

Science based Development of Viscous Eye Drop of Dorzolamide Hydrochloride and Timolol Maleate using Full Factorial Design

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ABSTRACT

Introduction: To develop and optimize viscous eye drop for dorzolamide hydrochloride and timolol maleate used in Glaucoma - the leading cause of irreversible blindness worldwide. **Methods and Materials:** 32 full factorial design was applied in formulation optimization to study the relationships between critical process parameters (concentration of HPMC K15 and Carbopol 934) and critical quality attributes, i.e. viscosity, mucoadhesion index and cumulative drug release for both the drugs. Further, the optimized formulation was identified through derringer's desirability approach of multi-criteria decision making technique and was fully characterized in terms of molecular interactions (DSC and FTIR) as well as *in vitro* and *in vivo* biological properties, including biological sensitivity/irritation studies performed on rabbits. Moreover, *in vivo* ocular pharmacokinetic study was performed using developed bioanalytical RP-HPLC method having mobile phase phosphate buffer (pH 6.8): methanol: Acetonitrile, 45:45:10 v/v/v in rabbit tear fluid employing protein precipitating sample preparation technique.

Results: Results revealed that optimized batch does not have any ocular irritancy on rabbit eye and shows increase in mean residence time compared to marketed formulation. **Conclusion:** Hence, the design planning methodology clearly showed its usefulness for optimizing the formulation and the formulated Viscous eye drop proved to be a promising formulation strategy for ophthalmic delivery of dorzolamide and timolol maleate making it a potential alternative for the marketed formulation.

Key words: Dorzolamide hydrochloride, Timolol maleate, Viscous eye drop, Full factorial design.

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INTRODUCTION

Glaucoma is an optical neuropathy disease resulting in optic nerve structural damage.¹ Glaucoma is second to cataract as the leading cause of irreversible visual loss and estimates 79.6 million population getting affected by glaucoma globally by 2020.² Immediate treatment for early-stage, open-angle glaucoma can delay progression of the disease.³ Glaucoma treatments include medicines, laser trabeculoplasty, conventional surgery, or combination of any of these. In recent years, the fixed-combination medications has increased substantially, because they offer the convenience of using a single medication bottle than a similar two-bottle regimen resulting in increased patient compliance. Although many different fixed-combination therapies are commercially available in various countries, a fixed dose combination that combines a carbonic anhydrase inhibitor with beta blocker, i.e., Dorzolamide hydrochloride (DZ) and timolol maleate (TM), seems to lower eye pressure better than the other medicines.^{4,6}

The available ophthalmic formulations are administered topically in the form of conventional eye drops. However, the rapid turnover of lacrimal fluid and extensive nasolacrimal drainage along with eyes blinking reflex rapidly eliminate the administered eye drops. This causes short pre-corneal residence time limiting effective transcorneal drug absorption. Thus, frequent instillation of eye drops is required to achieve therapeutic effect, and, this usually results in pulsed administration and patient non-compliance. In order to overcome these drawbacks of the conventional eye drops, many researchers have attempted to increase the pre-corneal residence time and improved bioavailability by increasing the viscosity of ophthalmic delivery systems.^{7,8}

The viscosity enhancers are hydrophilic polymers, includes various celluloses, polyvinyl alcohol and polyacrylic acid and could be incorporated into eye-drop formulations due to conceptual simplicity, ease of manufacture and promising data in animal models. Carbopols are essentially

non toxic and non irritant material with no evidence of hypersensitivity in human subjects when used topically. Their neutralized aqueous dispersion demonstrates a high viscosity hence popular for controlling the flow properties of topically applied dosage forms as they are inexpensive, transparent and harmless and easy to prepare and clean. Also, decrease in Carbopol concentration without compromising rheological property of the delivery system can be achieved by addition of viscosity enhancing polymer i.e. Hydroxy propyl methy cellulose (HPMC) which is water-soluble polymers derived from cellulose, the most abundant polymer in nature.⁹⁻¹¹ Moreover, solutions of HPMC is thermally a gel, a unique property that plays a key role in a surprising variety of applications. The fact is the presence of so many useful properties which are simultaneously present often acting in combination offering significant economic advantage. In addition, they are highly efficient, often yielding optimum performance at a lower concentration than that required with other watersoluble polymers.¹²

Design of Experiments (DOE) is a tool which allows formulators to understand the effects of formulation variables on desired performance outputs of the product. Using DOE, the relationship between different independent variables can be established and final product performance quality with respect to critical quality attributes (CQAs) can be determined⁸ giving the best levels of excipients that provide optimal levels of CQAs. CQA are properties of the product that is required to remain within certain range/limits to achieve reasonable shelf-life and product performance. For viscous ophthalmic formulation the excipients concentrations required is critical with respect to achieve the critical quality attributes (CQA). From the preliminary studies, CQAs which defines the quality of ophthalmic viscous formulation were identified and they were observed to be viscosity, mucoadhesion index and % drug release. The purpose of this study is to control critical process parameters (CPPs) affecting the CQAs defining an ophthalmic viscous formulation. Hence, this Quality

by Design (QbD) approach was used for screening the best compositions with best suited experimental conditions within short period of time and with minimum trial runs.¹³⁻¹⁵

In this study, we have hypothesised that the developed viscous formulation will provide better ocular retention in comparison to conventional formulation. To evaluate this hypothesis, we have compared the ocular pharmacokinetics of commercially available and prepared viscous ophthalmic formulation. Currently available bioanalytical method for the quantitation of timolol and dorzolamide simultaneously is high-performance liquid chromatography coupled with tandem mass spectrometry.¹⁶ But no methods are reported for their simultaneous estimation in tear fluid to study comparative elimination kinetics. So, RP-HPLC bio-analytical method in tear fluid was developed and validated after extracting the titled drugs by protein precipitation technique. The current method offers significant advantages in terms of sensitivity, selectivity and rapid throughput without the need for extensive or complex sample preparation procedures.

Hence, in line with the notion and keeping the current FDA requirements in mind while pursuing the research considering QbD based approach, the objective of our study was to formulate and optimize DZ and TM viscous eye drop solution employing 3² full factorial design, capable of delivering the drugs in a sustained manner, thus avoiding frequent instillation of the drops. In the meantime, the QbD abetted viscous eye drop formulation and marketed formulation were compared for their *in vivo* performance in rabbit using RP-HPLC.

MATERIALS AND METHODS

Materials

Pharmaceutical grade of DZ and TM were received as a gratis sample from Micro Labs Limited, Bengaluru, India. HPMC K15 and Carbopol 934 (CP 943) were received as gift samples from Mepro Pharmaceuticals, Vadhvan, India and Astron Chemicals, Ahmedabad, India respectively. All solvents and chemicals used were of analytical grade, purchased from Merck Specialities Pvt. Ltd., Mumbai, India and SD Fine Chemicals Ltd., Mumbai, India respectively.

Instruments and software

HPLC LC-2010 C HT, with Phenomenex column C₁₈ (5 μm, 250 mm x 4.6 mm), diode array detector (SPD-M20 A), Shimadzu, Japan as well as double-beam Shimadzu 1800 UV-Visible spectrophotometer, connected to computer and loaded with UV-Probe software version 2.34 were used for analysis. All data analysis of experimental design was performed by using the Design-Expert trial version 9.0.1 (Stat-Ease Inc., Minneapolis, USA). While other calculations were performed by use of Microsoft Excel 2010 software (Microsoft Corporation, USA).

Experimental Methods

pH dependent solubility of DZ

Dorzolamide has two pKa values i.e. 6.35 and 8.5 and has relatively low aqueous solubility as it exhibits nonionic behavior in this pH range.¹¹ To enhance solubility of DZ at physiological pH of eye, various concentration of PEG 400 were added to enhance the solubility of DZ at pH 7.4.^{17,18}

Identification of the Material Attributes and Potential CQAs required for development of ophthalmic viscous formulation

According to QbD, pharmaceutical development includes identifying potential CQAs of the drug product, determining material attributes, selecting an appropriate manufacturing process (CPPs) and defining

a control strategy. Preliminary trials were carried out for the selection of appropriate concentration and combination of viscosity enhancing polymers (material attributes). For the same, various polymers such as carboxy methyl cellulose sodium (Na CMC) 0.4, 0.7 and 1 %w/v; HPMC K15 0.25, 0.5 and 0.75 %w/v; CP 934 0.1, 0.2 and 0.3 % w/v as well as xanthan gum 0.5, 1 and 1.5 %w/v were screened on the basis of their rheological behaviour. Concentrations of above polymers were selected based on literature survey. Viscosity of the above prepared solutions were measured using Brookfield Viscometer (LVDV- 2 + pro, Germany) at shear rate 10, 25, 40, 50, 60, 75, 100, 120, 150 and 200 rpm by using LVDV Spindle No. 61, 62, 63 and 64.¹⁹

Drug - polymer compatibility study

Fourier Transform Infrared Spectroscopy (FTIR)

Infrared (IR) spectroscopy of pure DZ, TM, HPMC K15, CP 934 individually and their physical mixture were conducted using a FTIR Bruker optics alpha with opus 6.5 software and the spectrum was recorded in the wavelength region of 4000–400 cm⁻¹. The procedure consisted of dispersing a sample (drug alone, spherical agglomerates) in Zinc selenium crystal and instrument is attenuated total reflectance basis. The prepared mixture was placed in the light path and the spectrum of each sample was obtained.

Differential Scanning Calorimetry (DSC)

DSC thermogram of pure DZ, TM, HPMC K15, CP 934 individually and their physical mixture were recorded using Differential scanning calorimeter connected with thermal analysis data station system, computer and plotter interface. Samples weighed 2–3 mg were placed in open aluminium pans and heated from 40 to 500°C at a rate of 10°C per min. Nitrogen were used as a purge gas at a flux rate of 50 mL/min. The onsets of the melting points and enthalpies of fusion were recorded by the software.

Formulation optimization by full factorial design

To study all the possible combinations of factors at all levels, a two factor, three level full factorial design (FFD) was constructed and conducted in a fully randomized order. According to the FD, the total number of experimental combinations is 2^K + n₀, where K is the number of independent variables and n₀ is the number of repetitions of the experiments at the centre point.^{13,14} Based on the prior knowledge, product and process understanding as well as from preliminary trials results, formulation variables likely to affect the CQA of the product were identified. In the present study, two material attributes viz. concentration of HPMC K15(X₁) and CP 934 (X₂) were selected based on their criticality. The CQAs selected were viscosity of the formulation (Y₁), mucoadhesion index (MI) (Y₂) and % drug release of both the drugs, DZ and TM (Y₃ and Y₄).

All the batches were prepared according to the experimental design domain which summarizes an account of 13 experimental runs and their factor combinations. Various factorial computations for the current optimization study were performed employing Design Expert software (9.0.1). Polynomial models including interaction and quadratic terms were generated for all the response variables using multiple linear regression analysis approach.

The linear and quadratic models for predicting the optimal point was expressed using following equation 1 and 2, respectively:^{20,21}

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 \quad (\text{Eq. 1})$$

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_3 X_1^2 + \beta_4 X_2^2 +$$

$$\beta_5 X_1 X_2^2 + \beta_6 X_1^2 X_2 \quad (\text{Eq. 2})$$

Where, Y is the measured response associated with each factor level combination; β_0 is the intercept representing the arithmetic mean response of 13 runs; β_1 to β_6 are the regression coefficients whereas X_1 and X_2 are the coded levels of independent variables. The terms $X_1 X_2$ and X_i^2 ($i=1, 2$) represent the interaction and quadratic term respectively.²⁰⁻²² Statistical validity of the polynomials was established on the basis of ANOVA. Three dimensional response surface graphs and 2-D contour plots were constructed using the Design Expert software. Subsequently, two optimum check points were selected by intensive grid search, performed over the entire experimental domain, to validate the chosen experimental design and polynomial equations. The criterion for selection of optimum formula was primarily based on the constrains for CQAs i.e. viscosity (25-100 cps), mucoadhesion index (>1500 cPs/s) and cumulative percentage drug release (>90%) upto 8 h for DZ and TM. An optimum formulation was further selected employing the concept of desirability function.

Formulation and development of viscous eye drop

CP 934 was dispersed in 50 ml sterile water for injection (WFI) containing benzalkonium chloride (0.0075 %w/v) under constant stirring for 4-5 h. HPMC K15 was added to the Carbopol dispersion and the mixture was allowed to hydrate for 4 h and further placed on magnetic stirrer until clear solution was obtained. DZ (2% w/v) and TM (0.5 % w/v) were added separately in deionized double distilled water followed by addition of 1% w/w of PEG 400. The drug and polymer solutions were mixed at room temperature. Mannitol (0.5 %) as isotonic agent was added to maintain isotonicity and the pH was then adjusted to 7.4 with 1M NaOH and volume was made up to 100 ml with water for injection. Viscous eye drop formulations (13 batches) were prepared, and allowed to equilibrate for 24 h at room temperature prior to the evaluation both *in vitro* and *in vivo*.²²

Evaluation of viscous eye drops

Clarity, pH, osmolarity and refractive index

Clarity was assessed by visual inspection of each container under a good light and viewed against reflection into the eyes and also against a black and white background, with the contents set in motion with a swirling action. pH of prepared viscous eye drop was measured with pH meter. The osmolarity of optimized batch was determined with a freezing point Fiske Micro Osmometer. Refractive index was determined at 25 °C using refractometer to assess the formulation for clarity of vision.^{23,24}

Assay of DZ and TM

Assay of DZ and TM was performed by RP-HPLC analysis where 1 ml solution (2 % w/v DZ and 0.5 % w/v TM) was carefully transferred in a 10 ml volumetric flask containing about 4 ml of the mobile phase and it was sonicated for 5 min to obtain a clear solution followed by dilution up to the mark with the mobile phase, and was filtered using a 0.45 µm membrane filter. 20 µl of working solutions i.e. 2000 µg/ml and 500 µg/ml for DZ and TM respectively was then injected in HPLC system and study was replicated twice using optimized mobile phase, phosphate buffer (25 mM): methanol: acetonitrile adjusted pH 6.8 with triethylamine (45:45:10) at 275 nm.

Rheological evaluation

The viscosity measurements were done by using Brookfield LVDV II PRO+ viscometer. The formulations were poured into adapter of the viscometer and the angular velocity (shear rate) at increasing shear rate

of 10, 25, 40, 50, 60, 75, 100, 120, 150 and 200 rpm with a waiting period of 6 sec at each speed, by using LVDV Spindle No. 61, 62, 63 and 64. Graph of viscosity vs. shear rate was plotted and non-Newtonian flow pattern was evaluated. The viscosity was again determined as specified above after diluting the prepared formulations by adding artificial tear fluid (ATF) at the ratio of 40:7 with temperature 37°C, as the conventional commercial eye dropper delivers an average drop volume about 40 µl while available tear fluid is 7 µl. The measurements were performed in triplicate and the mean viscosity of the all batch was calculated.

Mucoadhesion index^{23,24}

The viscosity component due to bioadhesion η_b was obtained by employing the following equation:

$$\eta_b = \eta_t - \eta_m - \eta_p \quad (\text{Eq. 3})$$

Where, η_t is the viscosity of prepared sol/mucin system, η_m is viscosity of 15 % w/w mucin solution at 32°C and η_p is the viscosity of the prepared sol/phosphate buffer (pH 7.4).

The MI cPs/s was calculated using the shear rate $D[S^{-1}]$ and the viscosity component η_b [cPs] according to the following equation:

$$MI = \eta_b * D \quad (\text{Eq. 4})$$

In vitro drug diffusion study

The Franz diffusion cell was used for studying the *in vitro* release of drug from viscous eye drop. Cellulose acetate membrane was used as a permeability membrane for the donor compartment. 3 ml of viscous eye drop containing drugs, sufficient for establishing sink conditions for the assay, was placed into the donor compartment. The receptor compartment contained 15 ml of phosphate buffer solution of pH 7.4 and it was maintained at 37°C under mild agitation using a magnetic stirrer. At specified time intervals, one ml was withdrawn and immediately it was restored with the same volume of phosphate buffer.^{23,24} The amount of drug released was assessed by measuring the absorbance of first order derivative spectra at 255.46 nm and 315.11 nm for DZ and TM respectively using UV-Visible spectrophotometer.²⁵

Sterility testing

The sterility testing of optimized batch was performed for the aerobic, anaerobic bacteria and fungi by using alternative thioglycolate medium (AGTM) and soyabean casein digest medium (SBCD). The positive control (growth promotion), negative control (sterility) test was also carried out. All the samples were inoculated separately in to ATGM and SBCD media and it was incubated at 35°C and 20-25°C, respectively for 7 days.²⁶

Preservative efficacy test (PET)

Preservative efficacy test was performed as per USP method. Culture of bacteria (*Escherichia coli* (ATCC 4352), *Pseudomonas aeruginosa* (ATCC 9027), *Staphylococcus aureus* (ATCC 6538)) and fungi (*Candida albicans* (ATCC 10231), *Aspergillus Niger* (ATCC 16404)) were grown in Sabouraud glucose agar medium and SBCD agar medium for respective bacteria and fungi. Both culture of organism were diluted aseptically with sterile WFI to obtain 10-6 CFU/ml as per USP. All the cultures were transferred into 5 test tubes containing 10 ml prepared eye drops and 0.1% of prepared cultures in each. Initial counts were noted. These solutions were poured into petri plate containing SBCD agar medium for bacterial cultures and Sabouraud glucose agar medium for fungi. They were incubated at $32.5 \pm 2.50^\circ\text{C}$ and $22.5 \pm 2.5^\circ\text{C}$ for bacteria and fungi respectively. The numbers of colony of microorganism at 7th, 14th and 28th day were recorded. The criteria for preservative effectiveness for their acceptable

range were also checked.²⁷

In vivo animal study

Male New Zealand albino rabbits, with weight of 3 ± 0.5 kg were used in the study. The animals were kept under standard laboratory conditions at $25 \pm 1^\circ\text{C}$ and $55 \pm 5\%$ relative humidity. Food and water intake was allowed during the study. The experimental protocol (Protocol No-1341, dated 13th November, 2013) was approved by Institutional Animal Ethics Committee (IAEC) of animal house of Anand Pharmacy College as per guideline of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social and Empowerment, Government of India. The animals were checked by a veterinarian to examine their health and ensure the absence of clinical observable abnormalities.

Ocular Irritation study

All experiments were carried out under supervision by a veterinary doctor. Four animals were divided into two groups having two animals in each for ocular irritation study, and it was performed according to Modified Draize technique. The optimized formulation was instilled daily for a period of 21 days and the rabbits were observed for redness, swelling and watering.^{23,24,28}

Pharmacokinetic study

In vivo pre-corneal drainage of aqueous drug formulation was determined after an instillation of 20 μL (2% w/v DZ and 0.5% w/v TM) solution to the left cornea. A small plastic vial containing an aliquot of 100 μL solution to be tested was placed near the eye of the rabbit. Tear samples (10 μL) were collected immediately before dosing and at time intervals one hour for up to 12 h after dosing. The tubes containing tear samples were stored in deep freeze at -20°C until analysis. Test sample mixture was prepared by spiking 50 μL of internal standard (200 $\mu\text{g}/\text{mL}$) and vortexed to ensure complete mixing of contents (2 min). After adding acetonitrile, the mixture was vortexed for further 2 min to ensure uniform mixing. After centrifugation at 3000 rpm at 4°C for 25 min, the organic layer was transferred to a clear pre-labelled 1.5 ml of polypropylene microcentrifuge tube. The resulting solution (10 μL) was estimated by a developed and validated chromatographic method for quantification of DZ and TM in ATF and rabbit tear fluid based on the Guidance for industry, Bioanalytical Method Validation issued by USFDA.^{29,30} The separation was performed on column (250 \times 4.6 mm) filled with ODS chemically bonded to porous silica particles of 5 μm , with the mobile phase containing methanol: 25mM potassium dihydrogen orthophosphate: acetonitrile (pH 6.8 adjusted with trimethylamine) (45:45:10 %v/v). Flow rate was set at 1.0 mL/min and the elution was monitored at 275 nm. Under these conditions DZ, TM and internal standard (Tramadol) (ISTD) were eluted at retention time of 3.15 min, 5.17 min and 7.7 min respectively. The quantification of the chromatograms was performed using the ratio of the peak area of the DZ and TM to that of internal standard (ISTD) and Pharmacokinetic parameters, C_{max} elimination half-life, elimination rate constant and AUC (area under curve) were calculated.³⁰⁻³³

Stability studies

To assess the drugs and formulation stability, stability studies were done according to ICH guidelines. Optimized formulation was packed in 10 mL white low density polyethylene (LDPE) plastic dropper bottles and kept in a stability chamber at specified temperature and humidity ($40 \pm 1^\circ\text{C}$ and 75% RH) for three months. Various physico-chemical parameters viz. pH, viscosity, mucoadhesive index and assay of DZ and TM were evaluated at an interval of 10 days' during stability study, in triplicate.

RESULTS AND DISCUSSION

pH dependent solubility of DZ

In the present study, solubility of DZ is found to be 40 mg/mL at pH 4-5.5. This pH is irritating to eye while DZ has very low solubility at physiological pH of eye (pH 6.5-8.5), hence enhancement of solubility of DZ at this pH was attempted by addition of surfactant. For augmentation of solubility of DZ at pH 7 (1% phosphate buffer) different concentration of PEG 400 (0.5-2.5 %w/w) was added and amount of drug dissolved was calculated, and it was observed that solubility of DZ was increased as concentration of surfactant increased. Addition of 1 % PEG 400 resulted in an increase of solubility up to 26 mg/ml which satisfied the requirement for the preparation of viscous eye drop having concentration of DZ 22 mg/ml. Concentration of PEG 400 above 1% resulted in increased viscosity, hence was found unsuitable for the ophthalmic formulation though it showed increase in solubility of DZ.

Identification of the Material Attributes and Potential CQAs required for development of ophthalmic viscous formulation

Preliminary trials were carried out for the selection of polymers, concentration and combination ratio. The role of polymers used in an ophthalmic formulations is to increase the residence time on the ocular surface and thereby increasing permeability and bioavailability. For the same, various polymers such as Na CMC, HPMC K15, CP934 and xanthan gum were screened on the basis of their viscosity and rheological behaviour. All polymers showed that as shear rate increased their value of viscosity reduced (Non-Newtonian behavior). Na CMC, HPMC K15 and CP 934 were observed to form a clear solution after complete swelling while, xanthan gum was observed to produce dense liquid containing particles. From the obtained result, it was found that xanthan gum produced dispersion which was brown in color and was found to degrade within one week, hence was not used in further study. The solution prepared using HPMC K15 was found to be more clear than Na CMC and also HPMC K15 was selected as the best choice of polymer in the current study due to its common use in ophthalmic formulations, reliability, and the appropriateness of its physicochemical structure. HPMC K15, a non ionic polymer, forms viscous solution at all the pH values. However, it was observed that higher concentration (1.0 % w/v) of HPMC K15 it was possessing higher viscosity which may hinders the vision of the eye and generate difficulty during instillation, hence higher concentration of HPMC K15 was not used in further study (Figure 1). CP 934 was further selected because of its mucoadhesion property considering the ease of instillation of eye drop. Hence, from the results of preliminary trials, CP 934 and HPMC K15 were selected as rate controlling and mucoadhesive polymer. Further optimization was carried out via Full factorial experimental design. The potential CQAs for the development of ophthalmic viscous solution were identified and defined to be viscosity, MI, and % Cumulative release for both drugs.

Drug-polymer compatibility study Fourier Transform Infrared Spectroscopy (FTIR)

Figure 2 depicts FTIR spectra of physical mixture, where the peaks of DZ at 3378 cm^{-1} due to primary amino (-NH) group and TM with principal peaks at 3311 cm^{-1} indicating the presence of -OH group is clearly visible. And hence, Infrared Spectra of physical mixture revealed no considerable changes in the FTIR peaks of DZ and TM in the physical mixture thereby indicating the absence of any interaction at physical mixture level.

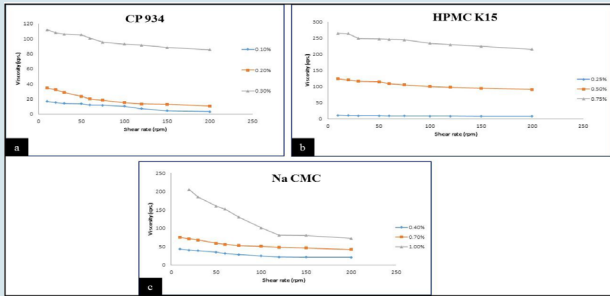


Figure 1: Rheograms of (a) Carbopol 934 (b) HPMC K15 (c) Sodium Carboxy Methyl Cellulose.

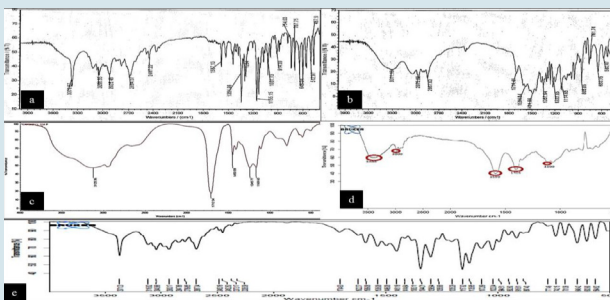


Figure 2: FTIR spectra of (a) DZ (b) TM (c) CP 934 (d) HPMC K15 (e) Physical mixture containing DZ, TM, CP 934 and HPMC K15.

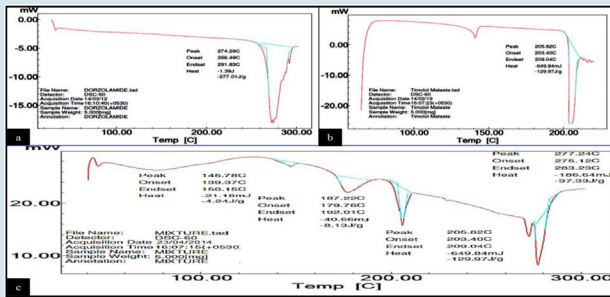


Figure 3: DSC thermogram of (a) DZ (b) TM physical mixture containing DZ, TM, CP 934 and HPMC K15.

Differential scanning calorimetry (DSC)

The thermogram of the investigated physical mixture exhibited the characteristic endothermic peaks of DZ and TM at 274.29°C and 205.82 °C respectively, indicating the absence of interaction between the drugs (Figure 3) thereby proving drug-excipient compatibility.

Formulation optimization by full factorial design

3² FFD included a total of 13 experiments for two factors (X₁ - HPMC K15 % w/v and X₂-CP 934 % w/v) with 4 center points. The experimental runs with independent variables and the observed responses for the 13

Table 1: Responses of various viscous eye drop formulations prepared as per the experimental design.

Batch	Factors			Responses		
	X ₁ : HPMC K15 (%w/v)	X ₂ : CP 934 (%w/v)	Y ₁ : Viscosity (cps)	Y ₂ : Mucoadhesive index (Cps)	Y ₃ : % CDR- at 12h for DZ (%w/w)	Y ₄ : % CDR at 12h for TM (%w/w)
F1	0.50	0.1	42.36	3323.3	99.01	99.04
F2	0.25	0.3	86.43	3752.0	90.95	79.02
F3	0.75	0.3	133.76	4192.0	69.99	67.55
F4	0.50	0.2	78.12	4105.0	90.20	85.65
F5	0.50	0.2	77.93	4109.0	89.70	83.87
F6	0.75	0.2	121.99	4251.0	75.73	73.45
F7	0.25	0.1	28.67	2621.0	82.47	83.29
F8	0.50	0.3	132.50	3810.0	74.30	79.16
F9	0.50	0.2	78.19	4103.0	90.14	85.23
F10	0.75	0.1	80.08	3999.0	90.89	90.17
F11	0.25	0.2	33.93	3298.0	88.44	81.78
F12	0.50	0.2	78.10	4100.0	88.88	86.09
F13	0.50	0.2	77.99	4020.0	89.70	86.02

Table 2: Polynomial equation with ANOVA for response.

Coefficient	Viscosity	MI	%CDR at 12h for DZ	%CDR at 12h for TM
β ₀	-48.687	-270.261	77.678	65.829
β ₁ (X ₁)	124.533	5738.56	41.927	162.514
β ₂ (X ₂)	335.967	21059.902	85.0167	109.531
β ₃ (X ₁ X ₂)	-	9380	293.8	185.35
β ₄ (X ₁ ²)	-	2015.228	-	308.378
β ₅ (X ₂ ²)	-	33380.172	-	-
r ²	0.9231	0.9552	0.6856	0.9566
Adjusted r ²	0.9077	0.9232	0.5809	0.9256
PRESS	2160.413	824129.6	888.7547	277.3309
Degree of freedom	2	5	3	5
Sum of Squares	12588.12	2560867	551.829	671.578
Mean Square	6294.061	512173.4	183.943	134.316
F value (Fischers ratio)	60.072	29.868	6.5446	30.864
p value	0.001	0.001	0.0122	0.001

MI is mucoadhesion index, CDR is cumulative drug release

batches are shown in Table 1.

For estimation of quantitative effects of the different factors and factor level on the response, models were computed by the Design-Expert software Trial Version (9.0.1) in terms of coded value. The quadratic model is tabulated in Table 2. A model is considered as significant if *p* < 0.05. The model selection was done on the basis of PRESS (predicted residual error sum of square) and R-square value of model. The model with lower PRESS value and R square near to 1 was selected for the

analysis of the response. The significant p-value, multiple correlation coefficient (r^2), adjusted multiple correlation coefficient (adjusted R^2), coefficient variation and the predicted PRESS of this model indicated that the evolved regression model was significant and valid for each considered response (Table 2)

The factor and response variables were correlated using polynomial equation with statistical analysis. The values of the coefficients of factor X_1 and X_2 exhibits the effect of these variables on the response. Mathematical relationships, generated using MLRA (Multiple linear regression analysis), gives an insight into the effect of the different independent variables. The full model consists the coefficients for intercept, first-order main effects, interaction term and second order effects. A positive sign of coefficient indicated a synergistic effect while negative term indicated an antagonistic effect upon the response. For estimation of significance of model, ANOVA was determined using Design Expert software at 5 % significance level. From this equation, the rank order of standardized co-efficient was $X_1 > X_2$ indicating that HPMC K15 had the greatest potential influence on the viscosity, mucoadhesion index and cumulative drug release of DZ and TM.

The 3D response surface plot (Figure 4a) shows the linear relationship at viscosity of 70 cps with respect to both factors i.e. X_1 (0.25-0.75 % w/v) and X_2 (0.1-0.3 % w/v). The Figure 4b shows the linear relationship at MI of 3500 with respect to both factors factor X_1 and X_2 . The plus sign (+) indicated that the higher concentration of HPMC K15 and CP 934 results in higher viscosity and higher MI. With DZ release, the Figure 4c shows the relationship between factor X_1 and X_2 . The concentration of HPMC K15 (0.25-0.35% w/v) and CP 934 (0.10-0.15 % w/v) resulted in greater than 90 % drug release of DZ. The Figure 4d shows the nonlinear relationship between factor X_1 and X_2 . The concentration of HPMC K15 (0.25-0.45 % w/v) and Carbopol 940 (0.1-0.20 % w/v) resulted in greater than 90 % drug release of TM.

Various constraints for factor and responses were set for obtaining optimum viscous eye drop formulation. The optimum values of the variables were obtained using the Design-Expert software based on criterion of desirability. The observed value of an optimized formulation was quite closer to the predicted value. The optimized formula for viscous eye drop formulation was achieved with 0.1 % of CP 934 and 0.55 % of HPMC K15 resulting in MI of 3532.57 with % drug release greater than 90% for both drugs viz. DZ and TM.

Evaluation of viscous eye drop

Clarity, pH, osmolarity and refractive index

The formulations were evaluated for clarity, pH and osmolarity. All the formulations were clear solution except the batches F2, F3, F6 and F8 having 0.3% CP 934 were found to be slightly opaque. As the concentration of CP 934 decreases, the opacity of the solution was found to decrease. In the science based development of formulation, the patient compliance will increase with clear formulation as it does not cause any problem with visibility after instillation of eye drop. The pH of F1 to F13 formulations was found to be 7.4 ± 0.25 . Ocular irritation moreover will not be experienced by the patient since the pH was close to the physiological pH. The osmolarity of optimized batch was found to be 345 mOsmol/kg, which was within the acceptable tonicity range 310-350 mOsmol/kg to avoid ocular irritation. Refractive index of tear fluid is 1.340 to 1.360. It is recommended that eye drop should have refractive index values not higher than 1.476. Formulated DZ and TM viscous eye drop had refractive index values ranging from 1.401 to 1.304 which were found to be within the recommended values.²⁴

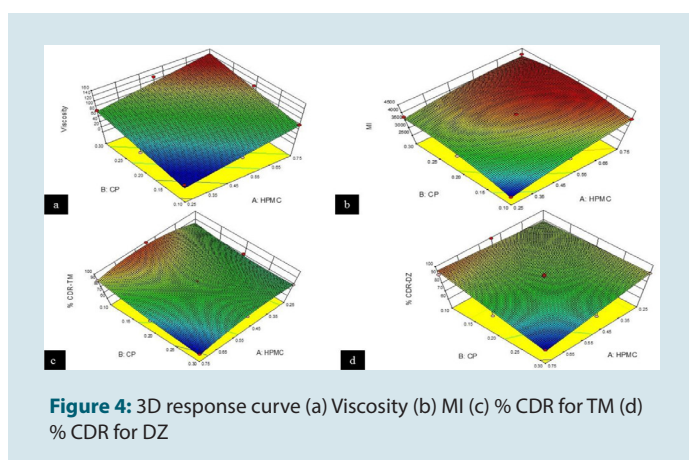


Figure 4: 3D response curve (a) Viscosity (b) MI (c) % CDR for TM (d) % CDR for DZ

Assay of DZ and TM

Assay of DZ and TM in viscous eye drop and in marketed formulation were done by developed RP-HPLC method with elution of DZ and TM at 3.18 and 5.15 min respectively at 1 mL/min flow rate. % recovery of DZ was found to be 99.73% and 99.68 % and % recovery of TM was found to be 100.20% and 99.41 % in viscous eye drop and marketed formulation (Dorzox-T (2% Dorzolamide, 0.5% Timolol) Cipla), respectively.

Rheological evaluation

Viscosity is one of the CQA that determines clinical performance and physical stability of the dispersed systems. It has been reported that increasing the viscosity of an ophthalmic preparation increases the contact time between the formulation and eye tissue, which results in increased ocular availability of drug and hence improved clinical outcome. The high viscous ophthalmic dispersion systems, to some extent, also inhibit the droplet fusion by reducing their movement.

The pseudo plastic character of precorneal tear film should be disturbed less by the administration of ophthalmic products. The ocular shear rate is about 0.03 s^{-1} during interblinking periods and $4250 - 2850 \text{ s}^{-1}$ during blinking. The viscoelastic fluid having high viscosity under low shear rates and low viscosity under high shear rates, called as pseudo plastic fluid, is often preferred. The viscosity of the formulations at pH 7.4 is shown in Figure 5a and 5b. All the formulations exhibited pseudo-plastic behaviour, i.e., decrease in the viscosity with increase in angular velocity. It was found that batches F3, F6 and F10 showed very high viscosity due to high concentration of HPMC (0.75 %w/w) while, batches F7 and F11 showed very low viscosity due to low concentrations HPMC (0.25 %w/w). To mimic physiological condition, formulations were mixed with artificial tear fluid (ATF) in a ratio of 40:7 and the scenario remained the same. Flow behaviour is an important CQA and control strategy shall be planned to minimize batch-to-batch variability. This can be achieved by monitoring the process conditions. Longer precorneal residence time will be observed due to increase in the viscosity, resulting in sustained clinical action.

Mucoadhesion Index

As shown in Table 1, MI was dependent on concentration of polymers and their combination. In combination of HPMC K15 and CP 934, batches showed excellent mucoadhesive strength. Higher viscosity of solutions due to HPMC was contributed for viscosity while CP 934 was responsible for mucoadhesion strength. Higher mucoadhesion index was not tolerated by eye and lower mucoadhesion index reflect poor prolong release of drug.

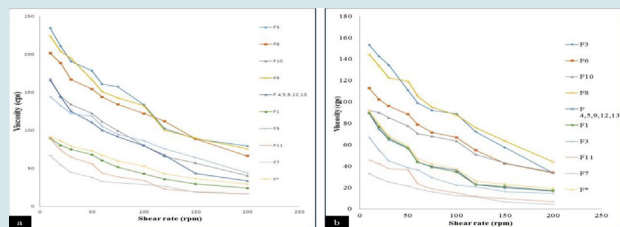


Figure 5: Rheogram (a) Formulations in WFI (b) Formulations in WFI after dilution with artificial tear fluid.

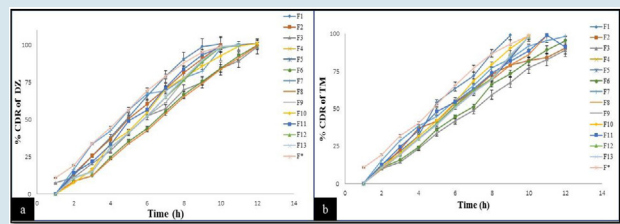


Figure 6: % CDR in 13 batches upto 12 h (a) DZ (b) TM.

Table 3: Microbial count at specified time interval for PET in F* batch.

Microorganisms	Initial	7 th day	14 th day	28 th day
<i>Candida Albicans</i>	14 x 10 ⁶	84 x 10 ⁵	36 x 10 ⁴	31 x 10 ³
<i>Aspergillus Niger</i>	8x 10 ⁶	35 x 10 ⁵	50 x 10 ⁴	11 x 10 ³
<i>Escherichia Coli</i>	23 x 10 ⁵	15 x 10 ⁴	298	298
<i>Pseudomonas Aeruginosa</i>	15 x 10 ⁵	09 x 10 ⁴	280	280
<i>Staphylococcus Aureus</i>	19 x 10 ⁵	10 x 10 ⁴	236	326

In vitro Drug Diffusion Study

An *in vitro* release mechanism was dependent on two simultaneous processes: water migration into the swollen polymer and drug diffusion. The swelling characteristics of HPMC and gelling characteristics of Carbopol are the probable reasons for the retardation of drug release. The diffusion path length is increased due to swelling of HPMC. The drug release from the formulations is shown in Figure 6. The formulations showed better performance in drug release studies and sustained the drug action upto 12 h. It is worthwhile to note that the F1, F10 and F13 showed complete drug release relatively faster, the reason accounted can be the lower concentration of the polymers. From the results it is concluded that the high viscosity plays an important role in controlling the release of drug from the formulations. When the polymer concentration increases, drug release decreases, and vice versa. The drug release shall be considered as a potential performance test and hence it should be given due weightage.

Sterility testing

The optimized formulation also was found to pass the sterility test, as there was no evidence of microbial growth when incubated for a period of not less than 7 days at 30- 35°C in AGTM medium and at 20-25°C in the SBCD medium.

Preservative efficacy test

PET was done to evaluate the efficiency of preservative. It revealed that fungi *Candida albicans* and *Aspergillus Niger* show inhibition in growth after 7th, 14th and 28th day from initial count. The microbial count for bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*) is shown in Table 3 and as per USP there should be 1 log reduction after 7 days, 3 log reductions after 14 days and no growth in population as compared to 14th day after 28 days. In case of fungi (Table 3), as per USP, there should be no growth/inhibition for PET. Optimized batch was obeyed the similar pattern of reduction in population as per standard limit and complies with the results.

In vivo Animal Study

Ocular irritation study

Ocular tolerability results showed no evidence of inflammation and/or discomfort in rabbit eyes. Thus, optimized batch (F*) is safe, provides therapeutically efficacious and suitable for the eye instillation. Daily instillation of the optimized batch in the rabbit eye for 21 days, showed no sign of redness, swelling or watering. Clinical efficacy is at the centre of formulation design. Therefore, these parameters should be consider as critical quality attribute.

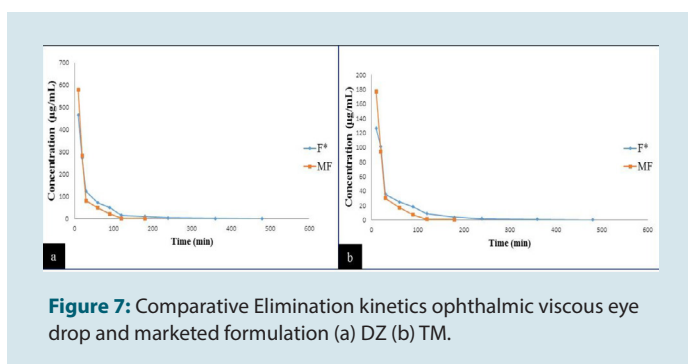
Table 4: Summary of Bioanalytical method validation parameters.

Parameters	DZ	TM
Linearity		
Linearity range (µg/ml) ^a	1-450	0.3-450
Correlation coefficient	0.9987	0.9964
Linear Regression Equation	Y=0.0971x + 0.7682	Y= 0.1306x + 0.4129
Slope ± SD	0.097 ± 0.002	0.130 ± 0.002
Intercept ± SD	0.768 ± 0.149	0.412 ± 0.061
Bartlett's Test ^b	0.00076	0.01497
Sensitivity at LLOQ^c		
(Mean recovered ± %CV)	87.29 ± 3.569	88.07 ± 7.059
Stability		
Freeze/thaw stability		
(Mean recovered ± %CV)		
LQC	89.40 ± 3.73	89.21 ± 5.17
HQC	95.64 ± 0.79	97.27 ± 1.60
Short term stability^d		
(Mean recovered ± %CV)		
LQC	93.70 ± 3.034	94.20 ± 4.412
HQC	95.14 ± 2.078	96.20 ± 1.90
Long term stability^d		
(Mean recovered ± %CV)		
LQC	65.79 ± 8.281	80.71 ± 6.284
HQC	80.78 ± 4.071	90.67 ± 0.856

^a5 replicates; ^b $\chi^2(0.05, 5)$ value less than 11.070 at 95% confidence interval level; ^c mean of 5 replicate; ^d mean of 3 replicates.

Table 5: Pharmacokinetics parameters for DZ and TM.

Pharmacokinetics parameters	DZ		TM	
	Viscous eye drop	solution	Viscous eye drop	solution
AUC ₀₋₁₂ (µg h/mL) ± SD	1186.151± 12.36	1023.742± 21.34	1248.909 ± 20.35	1076.955 ± 15.25
AUMC _{0-∞} (µg h/mL) ± SD	108520.67 ± 13.26	2030.34 ± 14.36	91628.65 ± 13.69	14652.28 ± 10.25
MRT (h)	1.524	0.330	1.222	0.226
RTmax (h) ± SD	1.333± 0.09	0.050 ± 0.001	1.333 ± 0.09	0.05 ± 0.001
C _{max} (µg/mL) ± SD	464.710 ± 9.58	577.690 ± 10.25	126.52 ± 1.58	177.42 ± 1.23
k _{el} (1/h)	0.925	0.842	2.376	2.224

**Figure 7:** Comparative Elimination kinetics ophthalmic viscous eye drop and marketed formulation (a) DZ (b) TM.

Pharmacokinetic study

The optimized viscous eye drop formulation was instilled to rabbit's eye and concentration of DZ and TM in tear (C_{tr}) fluid was estimated by developed and validated RP-HPLC method (Table 4).

Pharmacokinetic parameters give valuable information to the formulation and can serve as an important tool in an establishment of IVIVC, which is an important critical quality attribute. The results of the pharmacokinetic parameters are illustrated in Figure 7. The pharmacokinetic parameters of both the formulations are listed in Table 5. The rate of excretion of drug from viscous eye drop was lesser than that from marketed eye drop. The bioadhesive polymers (HPMC and Carbopol) helped in slowing drainage (Figure 7). The formulation of viscous eye drops prolonged drug precorneal residence time as reflected by T_{max} . The average peak elimination rate (K_{el}) of DZ and TM in viscous eye drop was lower than that of the eye drop (Table 4). Moreover, a significant difference was observed in the area under excretion rate curve (AUC₀₋₁₂) of the viscous eye drop and marketed formulation. The higher AUC of viscous eye drop formulation may contribute to better clinical efficacy (Table 5).

Stability studies

Stability study carried out with optimized batch F* for one month. The data of physicochemical parameters like pH, viscosity, MI, drug content was measured. There were no physical changes in formulation. Results of stability studied revealed that there was no significant change in viscosity and mucoadhesive index as compared to initial over 30 days under accelerated temperature and humidity condition. Also, there was no change in chemical property like pH, Assay of DZ, Assay of TM indicating no degradation of formulation over period of 30 days stability study.

CONCLUSION

Dorzolamide and timolol maleate (antiglaucoma agents) were successfully formulated as viscous eye drop. A 3² full factorial design was employed systemically to optimize formulation. The CQA, CMA and CPP were identified in the study. Processing conditions did not affect either of the drugs as evident from the assay. The range of CQAs chosen in this study will give us an effective overview of the key parameters involved in the formation of a viscous eye drop. This knowledge base will give a formulation design space from where carbopol 934 and HPMC K15 with a set of critical properties can be chosen to formulate a viscous eye drop specified CQAs. The present research work also vouches successful development and validation novel RP-HPLC bioanalytical method for the estimation of DZ and TM in artificial tear fluid according to USFDA guideline, where % extraction recovery, by employing protein precipitation technique, obtained was more than 85 %, with % CV of all parameters were less than 15 % which revealed that the method possess high degree of utility for estimation of the drugs in bioanalytical samples.

Pharmacokinetic parameters demonstrates that drugs were detected up to their LLOQ level in rabbit tear fluid indicating sensitivity of method. Elimination kinetics of viscous eye drop and marketed preparation indicated that drugs were more retained in viscous solution compared to marketed preparation. MRT of viscous eye drops was 4-5 folds higher and C_{max} was lesser compared to marketed formulation. Thus, the developed formulation is a viable alternative to conventional eye drops suspension by virtue of its ability to enhance bioavailability through its longer precorneal residence time and ability to sustain the drug release. Also, important is the ease of administration and decreased frequency of administration resulting in better patient compliance.

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CONFLICT OF INTEREST

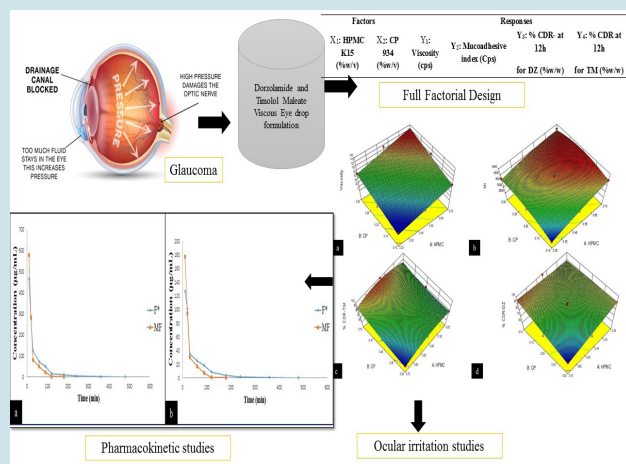
The authors have NO affiliations with or involvement in any organization or entity with any financial interest, or non-financial interest in the subject matter or materials discussed in this manuscript.

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PICTORIAL ABSTRACT



SUMMARY

- In this study, viscous formulation for dorzolamide and timolol maleate was prepared using bioadhesive polymers (HPMC and Carbopol) and optimized by 32 FFD. It was further investigated for its *in vivo* performance in comparison to conventional formulation. The drugs from the tear fluid were extracted and quantified by developed and validated bioanalytical HPLC method. The rate of excretion of drug from viscous eye drop was observed to be lesser than that from marketed eye drop with prolonged drug precorneal residence time. Hence, the developed viscous formulation will prove to be a viable alternative to conventional eye drops suspension by virtue of its ability to enhance bioavailability, facilitating the ease of administration and decreased frequency of administration resulting in better patient compliance.

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