pressure exerted by two different solutions separated by the semi-permeable membrane. It is extremely important for the ophthalmic formulation to be isotonic because non-isotonic formulations can cause irritation in the eyes and may cause excessive tearing.

The isotonicity of the formulation was evaluated by comparing against 0.9% NaCl as shown in fig. 11 and 12 respectively. The formulation didn't rupture the red blood cells and was found to be isotonic.

Ocular irritancy test

An equal amount of optimised formulation and standard marketed formulation has shown no ocular irritancy on rabbit eyes (New Zealand White) after exposure of consecutive 14 d duration. No watering from eyes, mucosal discharge and swelling were found during the specified period. This implies the biocompatibility and non-irritancy of optimised formulation in the biological system [20].

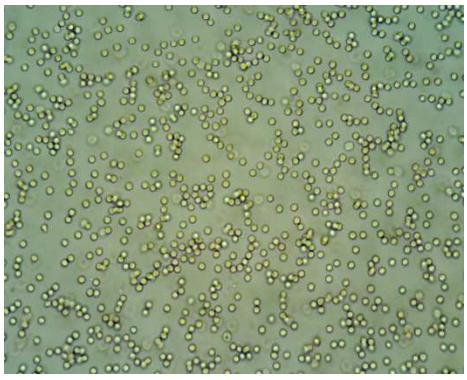

Fig. 11: Blood cells exposed with 0.9% NaCl

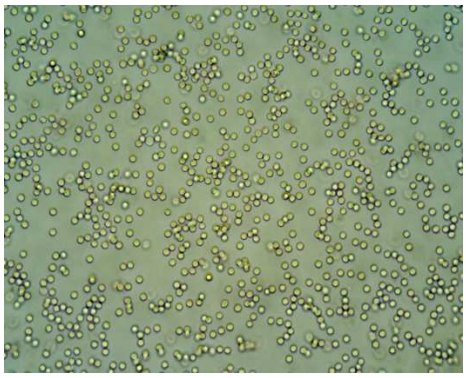

Fig. 12: Blood cells exposed with the formulation

CONCLUSION

Considering the prescribing trends in ocular infections and need for advanced drug delivery which could decrease the precorneal elimination of instilled drug and loss due to nasolacrimal drainage. Moxifloxacin hydrochloride *in-situ* gelling formulations were developed and analysed in the present study. As desired the prepared formulation was liquid at non-physiological conditions and was transformed to gel form under physiological conditions (pH 7.4 and 37 °C). Preformulation studies established the authenticity of the drug sample and its compatibility with the polymers used in the formulation. Taguchi method was used for pre-optimization to determine critical influential factors for the formulation. Optimization of the *in-situ* gelling formulation using influential factors Pluronic F-127, Carbopol and Gellan-gum was further done by BBD. A complex process since it involves many variables, which affect the characteristics of the final formulation. The concentration of Pluronic F-127, Carbopol, and Gellan-gum, play a critical role to influence MRT, GT and viscosity of the final formulation. GT was more concerned with Pluronic concentration and the MRT and viscosity of the product were more dependable on the concentration of Carbopol and Gellan-gum. The supportive study including compatibility, stability study, antimicrobial assessment, isotonicity and ocular irritancy test confirmed the long durability with significant potency of optimised formulation. The finalized formulation is a possible substitution to traditional eye drop by its proficiency to increased and extended antibacterial potency.

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AUTHORS CONTRIBUTIONS

All the author have contributed equally

CONFLICT OF INTERESTS

The authors declare no conflict of interest

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