A Colloidal Drug Delivery System for Antiallergic Drug

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1. Abstract

Allergic conjunctivitis (AC), one of the most eye sight-threatening infection defined by ocular itching, hyperemia, lacrimation and edema, impairs the quality of life across the globe. Ebastine is available as an oral antihistamine formula for allergic disorders such as tablets and syrup. Oral ebastine causes unfavorable effects on heart like QT prolongation, severe gastric distress, decreased tear production, resulting in dryness of the ocular surface, which exacerbates ocular discomfort and increasing susceptibility of eye to irritation. Topical antihistamines are preferred for treating ocular allergies over oral agents since their direct application at the site of action results in rapid onset and superior efficacy with less systemic side effects. Hence, topical formulation was developed to achieve onsite exposure of ebastine for ocular allergies. Moreover, conjunctiva is more accessible to hydrophilic molecules than lipophilic molecules. This creates challenge for a lipophilic molecule such as ebastine for topical ocular development. Successful dissolution of ebastine in o/w microemulsion allows its use in more convenient soluble form. Initially, solubility of drug in various oils, surfactant and cosurfactant was determined, followed by pseudo-ternary phase diagram to find microemulsion area. The D-optimal mixture design was employed for optimization of formulation. The optimized microemulsion formulation was characterized for its transparency, pH, drug content, droplet size, zeta potential, viscosity, osmolarity, refractive index and surface tension etc. The optimum physicochemical properties were observed to be eye-fitting. Carboxy methyl cellulose and sodium hyaluronate were used as gelling agents at different concentrations to increase residential time at the site of action. The antiallergic potential of optimized ebastine (1% w/v) colloidal ocular formulation was assessed by performing in vitro study like hen's egg chorioallantoic membrane test (HET-CAM) for tolerability and in vivo efficacy study in ovalbumin (OA)-induced allergic conjunctivitis (AC) with acute ocular irritation study and blinking index. Eye scratching behavior and edema were evaluated after topical antigen challenge. Edema was scored at periodic interval after the instillation of ovalbumin followed by histopathology. The results showed that ebastine (1% w/v) ocular formulation was effective in inhibiting symptoms of eye inflammation induced by ovalbumin. Acute ocular irritation test was performed using rabbits and results showed that developed formulation was non-irritant to the eye. The in-vivo pharmacokinetic studies revealed increased concentration of the drug in ocular tissue matrix with negligible systemic absorption. Hence, prepared microemulsion had great potential as an alternative to customary oral formulations of poorly soluble drug.
2. Introduction
Ophthalmic drug delivery generally involves the delivery of therapeutically active agents into anterior and posterior segments of the eye. The ophthalmic medical product market continues to grow at a strong pace, from approximately $12 billion in 2010 to an estimated $52.4 billion in 2017. [1] Conjunctivitis is a prevalent disease all over the world especially higher rate of infection was found in developing countries. Conjunctivitis may be bacterial, viral or chlamydial, allergic. Allergic conjunctivitis is caused by an allergen-induced inflammatory response in which allergens interact with IgE bound to sensitized mast cells resulting in the clinical ocular allergic expression. [2]
For the ailments of the eye, topical administration is usually preferred over systemic administration for obvious reasons:
- Systemic toxicity of many ophthalmic drugs.
- Rapid onset of action
- Smaller dose required, as compared to the systemic route.
Ebastine is official in British pharmacopoeia, second-generation H1 receptor antagonist, chemically 1-[4-(1, 1-dimethyl ethyl) phenyl]-4-[4-(diphenyl methoxy) piperidin-1-yl] butan-1-one indicated for various allergic manifestations of skin, nasal and ocular site by oral route. [3, 4, 5]

3. Definition of the problem
From the literature review on the subject related to ocular delivery and their available therapies, the challenges associated with the current therapies were identified, and from the review emerged the research problem to be addressed.
A major problem in ocular therapeutics is the attainment of an optimal drug concentration at the site of action. Poor bioavailability of drugs from ocular dosage forms is mainly due to the precorneal loss factors which include tear dynamics, non-productive absorption, transient residence time in the cul-de-sac and relative impermeability of the corneal epithelial membrane.[6] Additionally, most drugs with ocular therapeutic potential have the problem of poor solubility and hence less bioavailability. To overcome it, various technological strategies are reported in the literature including micronization, nanosuspension, polymeric micelles and cyclodextrin based formulation. [7]
Among various approaches, microemulsions are promising alternative to enhance the ocular
bioavailability of drugs by improved ocular retention, increased corneal/conjunctival drug absorption and reduced systemic side effects and maintain the simplicity and convenience of the dosage form as eye drops. Microemulsions are thermodynamically stable, surfactant-cosurfactant based system, form at low interfacial tension. They are good alternative for ophthalmic delivery as it offers the pseudo plastic rheology with increased viscosity after application and increased ocular retention and possibility of releasing drug in sustained and controlled way, increased shelf life, lastly reducing dose and dosing frequency. Microemulsions are also used to formulate poorly water-soluble drugs since their structure allows solubilization of lipophilic drugs in the oil phase.

[8, 9]

Hence, the objective of the present investigation was to design and develop microemulsion based gel of antiallergic drug ebastine with view to increase the topical bioavailability, improve residence time at eye site and provide sustained delivery of drug for longer period of time. This formulation offered as a promising strategy for topical drug delivery rather than systemic drug delivery for ocular allergic manifestation.

4. Objective and scope of work

The overall objectives of the research are summarized as:

- To select the right material, process and optimization design for preparation of Microemulsion
- To optimize the formulation microemulsion with low level of Surfactants, additionally the characterization parameters need to be eye fitting
- To target the drug to the ocular sac
- To avoid extensive first pass metabolism of the drug
- To increase the residential period of drug
- Prolonged drug release, reducing the need for repeated instillation and targeting toward affected tissues, reducing possible side effects and required dose
- To increase bioavailability of the drug
- To prove ocular tolerability and efficacy of said formulation
- To estimate the drug concentration in ocular tissue matrix post-instillation of formulation
Scope of the research work
In recent years, a dramatically higher occurrence of ocular diseases, allergic conjunctivitis demands an urgent need of a novel ocular drug delivery system, which can be effective and capable of providing sustained therapeutic effect and better patient compliance. After clinical trials and fulfillment of other regulatory requirements, the developed formulation may prove to be a boon to the society at large for the complete treatment of the allergic conjunctivitis.

5. Original contribution by the thesis
The entire work in this synopsis, as well as thesis is original. Extensive literature review was done to identify the challenges associated with the complete cure of allergic conjunctivitis and approaches which can resolve them. Although multitude researchers have worked on development of microemulsion for various drugs, the idea of the development microemulsion of anti-allergic drug by systematic approach of design of experiment for optimization of various parameters which fit to ocular administration (to avoid various drawbacks associated with oral route) for an attempt towards cure of disease of anterior chamber of eye was probably yet not investigated by any other researcher.

6. Methodology of research, Results
6.1 Preformulation study of drug
The procured drug sample was visually observed for its color and was compared with the reported appearance of the drug. Melting point is one of the identification test method for organic substances. Hence, it was determined for the sample by capillary method using melting point apparatus (VMP-D, Veego). The IR spectroscopy was conducted using an FTIR spectrophotometer and the spectrum was recorded in the wavelength region of 4000–400 cm⁻¹ [10, 11, 12] IR spectroscopy and DSC study of pure ebastine and physical mixtures with excipients were conducted using an FTIR spectrophotometer (Bruker Alpha-one, Bruker Optik, Germany) and DSC analyzer (Pyris-1 DSC, PerkinElmer) in order to detect the existence of a possible interaction between drug and excipients. IR study revealed that there is no interaction between drug and excipients as all characteristic peaks of pure ebastine were found in the physical mixture. The DSC thermogram of the pure ebastine and physical mixture exhibited the characteristic endothermic peaks at 88.69 °C and 87.50 °C respectively, indicating the absence of interaction between the
drugs thereby proving drug-excipient compatibility. Both the studies indicated the compatibility of the drug with the selected excipients.

6.2 Formulation development of microemulsion

Screening of Microemulsion Components

The solubility of ebastine was determined in various oils, surfactants and cosurfactants. Drug powder was added in excess to each of the oils, surfactants and cosurfactants, thereafter subjected to vortexing. After vortexing, the samples were kept for 24 h at ambient temperature for attaining equilibrium. [13] The equilibrated samples were then centrifuged at 3000 rpm for 20 min to remove the undissolved drug. The aliquots of supernatant were filtered through 0.45 μm membrane filters and solubility of ebastine was determined by analyzing the filtrate spectrophotometrically (Shimadzu 1800, Japan) after dilution with methanol at 252 nm.

Construction of Pseudo-ternary phase Diagrams

Pseudo ternary phase diagrams were constructed using aqua- titration method at ambient temperature (25°C). Pseudo-ternary phase diagrams were constructed by Prosim software. [14] Campul MCM EP selected as the oil phase. The blend of Labrasol with Tween 80 and blend of Propylene glycol with glycerol were selected as surfactant and co surfactant, respectively. Double distilled water was used as an aqueous phase.

Three phase diagrams were obtained for three different Smix individual ratios 1:1, 2:1, and 3:1. The comparatively maximum microemulsion area was obtained in 2:1 Smix ratio. The selected Smix ratio was further studied by Smix blend, 2(1:1):1, 2(1:1): 1(1:1).

6.3 Optimization of formulation by Design Expert

D-Optimal Mixture Design

D-optimal mixture design (Design-Expert 7.0.0 (Stat-Ease Inc., Minneapolis, USA) was selected because the generalized variance of the estimates of the coefficients is minimized. [15, 16] Different design constraints, i.e. A (amount of oil), B (amount of Smix), and C (amount of water) were taken at high and low levels. The sum of A, B, and C were kept fixed at 100%. The effect of these formulation variables was studied on response variables like % Transmittance, globule size and viscosity. ANOVA was applied to determine the significance and the magnitude of the effects of the variables and their interactions.
Experimental Validation of Design Space

Validity of experimental design was confirmed by plotting a standard error of design graph. The probability value ($\alpha$) for determination of statistical significance was set at 0.05, which indicated that a “hypothesis” theory would be rejected if their corresponding p-values were $\leq 0.05$. [16] Models were selected on the basis of sequential comparison and lack of fit test. Response surface, contour plot, residual plot and overlay plots were constructed for the response variables. It was observed from the response variables plots of Globule size that as the concentration of oil increases, globule size also increases while the concentration of Smix increase then globule size decreases. It was observed from the response variables plots of viscosity that as the concentration of Smix increases and decrease in amount of water, viscosity increases. It was observed from the response variables plots of % transmittance that as the concentration of oil increases % transmittance decreases and Smix increases % transmittance increases. Further, linear correlation was found analogous for actual response and predicated response. The reliability of these response surfaces was also confirmed by the corresponding residual plot between the experimental run and the internally studentized residuals for all response variables. These findings revealed that all points fall within a confidence interval of 95%. Experimental validation of DoE trials for formulation variables was undertaken by formulation and characterization of microemulsion formulation at the check point batch suggested by the software. The observed values (Globule Size (nm) 142 ± 0.16, Viscosity (cps) 13.19± 0.121 and Transmittance (%) 99.79± 0.134) were comparable with the predicted values (Globule Size (nm) 143.33, Viscosity (cps) 13.51 and Transmittance (%) 99.09) establishing the reliability of the optimization procedure. Calculated percentage prediction error was found to be less than 5 percent, confirming the validity of D-optimal mixture design for microemulsion formulation optimization.

6.4 Characterization of Microemulsion

Resultant developed formulation shows droplet size (142 ± 0.16 nm), Polydispersity Index (below 1), refractive index (1.369 ± 0.04). The pH value of the developed formulation was 6.9 ± 0.12, which can be easily buffered by tear fluid (pH 7.2-7.4), consequently, it is adequate to apply to the eye without causing irritation, reflex tear and rapid tear blinking. [17] An Osmolarity of developed formulation was found to be 291±0.301mOsm/L. Low microemulsion surface tension ensures good spreading effect on ocular surface and mixing with precorneal film components, thereby
improving contact with ocular surface. [18] The surface tension of the developed formulation was found to be 34.75 ± 0.13 mN/m. Zeta potential and viscosity of developed formulations was found to be -22.6 ± 0.39 mV and 13.19 ± 0.121 cps respectively. The percentage of drug content of optimized formulations was found to be 97.09 ± 0.12%. Studies of equilibrium solubility were conducted in different oils, surfactants and co-surfactants to rationally optimize the formulation using D-optimum mixture design. The developed microemulsion was found in the limit of acceptable droplet size range for ocular use and presented physical stability. Physicochemical parameters like pH, osmolarity, surface tension were found in the range which favors its ophthalmic suitability. Microstructures of microemulsion was studied by transmission electron microscopy, it directly produces high-resolution images. It can capture any co-existent structure and microstructural transitions, performed using Technai-20, Phillips, Holland, Electron source: LaB6, Tungsten Filament. The morphology of the droplets of optimized formulation measured using TEM showed spherical shape and uniform droplet size of optimized microemulsion. Because the loaded ebastine microemulsion globules are nanometric and morphologically spherical, they are not expected to cause ocular irritation. Further, the optimized microemulsion formulation was sterilized using membrane filtration unit by passing the formulation through 0.22 μm membrane filter under aseptic conditions. [19] The optimized formulation was found to pass the sterility test carried out using fluid thioglycollate media and soyabean casein digest media to detect aerobic bacteria and fungal organisms respectively. [20]

6.5 Formulation development of microemulsion based Gel
The addition of the gelling agent increased the viscosity in comparison to parent microemulsion. The results of the release study discussed further indicated that formulation could prolong the precorneal retention owing to mucoadhesion by polymer. Hence, bioavailability at the site of action of said drug was found to be significantly increased. Different gelling and mucoadhesive agents were screened based on desired viscosity. Carboxy Methyl Cellulose and Sodium hyaluronate were selected as polymers based on literature. Based on preliminary trial, 1% CMC and 1.5% SH polymers dispersion was found satisfactory to get optimum viscosity. The dispersion was formed by suspending the polymers in water. The polymer dispersion kept for overnight to form viscous gel matrix. Prepared microemulsion and polymer dispersion was mixed in 1:1 w/w ratio. [21] Smooth viscous, transparent gel was formed. The underlying procedure for preparation
of microemulsion based gel was carried out strictly in aseptic area to maintain the sterility of overall formulation.

6.6 Characterization of microemulsion based Gel

The microemulsion based gel was evaluated for physical examinations like homogeneity, consistency, texture, etc. Gel was also evaluated for appearance, pH, viscosity, drug content, mucoadhesive strength (determined by modified two-pan balance method) and spreadability (determined by taking 0.5 g gel between two cellophane membranes and placing 100 g weight on it for 1 minute. The diameter of the area in which the gel got spread was measured). The viscosity was determined by means of Plane and cone Viscometer at different shear stress. The pH value of the 1% aqueous solution of the prepared microemulsion based gel was found to be is 6.8 ± 0.44. The percentage of drug content of was found to be 98.22 ± 0.40 %. The mucoadhesive strength and spreadability of the gel was found to be 2.8 cm/gm gel and 15,401.7 dynes/cm² respectively. Proper spread of the gels on the ocular surface will ensure increased absorption of the drug after ocular administration. The pseudo plastic character of precorneal tear film should be disturbed less/or not disturbed by the administration of ophthalmic products. The ocular shear rate is about 0.03 s⁻¹ during interblinking periods and 4250 – 2850 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 for ophthalmic application. [22] The Rheogram of microemulsion gel and microemulsion gel diluted with tear fluid (To mimic physiological condition, formulations were mixed with artificial tear fluid (ATF) in a ratio of 40:7) exhibited pseudo-plastic behavior, i.e., decrease in the viscosity with increase in angular velocity exhibiting its suitability for ophthalmic use.

The in vitro drug release study was carried out using the dialysis bag method. (Molecular weight 12–14 kDa) [23, 24] The release mechanism was dependent on two simultaneous processes: water migration into the swollen polymer and drug diffusion. Maximum % ebastine released from microemulsion was found 89.19 ± 2.45% compared to Microemulsion gel 71.34 ± 2.34% within 8 hr. However, microemulsion gel was able to sustain the release of the remaining ebastine for up to 24 h. This might be possible matrix effect on release of ebastine due to incorporation of microemulsion in CMC and SH gel, a micro gel layer forms around the droplets that can hinder drug diffusion from the oil phase, so the rate and the amount of the released drug may decrease.
while the release rate of the drug from microemulsion depends on the rate of diffusion of the drug from oil droplets. The possibility of the drug partition between the oil and the water phases in the presence of the surfactant positioned at the oil–water interface prior to release. Formulation provided the highest in vitro drug release with the ability of providing a sustained release over 24 hr, thus reducing frequency of application and improving patient compliance.

6.7 In vitro/ In vivo study

The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) Reference No. 984/01/2017-07 for the use of animal in the study. Utmost care was taken to ensure that animals were treated in the most human and ethically acceptable manner. The ocular potential of optimized ocular formulation assessed by performing in vitro study like hen's egg chorioallantoic membrane test (HET-CAM) [25, 26, 27] and blinking index [28] for tolerability and in vivo antiallergic efficacy study in ovalbumin (OA)-induced allergic conjunctivitis (AC) in guinea pigs [29, 30] followed by histopathology. [31, 32] After sensitization period, animals were used for the experiments assessing efficacy of test formulation.

Test formulation (20μl), saline, (20μl) was instilled into the right eye of respective group and for oral, ebastine (3 mg/kg) in 0.5% CMC was given. At 0.5 and 24 h after the instillations, the eye was challenged with ovalbumin solution (100 mg/ml, 30μl). Edema was scored at 15, 30, 60, 90, and 120 min after the instillation of ovalbumin. For evaluation of edema, scoring system was used. In the same sensitization protocol, eye scratching behavior and edema were scored at periodic interval after the topical antigen challenge (instillation of ovalbumin) followed by histopathology. Eye scratching behavior was defined as fore-limb movements over two times directed to the ocular surface. [33, 34] The results shown that the test formulation (ebastine 1% w/v ocular) instilled 0.5 h and 24 h before the ovalbumin challenge caused significant inhibition of conjunctivitis symptoms. While the oral ebastine caused significant inhibition of conjunctivitis symptoms at 0.5 h only. It was also observed that compared to Ova challenge, test formulation showed 79.84%
inhibition at 0.5 h, the effect persist up to 24 h with 42.46% inhibition while oral ebastine showed 34.71% inhibition at 0.5 h. This result indicates that topical formulation of ebastine showed better efficacy in ova induce conjunctivitis model at very low dose as compared to oral.

The results showed that ebastine (1% w/v) ocular formulation was effective in inhibiting symptoms of eye inflammation induced by ovalbumin. Further, the study indicated that said formulation has a quick onset and the duration of effect sufficient to provide relief from symptoms for 24 hr. Ocular irritation by HET-CAM assay showed that the developed formulation does not cause any irritation to the blood vessels.

<table>
<thead>
<tr>
<th>Before Treatment</th>
<th>5 min After Treatment</th>
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<tbody>
<tr>
<td><img src="image1.png" alt="Before Treatment" /></td>
<td><img src="image2.png" alt="5 min After Treatment" /></td>
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<tr>
<td>a. Test Formulation</td>
<td>a. Test Formulation</td>
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<td><img src="image3.png" alt="b. Positive Control" /></td>
<td><img src="image4.png" alt="b. Positive Control" /></td>
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<tr>
<td>b. Positive Control (0.1N NaOH)</td>
<td>b. Positive Control (0.1N NaOH)</td>
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<tr>
<td><img src="image5.png" alt="c. Negative Control" /></td>
<td><img src="image6.png" alt="c. Negative Control" /></td>
</tr>
<tr>
<td>c. Negative Control (0.9% NaCl)</td>
<td>c. Negative Control (0.9% NaCl)</td>
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**Figure Ocular Irritation Study by Chorioallantoic Membrane Assay**

Acute ocular irritation test was performed using rabbits, the animals were observed up to 60 min for redness, swelling, watering of the eye. [35] and results showed that developed formulation was non-irritant to the eye. In histopathological evaluation, ocular formulation caused mild epithelium
damage with less eosinophil infiltration than in conjunctiva treated with ovalbumin alone. Data obtained in different groups expressed as mean ± Standard error of mean (SEM) were analyzed using one-way ANOVA followed by Dunett's test. Statistical significance was considered as p value < 0.05. Statistical analysis was performed using the Sigma Stat, Version 3.1. (SPSS Inc., USA)

6.8 Pharmacokinetic Study
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. From a high level perspective, ocular PK studies help elucidate a compound’s absorption into, distribution and metabolism within, and elimination from the eye (ADME). More precise objectives for these studies typically center on determining a compound’s ability to get to the target tissue, and in what concentration, by a specified dosing method. [36] Single dose pharmacokinetic study was performed by measuring drug concentration in ocular tissues. For reliable data quality, sampling time should be chosen carefully, 6-8 time-points with balanced sampling may be appropriate. For small molecules (≤1,000 Da), 1, 2, 4, and 8 hr sampling point recommended. [37] The animal experiment was carried out on SD rat. In study protocol, 10 μl test formulation was instilled into rat eyes, at 0.5, 1, 2, 4, and 8 h after the instillations, (N=3/time point), animals were euthanized and the eyes were removed. Weighed the ocular tissue samples for homogenization. Protein extraction reagent was added with a ratio of tissue to reagent of 1:5 (1g of tissue/5 ml of reagent). Homogenize the tissue using a pre-chilled micro homogenizer. Centrifuge the lysed sample for 10,000 rpm at 4°C for 10 min and transfer the supernatant to a chilled EPP tube. [38] The resultant samples were analyzed by developed and validated HPLC Method. The formulation achieved peak drug concentration (Tmax) within 30 min. The Cmax value was found 62.48μg/gm. The AUC of formulation was found 202.12 μg/gm*h. The higher AUC of formulation may contribute to better clinical efficacy. After single ocular instillation of 10 μl dose of 1% w/v ebastine ophthalmic formulation, the concentrations in the ocular tissue were more than that in the plasma showing negligible systemic absorption. These results are desirable for the ocular distribution of ebastine, because high-level distribution of ebastine was observed in the ocular tissue, which is a target tissue for pharmacologic effect (i.e., efficacy).
6.9 Stability Studies
Stress stability study of the microemulsion formulation was carried out by subjecting to centrifugation. [39] A formulation shows no sign of phase separation when subjected to centrifugation at 9,000 rpm for 20 minutes. Thus, it was concluded that the Microemulsion formulation was stable under stressful conditions. The stability of the microemulsion and microemulsion based gel were assessed under different storage conditions as per ICH guidelines, at room temperature (25°C ± 2°C / 60% RH ± 5% RH), at refrigeration condition (2-8°C), at 40°C ± 2°C / 75% RH ± 5%RH [40,41]

The samples of microemulsion were evaluated at 0, 1, 2, 3 and 6 months for pH, viscosity, globule size, zeta potential. Further, the samples of microemulsion based gel were evaluated at 0, 1, 2, and 3 months for pH, viscosity and drug content. All the studies were conducted in triplicate. The developed formulations (ebastine loaded microemulsion and gel formulation) were found to be stable over period of stability study. No significant changes were observed during the stability studies in measured parameters, while the microemulsion gel shown slight change in viscosity under accelerated temperature and humidity condition, hence recommended condition for proper storage for developed formulation is at room temperature and /or refrigeration.

6.10 Analytical Methods

UV- spectrophotometric method
UV spectrophotometric methods have been reported for the estimation of ebastine in formulations.[42,43] Calibration curves were plotted in methanol as solvent for determination of solubility of drug in different oils, surfactants and cosurfactants using UV-1800 spectrophotometer (Shimadzu) at 252 nm. Linearity for drug release studies was observed with the calibration curves plotted using methanolic phosphate bufferd saline pH 7.4 as solvent.

HPLC Method Development & Validation
A high performance liquid chromatographic method (HPLC) was developed and validated for the determination of ebastine in ocular tissue. The method was developed with HPLC using Waters X-Terra Shield, Phenomenex C18 (250mm×4.6mm i.d.) 5μm column and a mobile phase consisting of Methanol: Acetonitrile: Ammonium acetate pH 5.5 (80:10:10 v/v/v). The elution was monitored with the UV-Visible detector at 244 nm with a flow rate of 1.2 ml/min. Phenylephrine HCL was used as internal standard. The method was validated for linearity, precision, accuracy, specificity,
robustness and data obtained were statistically analyzed. Calibration curve was found to be linear over the concentration range of 2-400 µg/ml. The regression coefficient value was found to be 0.9965. The developed and validated HPLC method was used for determination of ebastine in ocular tissue.

7. Achievements with respect to objectives
Topical ocular therapy could prove to be superior to systemic therapy in treating ocular allergies. Hence, topical formulation was successfully developed to achieve onsite exposure of ebastine for ocular allergies. The use of surfactant –cosurfactant blend system resulted in formulation of drug delivery system with low surfactant level. The design expert allowed optimization of formulation and response variables as per ocular site application requisite. By the use of gelling and mucoadhesive polymer, the formulation residential time was successfully increased and the drug released for prolong period. The ocular presentation of model drug through micro emulsified form shown tolerability and efficacy as well as increased bioavailability due to site specificity. The analytical method was successfully developed and validated for the estimation of ebastine in ocular tissue.

8. Conclusion
With the present investigations, it may be concluded that microemulsion of a poorly soluble drug ebastine successfully formulated and optimized using the systematic approach of design of experiments (DoE) by water titration method. Studies of equilibrium solubility were conducted in different oils, surfactants and co- surfactants to rationally optimize the formulation using D-optimum mixture design. The developed microemulsion was found in the limit of acceptable droplet size range for ocular use and presented physical stability. Physicochemical parameters like pH, osmolarity, isotonocity were found in the range which favors its ophthalmic suitability. Mucoadhesive gel was prepared with the optimized microemulsion. The addition of the gelling agent increased the viscosity in comparison to parent microemulsion. The results of the release study indicated that formulation prolonged the precorneal retention owing to mucoadhesion by polymer. Hence, bioavailability at the site of action of said drug was found to be significantly increased. Further, formulation also proved its tolerability (no signs of irritation, bleeding, vessel lysis and coagulation) in vitro HET-CAM assay by preserving normal architecture of blood vessels
and in vivo acute irritation study evident by reduced lacrimation, blinking index and redness. The in vivo efficacy study revealed that the therapeutic role of formulation in allergic conjunctivitis by exhibiting statistically significant reduction in conjunctivitis symptoms like edema and scratching as compared to oral ebastine. In histopathological evaluation, ocular formulation caused mild epithelium damage with less eosinophil infiltration than in conjunctiva treated with ovalbumin alone. In a nutshell, the developed microemulsion based gel formulation using the design of experimentation approach held great potential as a possible alternative to traditional oral formulations of poorly soluble ebastine to improve solubility and bioavailability due to site specificity as well, fitting ocular application prerequisite. These findings further warrant in clinical investigation.

9. Publications from Research work

Papers published


10. References


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ABSTRACT

Ebastine is available as an oral antihistamine formula for allergic disorders such as tablets and syrup. Oral ebastine causes unfavorable effects on heart like QT prolongation, severe gastric distress, decreased tear production, resulting in dryness of the ocular surface, which exacerbates ocular discomfort and increasing susceptibility of eye to irritation. To avoid systemic side effects and ocular discomfort could prove to be superior to topical ocular therapy in treating ocular allergies. Hence, topical formulation was developed to achieve onsite exposure of ebastine for ocular allergies. Moreover, conjunctiva is more accessible to hydrophilic molecules than lipophilic molecules. This creates challenge for a lipophilic molecule such as ebastine for topical ocular development. Successful dissolution of ebastine in o/w microemulsion allows its use in more convenient soluble form. Initially, solubility of drug in various oils, surfactant and cosurfactant was determined, followed by pseudo-ternary phase diagram to find microemulsion area. The D-optimal mixture design was employed for optimization of formulation. The optimized microemulsion formulation was characterized for its transparency, drug content, droplet size, zeta potential, viscosity, isotonicity, osmolarity and surface tension etc. The optimum physicochemical properties were observed to be eye-fitting. Carboxy methyl cellulose and sodium hyaluronate were used as gelling agents at different concentrations to increase residential time at the site of action. In vitro drug release study revealed that ebastine release from microemulsion gel in a sustained manner up to 24 hrs. for the purpose of providing prolonged therapy for ocular allergy. Hence, prepared microemulsion had great potential as an alternative to customary oral formulations of poorly soluble drug.

Keywords: Ebastine, Microemulsion, D-optimal mixture design, Solubility

Graphical Abstract

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INTRODUCTION

Ocular drug delivery generally involves the delivery of therapeutically active agents into anterior and posterior segments of the eye. Conjunctivitis is a prevalent disease all over the world especially higher rate of infection was found in developing countries. Conjunctivitis may be bacterial, viral or chlamydial, allergic. Allergic conjunctivitis is caused by an allergen-induced inflammatory response in which allergens interact with IgE bound to sensitized mast cells resulting in the clinical ocular allergic expression. A major problem in ocular therapeutics is the attainment of an optimal drug concentration at the site of action. Poor bioavailability of drugs from ocular dosage forms is mainly due to the precorneal loss factors which include tear dynamics, non-productive absorption, transient residence time in the cul-de-sac and relative impermeability of the corneal epithelial membrane. Additionally, most drugs with ocular therapeutic potential have the problem of poor solubility and hence less bioavailability. To overcome it, various technological strategies are reported in the literature including micronization, nanosuspension, polymeric micelles and cyclodextrin based formulation.

Among various approaches, microemulsion is promising alternative to enhance the ocular bioavailability of drugs by improved ocular retention, increased corneal drug absorption and reduced systemic side effects and maintain the simplicity and convenience of the dosage form as eye drops. Microemulsions are thermodynamically stable, surfactant-cosurfactant based system, form at low interfacial tension and exhibit high solubilizing potential for hydrophobic drugs. They are good alternative for ophthalmic delivery as it offers the pseudo plastic rheology with increased viscosity after application and increased ocular retention and possibility of releasing drug in sustained and controlled way, increased shelf life, lastly reducing dose and dosing frequency.

Ebastine is official in British pharmacopoeia. It is a second-generation H1 receptor antagonist, chemically 1-[4-[[1,1-dimethyl ethyl] phenyl]-4-[4-(diphenyl methoxy) piperidin-1-yl] butan-1-one indicated for various allergic manifestations of skin, nasal and ocular site by oral route. Oral administration of antihistamine leads to dryness of eye which affects physiology of tear film. A successful attempt is made to prepared low dose and low concentration of the surfactant based Ophthalmic ebastine microemulsion formulations employing the concept of design of experiment with goal of solubility enhancement thereby boosting bioavailability due to site specificity as well reduces systemic side effects and hence will enhance the patient compliance.

MATERIALS AND METHODS

Ebastine was procured as a gift sample from, Bal Pharma Pvt. Ltd, Bommasandra, Bangalore, India. Campul MCM EP, Labrasol, Labrafac, Cremophor EL, Lauroglycol FCC was generously supplied by Gatetfosse, Saint-Priest, France. Oleic acid, Ethyl oleate, Isopropyl palmitate, Arachis oil, Lineseed oil, light liquid paraffin was purchased from Yarrow chemicals Pvt. Ltd Mumbai, India. Propylene glycol, Polycethyleneglycol 400 (PEG-200), Sorban mononoleate (Span-80), Polyoxyethyleneorbitan mononoleate (Tween-80), Polyoxyethylenesorbitan monolaurate (Tween-20), Isopropyl alcohol, butanol was purchased from S.D. Fine chemicals, Ahmadabad, India. All other chemicals used in the study were of highest analytical purity grade. The double distilled water is used throughout the study.

Screening of Microemulsion Components

The solubility of ebastine was determined in various oils, surfactants and co-surfactants. Drug powder was added in excess to each of the oils, surfactants and co-surfactants, thereafter subjected to vortexing. After vortexing, the samples were kept for 24 h at ambient temperature for attaining equilibrium. The equilibrated samples were then centrifuged at 3000 rpm for 20 min to remove the undissolved drug. The aliquots of supernatant were filtered through 0.45 μm membrane filters and the solubility of Ebastine was determined by analyzing the filtrate spectrophotometrically (Shimadzu 1800, Japan) after dilution with methanol at 252 nm. Appropriately diluted solutions of oils in methanol were taken as blank.

Construction of Pseudo-ternary phase Diagrams

In order to find out the concentration range of components for the existing range of microemulsion, pseudo ternary phase diagrams were constructed using aqua- titration method at ambient temperature (25°C). Pseudo-ternary phase diagrams were constructed by Prosim software. Campul MCM EP selected as the oil phase. The blend of Labrasol with Tween 80 and blend of Propylene glycol with glycerol were selected as surfactant and co surfactant, respectively. Double distilled water was used as an aqueous phase. Various phase diagrams were prepared with weight ratios of surfactant to co surfactant individual and blend. For each phase diagram at a specific surfactant/co surfactant weight ratio, the ratios of oil to the mixture of surfactant and co surfactant were varied as 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1. The mixtures of oil, surfactant and cosurfactant at certain weight ratios were diluted with water drop wise, under moderate magnetic stirring. After being equilibrated, the mixtures were assessed visually and determined as being microemulsion, crude emulsions or gels. No experimental attempt was made to distinguish between oil-in-water, water-in-oil or bicontinuous type microemulsion. Gels were claimed for those clear and highly viscous mixtures that did not show a change in the meniscus after tilted to an angle of 90°.

Three phase diagrams were obtained for three different Smix individual ratios 1:1, 2:1, 3:1. The comparatively maximum microemulsion area was obtained in 2:1 Smix ratio. The selected Smix ratio was further studied by Smix blend, (2:1:1), (2:1:2), (1:1:1). The Smix blend ratio which produced broader microemulsion region was selected for formulation optimization. This attempt was made to keep the surfactant concentration as low as possible in the ophthalmic formulation to avoid any associated toxicity.

Optimization of Microemulsion by D-Optimal Mixture Design

D-optimal mixture design (Design-Expert 7.0.0 (Stat-Ease Inc., Minneapolis, USA) was selected because the generalized variance of the estimates of the coefficients is minimized. The software selected a set of candidate points as a base design included factorial points (high and low level from the constraints on each factor, centers of edges, constraint plane centroids, axial checkpoint, and an overall center point).

It is commonly used to reveal main effects and interaction effects between the independent variables of the experiment. Moreover, the numbers of trials required are less. Twelve runs were carried out to optimize microemulsion formulation. Different design constraints, i.e. A (amount of oil), B (amount of Smix), and C (amount of water) were taken at high and low levels. The sum of A, B, and C were kept fixed at 100%. The effect of these
Preparation of Drug Loaded Microemulsion
The D-optimal design suggested different combinations of oil, Smix and water. The suggested quantity of oil and Smix was mixed using a magnetic stirrer to produce the oily phase, at this stage the Estabine was dissolved in the oily phase. Finally aqueous phase was added drop wise to obtain drug loaded microemulsion formulation.

Preparation of Drug Loaded Microemulsion Gel
The optimized microemulsion has very low viscosity, which may restrict its topical application. To overcome this, gel-forming agents were incorporated into formulation. The ocular delivery improved by adding mucoadhesive polymer in formulation. The weight ratio of CMC (1%) and SH (1.5%) was found satisfactory based on gel formation. The former polymer used in commercial ocular formulations, as it has desirable mucoadhesive and a high retention time on the ocular surface and the latter one exhibit excellent viscoelastic, lubricating and water retention properties.

Characterization and Evaluation of Microemulsion
Drug Excipient Compatibility
Fourier-transform Infrared Spectroscopy (FTIR) Study
IR spectroscopy was conducted using an FTIR spectrophotometer and the spectrum was recorded in the wavelength region of 4000– 400 cm−1.16, 17, 18 The procedure consisted of dispersing the samples in KBr, thus avoiding solid transition possibly inducing by extended grinding. The spectrums were scan at a resolution of 0.15 cm−1 and scan speed 20 scan/sec. The Infra-Red spectra’s of pure Estabine and optimized formulation were obtained on Fourier Transform Infrared Spectrophotometer in order to detect the existence of a possible interaction between drug and excipients.

Measurement of pH
For optimized formulation, pH was measured using pH meter which was previously calibrated using standard buffers of pH 4 and pH 7 as per the established procedure16, 17, 18.

Measurement of Refractive Index
After administration of eye drops, possible impairments of vision or discomfort to the patient is detected by refractive index measurements. Refractive index proved the transparency of formulation. The refractive index of the system was measured by Abbe Refractometer (RICO, Model RSR-1) by placing one drop of the formulation on the slide in triplicate and compared it with water16, 17, 18.

Measurement of Osmolarity
Evaluation of osmolarity using an osmometer is of vital importance for physiological acceptance of the formulation by ocular tissues16, 17, 18. Osmolarity of optimized formulation measured using Osmometer (Advanced Instruments Inc., USA; Model 3250).

Measurement of Surface Tension
Surface tension determination ensures the uniform spreading of the formulation on the corneal surface16, 17, 18.

Droplet size, Zeta potential and Viscosity measurement
The droplet size of the microemulsion was determined by photon correlation spectroscopy (which analyzes the fluctuations in light scattering due to the Brownian motion of the particles) using a Malvern zeta sizer (Nano ZS, Malvern instruments, UK). Zeta sizer able to measure sizes between 10 and 5000 nm. The measurements were performed at 25°C at a 90° angle. Each size value reported was the average of at least three independent measurements. Samples were suitably diluted with double distilled filtered water to avoid multi-scattering phenomena and then placed in quartz cuvettes. The real and imaginary refractive indexes were set at 1.59 and 0.0, respectively. Zeta Potential was determined by Zeta sizer (Malvern instruments UK) using clear disposable zeta cell and filed strength of 20 V/cm was employed. The electrophotometric mobility was converted into to the zeta potential. The viscosity of microemulsion was determined by Ostwald Type Capillary viscometer at room temperature.

Drug Content
0.5 ml optimized microemulsion formulation (1% w/v) containing drug equivalent to 5 mg was extracted with methanol followed by further appropriate dilution with methanol and the drug content was determined using UV spectrophotometer (Shimadzu 1800, Japan) at 252 nm in the formulation.

Transmission Electron Microscopy
To study the microstructures of microemulsion, transmission electron microscopy is the most important technique as it directly produces high-resolution images. It can capture any co-existent structure and microstructural transitions. The morphology of formulation was performed using (Technai-20, Phillips, Holland, Electron source: LaB6, Tungsten Filament) A drop of sample was placed onto a carbon coated grid on a single tilt sample holder to form a thin liquid film. The excess solution was removed followed by negative staining with 1% phosphotungstic acid. The sample was examined and simultaneously photographed at an accelerating voltage with point resolution 0.27nm and magnification up to 250x to 7,50,000x.
In Vitro Drug Release Study

The optimized microemulsion and microemulsion gel formulation was evaluated for drug release. The in vitro drug release study was carried out using the dialysis bag method. (Molecular weight 12–14 kDa) 22–23. Methanolic Phosphate-buffered saline pH 7.4 was used as release medium. The system was maintained at 32 ± 0.5°C to mimic conditions eye surface temperature with continuous stirring on magnetic stirrer at 150 rpm. The samples were collected periodically until 24hr. Etabistine content in the receptor chamber was determined by spectrophotometrically. Sink conditions were maintained in the receptor compartment during in vitro release studies. Each sample analysis was performed in triplicate.

Sterility Testing

Sterility test was performed to examine the growth of bacteria or fungus. The optimized microemulsion formulation was sterilized using membrane filtration unit by passing the formulation through 0.22 μm membrane filter under aseptic conditions 24. The media used to detect aerobic and anaerobic bacteria is fluid thioglycollate media and soyabean casein digest media is used to detect fungal organisms. For positive control aerobic bacteria Staphylococcus aureus and fungal organism Candida albicans were inoculated into fluid thioglycollate media and soyabean casein digest media respectively. The optimized formulation was incubated in an incubator at 37±1°C for a period of 14 days using both the medias. The gelling agent incorporated into microemulsion system in aseptic cabinet in between burners to avoid further possible contamination.

Accelerated Stability Tests by Centrifugation Stress Test

Stress stability study of the microemulsion sample was carried out by subjecting it to centrifugation. The formulation was centrifuged at 9,000 rpm for 20 min by Centrifuge (Make Remi) and examined for phase separation 25.

RESULTS AND DISCUSSION

Preliminary Screening of Microemulsion Components by Solubility Study

Amongst various oils tested, Etabistine showed low solubility in all oils except for Campul MCM EP (28.5 ± 0.2 mg/ml). Campul MCM EP is a mono-diglyceride of medium chain fatty acids (mainly caprylic and capric). After selection of Campul MCM EP as the oil phase, the goal was to identify the surfactant which shows the highest solubilization capacity for the drug. Etabistine shown maximum solubility in Labrasol (23.1 ± 0.3mg/ml) followed by Tween 80 (19.8 ± 0.1mg/ml). Therefore, blend of Labrasol and tween 80 was selected as the surfactant for microemulsion formulation. Amongst cosurfactant tested, maximum solubility in propylene glycol (18.2 ± 0.1mg/ml) followed by alcohols. Amongst various S mix blends, propylene glycol, isopropyl alcohol and ethanol forms transparent system with 98.21%T, 99.01%T, and 99.17% T respectively compared to other tested cosurfactants with selected surfactant. Due to ocular compatibility and volatility issue, selection of any alcohol as microemulsion component was prohibited. Therefore, blend of propylene glycol and glycerol was selected as the cosurfactant for microemulsion formulation. Moreover, glycerol will help in maintaining osmolarity of formulation. Figure 1 exhibited comparative account for solubility of drug in various components of microemulsion formulation. Various cosurfactants were screened for solubility as well miscibility with a surfactant. The toxicity of nonionic surfactants is generally lesser than ionic surfactants, besides they have lower critical micelle concentration (CMC) and offer better in vivo stability of o/w microemulsion dosage forms. Therefore the screening of surfactant was done from amongst the nonionic surfactants only.

![Figure 1: Solubility profile of drug in various Oils, Surfactants and Co surfactants](image)

Construction of Pseudo-ternary Phase Diagram

Initially, based on the results of maximum solubility, various pseudo ternary phase diagrams were constructed employing Campul MCM EP (Oil), Labrasol & Tween 80 (surfactants blend) and propylene glycol & glycerol (co-surfactant blend) for identifying the maximal region for formation of the thermo-dynamically stable microemulsion. Figure 2A and 2B illustrated pseudo ternary phase diagram for Smix individual system and Smix blend system respectively. Among the various combinations of individual and blend of surfactants and cosurfactants (i.e. 1:1, 2:1, 3:1) explored. The maximal region for microemulsion was observed at the ratio of 2:1 Smix blend system as compared to same ratio with Smix individual system. An o/w microemulsion region was found towards the water-rich apex of the phase diagram. As the surfactant concentration was increased in the S mix ratio, a higher microemulsion region was observed. The probable reasons are a reduction of the interfacial tension by surfactant and increased the fluidity of the interface by cosurfactant.
Optimization of Ebastine Microemulsion using D-Optimal Mixture Design

D-optimal mixture experimental design was applied in the present study. Campul MCM EP (X1), Smix (X2), and water (X3) were chosen as formulation variables and Globule size (nm) (Y1), Viscosity (cp) (Y2) and Transmittance (%) (Y3) were selected as response variables. The data obtained from globule size (response Y1), viscosity (response Y2), and transmittance (response Y3) was analyzed using Design Expert® Software. The polynomial equations comprise the coefficients for intercept, main first-order effects, interaction term. The value of the coefficients exhibits the effect of these variables on the response. A positive sign of coefficient indicates a synergistic effect while negative term indicates an antagonistic effect on the response. The data summarized in Table 1. After generating the polynomial equations through MLRA (Multiple linear regression analysis) relating the...
dependent and independent variables, mixture components were optimized for the responses. The values of all the responses were fitted to models viz linear, quadratic, special cubic and cubic model where the best fit model was found to be cubic model for all the responses as compared to other models (Table 2). $R^2$ values were reported resemble to unity indicating the high predictive ability of Response Surface Methodology (RSM) of underlying study. Further, the higher values (>4) of “Adequate Precision” indicate adequate signal.

Figure 3A, 3B, 4A, 4B and 5A, 5B shows contour, 3D response curve for dependent variables viz globule size (nm), viscosity (cps) and transmittance (%) respectively. It can be observed from the response variables plots of Globule size that as the concentration of oil increases, globule size also increases while the concentration of Smix increase then globule size decreases. It can be observed from the response variables plots of viscosity that as the concentration of Smix increases and decrease in amount of water, viscosity increases. It can be observed from the response variables plots of transmittance that as the concentration of oil increases % transmittance decreases and Smix increases % transmittance increases. Further, linear correlation was found analogous for actual response and predicated response (Figure 3C, 4C, 5C). The reliability of these response surfaces was also confirmed by the corresponding residual plot between the experimental run a and the internally studentized residuals for all response variables, as shown in Figure 3D, 4D, 5D. The vertical distribution of the internally studenized residuals was in line from top to bottom under the completely randomized run. These findings revealed that all points fall within a confidence interval of 95%.

Table 1: Coefficient of Cubic equation for each independent variable

<table>
<thead>
<tr>
<th>Coefficient</th>
<th>Globule size (nm)</th>
<th>Transmittance (%)</th>
<th>Viscosity (cps)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A(Oil)</td>
<td>+123.323.37</td>
<td>-17.42</td>
<td>+319.48</td>
</tr>
<tr>
<td>B(S mix)</td>
<td>-183.07</td>
<td>+96.57</td>
<td>+0.16</td>
</tr>
<tr>
<td>C(Water)</td>
<td>+395.14</td>
<td>+98.00</td>
<td>-9.28</td>
</tr>
<tr>
<td>AB</td>
<td>-21162.35</td>
<td>+222.24</td>
<td>-52.87</td>
</tr>
<tr>
<td>AC</td>
<td>-20873.16</td>
<td>+188.96</td>
<td>-5.493.36</td>
</tr>
<tr>
<td>BC</td>
<td>+267.36</td>
<td>+9.88</td>
<td>+29.37</td>
</tr>
<tr>
<td>ABC</td>
<td>+19689.51</td>
<td>-211.96</td>
<td>+512.18</td>
</tr>
<tr>
<td>AB (A-B)</td>
<td>-11469.5</td>
<td>+122.26</td>
<td>-33.81</td>
</tr>
<tr>
<td>AC (A-C)</td>
<td>-9475.61</td>
<td>+65.83</td>
<td>-274.17</td>
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<tr>
<td>BC (B-C)</td>
<td>+991.69</td>
<td>+12.02</td>
<td>+23.55</td>
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</table>

Table 2: Summary of regression analysis for all response

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<thead>
<tr>
<th>Model</th>
<th>Std. Dev.</th>
<th>R-Squared</th>
<th>Adjusted Squared</th>
<th>Predicted Squared</th>
<th>R- Squared</th>
<th>Remark</th>
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</thead>
<tbody>
<tr>
<td><strong>Response 1 Globule size (nm)</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Linear</td>
<td>4160808297</td>
<td>0.837278282</td>
<td>0.8011179</td>
<td>0.715878822</td>
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</tr>
<tr>
<td>Quadratic</td>
<td>32.84677275</td>
<td>0.93294153</td>
<td>0.87605946</td>
<td>0.63422757</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Special Cubic</td>
<td>14.55714352</td>
<td>0.98934528</td>
<td>0.97565962</td>
<td>0.936588798</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cubic</td>
<td>1.573384878</td>
<td>0.999948293</td>
<td>0.999715613</td>
<td>0.88135238</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Response 2 Viscosity (cps)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linear</td>
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<td>0.95745716</td>
<td>0.941721624</td>
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<tr>
<td>Quadratic</td>
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<td>0.95571089</td>
<td>0.933770144</td>
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<td></td>
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<tr>
<td>Special Cubic</td>
<td>0.39252352</td>
<td>0.97656627</td>
<td>0.948445801</td>
<td>0.904700682</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cubic</td>
<td>0.321333363</td>
<td>0.99318236</td>
<td>0.9654503</td>
<td>0.954526667</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Response 3 Transmittance (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linear</td>
<td>0.438370154</td>
<td>0.65729547</td>
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<td></td>
<td></td>
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<tr>
<td>Quadratic</td>
<td>0.476286834</td>
<td>0.73029823</td>
<td>0.505546771</td>
<td>-0.520492351</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Special Cubic</td>
<td>0.459194362</td>
<td>0.791090361</td>
<td>0.540398794</td>
<td>-0.908508779</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cubic</td>
<td>0.024307431</td>
<td>0.999765845</td>
<td>0.998712147</td>
<td>0.464988912</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3: Response variable Globule size (Y1)
A. Contour plot B. Surface response curve C. Predicted Vs. Actual response and D. Residual Vs. run

Figure 4: Response variable Viscosity (Y2)
A. Contour plot B. Surface response curve C. Predicted Vs. Actual response and D. Residual Vs. run
Experimental Validation of Design Space

Experimental validation of DoE trials for formulation variables was undertaken by formulation and characterization of microemulsion formulation at the check point batch suggested by the software. Figure 6 shows the overlay plot displaying the design space and optimized parameters as check point suggested by DoE software to obtain the desired responses. The observed values were comparable with the predicted values establishing the reliability of the optimization procedure as shown in Table 3. Calculated percentage prediction error was found to be less than 5 percent, confirming the validity of D-optimal mixture design for microemulsion formulation optimization.
Table 3: Checkpoint analysis of optimized formulation

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Predicted value</th>
<th>Experimental value</th>
<th>% Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Globule Size (nm)</td>
<td>143.33</td>
<td>142 ± 0.16</td>
<td>0.92</td>
</tr>
<tr>
<td>Viscosity(cps)</td>
<td>13.51</td>
<td>13.19± 0.121</td>
<td>2.36</td>
</tr>
<tr>
<td>Transmittance (%)</td>
<td>99.09</td>
<td>99.79± 0.134</td>
<td>0.70</td>
</tr>
</tbody>
</table>

Error (%) = (predicted value – experimental value)/ predicted value × 100.

Data expressed were of mean ±SEM (n=3)

Characterization and Evaluation of Microemulsion

Drug-Excipient Compatibility

FTIR studies were carried out for pure drug alone and optimized formulation. The all characteristic peaks of pure ebastine were found in the optimized formation, suggested that there is no interaction between drug and excipients as shown in Figure 7A and 7B.

Measurement of pH

Without much discomfort, the eye can tolerate pH of 6.5-8.0. The pH value of the developed microemulsion is 6.9 ± 0.12, which can be easily buffered by tear fluid (pH 7.2-7.4); consequently, it is adequate to apply to the eye without causing irritation, reflex tear and rapid tear blinking.

Measurement of Refractive Index

Refractive index measurements detect possible impairment of vision or discomfort to the patient after administration of eye drops. Refractive index of tear fluid is 1.340 to 1.360. It is recommended that eye drops should have refractive index values not higher than 1.476. The optimized formulation had refractive index values ranging from 1.369 ±0.04 which is resemble to the recommended values.

Measurement of Osmolarity

In formulating ophthalmic preparations consideration of isotonicity is of prime concern. The osmolarity of human tear film after prolonged eye closure is 288-293 mOsm/L and as eye is open, it progressively rises up to 302-318 mOsm/L. An Osmolarity of optimized formulation was found to be 291 ± 0.301mOsm/L indicating appropriateness for ocular application. The glycerol used in said formulation performed dual role of imparting osmolarity to formulation and act as a cosurfactant also.

Measurement of Surface Tension

Ophthalmic formulation had the surface tension range at the surface to air interface of 34.3-70.9 mN/m. Formulations indicated for treatment red eye had surface tensions below normal tear. The surface tension of the optimized microemulsion formulation was found to be 34.75 ±0.13 mN/m. Low microemulsion surface tension ensures good
spreading effect on the conjunctive, cornea and mixing with precorneal film components, thereby improving contact between the drug and the conjunctival tissue.

**Droplet size, Zeta potential and Viscosity measurement**

The droplet size of prepared microemulsion formulation was found to be 142±0.16 nm as shown in the Figure 8. The particle size that human eyes can tolerate is about 10 micrometer\(^2\), indicating suitability of developed formulation for ocular use. The Polydispersity Index (Pdi) was found to be well below 1.0 which confirms that the optimized microemulsion remains stable upon dilution. Zeta potential of prepared microemulsion formulations was found to be -22.6±0.39 mV as shown in the Figure 9 indicating that dilution does not have a significant impact on the microemulsion zeta potential. The Viscosity of optimized formulation was found to be 13.19±0.121cps. The residential capacity of formulation at physiological site (eye) can be increased by adding gelling agent.

**Drug Content**

Microemulsion of Ebastine with blend of surfactant and cosurfactant were prepared by Phase Titration Method (Water titration) method. The percentage of drug content of optimized formulations was found to be 97.09±0.12%.

**Transmission Electron Microscopy**

The morphology of the droplets of optimized formulation measured using TEM showed spherical shape and uniform droplet size of optimized microemulsion. Because the loaded ebastine microemulsion globules are nanometric and morphologically spherical, they are not expected to cause ocular irritation. (Figure 10)
In vitro Drug Release Study

It is difficult to mimic diffusion cell in vitro method with the real situation in vivo because cellulose membrane cannot exhibit the barriers of ocular multilayered epithelium as well as the constant volume of diffusion cell will not be able to eliminate the drug released by tear fluid turnover and nasolacrimal leakage. This phenomenon affects the concentration gradient and the diffusion of the drug through the epithelium. Therefore, possibility exists that formulations would have a different release in vivo.

In drug release profile (Figure 11), Maximum % Ebastine released from Microemulsion was found 89.19 ± 2.45% compared to Microemulsion gel 71.34 ± 2.34% within 8 hr. However, microemulsion gel was able to sustain the release of the remaining Ebastine for up to 24 hr. It is found that drug release from Microemulsion is comparatively more than Microemulsion gel. This might be possible matrix effect on release of Ebastine due to incorporation of microemulsion in CMC and HA gel, a micro gel layer forms around the droplets that can hinder drug diffusion from the oil phase, so the rate and the amount of the released drug may decrease, while the release rate of the drug from microemulsion depends on the rate of diffusion of the drug from oil droplets. The possibility of the drug partition between the oil and the water phases in the presence of the surfactant positioned at the oil–water interface prior to release. Formulation provided the highest in vitro drug release with the ability of providing a sustained release over 24 hr, thus reducing frequency of application and improving patient compliance. But, microemulsion gel has better consistency for topical drug delivery.

Sterility Testing

After specified incubation period, both fluid thioglycolate and soybean casein digest media showed absence of turbidity which is a sign of growth of microorganisms in the test sample of the optimized formulation and negative control while the turbidity or growth was found in the sample for positive control of Staphylococcus aureus and Candida albicans.

Accelerated Stability Tests by Centrifugation Stress Test

Stress stability study of the microemulsion sample was carried out by subjecting to centrifugation. A formulation shows no sign of phase separation when subjected to centrifugation at 9,000 rpm for 20 minutes. Thus, it was concluded that the Microemulsion formulation was stable under stressful conditions.

CONCLUSION

Studies of equilibrium solubility were conducted in different oils, surfactants and co-surfactants to rationally optimize the formulation using D-optimum mixture design. The developed microemulsion was found in the limit of acceptable droplet size range for ocular use and presented physical stability. Physicochemical parameters like pH, osmolarity, isotonicity were found in the range which favors its ophthalmic suitability. The addition of the gelling agent increased the viscosity in comparison to parent microemulsion. The results of the release study indicated that formulation could prolong the precorneal retention owing to mucoadhesion by polymer. Hence, bioavailability at the site of action of said drug was found to be significantly increased. In conclusion, Ebastine microemulsion could be offered as a promising strategy for ocular drug delivery for allergic manifestation. These findings further warrant in vivo investigation.

CONFLICT OF INTEREST

The authors report no conflicts of interest.

ACKNOWLEDGEMENT

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Ocular Tolerability and Efficacy of Ebastine Colloidal Formulation in Allergic Conjunctivitis

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ABSTRACT
Allergic conjunctivitis (AC), defined by ocular itching, hyperemia, lacrimation and edema, impairs the quality of life across the globe. Ebastine is available as an oral antihistamine formula, such as tablets and syrup, for allergic disorders. Topical antihistamines are preferred over oral agents since their direct application at the site of action results in rapid onset and superior efficacy with less systemic side effects. The objective of the present work was to evaluate the antiallergic potential of optimized ebastine (1% w/v) colloidal ocular formulation by performing in vitro study like hen’s egg chorioallantoic membrane test (HET-CAM) for tolerability and in vivo efficacy study in ovalbumin (OA)-induced allergic conjunctivitis (AC) with acute ocular irritation study. Eye scratching behavior and edema were evaluated after topical antigen challenge. Edema was scored at periodic interval after the instillation of ovalbumin followed by histopathology. The results showed that ebastine (1% w/v) colloidal ocular formulation was effective in inhibiting symptoms of eye inflammation induced by ovalbumin. Further, the study indicated that said formulation has a quick onset and the duration of effect sufficient to provide relief from symptoms for 24 hr. Ocular irritation by HET-CAM assay showed that the developed formulation does not cause any irritation to the blood vessels. Acute ocular irritation test was performed using rabbits and results showed that developed formulation was non-irritant to the eye. The present study revealed that the ocular ebastine formulation could offer a novel therapeutic opportunity against IgE-mediated allergic conjunctivitis.

Keywords: Allergic Conjunctivitis, Colloidal Formulation, Ovalbumin, Chorioallantoic Membrane.

INTRODUCTION
Allergic conjunctivitis is most common immune-mediated diseases of the eye, clinically characterized by ocular itching, redness, lacrimation, edema and presence of inflammatory cells specifically eosinophil’s in the conjunctiva. [1-3] The incidence of allergic conjunctivitis has already increased dramatically over the past years. The diseases are often concomitant with
other allergic diseases such as allergic rhinitis, atopic dermatitis and allergic asthma. [3] Allergic conjunctivitis categorized into acute and late phase disease. The acute phase is described clinically by ocular itching, hyperemia, and edema, supported by cellular infiltration into the conjunctiva. The late phase reaction is activated by immunoglobulins and characterized by the presence of inflammatory sequel and clinical symptoms after the acute phase. [4] Histamine is the dominant allergic reaction mediator. [5-6] Histamine H1 receptor antagonists are the prime class of medication in the therapy of allergic and non-allergic conjunctivitis. For eye application, topical antihistamines are preferred over oral agents since their direct application at the site of action results in rapid onset and superior efficacy caused by high local concentrations achieved after their instillation into the eye. The topical application also minimizes the risk of systemic side effects. [7-8] The test formulation used in the present study was laboratory made surfactant-based ocular ebastine colloidal formulation. Ebastine is the second-generation H1 receptor antagonist, chemically 1-[4-(1, 1-dimethyl ethyl) phenyl]-4-[4-(diphenyl methoxy) piperidin-1-yl] butan-1-one indicated by oral route for various allergic manifestations of skin, nasal and ocular site. [1-2] Ebastine (1% w/v) oil in water microemulsion formulation was formulated by phase titration method using D-optimal mixture design, with the goal of enhancing solubility, thereby enhancing bioavailability due to site specificity as well as reducing systemic side effects and improving patient compliance. Campul MCM EP selected as the oil phase. The blend of Labrasol with Tween 80 and blend of Propylene glycol with glycerol were selected as surfactant and co surfactant, respectively. A main challenge in developing a sterile ophthalmic formulation is the capacity to guarantee that the formulation has acceptable pH, clarity, zeta potential, globule size, osmolarity, surface tension, viscosity etc. Resultant developed formulation showed droplet size (142 ± 0.16 nm), polydispersity index (below 1), refractive (1.369 ± 0.04) and osmolarity (291 ± 0.301 mOsm/L). The pH value of the developed formulation was 6.9 ± 0.12, which can be easily buffered by tear fluid (pH 7.2-7.4). Low microemulsion surface tension ensures good spreading effect on ocular surface and mixing with precorneal film components, thereby improving contact with ocular surface. The surface tension of the developed formulation was found to be 34.75 ± 0.13 mN/m. Zeta potential and viscosity of developed formulations was found to be -22.6 ± 0.39 mV and 13.19 ± 0.121cps respectively. These determined optimum physicochemical properties were observed to be eye-fitting, which is published in our previous paper. [9] In vitro results suggest that the developed formulation is suitable for further investigation in animal models to elucidate the safety and efficacy in treatment of allergic conjunctivitis. Hence, the present study was planned for determination of tolerability and assessment of efficacy of previously developed ebastine (1% w/v) colloidal ocular formulation for its onset and duration of effect in ovalbumin-induced conjunctivitis models in guinea pig.

MATERIALS AND METHODS

Animals
New Zealand white rabbits approximate weighing 1.5-2 kg was used for studying acute ocular irritation symptoms like blinking, redness, lacrimation etc. Dunkin-Hartley guinea pigs approximate weighing 300-350 g was used for studying efficacy study by ovalbumin-induced conjunctivitis model and scratching behavior. Rabbits were housed as one animal per cage while guinea pigs were housed as 3-4 animals per cage (polypropylene cage of 421 × 290 × 190 mm) and both maintained at 20-30°C and 50-55% relative humidity in a natural light and dark cycle of 12 h/12 h. They were allowed free access to certified pelleted food (Harlan, USA) and potable water in water bottles. The animals were acclimatized to animal holding laboratory area for 7 days prior to experiments. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) Reference No. 984/01/2017-07 for the use of animal in the study. Utmost care was taken to ensure that animals were treated in the most human and ethically acceptable manner. Fertilized Chick Eggs for Chorioallantoic membrane assay was obtained from Government Poultry House, Vadodara. Reagents like Isopropyl alcohol (70%), Normal saline (0.9% sodium chloride) and 1N NaOH used in experiment are of analytical grade.

Drug
Laboratory made ebastine (1% w/v) colloidal ocular formulation

Ocular Irritation Study by Hen’s Egg Test Chorioallantoic Membrane (HET-CAM)

Testing protocol for CAM Test

Selection of Eggs
The eggs were collected less than 1 week after lying and incubated for about 9 days; on 10th day, their blunt ends are tested by the candling lamp. Only the eggs with emergent vascular system were selected for the test.

Preparation of the Eggs for Test
Candling procedure helps in identifying the air space and it was marked on the eggs; then, after wiping with 70% IPA and a small window was made on the shell at the pointed end of the egg. The shells of the egg are opened at that marked portion on the blunt ends. The underlying membrane was carefully removed in such a way that underlying blood vessels are not damaged. Exposed chorioallantoic membrane (CAM) was treated with 10 microliter of the test formulation. The chorioallantoic membrane was also treated with 10 microliter of 1 N NaOH and considered as positive
control and 10 microliter of 0.9% w/v NaCl as negative control. The effects were observed near the surroundings of the applied sample within 5 min. After 5 min, change in CAM was observed for parameters like hemorrhage, coagulation and lysis.[10-12]

**Ocular Tolerability Test**

**Blinking Index**

**Testing Protocol for Blinking Index (B.I.)**

The animals were held on top of a lab table with a thick absorbent paper covered by hand. Blinking counts were performed with an electronic count-up timer over a 5-minute period. Using an adjustable volume digital pipette, saline and test formulation were applied to the lower cul-de-sac while pulling the upper eyelid gently and tilting the head of the animal slightly, making sure that the formulation did not spill out before the first blink. A volume of 25 microliters was used here as a substantial stimulus for blinking was found in preliminary tests. The right and left eyes were tested with saline, and the test solution was tested 30-60 minutes later. [13]

**Mathematical Expression**

The ratio between the number of blinks, counted over a 5-minute period following the instillation of a test solution, and the corresponding number of blinks, counted over a 5-minute period, in the same animal following the instillation of a normal saline solution, gives the Blinking Index (B.I.).

The average result obtained consecutively from both eyes of the same animal was entered as a single value. The results are presented as Mean ± Standard Error of the Mean (S.E.M.).

**Acute Ocular Irritation Study**

The animals were held in position same as that of previously mention in 2.2.1. The saline and test formulation were applied to the lower cul-de-sac while pulling the upper eyelid gently and the animals were observed up to 60 min for redness, swelling, watering of the eye. [14]

**Ocular Efficacy Study**

**Ovalbumin-Induced Allergic Conjunctivitis**

**Testing Protocol for Ovalbumin-Induced Allergic Conjunctivitis**

Allergic conjunctivitis was induced as per previously reported protocol. [15-17] Briefly, animals were sensitized on day 1, 7, 14, and 21 by intraperitoneal injection of ovalbumin (100µg/0.5 ml/animal), suspended in aluminium hydroxide gel as an adjuvant. Non-sensitized animals used for the experiment received only aluminium hydroxide gel. Seven days after the last sensitization, animals were used for the experiments assessing efficacy of test formulation.

At the time of experiment, a 20µl test formulation, saline, was instilled into the right eye of respective group using a micropipette and for oral, ebastine (3 mg/kg) in 0.5% CMC. At 0.5 and 24 h after the instillations, the eye was challenged with ovalbumin solution (100 mg/ml, 30µl). Edema was scored at 15, 30, 60, 90, and 120 min after the instillation of ovalbumin. For evaluation of edema, scoring system was used.

**Scratching Behavior**

Eye scratching behavior was defined as fore-limb movements over two times directed to theocular surface. [18] In the same sensitization protocol as describe earlier; along with edema, the number of eye scratches was counted for 30 min. The scratching response was assessed after topical antigen challenge at 0.5 h. [19]

**Histopathological Study**

The eyeballs together with the conjunctiva and lids of animals from the saline, saline + ovalbumin and test formulation group were exenterated and fixed in 10% buffered formalin. Tissues were subsequently processed for dehydration in a series of ascending alcohol concentrations. The samples embedded into paraffin wax and stained with hematoxylin and eosin. [20-29] DPX was used as mounting medium and micro toming was performed using microtome (model 0126, Yorco, India). The histopathological examinations for determination of damage/irritation due to the formulation were performed using inverted microscope (Nikon TS-100).

**RESULTS AND DISCUSSION**

**Ocular Irritation Study by Hen’s Egg Test**

**Chorioallantoic Membrane (HET-CAM)**

HETCAM (Hen’s chorioallantoic membrane) experiment was used for testing the potential of optimized formulation for eye irritation. The effects of test formulation as well as positive and negative controls on the chorioallantoic membrane were noted before and after the treatment as shown in Fig. 1. There was a remarkable difference between the test formulation and positive control by observing the changes in the chorioallantoic membrane. The positive control (0.1 N NaOH) induced major damage to the CAM. The 0.1 N NaOH causes hemorrhage followed by the lysis of blood vessels, whereas test formulation and negative control (0.9% NaCl) does not show severe changes in the chorioallantoic membrane after the application.

The severity of ocular irritation of the formulations was compared with that of positive and negative controls. The images showed that there was no considerable change in the blood vessel morphology of isolated CAM and formulation did not cause any damage or irritation to the eye upon application.

**Blinking Index**

The B.I. is defined as the ratio of the number of blinks (drug) divided by the number of blinks (saline) and it is used as an indication of the drug irritability. As shown in Table 1, the blinking index of saline solution and test formulation is 1.5 ± 0.4 and 2.4 ± 0.6 respectively. After topical application the strong correlation between the osmolarity of the solution and the irritation/pain
discomfort was observed. Obviously, other factors such as pH, the presence of other chemicals (i.e. preservatives) and the drug's own chemical nature can greatly affect its potential for eye discomfort/irritation.

Before Treatment

5 min After Treatment

a. Test Formulation

b. Positive Control (0.9% NaOH)

Positive Control (0.9% NaOH)

c. Negative Control (0.9% NaCl)

c. Negative Control (0.9% NaCl)

Table 1: Blinking Index and Clinical Symptoms

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Blinking Index (Mean ± SEM)</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>1.5 ± 0.4</td>
<td>0/4</td>
</tr>
<tr>
<td>Test formulation</td>
<td>2.4 ± 0.6</td>
<td>0/4</td>
</tr>
</tbody>
</table>

Fig. 1: Ocular Irritation Study by Chorioallantoic Membrane Assay

a. CAM treated with Test formulation
b. CAM treated with positive control (0.1 N NaOH)
c. CAM treated with Negative control (0.9% NaCl); Test formulation: Ebastine (1% w/v) ocular formulation

Table 2: Edema Score (At 0.5 h and 24 h after Topical Antigen Challenge)

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Sum of edema score (Mean ± SEM)</th>
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<td></td>
<td>Non sensitized</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>Sensitized</td>
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</tr>
<tr>
<td></td>
<td>Sensitized</td>
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</tr>
<tr>
<td></td>
<td>Sensitized</td>
<td>11.53 ± 0.16*</td>
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Significant compared to saline + ovalbumin treated sensitized animals, ANOVA followed by Dunnett’s test, p<0.05. Each value represents mean ± SEM of 4 animals /treatment

Fig. 2: Acute Ocular Irritation Study; Test formulation: Ebastine (1% w/v) ocular formulation

Acute Ocular Irritation Study

Comparable scoring in symptoms like eye closure, lacrimation and redness with respect to saline in rabbits indicates that the test formulation was well tolerated by the rabbits causing less discomfort. Thus the test formulation was found to be nonirritating with no ocular damage or abnormal clinical signs to the cornea, iris or conjunctiva observed. Hence the test formulation was suitable for the eye instillation and viable alternative to conventional ocular formulation (Fig. 2 and Table 1).

Ovalbumin-Induced Allergic Conjunctivitis

At 0.5 and 24 h after the instillations, the eye was challenged with ovalbumin solution (100 mg/ml, 30µl). Edema was scored at 15, 30, 60, 90, and 120 min after the instillation of ovalbumin. (Table 2) The edema scoring was done according to graded scale. Following system was used for assigning the edema scores

0-No edema

1-Slight edema

2-Partial eversion of eye

3-Eyelid half-closed

4-Eye swelling, more than half eyelid closed

The observations which did not match exactly with the score mentioned in the following scoring system were assigned a value between two adjacent scores up to 0.5. E.g. for scoring eyelid edema a value of 1.5 was assigned in case of the score falling between 1 and 2.

Table 2: Edema Score (At 0.5 h and 24 h after Topical Antigen Challenge)

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The Fig. 3 and Fig. 4 indicate graphical presentation of scoring at 15, 30, 60, 90, and 120 min after topical antigen challenge at 0.5 h and 24 h. Guinea pigs in the OA induced AC model observed for clinical symptoms as shown in Fig. 5. The results presented in Table 2 indicate that the test formulation (ebastine 1% w/v ocular) instilled 0.5 h and 24 h before the ovalbumin challenge caused significant inhibition of conjunctivitis symptoms. While the oral ebastine caused significant inhibition of conjunctivitis symptoms at 0.5 h only. It was also observed that compared to Ova challenge, test formulation showed 79.84% inhibition at 0.5 h, the effect persist up to 24 h with 42.46% inhibition while oral ebastine showed 34.71% inhibition at 0.5 h. This result indicates that topical formulation of ebastine showed better efficacy in ova induce conjunctivitis model at very low dose as compared to oral.

**Scratching Behavior**

After the instillation of 30μl/site of ovalbumin dissolved in normal saline solution (0.9% NaCl) into the eye, guinea pigs were placed into the observation cage (1 animal/cage), and the number of eye scratches was counted for 30 min. The scratching response was assessed after topical antigen challenge at 0.5 h. Animals treated with topical ebastine showed a significant reduction in itch-scratch response as compared to ova challenge and oral ebastine (Fig. 6).
Fig. 5: Instillation of ovalbumin in non-sensitized animal do not induce edema, hence no eyelid swelling (A). Severe edema, redness, and lacrimation were observed in sensitized animal after ovalbumin challenge (B). Ebastine (1% w/v) ocular formulation + ovalbumin (C). Ebastine (3 mg/kg) suspension oral + ovalbumin (D).

**Histopathological Study**

Histopathological conditions of conjunctiva after treatment with saline (negative control), saline+ ovalbumin (positive control), and test formulation (ebastine 1% w/v ocular) are shown in Fig. 7. No significant damage/harmful or mild epithelium damage effects on the microscopic structure of the conjunctiva treated with test formulation was observed in comparison to that of sample treated with topical allergen ovalbumin indicating the safety of the test formulation for ocular application. Conjunctiva treated with topical allergen showed severe damage epithelium cellular layer and edema, as well as neutrophil and eosinophil infiltration.

**Data Analysis**

Data obtained in different groups expressed as mean ± Standard error of mean (SEM) were analyzed using one-way ANOVA followed by Dunett's test. Statistical significance was considered as $p$ value < 0.05. Statistical analysis was performed using the Sigma Stat, Version 3.1. (SPSS Inc., USA)

Allergic conjunctivitis is a type-I hypersensitivity reaction resulting from the cascade of events initiated by allergen crosslinking of IgE molecules on mast cells in the conjunctiva. Ocular irritation is a prevalent adverse effect when new ocular formulations are developed.

To conclude, our previously developed ebastine (1% w/v) colloidal ocular formulation proved its tolerability in *in vitro* HET-CAM assay by preserving normal architecture of blood vessels and *in vivo* acute irritation study evident by reduced lacrimation, blinking index and redness. Further, the present study revealed the therapeutic role of formulation in allergic conjunctivitis by exhibiting statistically significant reduction in conjunctivitis symptoms like edema and scratching as compared to oral ebastine. In histopathological evaluation, ocular formulation caused
mild epithelium damage with less eosinophil infiltration than in conjunctiva treated with ovalbumin alone.

![Saline (Negative Control)](image1)

Normal epithelium cell layer and no edema, as well as neutrophil and eosinophil exudations in the proper lamina (40x)

![Saline + Ovalbumin (positive control)](image2)

Damage epithelium cellular layer and edema, as well as neutrophil and eosinophil infiltration (40x)

![Test formulation (Ebastine 1% w/v ocular formulation) + Ovalbumin](image3)

Mild epithelium damage and, there was less eosinophil infiltration than in conjunctiva of guinea pigs treated with ovalbumin alone (40x)

Fig. 7: Histopathological Photomicrographs of the conjunctival tissues in Ovalbumin induced Allergic conjunctivitis in guinea pigs treated with (a) Saline (b) Saline + Ovalbumin (c) Test formulation (Ebastine 1% w/v ocular formulation) + Ovalbumin.

In future, pharmacokinetic study and ocular tissue distribution study need to be performed to precisely define ADME pattern of said formulation after ocular application.

ACKNOWLEDGEMENT
The authors are also grateful to Parul University and ITM School of Pharmacy, Gujarat Technological University for providing research related facilities. We want to thanks Government Poultry House, Vadodara for providing fertilized eggs for CAM assay. We would like to thank Dr. Prachi Karia, Assistant Professor for assisting in CAM assay. We would like to thank Mr. Sameer Mehetre, Senior Manager, Drug Discovery (Pharmacology), Sun Pharma Advanced Research Company Ltd (SPARC), Vadodara for his kind help in data analysis.

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