IJPSR (2021), Volume 12, Issue 2 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 28 January 2020; received in revised form, 25 April 2020; accepted, 10 May 2020; published 01 February 2021

FORMULATION OF DORZOLAMIDE HYDROCHLORIDE *IN-SITU* PREPARATION FOR TREATMENT OF GLAUCOMA; *IN-VITRO, EX-VIVO* AND *IN-VIVO* CHARACTERIZATION

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Keywords:

In-situ, Factorial design, Histological study, Transcorneal permeation, *In-vivo* study

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ABSTRACT: Present study was planned to prepare a dorzolamide hydrochloride *in-situ* gel by using Carbopol 974 and HPMC K4M to reduce dosing frequency by increasing residence time as well as sustained drug release from the formulation in cul-de-sac. The concentration of both polymers was optimized by using 3^2 factorial designs with correlating its impact on dependent variables as cumulative *in-vitro* drug diffusion at the end of 8 h and viscosity at pH 7.4. Polymer composite showed a quadratic model for drug diffusion as well as for viscosity. Comparative *in-vitro* drug release study by using type II dissolution apparatus confirms sustained drug release characteristics of optimized formulation which showed 89.41% drug release at the end of 8 h as compared to a marketed formulation which showed complete drug release at the end of 3 h. *Ex-vivo* transcorneal permeability study ensured permeability of drug through the cornea. Histological study on goat eye cornea proved non-irritation potential of the prepared formulation. A comparative *in-vivo* study between optimized formulation and marketed formulation on normotensive rabbits confirms sol to gel transition of prepared formulation with percent IOP reduction of 32.87 ± 1.54 at the end of 8 h, whereas; marketed formulation failed to control IOP beyond 3 h. Thus, prepared *in-situ* gel offers more intensive treatment for glaucoma with a reduction in dosing frequency and enhanced patient compliance.

INTRODUCTION: Glaucoma is a dynamic optic neuropathy aligned with irreversible visual impairment, which has been already affected more than 70 million people worldwide [1, 2, 3]. Glaucoma is a consequence of awkwardness among generations and drainage of aqueous humor, which predominantly increases intra ocular pressure (IOP). It further results in progressive degeneration of the retinal ganglion cell layer and ultimately results in irreversible visual field loss with damage to optic nerve [1]. Glaucoma is treated with an aim to maintain intra ocular pressure to normal value by either increasing surge of aqueous humor or reducing its production [4].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.12(2).830-37

The article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.12(2).830-37

In the most recent couple of years, several classes of drugs have been developed with the potential to lower IOP, which comprises of beta-blocker, prostaglandin analogue (PGA), alpha-adrenoceptor agonist (AA), and topical carbonic anhydrase inhibitors (CAI's) [5].

These drugs are preferably administered in the form of eye drops. Nevertheless, an inimitable structure of eye and its defense mechanism renders drug targeting to eye site as challenging to formulate scientist [6]. Dorzolamide and acetazolamide belong to a class of carbonic anhydrase inhibitors responsible for lowering IOP by down regulation of carbonic acid anhydrase isoenzyme, which plays a foremost role in aqueous humor secretion. However, dorzolamide hydrochloride is 20 times more potent than acetazolamide and hence, potentially used in the treatment of glaucoma [7]. Presently it is delivered in the form of simple eye drop, from which the drug rapidly undergoes precorneal loss by a different protective

mechanism of eye and results in poor ocular bioavailability as well as several side effects by systemic absorption through naso-lachrymal drainage [8, 9]. As a result of poor corneal contact time of drug, three instillations of the solution are required to accomplish the ideal helpful impacts. A high frequency of administration of formulation is associated with patient non-compliance, and a study has put forth that nearly 50% of glaucoma patients discontinued topical ocular therapy within six months [10].

There is a need to develop a novel formulation to augment retention of formulation at the eye site. *An in-situ* gel system is the best approach to protract the contact time of formulation with the ocular tissues. In these types of formulations, the polymer undergoes conformational changes in physiological environment response and converts from low viscosity solution to viscoelastic gel [11].

The objective of presented work was to prepare pH sensitive *in-situ* gel of dorzolamide hydrochloride by using Carbopol 974P and HPMC K4M and to explore its influence on treatment of glaucoma by *in-vitro*, *ex-vivo* and *in-vivo* characterization. Carbopol 974 P is chemically polyacrylic acid (PAA) which required in high concentration to form stiff gel. Such highly acidic solution failed to get neutralized by buffer action of tear fluid. However; Reduction in its concentration without affecting the gelling capacity and viscosity was attained by addition of viscosity increasing polymers such as HPMC K4M.

MATERIALS AND METHODS:

Materials: Dorzolamide hydrochloride was obtained as a gift sample from FDC Ltd. Aurangabad. Carbopol 974 P was gifted by Lubrizol Ltd., Mumbai and HPMC K4M was obtained from S. D. Fine, Mumbai. All other ingredients and reagents were of analytical grade.

Analytical RP-HPLC Method for DZH: To quantitate the content of dorzolamide hydro-chloride in samples, reversed-phase (RP)-HPLC method was developed and validated as per ICH guidelines Q2 (R1). Shimadzu RP-HPLC instrument (CFR-21) equipped with photodiode array detector (PDA) and C18 column of Kromasil (250 mm × 4.6 mm, 5 µm particle size) was used. Acetonitrile: potassium dihydrogen phosphate (10:90 v/v) having pH 3.5 was used as mobile phase. Elution was measured at 254 nm with a flow rate of 1.0 ml/min [12].

Preparation of DZH pH Triggered *In-situ* Gel: Dorzolamide hydrochloride *in-situ* gel was formulated using carbopol 974P and HPMC K4M. These polymers were placed in half of the desired volume of purified water for eight hours to soak. A solution of drug, sodium chloride, and benzalkonium chloride was prepared in purified water. Drug (0.5% w/v), sodium chloride (0.85% w/v), and benzalkonium chloride (0.01% v/v) were dissolved in a small possible quantity of water, and it was transferred to the polymeric solution under constant stirring to obtained a uniform solution. The final volume was adjusted to 100 ml with purified water. At last prepared formulations were subjected for terminal sterilization by autoclaving at 121 ºC, 15 p.s.i. for 20 min [13]. The addition of sodium chloride was based on the required value of tonicity of the formulation. With the addition of 0.85% of sodium chloride, the osmolarity of the prepared formulation was found to be 305 mOsmol/Kg, which when determined by using digital osmometer 'Osmomat 030/050 Terminal'.

TABLE 1: COMPOSITION OF pH TRIGGERED *IN-SITU* GEL OF DZH

Name of excipients	Composition of DZH *in-situ* gel (%w/v)								
	F1	F2	F3	F4	F5	F6	F7	F8	F9
Dorzolamide Hydrochloride	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Carbopol 974 P	0.15	0.15	0.15	0.30	0.30	0.30	0.45	0.45	0.45
HPMC K 4M	0.50	1.0	1.50	0.50	1.0	1.50	0.50	1.0	1.50
Benzalkonium chloride*	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Sodium chloride	0.85	0.85	0.85	0.85	0.85	0.85	0.85	0.85	0.85
Water	100	100	100	100	100	100	100	100	100

*Amount of Benzalkonium chloride in terms of % v/v

Factorial Experimental Design: The dorzolamide hydrochloride gel was optimized by using 3^2 randomized full factorial design. Amount of *in-situ* gelling polymer, Carbopol 974P (X1) and viscosity enhancer, HPMC K4M (X2) were selected as independent variables, whereas % drug release at 8

h (Y1) and viscosity at pH 7.4, 20 rpm (Y2) were selected as the dependent variable. Independent variable were fitted at three level of concentration *viz.* higher, middle and lower levels of factor X_1, 0.15%, 0.3% and 0.45% and for factor X_2, 0.5%, 1% and 1.5% respectively. The composition of different batches with the application of optimization design is shown in **Table 1**.

Characterization of *In-situ* Gel:

Physicochemical Characterization: Prepared all batches were primarily subjected for evaluation of clarity, pH, and gelling capacity. The clarity of formulation in sol and gel state was determined by visual inspection under the black and white background.

The pH of the formulation was determined immediately after preparation and after 24 h using a digital pH meter. The gelling capability was determined by placing 100 µl of a sample in a vial containing 2 ml artificial tear fluid (ATF), and gelation time was recorded [13].

***In-vitro* Drug Diffusion Studies:** The *in-vitro* diffusion study of the *in-situ* gel was carried out by using Franz Diffusion Cell. Freshly prepared artificial tear fluid (ATF) having pH 7.4 filled in a receptor chamber. Dialysis membrane was placed in between donor and receptor compartment. The testing assembly was placed on a thermostatically controlled magnetic stirrer, which maintained temperature and stirring rate at 37 ± 1 °C and 50 RPM, respectively. In the donor compartment, 1 ml *in-situ* gel was placed. From the receptor compartment, samples were withdrawn at regular intervals and equal volume replaced by artificial tear fluid. The samples were analyzed by a validated RP-HPLC method of analysis [3, 13].

Viscosity Study: The viscosity of prepared formulations was determined at formulation pH (sol state) and pH raised to 7.4 by adding 0.5 M NaOH solution (gel state) by using Brookfield viscometer (Oswal's Scientific PES / Mcop). Angular velocity was gradually increased from 5 to 200 rpm [14].

***In-vitro* Drug Release Study and Release Kinetics:** Comparative *in-vitro* drug release test between optimized formulation and the marketed solution was carried out by using a Type II USP dissolution test apparatus. A 1 ml volume of the optimized formulation and the marketed solution was accurately filled separately into a dialysis bag (Himedia, India) and subjected in 500 ml freshly prepared artificial tear fluid. The rotating speed and temperature were maintained at 50 rpm and 34 ± 1 °C, respectively. During each sampling time, 5 ml of aliquots were withdrawn and subjected to HPLC analysis to determine drug concentration. Percentage cumulative drug release was calculated. Drug release from the optimized batch was also subjected to describe drug release kinetics [15].

***Ex-vivo* Drug Permeation Studies:** The optimized formulation was subjected to permeation study through the goat cornea in order to find out the extent of drug permeability in terms of permeation flux and permeation coefficient. Goat eyeballs were obtained from the local slaughterhouse. The cornea was separated surgically and washed with saline solution. The excised goat cornea was mounted in between the donor and receptor compartment of the Franz diffusion cell. The receptor compartment was filled with freshly prepared artificial tear fluid. An aliquot of 1 ml of the formulation was placed on the cornea.

The cell content was stirred at 50 rpm using a magnetic bead at 37 °C. The samples were withdrawn from the receptor compartment at regular intervals and replaced with the same volume of artificial tear fluid. The samples were analyzed by the RP-HPLC method of analysis. The graph of the cumulative amount of DZH permeated per unit of corneal surface area (cm2) against time (h) was plotted. From data, permeability coefficient (P) and permeation flux (J) were calculated [3, 15].

Assessment of Local Irritation Potential on Goat Cornea: Ocular irritation potential of optimized formulation was checked by using histological study on isolated goat eye cornea. The cornea was incubated for 5 h at 37 °C in a formulation (test), saline solution (negative control), and 0.1% (w/w) sodium dodecyl sulfate (SDS) in phosphate buffer saline (PBS) (positive control). Following incubation time, corneas were washed with PBS and immediately fixed in formalin (8%, w/w). Tissues were dehydrated in an alcohol gradient, placed in melted paraffin, and solidified in block form.

Cross-sections were cut, stained with hematoxylin and eosin (H&E). Cross-sections were observed microscopically for any modifications [3, 10].

***In-vivo* Study:** Sterile optimized formulation which proved an absence of ocular irritation potential was at last subjected to explore its role in the management of intraocular pressure on normotensive male albino rabbits with weight 2-2.5 kg as per the experimental protocol approved by Animal Ethical Committee. An experiment was designed and conducted in accordance with the guidelines laid by CPCSEA, New Delhi (IAEC/CPCSEA/RCPIPER/2018-22). Six healthy New Zeland albino rabbits were divided into two groups and kept in individual cages with free access to food and water. A local anesthetic, xylocaine was administered into rabbit's eyes. To the right eye cul-de-sac of rabbits from the first group, 50 µl prepared formulation, and the second group, 50 µl marketed formulation were instilled while the left eye was used as control. Intraocular pressure was measured at a time interval of 1h to 8h by using Schiotz tonometer [7]. IOP was measured three times in each case, and the mean value was taken to determine % decreased IOP by using the following formula;

% decrease IOP = ((IOP of controlled eye-IOP of trated eye) / (IOP of controlled eye)) × 100

TABLE 2: EXPERIMENTAL DESIGN LAYOUT OF DZH *IN-SITU* GEL FORMULATIONS

Run	FC	Coded levels of variables		Clarity	pH		Gelling capacity*	Response 1 (Y_1) % Drug release at 8 h	Response 2 (Y_2) Viscosity at pH 7.4 (cp), 20 rpm
		Factor X_1 (Carbopol 974 P)	Factor X_2 (HPMC K4M)		0 h	24 h			
1	F1	-1	-1	Clear	4.03	4.0	-	96.54 ± 0.25	7500 ± 122
2	F2	-1	0	Clear	4.60	4.56	+	94.18 ± 0.36	7900 ± 139
3	F3	-1	1	Clear	4.20	4.17	+	92.24 ± 0.42	8450 ± 125
4	F4	0	-1	Clear	3.80	3.79	+	96.44 ± 0.55	8175 ± 119
5	F5	0	0	Clear	3.88	3.86	+	92.15 ± 0.39	8350 ± 131
6	F6	0	1	Clear	3.80	3.77	++	91.79 ± 0.26	9100 ± 109
7	F7	1	-1	Clear	3.70	3.70	++	90.17 ± 0.61	8400 ± 141
8	F8	1	0	Clear	3.40	3.41	+++	89.86 ± 0.29	8700 ± 121
9	F9	1	1	Clear	3.50	3.47	+++	86.74 ± 0.41	9425 ± 126

*Grades of gelling capacity: –, No gelation; +, gels after a few minutes, dissolves rapidly; ++ gelation immediate, remains for few hours; +++ gelation immediate, remain for extended period.

RESULTS AND DISCUSSION:

Full Factorial Experimental Design: Experimental design layout developed for optimization of *in-situ* gel formulations is shown in **Table 2**. The effect of independent variables on the dependent variable was studied by using Design Expert 9.0.

Various models such as linear, 2FI, Quadratic, and Cubic were fitted to the data, and the model which fit well was suggested by software and was tested for analysis of variance (ANOVA). Regression polynomials were calculated for the dependent variables.

Characterization of *In-situ* Gel:

Physicochemical Evaluation: Clarity is one of the imperative parameters of the ophthalmic formulations. The clarity of all formulations was found clear at pH 7.4.

Formulation pH was measured immediately and after 24 h of preparation, which was found to be satisfactory in the range of 3.5-5.0. Gelling capacity for formulations was evaluated and graded as shown in **Table 2**.

Effect of Formulation Variables on *in-vitro* Drug Diffusion Study at 8 h: To optimize concentration of selected polymers, an *in-vitro* drug diffusion study was carried out. Cumulative % drug diffusion at the end of 8 h is shown in **Table 2**. Although *in-vitro* conditions may vary from the *in-vivo* condition at eyesight but *in-vitro* diffusion study clearly indicates sustain release characteristic of prepared formulations.

On applying factorial design, software suggested the quadratic model for response Y1, and it was found to be significant with a model F value of model F value 65.81, *p-value* 0.0019, an R^2 value of 0.9910, and there was only 0.02% chance that a model "F value" this large could occur due to noise.

Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case, X_1, X_2, and X_{12} were significant model terms.

The model for response Y1 is as follows

$Y_1 = 94.92 - 2.57\ X_1 - 2.27\ X_2 + 0.023\ X_1X_2 - 2.15\ X_{12} - 0.06\ X_{22}$.....(1)

From Eq. (1) it is clear that the drug diffusion get decreases with an increasing amount of factor X_1 (concentration of Carbopol 974 P) and factor X_2 (concentration of HPMC K 4 M). Hence, formulation containing lowest concentration of carbopol 974 P (0.1%) and HPMC K 4M (0.5%) shows highest release (96.54%) whereas formulation containing highest concentration of carbopol 974 P (0.45%) and HPMC K 4 M (1.5%) shows lowest release of drug (86.74%).

The combined effect of factor X_1 and factor X_2 can be further interpreted with the help of a counter plot and 3D response surface plots **Fig. 1** showing the effect of concentration of Carbopol 974 P and HPMC K 4 M on % drug release at 8 h **Table 2**.

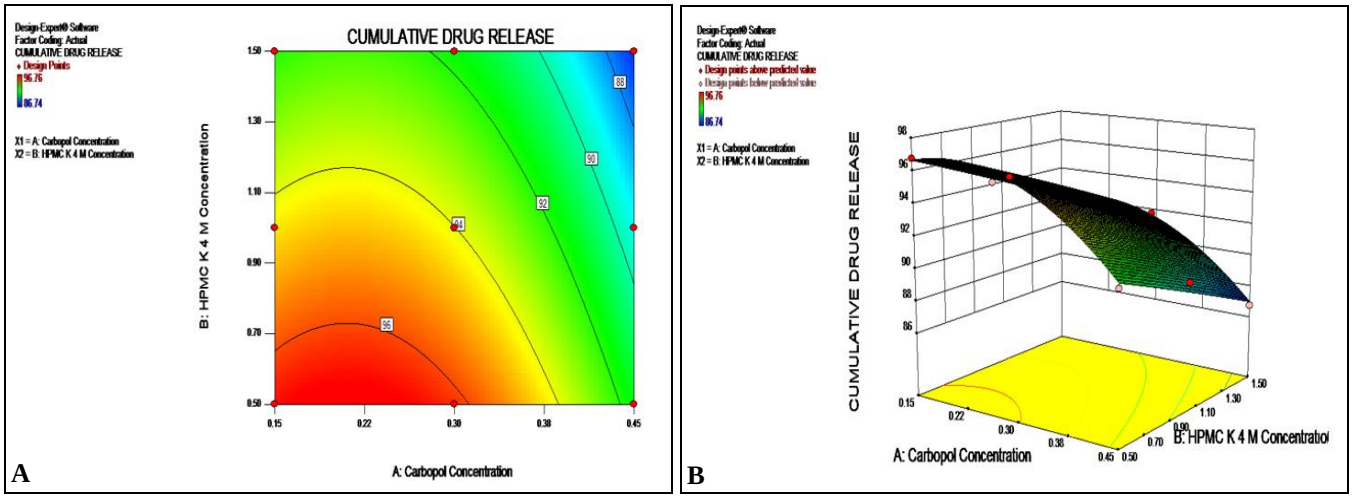

FIG. 1: (A) TWO DIMENSIONAL COUNTER PLOT, (B) THREE DIMENSIONAL (3 D) RESPONSE SURFACE PLOT FOR RESPONSE Y1 (% DRUG RELEASE AT 8 H)

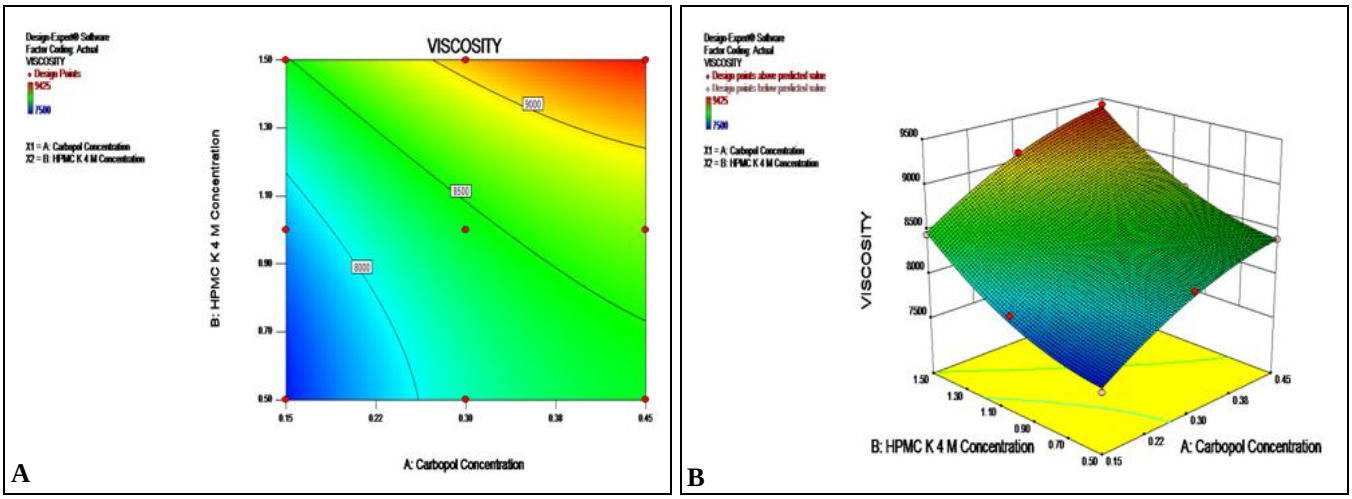

FIG. 2: (A) TWO DIMENSIONAL COUNTER PLOT, (B) THREE DIMENSIONAL (3 D) RESPONSE SURFACE PLOT FOR RESPONSE Y2 (VISCOSITY RATE AT pH 7.4)

Effect of Formulation Variables on Viscosity: Viscosity is crucial property of *in-situ* gel in context with retention time and hence ocular bioavailability of drug from formulation. Quadratic model was suggested by software for response Y2, and it was found to be significant with model F value 57.99, *p-value* 0.00016, and R^2 value of 0.9938 and there was only a 0.16 % chance that a "Model F- Value" this large could occur due to noise. Values of 'Prob > F' less than 0.0500 indicate model terms are significant. In this case, X_1, X_2 and X_{22} were significant model terms.

The model for response Y_2 is as follows

$Y_2 = 8413.89 + 445.83X_1 + 483.33X_2 + 18.75\ X_1X_2 - 145.83X12 + 191.67\ X_{22}$.....(2)

From Eq. (2) it is clear that the viscosity rate appeared to increase with an increasing amount of factor X_1 (concentration of Carbopol 974 P) and factor X_2 (concentration of HPMC K 4 M). The combined effect of factor X_1 And factor X_2 can be further interpreted with the help of a counter plot and 3D response surface plots **Fig. 2** showing the effect of concentration of Carbopol 974 P and HPMC K 4 M on gel viscosity at pH 7.4 **Table 2**.

Optimization of Formulation: For optimization of response variable Y_1 and Y_2, 3^2 factorial designs were applied. A study has been reported that viscosity plays a decisive role in ophthalmic formulations. As when viscosity increases, it sustains the drug release and reduces the swipe of formulation, but at the same time, high viscosity may also reduce patient adherence to therapy by increasing patient discomfort [14]. Therefore, a formulation with optimum viscosity with required drug diffusion is to be selected. Formulation F8 showed all these desirable characteristics with 8700 cps viscosity with 89.86% drug release at 8 h was considered as optimized batch and was used for further evaluation.

***In-vitro* Drug Release Study and Release Kinetics:** Comparative *in-vitro* drug release study between optimized formulation and the marketed solution was carried out by using USP Type II dissolution apparatus. A comparative plot of cumulative percent drug release by optimized formulation and marketed solution is shown in **Fig. 3**. Marketed formulation showed complete drug release within 3 h whereas optimized formulation proved sustained drug release property with 89.41% cumulative drug release at the end of 8 h.

This drug release retardation may be observed because of increased viscosity during gel formation and formation of hydrogen bond between drug and polymeric system. Data obtained from *in-vitro* drug release study was further subjected to mathematical treatment to determine drug release kinetic profile. Korsmeyer-Peppas model was found to be suitable to explain release kinetic of drug with a value of r^2= 0.992 and n = 0.226. This study indicates drug movement from gel by Quasi Fickian diffusion. Similar model was observed by Pund *et al.*,

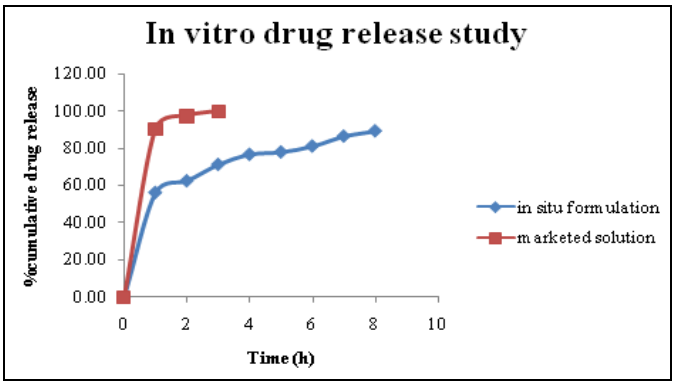

FIG. 3: CUMULATIVE PERCENT DRUG RELEASE BY OPTIMIZED FORMULATION AND MARKETED SOLUTION

***Ex-vivo* Drug Permeation Studies:** The *ex-vivo* transcorneal permeation studies of the optimized batch was carried out on goat eye cornea. Results is shown in **Fig. 4**. Optimize formulation showed 45.21% drug permeation at the end of 5 hr with permeability coefficient (P) 4.45 (cmh^{-1}) × 10^{-3} and permiation flux (J) 11.14 µg cm-2h-1. Transcorneal permeation study indicates the movement of drug through cornea as well as drug release retardant property of the polymeric system.

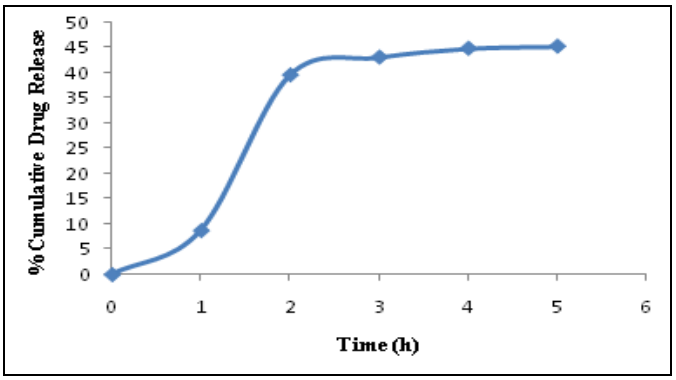

FIG. 4: *EX-VIVO* DRUG PERMEATION STUDY THROUGH OPTIMIZED *IN-SITU* GEL FORMULATION

Assessment of Local Irritation Potential on Goat Cornea: Ocular irritation potential of optimized formulation was evaluated by the histological study of the goat eye cornea. The structure of the cornea was well retained by formulation treated (test) and saline solution treated (negative control) cornea. However, the marked ruin of corneal structure was observed with SDS treated cornea (positive control), as shown in **Fig. 5**. Histology study confirms the non-irritation nature of the prepared optimized formulation.

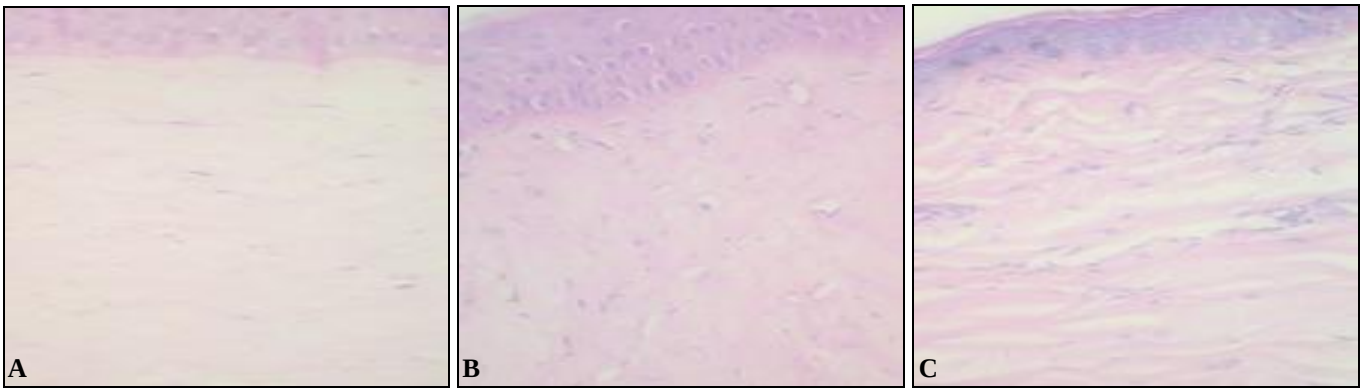

FIG. 5: HISTOLOGICAL SECTIONS OF GOAT CORNEA (MAGNIFICATION 40 X) (A) NEGATIVE CONTROL (UNTREATED CORNEA) (B) TEST SAMPLE (TREATED WITH *IN-SITU* GEL) (C) POSITIVE CONTROL (CORNEA TREATED WITH 0.1 % SDS)

***In-vivo* Study:** Impact of prepared formulation on intra ocular pressure in comparison with marketed formulation was studied by using normotensive New Zeland albino rabbits. Optimized formulation showed an 8.59 ± 1.14 % decrease in IOP after single-dose administration, and this effect was last up to 8 h with a percentage drop of IOP 32.87 ± 1.54 ($P<0.001$).

However, the marketed formulation showed an initial rapid drop in IOP as 11.55 ± 1.17%, which last for 3 h only. Results clearly indicate the *in-situ* gel formation ability of optimized formulation by a change in pH at cul-de-sac, which further increases retention time of formulation as well as sustained the release of drug. It was also observed that initially drop in IOP by prepared formulation was rapid as compared to later time-lapse, which may be due to the required time for sol to gel transition.

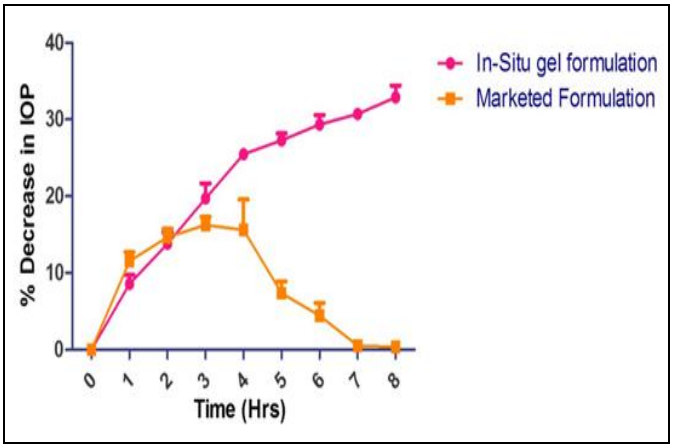

FIG. 6: PERCENTAGE DECREASE IN IOP AFTER ADMINISTRATION OF DEVELOPED *IN-SITU* GEL AND. MARKETED PRODUCT (n= 3, MEAN ± SD)

On the other hand, marketed formulation failed to maintain IOP beyond 3 h, which indicates poor retention of formulation at eye site due to its liquid nature. Comparative percentage drop of IOP in-between prepared formulation and marketed formulation is shown in **Fig. 6**.

From the study it can be concluded that prepared formulation undergoes gel formation and further sustained release of a drug due to which one dose of prepared formulation is enough to maintain IOP as compared to a marketed formulation which failed to retain at the cul-de-sac and hence, required three doses in a day.

CONCLUSION: Dorzolamide hydrochloride *in-situ* gel was successfully formulated by using Carbopol 974P and HPMC K4M. The concentration of Carbopol and HPMC was optimized by using 3^2 factorial designs, which confirms the role of Carbopol and HPMC to increase the viscosity of formulation and to sustain the drug release. Comparative *in-vitro* drug release study further confirms the sustained drug release property of prepared formulation, which lasts up to 8 h as compared to a marketed formulation, which showed complete drug release within 3 h. *Ex-vivo* study on goat eye cornea proved transcorneal permeability of drug through optimized formulation. A histopathology study showed a safe and ocular tolerance approach of optimized formulation with no damage to the corneal structure. *In-vivo* study leads to confirm sustained drug release property and enhanced ocular retention potential of prepared formulation which is even not terminated by the end of 8 h as compared to a marketed formulation which failed to retain at eyesight and hence unable to control IOP after 3 h. Therefore, it can be concluded that prepared

formulation is safe, non-irritating, and possesses sustained drug release ability, which helps it overcome drawbacks associated with conventional treatment.

ACKNOWLEDGEMENT: The first author is thankful to the Trustees of Mumbai Education Trust, Nashik, Maharashtra, India, for providing infrastructure and research facilities.

CONFLICTS OF INTEREST: The authors declare that they have no conflict of interest

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How to cite this article:
Laddha UD and Kshirsagar SJ: Formulation of dorzolamide hydrochloride *in-situ* preparation for treatment of glaucoma; *in-vitro*, *ex-vivo* and *in-vivo* characterization. Int J Pharm Sci & Res 2021; 12(2): 830-37. doi: 10.13040/IJPSR.0975-8232.12(2).830-37.