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Design, Development and Characterization of Ketorolac Tromethamine Nanosuspension Loaded *In-Situ* Mucoadhesive Ocular Gel

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ABSTRACT

Currently, a variety of ophthalmic products illustrate low bioavailability after topical administration because of anatomical and physiological barriers of eye. Ketorolac tromethamine (KT) is a BCS class I, potent anti-inflammatory drug. The rationale of present work was to design and develop KT nanosuspension loaded *in situ* gel with sustained effect and greater permeability for ocular drug delivery through increased ocular residence time of drug. KT nanosuspension loaded *in situ* gel was designed by using 3^2factorial design. Polymers and surfactant were optimized through trial batches exhibiting better drug content (%), *In Vitro* trans-corneal permeation (%) and corneal hydration (%).Optimized formulation was evaluated for clarity, pH, gelling capacity, rheological behavior, drug content (%), *Ex-vivo* trans-corneal permeation, corneal hydration, HET CAM assay and physical stability. The resultant formulations revealed optimum viscosity, pH and drug content; as well as higher trans-corneal permeability when compared to the marketed eye drop. Optimized formulation was found as nonirritant to eye with sustained effect and good stability. So, current system can be considered as an efficient ocular drug delivery system for the treatment of postoperative inflammation, which would improve patient compliance and ocular bioavailability.

Keywords: Ketorolac tromethamine, *in situ* gel, corneal hydration, mucoadhesive, trans-corneal permeability

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1. INTRODUCTION

Ketorolac tromethamine (KT) is a BCS class I drug having potent anti-inflammatory activity. Chemically it is a pyrrolizine carboxylic acid; NSAID used for the treatment of post-operative eye inflammation and conjunctivitis[1-2]. Being water soluble agent; to formulate nanosystem is quite difficult by entrapment in polymeric vehicle[3]. Generally the basic problems for topical application in the treatment of ocular infection is drug loss from pre-corneal surface, conjunctival uptake due to poor bioavailability and rapid drainage through naso-lacrimal areas[4-5]. However, short pre-corneal contact time combined with corneal impermeability result in low bioavailability, and frequent dosing is usually needed[6]. Nanosuspension by nanoprecipitation is the novel drug delivery approach for sustaining the drug in its crystalline state[7-9]. Selection of polymers and stabilizers are very essential in the development of nanosuspensions to avoid particle aggregation, and crystal growth[10-11]. Design of experiment has proven effective optimization of formulations[10-11]. In present investigation; formulation was optimized by using 3^2 factorial design. Hence, based on above challenge, KT nanosuspension loaded *in situ* gel increases ocular bioavailability, and residence time on the corneal surface. The rationale of present work was to design and develop KT nanosuspension loaded *in situ* gel with sustained effect and greater permeability for challenging ocular drug delivery.

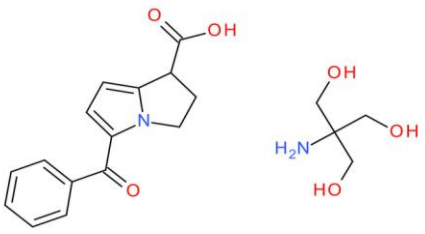

Figure 1: Chemical structure of ketorolac tromethamine

2. MATERIALS AND METHODS

2.1. Materials

Ketorolac tromethamine (KT) gifted from Zhejiang Medicines and Health Products Imports & Exports Co. Ltd. China, was used as a model drug in the study. Eudragit RL-100 was received as a gift sample from Evonik Industries, Mumbai. Carbopol 934, sodium alginate and poloxamer 188 were procured from Sigma Aldrich. All other chemicals and reagents were of analytical grade. Double distilled water was used throughout the investigation.

2.2. Methods

2.2.1. Selection and preliminary optimization of gelling agent and preservatives by formulation of trial batches

Initially, trial batches were formulated using two different gelling agents (sodium alginate and carbopol 934) at different concentrations. Benzalkonium chloride was used as a preservative at various concentrations. Freeze dried powder of optimized KT loaded nanosuspension was dispersed into *in situ* gelling system of different gelling agents, and then subjected for determination of trans-corneal permeation and corneal hydration study. Based on results, gelling agent and preservative were optimized for design of formulations.

2.2.2. Experimental Design

Initially screening studies were performed to test the effect of process parameters and formulation parameters to fabricate stable KT nanosuspension loaded *in situ* gel. The concentration of sodium alginate (% w/w) and concentration of benzalkonium chloride (% w/w) were acknowledged as significant formulation parameters (Table 1). Design of experiment was used thoroughly to evaluate and optimize the selected formulation parameters at three levels (-1, 0, +1) using 3^2 factorial design to find out their effects on critical quality attributes of KT nanosuspension loaded *in situ* gel.

2.2.3. Preparation of KT nanosuspension loaded in situ gel

Nanoprecipitation followed by probe sonication technique was used to prepare KT loaded nanosuspensions. Drug and polymers were co-dissolved in 10 ml organic solution (methanol: acetone in 1:1 ratio). The solution was slowly injected drop wise in 100 ml of distilled water containing surfactant using high speed homogenizer at 2500 rpm. Organic solvent was evaporated completely. To the resulting uniform nanosuspension, probe sonication was used at 20-25 kHz for 5 min. The optimized formulation KT6 was lyophilized with mannitol as cryoprotectant by lyophilizer (Christ, Alpha, 12LD PLUS). The freeze dried product was kept in air tight container.

Accurately weighed sodium alginate was dissolved in hot distilled water, cooled at room temperature and lyophilized powder of optimized KT nanosuspension was dispersed into it. To above system, benzalkonium chloride was added as a preservative at the end. HPMC E15 (1 %) is used as co-polymer using a magnetic stirrer to dissolve polymer completely. Sodium chloride (0.9 %) was added as isotonicity adjuster. The pH of the solution was adjusted to 6.5 using 0.1 N NaOH/0.1 N HCl.

2.2.4. Evaluation of optimized KT nanosuspension loaded in situ gel

2.2.4.1. Test for Clarity and pH

Clarity was observed for the presence of any particular matter visually. The pH of formulations were determined, using a calibrated pH meter (Digital Systronic, Mumbai, India).The average reading was recorded (n=3).

2.2.4.2. Gelling capacity determination

Gelling capacity of the formulation was determined by insertion of a drop of the formulation in a vial having 2 ml of fresh simulated tear fluid, and visually observed. The time taken for gelling was noted[12-14].

2.2.4.3. Rheological study

The viscosity of the gels was determined on Brookfield Viscometer (Brookfield Ametek) by using L1 spindle at 100 rpm.

2.2.4.4. Drug content (%) determination

Drug content was determined by taking 1 ml gel in 50 ml volumetric flask. It was dissolved in distilled water properly and the final volume was made up to 50 ml with it. After appropriate dilution, the absorbance of prepared solution was measured at 322 nm by using UV spectrophotometer (UV-1800, Shimadzu, Japan) and % drug content in the formulation was calculated.

2.2.4.5. Ex-vivo trans-corneal permeation and corneal hydration study

The corneal area available for diffusion was 0.5 cm^2 which was placed between donor and receptor compartments of modified Franz diffusion cell with epithelial surface faced the donor compartment. Receptor compartment was filled with 10 ml fresh; simulated tear fluid (pH 7.4) and all air bubbles were removed from the compartment. An aliquot (1 ml) of prepared KT nanosuspension loaded *in situ* gel was placed on the cornea and the opening of the donor cell was sealed with a glass cover slip; receptor fluid was kept at 37 °C with constant stirring using a Teflon-coated magnetic stir bead. Permeation study was continued for 120 minutes, and samples were withdrawn from receptor and analyzed for ketorolac tromethamine content by measuring absorbance at 322 nm using UV spectrophotometer (UV-1800, Shimadzu, Japan). Results were expressed as percentage permeation or *In vitro* ocular availability. Marketed formulation of KT was used for comparative analysis. The permeation (%) or *In vitro* ocular availability was calculated as follows[15-16].

$$\text{Permeation (\%)} = \{\text{Qty. of drug permeated/Initial Qty.}\} \times 100$$

In brief, at the end of the experiment, the scleral tissue was removed from cornea; its epithelial surface was wiped with filter paper and weighed (Initial weight). The cornea was then soaked in 1ml of methanol, dried overnight at 90 °C, and reweighed (Final weight). From the difference in weight, corneal hydration (%) was calculated as follows[15-16].

$$\text{Corneal hydration} = \{(\text{Initial weight-Final weight})/\text{Initial weight}\} \times 100$$

2.2.4.6. HET-CAM assay

HET-CAM assay was completed following ICCVAM recommendations (Appendix G, November 2006)[17-19]. Clean and fertile chicken eggs weighing 40-50 gm were purchased from commercial sources. Those eggs were candled to detect the viability and development of embryo before use. Nonviable or defective eggs were discarded. Finally, six eggs (after 9th day of incubation) per group were used in the study to evaluate the potential of a test substance to produce ocular irritation using their chorio-allantoic membrane (CAM) [17-19]. Care should be taken while removing the eggshell to ensure that the inner membrane was not injured.

The NaCl (0.9 %) solution was applied in each experiment to provide a baseline for the assay. Negative Control (0.3 ml of 1 % SDS + 0.3 ml of 0.1 N NaOH) and 0.3 ml of test formulation was applied on the CAM of treatment group ensuring that at least 50 % of the CAM surface area was covered. Marketed formulation (0.3 ml, Acular LS) directly applied on the CAM of standard group. Effects were checked for blood vessel lysis, hemorrhage and coagulation. Accordingly, combined score was derived to classify the irritancy level of the test substance. The reactions on the CAM were observed over a period of 5 min (0.5 min, 2 min & 5 min) and time for the appearance of each of the noted endpoints should be recorded, in seconds[17-19].

2.2.4.7. Accelerated stability studies

Optimized sterile gel formulation was evaluated for its stability. Formulation was filled in glass vials, closed with rubber closures and sealed with an aluminum caps. It was kept in the stability chamber (Remi) at 40 ± 2°C temperature and 75 ± 5 % RH for one month[20]. Samples were withdrawn at specific time intervals and analyzed for visual appearance, drug content, pH, gelling capacity and trans-corneal permeation.

2.2.4.8. Statistical analysis

Statistically data were analyzed using Microsoft Excel 2007 and Design Expert 7.0.0 software. The results were documented as mean ± SD (n=3).

3. RESULTS AND DISCUSSION

3.1. Selection and preliminary optimization of gelling agent and preservatives by formulation of trial batches

Sodium alginate in the range of 0.5 % w/w to 1.5 % w/w was selected as mucoadhesive gelling agent based on results[21-22]. Benzalkonium chloride in the range of 0.05 % w/w to 0.1 % w/w was optimized as a preservative. All formulations in that range showed trans-corneal permeation in between 5.1 ± 0.04 % to 9.3 ± 0.17 %. Corneal hydration of all formulations were found in between 77.05 ± 0.06% to 79.77 ± 0.08 %; depicting normal range without damage to the corneal tissue. The concentration range was optimized based on highest % permeation and normal value of corneal hydration.

3.2. Experimental Design

Regression analysis of data acquired from the experimental runs generated following equations in which F ratios were statistically significant ($p < 0.05$) with Adj-R^2 value in the range of 0.9-1 (Table 2) and with a statistically non-significant lack of fit values ($p > 0.05$).

Table 1: Preparation of KT nanosuspension loaded *in situ* gel using 3^2 factorial design

Formulation Code	Independent Variables				Dependent Variables		
	Formulation Variable				Y_1	Y_2	Y_3
	X_1	Conc. of Sodium Alginate (% w/w)	X_2	Conc. of Benzalkonium Chloride (% w/w)	Drug Content (%)	Trans-corneal Permeation (%)	Corneal Hydration (%)
KTNG1	− 1	0.5	− 1	0.5	76.18 ± 1.17	2.9 ± 0.2	76.81 ± 0.96
KTNG2	0	1	− 1	0.5	79.11 ± 1.09	3.1 ± 0.2	77.19 ± 0.87
KTNG3	+1	1.5	− 1	0.5	83.25 ± 1.31	3.6 ± 0.1	78.41 ± 0.81
KTNG4	− 1	0.5	0	0.75	77.12 ± 0.97	3.0 ± 0.3	76.91 ± 0.93
KTNG5	0	1	0	0.75	79.91 ± 1.18	3.3 ± 0.2	77.12 ± 1.07
KTNG6	+1	1.5	0	0.75	84.19 ± 0.83	3.7 ± 0.1	78.34 ± 1.05
KTNG7	− 1	0.5	+1	1	76.32 ± 0.98	2.8 ± 0.3	76.55 ± 0.77
KTNG8	0	1	+1	1	78.47 ± 0.99	3.1 ± 0.2	77.07 ± 1.12
KTNG9	+1	1.5	+1	1	83.92 ± 1.28	3.6 ± 0.2	78.41 ± 0.81

Where +1 is higher level, −1 is lower level and 0 is mid level for the independent variable and all values are expressed as mean ± SD (n = 3).

These model equations fitted the data well. A positive sign indicates a synergistic effect, while negative sign indicates an antagonistic effect[21-23, 27].

Drug Content (%) = $79.74 + (3.62 \times X_1) + (0.028 \times X_2) + (0.13 \times X_1.X_2) + (1 \times X_1^2) - (0.86 \times X_2^2)$ (Quadratic model)…… (1)

Trans-corneal Permeation (%) = $3.27 + (0.37 \times X_1) - (0.017 \times X_2) + (0.025 \times X_1.X_2) + (1 \times X_1^2) - (0.15 \times X_2^2)$ (Quadratic model)…… (2)

Corneal Hydration (%) = $77.16 + (0.82 \times X_1) - (0.063 \times X_2) + (0.065 \times X_1.X_2) + (0.44 \times X_1^2) - (0.05 \times X_2^2)$ (Quadratic model)…… (3)

Where X_1 and X_2 are concentration of sodium alginate (% w/w) and concentration of benzalkonium chloride (% w/w) respectively.

Equation (1) represents that, increase in concentration of both sodium alginate and benzalkonium chloride increases % drug content. According to equations (2) and (3), concentration of sodium alginate is directly proportional and concentration of benzalkonium chloride is inversely proportional to % trans-corneal Permeation and % corneal Hydration respectively. Response surface plots displayed in figure 2 clearly supports the results.

Table 2: Results of statistical analysis of the experimental design

Responses	Sources		
	Model *p* value	Adj-R^2	Lack of fit test *p* value
Drug Content (%)	0.0014	0.9371	0.3791
Trans-corneal Permeation (%)	0.0011	0.9558	0.7746
Corneal Hydration (%)	0.0022	0.9119	0.3272

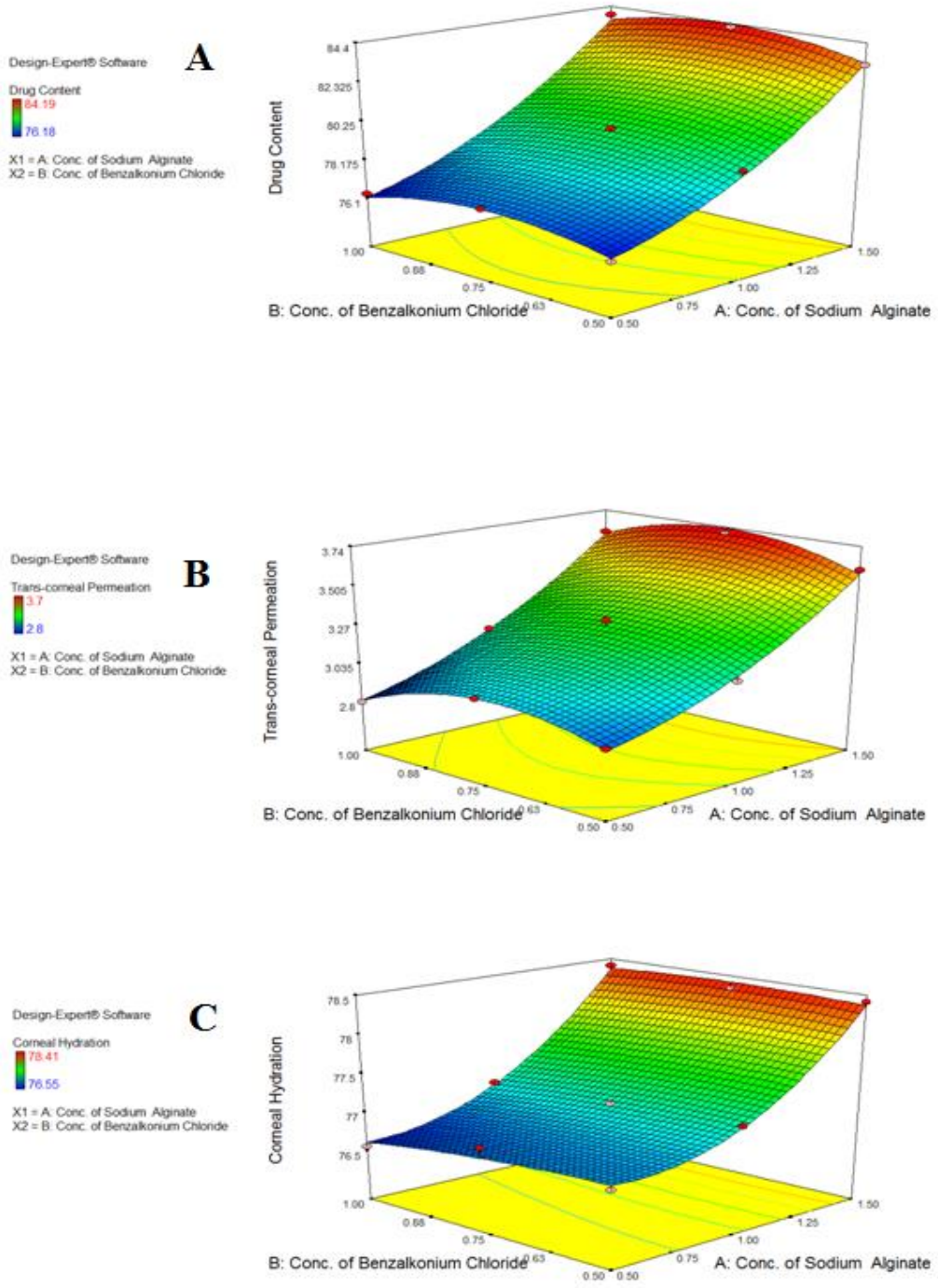

Figure 2: Response surface plots showing the effect of conc. of sodium alginate (% w/w) and conc. of benzalkonium chloride (% w/w) on (A) Drug content, (B) Trans-corneal permeation and (C) Corneal hydration

The desirability function was evaluated by Design-Expert software to obtain the optimized KT nanosuspension loaded *in situ* gel. The model verification results are displayed in table 3; that comparing observed and predicted values of drug content, trans-corneal permeation and corneal hydration by using model equations.

Table 3: Comparison of observed and predicted values of responses of optimized KT nanosuspension loaded *in situ* gel

Factors		Predicted value			Observed value*		
Conc. of Sodium Alginate (% w/w)	Conc. of Benzalkonium Chloride (% w/w)	Drug Content (%)	Trans-corneal Permeation (%)	Corneal Hydration (%)	Drug Content (%)	Trans-corneal Permeation (%)	Corneal Hydration (%)
1.5	0.75	84.31	3.73	78.41	84.19 ± 0.83	3.7 ± 0.1	78.34 ± 1.05

* All values are mean ± SD (n=3).

3.3. Evaluation of optimized KT nanosuspension loaded in situ gel

3.3.1. Test for Clarity and pH

All formulations found as clear in appearance. The pH values for all the formulations are within adequate range 6.51- 6.53 and hence there will be no irritation occurs upon administration in the eye.

Table 4: Evaluation parameters of optimized KT nanosuspension loaded *in situ* gel

Formulation Code	Appearance	Clarity	pH	Drug Content (%)	Gelling Capacity	Viscosity (cp)
KTNG1	Transparent	Clear	6.51 ± 0.01	76.18 ± 1.17	++	618.3
KTNG2	Transparent	Clear	6.51 ± 0.01	79.11 ± 1.09	++	1177.9
KTNG3	Transparent	Clear	6.50 ± 0.02	83.25 ± 1.31	++	1873.2
KTNG4	Transparent	Clear	6.53 ± 0.01	77.12 ± 0.97	++	633.7
KTNG5	Transparent	Clear	6.51 ± 0.00	79.91 ± 1.18	++	1187.3
KTNG6	Transparent	Clear	6.49 ± 0.01	84.19 ± 0.83	++	1789.5
KTNG7	Transparent	Clear	6.49 ± 0.01	76.32 ± 0.98	++	627.9
KTNG8	Transparent	Clear	6.52 ± 0.01	78.47 ± 0.99	++	1203.5
KTNG9	Transparent	Clear	6.52 ± 0.02	83.92 ± 1.28	++	1810.6

++ Immediate gelation remains for few hours.

3.3.2. Gelling capacity determination

The gelling capacity data of all formulations (Table 4) depicted immediate gelation that existed for 2 to 3 hr.

3.3.3. Rheological study

Rheological evaluation of all the formulations exhibited moderate viscosity in the range of 618.3 to 1873.2 cp. It is favorable to form *in situ* gel with its integrity[26-28, 30].

3.3.4. Drug content (%) determination

The drug content values for all the formulations were found within the range of 76.18 to 84.19 %. Optimized batch KTNG6 showed 84.19 % drug content.

3.3.5. Ex-vivo trans-corneal permeation and corneal hydration study

The *ex-vivo* trans-corneal permeation after 2 hr of all gel formulations were depicted in table 1 (2.8 to 3.7 %). Marketed KT formulation showed 73.23 % trans-corneal permeation for the same time. Such large difference might be due to the slow release of drug from nanoparticles incorporated in gelling matrix. Corneal hydration values of all formulations were observed in between 76.55 to 78.41%. It was indicating normal corneal hydration without any damage to the corneal tissue.

3.3.6. HET-CAM assay

Irritancy Potential of optimized formulation was evaluated by using Hen's Egg Test Chorio-Allantoic Membrane Assay (HET-CAM Assay) and the time for the appearance of each of the observed endpoints on the CAM over a period of 5 min (0.5 min, 2 min & 5 min) were recorded, as in table 5. Effects observed were displayed in figure 3.The test sample was observed as non irritant when compared to negative control.

Table 5: HET-CAM Test Scores

Egg No.	0.9 % NaCl (Control)			1% SDS + 0.1 N NaOH (Negative Control)			Test Sample (Test Group)			Acular LS (Standard Group)		
	0.5 min	2 min	5 min	0.5 min	2 min	5 min	0.5 min	2 min	5 min	0.5 min	2 min	5 min
Egg 1	0	0	0	5	5	5	0	0	0	0	0	0
Egg 2	0	0	0	8	6	5	0	0	0	0	0	0
Egg 3	0	0	0	7	5	3	0	0	0	0	0	0
Egg 4	0	0	0	5	6	5	0	0	0	0	0	0
Egg 5	0	0	0	9	5	5	0	0	0	0	0	0
Egg 6	0	0	0	7	5	3	0	0	0	0	0	0
Mean (Time in Second)	0	0	0	6.83	5.33	4.33	0	0	0	0	0	0

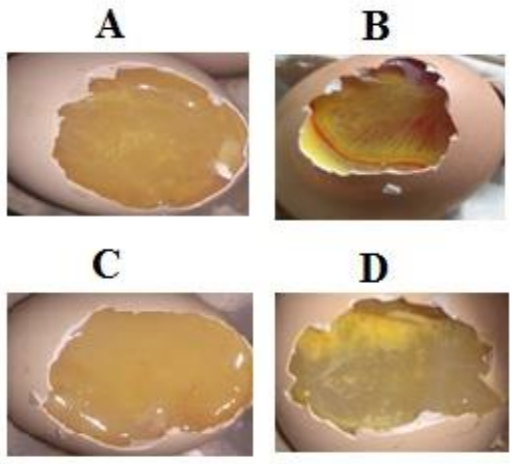

Figure 3: Effects observed; A) Positive control, B) Negative control, C) Test Solution & D) Acular LS during HET-CAM assay

3.3.7. Accelerated stability studies

During accelerated stability studies; optimized sample was analyzed periodically for visual appearance, clarity, pH and gelation. No significant changes were observed indicating stable formulation. Trans-corneal permeation profile was found as almost similar as that of its initial value.

4. CONCLUSIONS

In the present investigation, sustained release ketorolac tromethamine nanosuspension loaded *in situ* gel forming ophthalmic solutions were prepared using sodium alginate and HPMC E15. Sodium alginate, a mucoadhesive polymer, which gets converted to gel effectively in the presence of divalent-cations (Ca^{++}) present in the lachrymal fluid. Benzalkonium chloride was proven the suitable preservative for the preparations. During process; 3^2 factorial design was used to optimize the best formulation. All resultant formulations exhibited optimal viscosity, pH, drug content, corneal hydration and nonirritant nature. Optimized gel demonstrated excellent trans-corneal permeability and physical stability. It can be concluded that, ketorolac tromethamine nanosuspension loaded *in situ* gel will be the efficient ocular drug delivery system for the treatment of postoperative inflammation through improved patient compliance and ocular bioavailability.

CONFLICTS OF INTEREST

Authors have no any conflicts of interest.

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