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Concomitant delivery of Ofloxacin and Diclofenac sodium via pH triggered *in-situ* ophthalmic gel: *in-vitro* and *in-vivo* consideration

Vibhas Kumar, Anubhav Anand*, Sandeep K. Singh and Koshy M Kymonil

Department of Pharmaceutics, Faculty of Pharmacy, Babu Banarasi Das National Institute of Technology & Management, Lucknow (227105), Uttar Pradesh, India.

ABSTRACT

The aim of present study was to develop and evaluate in-situ ophthalmic gel system based on sol-to-gel transition for concomitant delivery of ofloxacin (OFL) and diclofenac sodium (DS) to facilitate sustained release to improveocular bioavailability and therapeutic response exhibited by conventional formulations.A 3^2 full factorial design was adopted to optimize the experimental conditions for development of formulation using Carbopol 940 and HPMC as independent variable. Developed formulations were characterized in terms of physical appearance, pH, viscosity, drug content, gelling capacity, and in-vitro drug release. Further, ex-vivo permeation and in-vivo irritation studies were conducted. The pH, viscosity and drug content of all developed formulations were found in the range of 5.2±0.02-6.5±0.04, 12±1.26-114±2.05cps and 88.32±2.26-98.46±1.36% respectively. The gel p rovided sustained drug release over an 8 h period. Furthermore, in-vivo ocular irritation study and antimicrobial efficiency of optimized formulation suggest that developed formulation was therapeutically efficacious and non-irritant. In conclusion, developed in-situ gelling system would be used to enhance ocular bioavailability of the loaded drugs and could reduce the frequency of instillation thereby improving patient compliance.

***Key Words*:** *In-situ* gelation; Ofloxacin; Diclofenac Sodium; Carbopol 934; HPMC

INTRODUCTION

Ophthalmic drug delivery is one of the most fascinating and challenging endeavour facing by the formulation scientists[1].The major hurdles of ophthalmic drug delivery system (ODDS) is associated with low bioavailability within eye cavity due to rapid tear turnover, lacrimal drainage, reflex blinking and drug dilution by tears. In clinical practice, the anterior segment of the eye cavity can be treated with many conventional ophthalmic preparations such as solutions (eye drops), suspensions, gels and ointments which show poor bioavailability and therapeutic response[2]. The most common method of ODDS is the instillation of eye drops into the lower *cul-de-sac.* However, eye drops are rapidly drained away from the ocular cavity due to tear flow when instilled into the *cul-de-sac*. Only a small amount (<1%) is available for its therapeutic effect resulting in frequent dosing. It may also cause systemic effect due to nasolacrimal drainage[3]. Alternatively, disadvantages of ointments include interference with vision and precorneal disappearance of drug. Furthermore, erodible and non-erodible inserts have been confirmed long duration of action however they are not well tolerated by patients. Hence, inserts are not supposed as desirable next-generation topical ocular drug delivery system[4].

To optimize topical ocular drug delivery system, prolonged contact time with the corneal surface and better penetration through cornea is necessary[5][6][7].An *in-situ* gel system utilizesadvantages of both solutions and gels such as easy administration, accurate dosing and prolonged residence time to improved efficacy. *In-situ* gelling system involves phase transition of installed liquid to the viscoelastic gel or solid phase once administered in the *cul-de-sac* of the eye. *In-situ* gelling systems consist of viscous polymer-based liquid formulation that exhibit sol-to-gel phase transition on the ocular surface due to change in a specific physicochemical parameter (pH or temperature)[8][6, 9].Depending on the method employed to cause sol-to-gel phase transition on the eye surface, the following three types of systems are recognized: pH triggered systems (e.g. cellulose acetate hydrogen phthalate latex[10][11], temperature-dependent systems (e.g. pluronics [12] and tetronics[13][14] and ion-activated systems(e.g. GelriteE and gellan) [15][16][17].

DS (0.1%) is one of non-steroidal antiinflammatory drug indicated for the treatment of postoperative ophthalmic inflammation as well as for the temporary relief of pain in patients undergoing corneal refractive surgery and in conjunctivitis. The ophthalmic solution of DS should be applied to the affected eye, with 3 to 4 times daily and continuing throughout the first 2 wk of the postoperative period after cataract surgery [18]. However, OFL ophthalmic solution (0.3%) is indicated for the treatment of bacterial infections of the eye including conjunctivitis and corneal ulcers. Following a loading dose of one to two drops in the affected eye, every 2 to 4 h, the drug is instilled one to two drops 4 times daily[8]. Thus, minimized frequency of administration of both drugs, once or twice a day, is crucial to increase patient compliance.

An important strategy for improved treatment of ophthalmic diseases could be combination therapy. Combination therapy has become a common practice used effectively to treat bacterial, fungal, proliferative and inflammatory eye diseases and vascular proliferation. Furthermore, combination therapy also encompasses the synergistic effect of electromagnetic radiation and medications. The objective of present study was deals with development and evaluation of pH triggered *in-situ* gel forming solution containing OFL and DS for sustained ophthalmic delivery. This system will ensure enhanced drug retention time leading to improved absorption of drug across membrane, improve bioavailability and dose reduction.

MATERIALS AND METHODS

Materials

Ofloxacin (OFL) and Diclofenac sodium (DS) were procured as a gift sample from Moraceae Pharmaceutical (P) Ltd. Lucknow, India and Kairav Chemical Ltd. Ahmedabad, India, respectively. Hydroxypropylmethyl cellulose (HPMC), Tween 20 and Benzalkonium chloride (BKC) were purchased from SD Fine Chemicals Limited, Mumbai, India and Carbopol 940 was procured from Himedia Laboratories Pvt. Limited, Mumbai, India. All other chemicals/reagents/solvents used were of analytical grade, available commercially and used as such without further processing. Triple distilled Water (TDW) was obtained from Milli-Q water purification system, Millipore, Synergy, Bangalore, India.

Table 1:Composition of DS and OFL*in-situ* gelling system prepared as per 3^2 factorialdesigns.

Ingredients (gm)	B1	B2	B3	B4	B5	B6	B7	B8	B9
DS (%w/v)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
OFL (%w/v)	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Carbopol 934 (%w/v)	0.2	0.2	0.2	0.3	0.3	0.3	0.5	0.5	0.5
HPMC 15cps (%w/v)	0.5	1.0	1.5	0.5	1.0	1.5	0.5	1.0	1.5
Citric acid (gm)	0.407	0.407	0.407	0.407	0.407	0.407	0.407	0.407	0.407
Disodium hydrogen phosphate (gm)	1.125	1.125	1.125	1.125	1.125	1.125	1.125	1.125	1.125
Tween 20 (ml)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Benzalkonium chloride (%v/v)	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Distilled water (q.s.) (ml)	100	100	100	100	100	100	100	100	100

Experimental design

A 3^2 full factorial design was used where in 2 factors were evaluated, each at 3 levels and experimental trials were performed at all 9 possible combinations. The amount of Carbopol 934 (X1) and amount of HPMC (15cps) (X2) were selected as independent variables. Furthermore, viscosity, gelation capacity and drug content were selected as dependent variables. The experimental design is given in Table 1.

Preparation of Formulations

The formulations were prepared by using OFL (0.3%) and DS (0.1%) as model drug with benzalkonium chloride (BKC), carbopol 934P and HPMC in different concentrations ratio. Solution 1 was prepared by dissolving appropriate quantity of HPMC (0.1 g)in 25 mL of phosphate buffer solution(PBS, pH 6.0).Weight quantity of carbopol 934 was sprinkled over this solution 1and allowed to hydrate overnight. Tween 20 and DS were added to above solution 1 with continuous stirring. In case of solution 2, OFL was dissolved in small quantity of acidic medium (glacial acetic acid) followed by addition of BKC and distilled water. Afterwards, solution 2 was added to the carbopol–HPMC-DS containing solution 1 under continuous stirring until a uniform solution was obtained. Finally, purified distilled water was added to make up the volume to 100 mL. The developed formulations were sterilized by filtering through 0.22 µm membrane filter. Ingredients of different formulations(F1 to F9) are represented in Table 1.

***In-vitro* evaluation of formulations**

Physical appearance

The physical appearance of developed formulations was visually observed for their color, homogeneity, consistency, spreadability and phase separation[19, 20].

Determination of pH

The pH of a product may alter on storage due to chemical change. The pH of each formulation was recorded using a digital pH meter (pH Meter, E I Instruments, Model 111E).The pH meter was calibrated before use with buffer solutions (pH 4 and pH 7).Weight quantity of gel (0.3g) was dissolved in 100mL distilled water and the pH was measured in triplicate [21].The pH of all the formulations was recorded immediately after preparation as well as after 24 h of storage at room temperature.

Rheological studies

Viscosity of the instilled formulation is an important factor in determining residence time of drug in the eye. The viscosity of various formulations was determined by using a Brookfield digital viscometer (Model-RVT) with spindle number 3 and angular velocity run from 10-100 rpm. The developed formulations (F1 to F9) were poured into the small sample adaptor of the Brookfield viscometer and the angular velocity increased gradually from 5 to 100 rpm. The hierarchy of the angular velocity was reversed and average of the two readings was used to calculate the viscosity of developed formulations. [20]

Gelation capacity

In-vitro gelling capacity was determined by visual method. For assessing gelation capacity, 100 µL of prepared formulations(F1 to F9) was transferred in a glass test tube containing 2 mL of simulated tears fluid (STF)freshly prepared (pH 7.2)and equilibrated at 37±1^{o}Ctemperature. The tubes were gently shaken for manually simulation of eye blinking and to observe gel dissolution. The gel formation was visually assessed. Elapse time in sol to gel and gel to sol was noted. The gelling capacity of solution was evaluated on the basis of stiffness of formed gel and time period for which the formed gel remains as such. The composition of STF used for this study was as follow: sodium chloride (0.670g), sodium bicarbonate (0.200g), calcium chloride.2H_2O (0.008g), and purified water q.s. (100g). The in-vitro gelling capacity was graded in three categories on the basis of gelation time and time period for which the formed gel remains as such. The lowest scores (+) were assigned to those formulations in which the phase transition occurred only after 60–90 s and the formed gels collapsed within 1–2 h. The highest scores (+++) were assigned to those products in which the phase transition commenced within 60–90 s and the gels so formed were stable for about 7–8 h. The moderate scores (++) were assigned to the products, which formed the gel in 60–90 s but failed to maintain gel structure for more than 4 h[22].

Drug content

The concentration of OFL and DS present in ophthalmic gel was determined by simultaneous method as reported in **Kumar *et al.*, 2011** with slight modification. The weight quantity of formulation was diluted with 100 mL PBS (pH 6.8) and resulting solutions were filtered through 0.45 µm membrane filters. The samples were suitably diluted and analyzed spectrophotometrically. A simultaneous estimation method for OFL and DF at 287 nm and 277 nm, respectively was employed using UV-visible spectrophotometer (UV-1700 Pharmaspec, Shimadzu) [23]

In-vitro release study

In-vitro release studies can help in investigating mechanisms behind skin permeation of the drug. Modified K-C diffusion cell was used to determine *in-vitro* drug release profile of OFL and DS concurrentlyin15mL of STF at pH 7.4 which was added to the acceptor chamber. The temperature within the chamber was maintained at 37±0.5^{o}C. The dissolution medium was stirred with magnetic bead at 50 rpm using magnetic stirrer, to prevent the formation of concentrated drug solution layer below the standard membrane. At predetermined time intervals, 1mL of sample was withdrawn from the acceptor compartment and replaced the sample volume with TDW. The samples were diluted with STF pH 7.4, filtered and the amount of drug release was analyzed as mention above[10].

Kinetics of Drug Release

Zero order release (Eq. (1)), Higuchi (Eq. (2)), and first order (Eq. (3)), as well as Korsmeyer-Peppas model (Eq. (4)) release kinetic models were applied to process the in-vitro release data of optimized formulation (F5) to find mechanism behind the drug release form the developed system.

$$Q = k_1 t \quad \text{…….....eq.1}$$
$$Q = k_2 (t)^{0.5} \text{…….....eq.2}$$
$$Q = 100(1 - e^{-k3t}) \text{ …….eq.3}$$
$$M_t/M_\alpha = K_p t^n \text{ ……..eq.4}$$

where Q is the percentage release at time t. k_1, k_2 and k_3 are the rate constants of zero order, Higuchi, and first order model, respectively. Whereas, M_t/M_α is the fraction of the drug release at time t, K_p is the rate constant and n is the release exponent. The n value is used to characterize different release mechanisms and is calculated from the slope of the plot of log of fraction of drug released (M_t / M_α) vs log of time (t)[24].

Ex-vivo drug permeation study

Goat cornea was used to find the permeation across the corneal membrane which was arranged from local slotter house. Isolated corneas were mounted by sandwiching the surrounding scleral tissue between clamped donor and receptor compartments of a modified K-C diffusion cell in such a way that its epithelial surface faced the donor compartment. The receptor compartment was filled with 10 mL of freshly prepared STF pH 7.4, after expelling all the air bubbles by inverting the diffusion cell and then allowing the bubbles to travel through the sampling port. An aliquot (1 mL) of optimized formulation (F5) was placed on the cornea and the opening of the donor compartment was sealed with a glass cover slip, while the receptor fluid was maintained at 37ºC ± 1ºC with constant stirring using a Teflon-coated magnetic bead. The samples were withdrawn at predetermined time interval upto 8h and analyzed spectrophotometrically as mention above. The receptor phase was replenished with an equal volume of STF pH 7.4 at each time interval. The amount of drug permeated was determined by the following relationship

$$\text{Drug permeation (\%)} = \frac{\text{Amount of drug permeated in receptor}}{\text{Initial amount of drug in donor}} \times 100 \qquad (1)$$

Sterility test

The optimized formulation was evaluated for sterility test using direct inoculation method. Test formulation F5 and standard OFL solution (S) (each, 2 mL) was aseptically transferred to fluid thioglycolate medium (20 ml) and soyabean-casein digest medium (20 mL) separately, which were used as culture media. The inoculated media were incubated for 14 days at 30±5°C for fluid thioglycolate medium and at 20±5°C for soyabean-casein digest medium. The media was observed for microbial growth.

Antimicrobial efficiency study

Antimicrobial efficiency studies were carried out to ascertain the biological activity of sol-to-gel systems against micro-organisms by the agar disk diffusion test using the cup-plate method. Muller Hinton agar was employed as culture medium and PBS was used as solvent control for antimicrobial activity. A 100 µL of sterile solution of OFL in PBS of pH 6.4(standard solutions) and the developed formulation diluted suitably with PBS(test solutions)were poured into separate wells (4 mm diameter) bored into sterile nutrient agar previously seeded with test organism *Staphylococcus aureus*. After allowing diffusion of the solutions for 2 h, the agar plates were incubated at 37±0.5ºC for 24 h. The entire operation except the incubation was carried out aseptically in a laminar flow unit. Each solution was tested in duplicate. Both positive and negative controls were maintained through the study. The zone of inhibition (ZOI) was measured and compared with standard.

In-vivo ocular irritation study

The eye irritation study was performed according to Draize technique to find the irritation potential of optimized formulation on female albino rabbits. Six albino rabbits of uniform body weight (2-3 kg) with no prior drug treatment were maintained on normal diet.The eyes were marked as test (Right eye) and control (Left eye). The control group received no sample and the test eye received the 100 µL of sterile formulation F5 and marketed DS eye drop (Voveron® by Novartis).During the experiments, the rabbits were placed in restraining boxes.Sterile formulations were instilled twice a day up to a period of 7 days and the rabbits were observed periodically for redness, swelling and watering of the eye. The eyes were observed for ocular irritancy (includes the macroscopic observation of cornea, iris, and conjunctiva). Evaluation was done as per *Draize's Scale of Weighted Scores for Grading the Severity of Ocular Lesions*.

RESULTS AND DISCUSSION

The formulation F5 was selected as optimized formulation on the basis of 3^2full factorial design and studied for performance characteristics and in vivo examination. The formulations were optimized on the basis of pH, viscosity and gelation capacity. An alternative pH responsive gel with different phase transition mechanisms for co-administration of two drugs was successfully formulated as an ophthalmic vehicle. Combination of carbopol 934P and HPMC was investigated as vehicle for ophthalmic drug delivery. Apart from in situ gelling capacity, both polymers have good mucoadhesive strength.

Physical examination

Formulations were examined against black and white background for transparency, smoothness and homogeneity in appearance. On the basis of physical appearance, formulations F1, F2, F3 and F9 were found to be transparent in nature, homogenous and easily spreadable while formulations F6, F7, F8 and F9 were found to be not suitable due to their cloudy appearance(**Table 2**). However, all the formulations were odourless and free from foreign suspended particles.

Determination of pH

The pH of all the formulations(F1 to F9) was found to be in the range 5.2±0.4-6.5±0.2which is considered acceptable to avoid the risk of irritation upon application in the eye.Optimized formulation F5 had a pH of 6.5±0.2, which might be the triggering factor for conversion of sol to gel state (**Table 2**). Generally, the ophthalmic solutions should have pH in the range of 4.5–11.0 for better ocular tolerance and patient compliance.

Table 2: Results of response variables for 3^2full factorial design

S.No.	Formulation Code	Variable Levels in Coded Form				Appearance	Viscosity of gel (cps) (20 rpm)	pH of solution (Y1)	Viscosity of sol (cps) (20 rpm) (Y2)	Gelling capacity (Y3)*	Drug content (%)
		X_1	Gelling agent (% w/v)	X_2	Viscosifying agent (% w/v)						
1	B1	-1	0.2	-1	0.5	Clear Transparent	750	6.2±0.9	12	-	90.46±3.16
2	B2	-1	0.2	0	1.0	Clear Transparent	860	6.3±0.2	14	-	88.32±2.64
3	B3	-1	0.2	+1	1.5	Clear Transparent	940	6.3±0.8	15	+	86.94±2.45
4	B4	0	0.3	-1	0.5	Clear Transparent	1375	6.4±0.3	28	++	90.31±1.98
5	**B5**	**0**	**0.3**	**0**	**1.0**	**Clear Transparent**	**1500**	**6.5±0.2**	**35**	**+++**	**98.46±1.26**
6	B6	0	0.3	+1	1.5	Cloudy	1675	6.4±0.2	48	++	93.72±2.28
7	B7	+1	0.5	-1	0.5	Cloudy	2834	5.4±0.4	75	+++	89.16±2.98
8	B8	+1	0.5	0	1.0	Cloudy	3765	5.3±0.2	88	++	90.21±2.88
9	B9	+1	0.5	+1	1.5	Cloudy	4056	5.2±0.4	114	++	95.41±2.53

**(−) - No gelation, (+) - gelation occurs within 90 sec. but dissolve within 1 hours, (++) - gelation occurs within 90 sec. but dissolve within 3 hours, (+++) gelation occurs within 90 sec. and the gel remain stable for about 7-8 h.*

Gelation capacity

The comparative scores for gelling capacity of all developed formulations (F1 to F9) are represented in **Table 2**. The formulations F1 and F2 showed no gelation when contacted with STF whereas formulation F3 showed gelation within 90 sec but dissolved within 1h. The formulations F4, F6, F8 and F9 showed gelation within 90 sec but

dissolved within 3h. The formulations F5 and F7 showed immediate gelation and remained for extended period (7 - 8h). However, the nature of the gel formed depended on the concentration of polymers used. The formation of instantaneous gels can be attributed to the buffering capacity of the STF. Based on *in-vitro* gelation capacity, formulations F5 and F7 shown satisfactory gelling property. The gel structure was stable for 7–8 h which suggests that it can prolong the therapeutic effect of drug by increasing corneal contact time[25].

Drug content

The amount of OFL as well as DS was determined at 287 and 277nm respectively using UV-visible spectrophotometer (1200, Shimadzu, Japan) as reported in **kumar *et al.*, 2011** with slight modification. Drug content of OFL and DS in all the formulations was between 86.94±2.45-98.46±1.26%. Furthermore, optimized formulation F5 showed maximum drug content (98.46±1.26%) compared with other formulations which might be due to high capacity of polymer to hold the drug (Table 2).

Rheological studies

The average viscosity of formulations from F1 to F9 at 20 rpm is shown in Table 2.On the basis of viscosity values, formulation F4, F5 and F6 were found within desired range. Viscosity of carbopol 934 based *insitu* gels was increased in proportion with viscosifying agent. The rheological profiles of *in-vitro* formed gel showed that formulations were shear thinning and an increase in shear stress was observed with increase in angular velocity (pseudo-plastic rheology) as shown in **Table 3**.Results obtained from the rheological study of prepared *in-situ* gelling system F1 to F9 revealed that the viscosity decreases as the angular velocity increases. Generally, viscosity values in the range of 15-50 cps for sol (or more than1000 cps for *in-situ* formed gel) significantly improve upon contact with eye. However, higher viscosity values offer no significant advantage and have a tendency to leave a noticeable residue on the lid margin.

Table 3: Rheological profiles of the developed *in-situ* gel formulations at various angular velocity.

Angular Velocity (rpm)	Viscosity of gel (cps)								
	Formulations code								
	F1	F2	F3	F4	F5	F6	F7	F8	F9
5	980	1280	1310	1545	1800	2038	4050	6285	7650
10	900	1058	1150	1410	1654	1862	3260	4965	6552
20	750	860	940	1375	1500	1675	2834	3765	4056
50	675	735	890	1084	1248	1472	1974	2540	3840
100	350	550	745	950	1095	1250	1580	2046	2750

In-vitro release study

Figure1 shows the cumulative amount of OFL and DS release as a function of time for optimized formulation F5. *In-vitro* release study was performed using STF; pH 7.4 as the dissolution medium. It was found that cumulative percent drug release was 96.2 ±2.06% and 96.8±3.86% for OFL and DS respectively after 8 h. The formulation F5 showed almost constant release for both the drugs. Although all formulations were tested for drug release study, only formulation F5 shows desired release pattern, i.e. drug release up to 8h. Standard solution of drug without polymers was also tested which showed complete drug release within 5 min of study (Data not shown). During *in-vitro* release studies, developed formulation (F5) showed initial burst release due to low viscosity of the formulations. As the diffusion study continued, there was an increase in the viscosity of the formulation and drug release was retarded, since the release of drug from gel is inversely proportional to the gel strength. Almost 50% drug was released within 2 h, but the release rate was gradually retarded up to ≥96% after 8 h **(Figure 1)**. The prolonged release of drug from formulation can be attributed to the slow diffusion of the drug through polymer matrix. Results clearly showed that the gels have ability to retain drug for prolonged period of time (8 h) and that premature drug release will not occur. In the *cul-de-sac*, the gels will probably undergo faster dissolution due to the shearing action of the eyelid and eyeball movement. It was also observed that the dissolution in the *cul-de-sac* will proceed more slowly than that seen in the *in-vitro* experiments, as the normal resident volume of the lacrimal fluid in the human eye is 7.5-10µL.

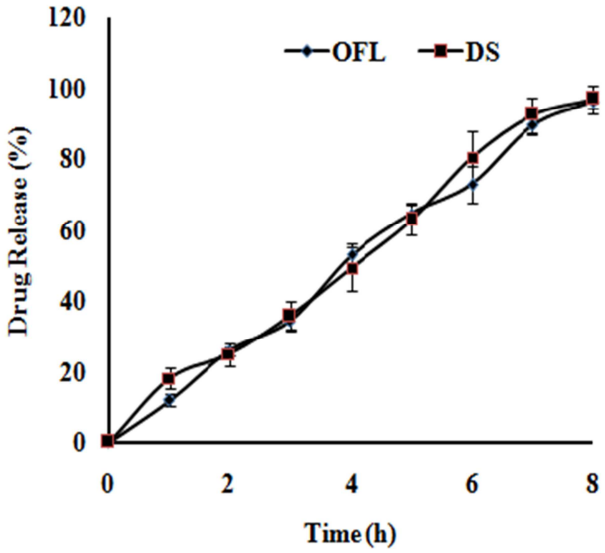

Figure: 1 *In-vitro* release profile of OFL and DS from developed formulation F5 in simulated tear flued (STF, pH7.4)

Table 4: Release kinetics of the developed optimized formulation F5 containing OFL and DS

Formulation Code	Zero Order		First Order		Higuchi Matrix		Korsmeyer-Peppas		Hixon-Crowell
B5(OFL)	K	R	K	R	K	R	K	n	R
	12.657	0.9931	0.1604	0.8675	48.347	0.9571	1.0222	0.9927	0.8945
B5(DS)	12.545	0.9967	0.1389	0.9001	48.739	0.9796	0.8777	0.9712	0.8831

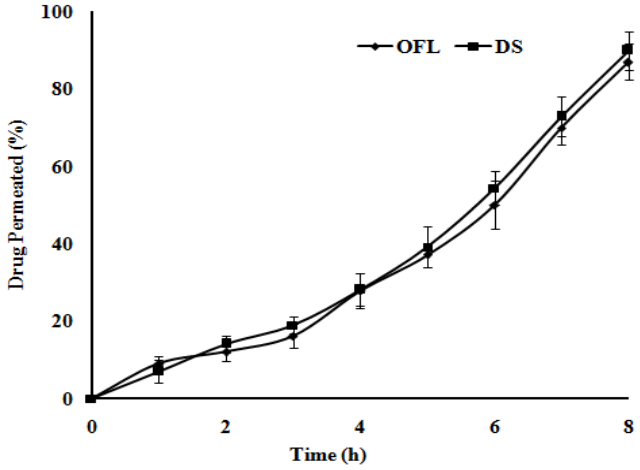

Figure: 2 *Ex-vivo* percentage permeations of OFL and DS from developed optimized formulation F5 using goat cornea

Kinetics of release

The *in-vitro* release profiles of DS and OLF from *in-situ* gel were fitted to various kinetic models in order to find out the mechanism of drug release. The interpretation of data as well as rate constants was calculated from the regression coefficients and slope of the respective plots**(Table 4)**. High correlation (R^2=0.9031) was observed in the Higuchi plot rather than first order (R2=0.3273) and zero order (R^2=0.6485) models. The drug release was proportional to square root of time, indicating that the mechanism of drug release from Carbopol 934 and HPMC 15cps based pH triggered *in-situ* gel was might be diffusion coupled with erosion controlled. The data obtained was also fit in Korsmeyer-Peppas model in order to find out n value, which describes the drug release mechanism. The n value (0.8029) obtained from Korsmeyer-Peppas was more than 0.5, which indicated that the mechanism of the drug release was Anomalous and Non Fickian diffusion controlled.

Ex-vivo drug permeation studies

Corneal permeation studies were performed using isolated goat's cornea on K-C diffusion cell using STF (pH 7.4) at 37ºC ±1ºC temperature. It was found that cumulative percent drug permeation was 86.96±4.64% and 89.97±4.92% for OFL and DS, respectively after 8 h from optimized formulation F5**(Figure 2)**.

Sterility test

The visual examination of the two media against dark and light background for 1W showed that there was no appearance of turbidity was found in tested formulation and standard solution as represented in **Figure 3** and hence no evidence of microbial growth was observed. Therefore, preparations were passed the test for sterility.

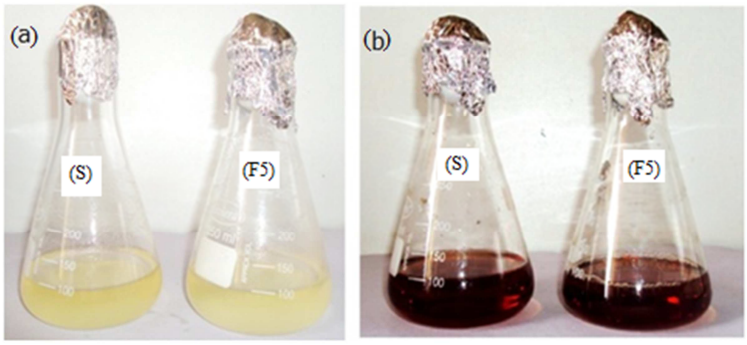

Figure: 3 Comparative sterility test of developed formulation F5 with standard Solution (S) in soybean casein media (a) and thioglycolate media (b)

Antimicrobial activity

The results of the antimicrobial efficiency of developed formulation F5 (test) and OFL solution (standard, 0.3%w/v), diluted with PBS; pH 6.4 are shown in **Figure4**. The results of the antimicrobial efficiency test revealed that there was no significant change in the antimicrobial activity of drug due to formulation ingredients and conditions, when compared with OFL standard solution. Antimicrobial efficacy of tested formulation was found to be increase with increased drug concentration. Developed formulation showed ≥86% antimicrobial efficacy against *Staphylococcus aureus* compared with standard OFL solution.

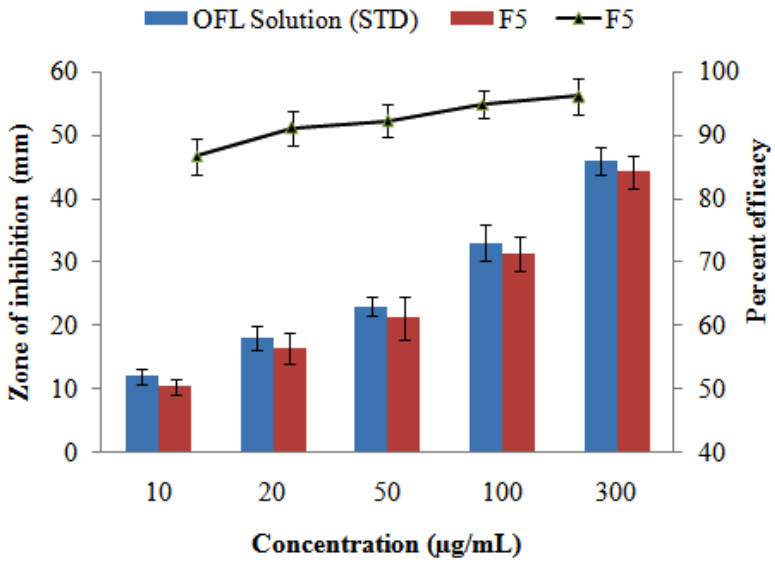

Figure: 4 Comparative *in-vitro*antimicrobial activity of tested formulation *F5 with standard OFL solution.*

In-vivo ocular irritation study

The protocol was approved from Institutional Animal Ethical Committee (IAEC) of Faculty of Pharmacy, Babu Banarasi Das National Institute of Technology and Management, Lucknow, India. All the protocols and the experiments were conducted in strict compliance to the ethical principles and guidelines by committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA). The comparative *in-vivo* eye irritation test for optimized formulation F5 (containing 0.3% OFL and 0.1% DS) with marketed preparation was carried out using rabbits and as per Draize test protocol as represented in **Table 5**. The formulation was found to be non-irritating with no ocular damage or abnormal clinical signs to the cornea, iris or conjunctivae. However, increased blinking of eye was observed with marketed DS eye drop (Voveron®), marketed eye drop Zenflox©as well as with formulation F5. This phenomenon suggests appropriateness of developed formulation for instillation in the eye cavity.

Table 5: Comparative *in-vivo* ocular irritation test of developed formulation F5 with marked eye drops

S No.	Preparations	Scores
1	Marketed eye drop Voveron©	0
2	Marketed eye drop Zenflox©	0
3	Blank optimized formulation F5	0
4	Developed optimized formulation F5	0

CONCLUSION

In-situ gel forming based combined dosage form of ofloxacin and diclofenac sodium was successfully developed as pH triggered ophthalmic solution using 3^2 full factorial designs. Concentration of the gelling agent (Carbopol 934) with HPMC was found to be significantly affected the residence time and drug release properties of the formulation.The developed formulation showed a strong pH dependent gelation which favors its ophthalmic application. The methodology used for the preparation of formulation was simple and economic. Compared to conventional ODDS,it would enhance the bioavailability of loaded drug through its longer precorneal residence time and ability to sustain the drug release. The developed formulation would be a viable alternative of the marketed formulation to treat ocular diseases.

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