Chapter 4
Design and Evaluation of Aceclofenac Nanoparticulate Formulations Using Eudragit RL100 Polymer
1. Introduction
The most common disease affecting the eye is inflammation. Inflammation is manifested as cellular and vascular response to the injury, infection, ischemia and excessive or inappropriate operation of immune mechanism. The response is amplified by activation of inflammatory cells and production of chemical mediators like acidic lipids e.g. prostaglandins, thromboxanes, leukotrienes, vasoactive amines, cytokines etc. The acidic lipids are produced through arachidonic acid metabolism. Arachidonic acid is released from the phospholipids component of cell membrane by the action of phospholipase A2. The arachidonic acid is fed into the cyclooxygenase and lipoxygenase pathways resulting in production of pro-inflammatory prostaglandins and leukotrienes [1, 2]. Topical therapy with corticosteroids is quite common in the treatment of ocular inflammatory disorders but their use is often associated with severe side effects such as increase in intraocular pressure, cataract formation and risk of infection [3]. Non-steroidal anti-inflammatory drugs (NSAIDs) like indomethacin [4], flurbiprofen [5], ketorolac [6] and diclofenac [7] which are devoid of these side effects have been found to be safer alternatives to steroids in treating ocular inflammation. Aceclofenac, 2-[(2-[(2,6-dichlorophenyl)amino]phenyl)acetyl]oxy]acetic acid, is a NSAID of the phenyl acetic acid group which is structurally related to diclofenac. It possesses good anti-inflammatory and analgesic activities, while maintaining better gastric tolerance in comparison with other NSAIDs such as indomethacin and diclofenac. Aceclofenac acts as such by inhibiting the secretion of tumor necrosis factor (TNF-α) and interleukin-1 along with preferential selective cyclooxygenase-2 (COX-2) inhibition after conversion into active metabolite [8-10].
Mostly, all ocular therapeutics has been administered to the eye as aqueous solution. About 90% of the dose applied topically from such solutions is lost due to pre-corneal losses (lacrimation and drainage) which lead to poor ocular availability [11]. Accordingly, there is a need for an appropriate delivery system which could increase the contact time of the drug with the eye surface and facilitate the transport of drug
molecules into the eye tissue. In this role, a controlled or sustained delivery of ophthalmic drugs would be beneficial.

A number of colloidal drug delivery systems such as liposomes [12], polymeric micelles [13], nanocapsules [14] and nanoparticles [15] have been evaluated for improved ocular bioavailability. Nanoparticles, because of their submicron size are well tolerated and have the tendency to deposit in the cul-de-sac for prolonged period. Nanoparticles of several synthetic polymers, e.g. poly(alkyl cyanoacrylate) [16], poly(lactic-co-glycolic acid) [17], poly(epsilon-caprolactone) [18], as well as natural polymers such as chitosan [19] and gelatin [20] have demonstrated promising results for efficient drug delivery to the ocular tissues. Despite the positive results, these polymers have their own disadvantages like, poly (alkyl cyanoacrylate) causes disruption of corneal epithelium [21]. The higher cost and slow degradability of poly (lactic-coglycolic acid) and poly (epsilon-caprolactone) limit their use. Eudragit RL 100 polymer is a copolymer of poly (ethylacrylate, methylmethacrylate, and chloro trimethyl-ammonioethyl methacrylate) containing an amount of quaternary ammonium groups between 8.8% and 12%. Eudragit RL 100 is insoluble at physiological pH and capable of limited swelling, thus appears to be a good polymeric carrier for the dispersion of drugs. The presence of quaternary ammonium group renders positive charge to the polymer by which it can interact with anionic drugs and mucin. The positive charge on the polymer may also impart mucoadhesion to the anionic cornea having isoelectric point (pI) of 3.2 and thereby increase its residence on corneal surface.

Polymeric nanosuspensions prepared from Eudragit RL 100 and RS 100 have been investigated for the ocular delivery of flurbiprofen [22], cloriocromene [23], amphotericin B [24], methylprednisolone [25] and piroxicam [26]. It has already been stated that for treatment of ocular inflammation, NSAIDs are preferred over steroid like prednisolone due to lack of ocular side effects. Among the NSAIDs, one which selectively inhibits COX-2 could offer therapeutic advantage, as COX-2 is involved in prostaglandin production at the site of inflammation. Thus, aceclofenac being a COX-2 inhibitor appears to be an ideal candidate for ocular inflammation.
Hence, attempts were made to formulate and characterize Eudragit RL 100-based nanoparticles of aceclofenac and evaluate the anti-inflammatory activity of selected formulation against arachidonic acid-induced ocular inflammation in rabbits.

2. Materials and methods

2.1. Materials

Aceclofenac and Eudragit RL 100 (Evonik Degussa India Pvt. Ltd., Mumbai, India) were received as gifts from Ranbaxy Research Laboratories (Gurgaon, India) and Jubilant Organosys Ltd. (New Delhi, India), respectively. Acetone and methanol were purchased from S. D. Fine Chemical Limited (Mumbai, India). Mannitol molecular grade was supplied by S. D. Fine Chemical Ltd. Arachidonic acid was purchased from Merck chemical Ltd. (Darmstadt, Germany). All other chemicals purchased were of analytical grade and were used as received. Fresh eyeballs of goat were obtained from local butcher shop (Ambedkar Nagar, New Delhi, India). Rabbits were obtained from the disease-free small animal house of Delhi Institute of Pharmaceutical Sciences and Research, University of Delhi.

2.2. Methods

2.2.1. Preparation of nanoparticles

Polymeric nanoparticles (NPs) of aceclofenac were prepared with Eudragit RL 100 by nanoprecipitation technique [15]. In brief, accurately weighed quantity of Eudragit RL 100 (50, 100 or 200 mg) and aceclofenac (10 mg) were dissolved in 5 mL acetone. This solution was poured into 20 mL distilled water containing 0.02%, w/v Tween 80 as hydrophilic surfactant under constant stirring by mechanical stirrer at 2200 rpm (Remi, Mumbai, India). Nanoparticles were spontaneously formed and turned into a milky colloidal solution with a bluish opalescence. The resulting dispersion was stirred at room temperature for 16-18 h with a magnetic stirrer to allow evaporation of acetone. Subsequently, the solvent was evaporated under reduced pressure at 60 °C to 10 mL by Rota evaporator (Hiedolph, Germany). To this aqueous dispersion, 5%, w/v mannitol was dissolved as a cryoprotectant and the Eudragit NPs were lyophilized to get free flowing powder. The Freeze-Dryer (Allied Frost, New Delhi) was operated for 24 h at -60 °C, at a
0.02 mm Hg pressure. The process variables involved in NPs preparation are presented in Table 1. Replicate batches of different formulations in varying drug: polymer ratios were prepared for experimentation. One batch of Eudragit NPs having drug to polymer ratio of 1:10, was also prepared without the addition of mannitol.

2.2.2. Nanoparticles- size, zeta potential and surface morphology
Freeze-dried nanoparticles were dispersed in distilled water after treatment in an ultrasonicator for 30 s. The mean particle size (z average), zeta potential, and polydispersity index (PDI) of the aqueous dispersion of aceclofenac-loaded nanoparticles were measured by a dynamic light scattering method using a Zetasizer Nano ZS-90 (Malvern Instruments, Worcestershire, UK) equipped with the DTS software. Each value quoted was the average of determinations of three independent samples. Morphological evaluation of the freeze-dried nanoparticles was performed using transmission electron microscopy (268D, FEI, Holland). Samples of the nanoparticle suspension (5-10 µL) were dropped onto copper grids coated with colladion in amyl acetate (Plano GmbH, Wetzlar, Germany). After complete drying, the samples were stained using 2% w/v phosphotungstic acid. Digital micrograph and soft imaging viewer software (Olympus, Singapore) were used to perform the image capture and analysis.

2.2.3. Entrapment efficiency of nanoparticles
It is the percentage of the actual mass of drug entrapped in the polymeric carrier, relative to the initial amount of loaded drug and was calculated using the following equation:

Entrapment efficiency % = Actual loading/Theoretical loading ×100 …… (1)

Theoretical drug loading was calculated from the amount of drug taken relative to the amount of total drug and excipients used in the preparation of nanosuspension as follows:

Theoretical loading (%) = Total drug/ Total drug + Total excipients …… (2)

For actual drug loading, the nanosuspension prepared by dispersing 25 mg of the lyophilized powder in 2 mL of distilled water was centrifuged at 13000 rpm (Superspin, Mumbai) for 20 min. The clear supernatant was analyzed for free aceclofenac content by measuring absorbance at 270 nm in an ultraviolet-visible spectrophotometer (Hitachi, Japan). The total amount of drug present in the nanosuspension was determined by
dispersing 25 mg of the lyophilized powder in 10 mL methanol by sonication and filtering through a micro syringe filter (0.2 µm) and analyzing the filtrate for aceclofenac by measuring absorbance at 270 nm in an ultraviolet-visible spectrophotometer. The following formula was used to calculate actual loading:

\[
\text{Actual loading} (\%) = \frac{\text{Total drug} - \text{Free drug}}{\text{mg of lyophilized powder}} \times 100 \quad \ldots \ldots (3)
\]

2.2.4. Physicochemical characterization

2.2.4.1. Fourier transform infrared spectroscopy (FT-IR). FT-IR spectra of samples were recorded using a Shimadzu FT-IR 8300 Spectrophotometer (Shimadzu, Tokyo, Japan) in the range of 4000-400 cm\(^{-1}\) as KBr pellets.

2.2.4.2. Powder X-ray diffraction (PXRD). PXRD patterns of samples were recorded with an X'Pert-PRO multipurpose X-ray diffractometer (PANalytical, Netherland) using Cu K\(\alpha\) radiation generated at 45 kV and 40 mA in the diffraction angle range of 5-40° 2θ.

2.2.4.3. Differential scanning calorimetry (DSC). DSC analysis was performed using a DSC TA-60 (Shimadzu, Tokyo, Japan) calorimeter. Samples were heated in sealed aluminum pans under nitrogen flow (50 mL/min) at a scanning rate of 10°C/min from 40°C to 200°C. An empty aluminum pan was used as the reference pan.

2.2.5. \textit{In-vitro} drug release

Required quantities of lyophilized powders were dispersed in distilled water to prepare nanosuspensions containing the drug equivalent to aceclofenac (0.1%, w/v). To study the \textit{in-vitro} release of aceclofenac from nanosuspension, 2 mL of the freshly prepared nanosuspension was placed in a dialysis sac (spectra pore membrane cut off; 6000-8000 MW). The sac was immersed with the help of a sinker into 200 mL of Sorenson’s phosphate buffer (pH 7.4) at 37°C in a USP dissolution rate test apparatus (type-2) (Lab India, India). The buffer was stirred by means of a paddle at 25 rpm. Aliquots of 4 mL sample were withdrawn at various time intervals and analyzed for aceclofenac content at 270 nm using an ultraviolet-visible spectrophotometer. The withdrawn samples were replaced with an equal volume of buffer.
2.2.6. *In-vitro* transcorneal permeation studies

*In-vitro* transcorneal permeation studies were carried out using freshly excised goat corneas (paired). The cornea was fixed between clamped donor and receptor compartments of an all-glass modified Franz diffusion cell in such a way that its epithelial surface faced the donor compartment. The corneal area available for diffusion was 0.50 cm$^2$. The receptor compartment was filled with 10 mL freshly prepared bicarbonate ringer solution (pH 7.2), and all air bubbles were expelled from the compartment. An aliquot (1 mL) of formulation [aceclofenac (0.1%, w/v) ophthalmic solution in isotonic phosphate buffer (pH 7.2), or aceclofenac (0.1%, w/v) nanosuspension in phosphate buffer (pH 7.2) (A2)] 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 min, and samples were withdrawn from receptor and analyzed for aceclofenac content by measuring absorbance at 270 nm in an ultraviolet-visible spectrophotometer. Results were expressed as amount permeated and percentage permeation or *in-vitro* ocular availability. The permeation (%) or *in-vitro* ocular availability was calculated as follows:

\[
\text{Permeation (\%)} = \left( \frac{\text{Amount of drug permeated in receptor}}{\text{Initial amount of drug in donor}} \right) \times 100 \quad \ldots \quad (4)
\]

At the end of the experiment, each cornea (freed from adhering sclera) was weighed, soaked in 1 mL methanol, dried overnight at 90 °C, and reweighed. From the difference in weights, corneal hydration was calculated.

2.2.7. *In-vivo* efficacy of aceclofenac formulation on arachidonic acid-induced ocular inflammation in rabbits

The arachidonic acid-induced rabbit ocular inflammation model [27] was used to compare the anti-inflammatory activity of aceclofenac-loaded NPs with the aqueous solution containing equivalent amount of aceclofenac. The experimental protocol was designed and approval of Institutional Animal Ethics Committee (IAEC 2010-1/Prot.no.14) was obtained. Six albino rabbits of either sex weighing 1-1.5 kg were
divided randomly into two groups of three animals each. Animals were housed in institutional animal house under standard conditions with free access to food and water. Left eye of each rabbit served as the control and received 50 µL of isotonic phosphate buffer (pH 7.2) vehicle while the right eye of the rabbit received 50 µL of aceclofenac (0.1%, w/v) ophthalmic solution in isotonic phosphate buffer (pH 7.2) (Group-I) or 50 µL of aceclofenac (0.1%, w/v) nanosuspension in phosphate buffer (pH 7.2) (A2) (Group-II). After 10 min, following administration of control vehicle or aceclofenac formulation in the respective eyes, 50 µL of arachidonic acid (0.05%, v/v prepared in phosphate buffer pH 7.0) was instilled in both eyes. All eyes were then evaluated for lid closure and polymorphonuclear leukocytes (PMN) migration. Lid closure was scored as follows: 0, fully open; 1, two-third open; 2, one-third open; and 3, fully closed. PMN migration was evaluated by counting PMN in tear fluid. Two drops of normal saline were instilled into inferior cul-de-sac of rabbit’s eye and after gentle mixing 50 µL of the tear fluid was withdrawn in WBC pipette at periodic intervals following arachidonic acid instillation. The tear fluid so withdrawn was diluted with Turke’s fluid and the number of PMN was counted in a Neubauer haemocytometer [28].

2.2.8. Stability studies on optimized formulation
Freeze dried NPs were stored in screw capped glass bottles wrapped with aluminum foil and subjected to accelerated stability testing by exposing the particles at 40 °C and 75% RH. The long term stability study was conducted by storage at room temperature. Samples kept under accelerated storage condition were withdrawn at 0, 1.5, 3 and 6 months and drug content was estimated. Similarly, samples stored at room temperature were withdrawn at 0, 3, 6 and 12 months and analyzed for drug content.

2.2.9. Statistical analysis
Statistical calculations were done by one-way analysis of variance (ANOVA) followed by Dunnett’s test or Student’s t-test using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA). A p value < 0.05 was considered significant.
3. Results and Discussion

3.1. Nanoparticles-size, zeta potential and surface morphology

The effect of drug-polymer ratio on particle size, zeta potential and entrapment efficiency of nanoparticles is shown in Table 1. The particle size ranged from 75.52 ± 6.7 nm (Formulation A1) to 184.36 ± 20.2 nm (Formulation A3) which was prepared using higher amount of polymer in the organic phase. The increase in amount of polymer in the organic phase resulted in significant differences ($p < 0.05$) in particle size (Table 1). The larger particle size with increase in polymer concentration was probably due to increased viscosity of dispersed phase (polymer solution in organic phase), resulting in larger nanodroplets formation. Similar findings were reported in earlier studies on Eudragit RL 100-based nanoparticles [24].

The polydispersity index (PDI) is an indicator of particle size distribution. Its value in case of submicron particles in the range of 0.15-0.3 indicates size homogeneity, while a polydispersity index greater than 0.3 indicates heterogeneity [29]. The mean PDI values for the drug loaded formulations varied in the range of 0.186-0.380. All batches showed a smaller mean size, well suited for possible ocular application.

Zeta-potential measurement is an important surface characterization technique which provides information regarding the surface charge of nanoparticulate systems. The magnitude of zeta potential gives an indication of the potential stability of colloidal system. A zeta potential of ±30 mV is considered sufficient to ensure physical stability of suspension [30]. The nanosuspensions exhibited positive zeta potential values (Table 1) ranging from +22.5 to +32.6 mV. The surface of nanoparticles attains positive charge due to the presence of the quaternary ammonium groups on Eudragit RL 100. This is consistent with the findings of Pignatello et al. [22]. As mucin layer on corneal surface is negatively charged, the positively charged nanoparticles could help in effective adhesion to the corneal surface which in turn would enhance ocular bioavailability.

TEM images for Eudragit RL 100 nanoparticles of different drug polymer ratio are shown in Fig. 1. The prepared Eudragit RL 100 nanoparticles were nearly spherical in shape with a smooth surface. These particles are not expected to cause any irritation to
ocular surface, as it is known that isometric particles with obtuse angles and edges cause less irritation than particles with sharp angles and edges [31].

3.2. Entrapment efficiency of nanoparticles

In the present experiment, the actual drug loading values of aceclofenac in the Eudragit RL 100 nanoparticles with different drug polymer ratios (1:5, 1:10, 1:20) were 9.71%, 8.69% and 4.17% and the corresponding entrapment efficiencies were 58.33%, 95.73% and 87.77%, respectively (Table 1). The result suggests that as the drug: polymer ratio was changed from 1:5 to 1:10, the drug entrapment efficiency increased significantly ($p < 0.05$). Further increase in polymer concentration (1:20), however, showed decrease in entrapment efficiency. As the polymer concentration in organic phase increases it results in significantly higher drug entrapment efficiency due to increase in organic phase viscosity (Table 1), which hinders the movement of drug molecules from organic phase to aqueous phase. The decrease in entrapment efficiency with drug: polymer ratio of 1:20 appears to be due to decrease in actual drug loading, as entrapment efficiency is the ratio of actual drug loading and theoretical drug loading. Based on the results, formulation made with 1:10 drug-polymer ratio (A2) was selected for further studies.

3.3. Physicochemical characterization

The FT-IR spectrum of aceclofenac showed carbonyl stretching (C=O) at 1717.18 cm$^{-1}$, aromatic C=C stretching at 1589 cm$^{-1}$, 1578 cm$^{-1}$, 1508 cm$^{-1}$ and 1452 cm$^{-1}$, C-H bending for substituted benzene ring at 781 cm$^{-1}$ and 749 cm$^{-1}$ and N-H stretching at 3319 cm$^{-1}$. Eudragit RL 100 exhibited IR absorption at 3434.91 cm$^{-1}$ (OH stretch), 2955.11 cm$^{-1}$ (CH stretch), 1735.88 cm$^{-1}$ (CO stretch for ester). Spectrum of mannitol showed OH stretching at 3400 cm$^{-1}$, OH in plane bending at 1420 cm$^{-1}$, C-O stretching at 1081 cm$^{-1}$ and OH out of plane bending for alcohol at 701 cm$^{-1}$. The spectrum of freeze dried NPs (1:10 ratio) without mannitol showed the characteristic peaks of Eudragit RL 100 at 3432 cm$^{-1}$, 2953 cm$^{-1}$ and 1734 cm$^{-1}$ while freeze dried NPs (1:10 ratio) containing mannitol showed the peaks of Eudragit RL 100 and mannitol. The characteristic peaks of drug could not be located due to dilution by the stated excipients.
Fig. 2 displays the X-ray diffractograms of samples. Aceclofenac showed the characteristic peaks at 11.64°, 18.39°, 22.07°, 24.43°, 25.93° and 32.09° 2θ. The presence of sharp peaks in the diffractogram of aceclofenac indicated its crystalline nature while the diffractogram of polymer (Eudragit RL 100) indicated amorphous structure. The physical mixtures of aceclofenac and Eudragit RL 100 (1:10) resulted in a relatively less crystalline form which showed the characteristic peaks of aceclofenac at 18.68°, 22.37°, 24.78° and 26.24° 2θ. Mannitol showed crystalline nature and manifested several distinct peaks at 10.55°, 14.55°, 18.72°, 20.89°, 23.29°, 24.72°, 29.53°, 33.69°, 35.98° and 38.53° 2θ which correspond to its β polymorphic form (mannitol exists in α, β and δ polymorphic forms) [32]. The freeze dried NPs (1:10 ratio) with mannitol exhibited peaks at 9.43°, 20.29°, 20.89°, 21.85°, 24.73° and 35.81° 2θ which appears to be contributed by mannitol, where the peak at 9.43 °2θ was relatively intense. It has been reported that the α polymorph of mannitol shows a weaker peak at 9.57° 2θ and the δ form shows an extremely intense peak at 9.74 °2θ while the β form does not show any peak at the said position [32]. Thus, the presence of a relatively intense peak at 9.43 °2θ in the diffractogram of freeze dried NPs with mannitol suggests possible existence of δ polymorphic form induced by lyophilization. No distinct peak was observed for freeze dried NPs without mannitol. The diffractogram of nanoparticles without mannitol indicated amorphous structure which was devoid of the crystalline peaks of aceclofenac. Therefore, the absence of the drug crystalline peaks is attributed to the presence of the drug in the amorphous state within the polymer.

The DSC thermograms of samples are presented in Fig. 3. The thermogram of aceclofenac is characterized by a sharp melting endotherm at 152.02 °C and heat of fusion of 91.61 J/g. The thermal curve of Eudragit RL 100 is characterized by a broad endotherm at 65.69 °C with heat of fusion of 3.29 J/g. The thermogram of aceclofenac and Eudragit RL 100 physical mixture (1:10) showed a depressed endotherm of drug at 151.55 °C with heat of fusion of 0.41 J/g. The thermal behavior of mannitol is characterized by an endothermic peak at 170.05 °C with heat of fusion of 367.03 J/g. The DSC curve of aceclofenac containing Eudragit RL 100 nanosuspension lyophilized with
mannitol showed a small endotherm at 155 °C which corresponds to the melting point of δ polymorph of mannitol [33] followed by a sharp endotherm at 168.58 °C (with heat of fusion of 87.74 J/g), which appears to be the depressed endothermic peak of β polymorph of mannitol. The thermogram of nanosuspension lyophilized without mannitol showed no endothermic peak of aceclofenac because of the decreased crystallinity in the formulations or due to the presence of very small quantity of aceclofenac in the lyophilized powder. Concisely, XRD and DSC studies verified decrease in crystallinity of drug in polymeric matrix.

3.4. In-vitro drug release

Fig. 4 compares the in-vitro release of aceclofenac from the Eudragit RL 100-based nanosuspensions. The release of aceclofenac was evaluated by dialysis, using Sorenson’s phosphate buffer (pH 7.4) as the release medium. The NP formulations made with drug:polymer ratios of 1:5, 1:10 and 1:20 showed 32.55, 29.18 and 23.88% drug release in 1 h and 80.67, 74.55 and 65.98% release in 8 h while 97.61% drug diffused into the release medium from an aqueous solution of the drug (0.1%, w/v) used as a control. The result suggests entrapment of the drug in the nanoparticles hinders the drug release which is biphasic: an initial burst release followed by a slow release phase. As the drug:polymer ratio is changed from 1:5 to 1:20 (i.e. as the polymer concentration is increased) the drug release is sustained due to increase in particle size or reduction in surface area per unit volume and increase in diffusional path length.

The release data were fitted into various kinetic models [34] like zero-order, first-order, Higuchi and Korsmeyer-Peppas equations in order to determine the release mechanism and regression coefficients ($R^2$). The release of aceclofenac from Eudragit RL 100 NPs fitted best to Higuchi-square-root release kinetics, which can be confirmed by comparing the values for the regression coefficient of the zero order ($R_0^2 = 0.950$, 0.947, and 0.952 for A1, A2, and A3), first order ($R_1^2 = 0.986$, 0.976, and 0.991), Higuchi matrix ($R^2 = 0.992$, 0.987, and 0.997) and Korsmeyer-Peppas ($R_k^2 = 0.980$, 0.983, and 0.987) equations. The value of ‘n’, (0.43< n < 0.85) the diffusion exponent of Korsmeyer-
Peppas equation indicated that the release of aceclofenac from Eudragit RL 100 NPs is anomalous, i.e. contributed by combination of dissolution and diffusion.

3.5. *In-vitro* transcorneal permeation studies

Transcorneal permeation studies through excised goat cornea indicated about 2-fold increase (statistically significant, $p < 0.05$) in permeation of drug from nanosuspension formulation compared with an aqueous solution of aceclofenac of same concentration (Table 2). The results suggest possible corneal uptake of the NPs due to positive charge and small size. Corneal hydration remained in the normal range of 75-80% which showed the eye friendly behavior of developed formulation.

3.6. *In-vivo* efficacy of aceclofenac formulation on arachidonic acid-induced ocular inflammation in rabbits

Topical instillation of arachidonic acid induces characteristic signs of ocular inflammation, including conjunctival vasodilation, edema, mucous discharge, lid closure, increased intraocular pressure, PMN migration and increased aqueous humor protein [35]. Thus, we assessed the effect of aceclofenac nanosuspension and aqueous solution on arachidonic acid-induced PMN migration and lid closure in rabbit eyes (Fig. 5 and Table 3). The results of the *in-vivo* ocular anti-inflammatory study indicated that the lid closure was prominent up to 3 h after which it decreased. The eyes, which were treated with aceclofenac formulation, showed smaller lid closure scores as compared to their respective controls. Further, the lid closure score in eyes treated with aceclofenac nanosuspension was observed to be much smaller than that in the eyes treated with aqueous solution of aceclofenac. The PMN count in the tear fluid of rabbits also increased up to 3 h and decreased afterwards. The third hour PMN count in the tears of rabbit eyes treated with aqueous solution of aceclofenac and its control were 700 ± 28 and 900 ± 28 respectively, whereas in case of eyes treated with nanosuspension and its control, the counts were found to be 533.33 ± 28 and 883.33 ± 60 respectively. The administration of both the aqueous solution and nanosuspension of aceclofenac caused significant ($p < 0.05$) reduction in the PMN migration in the tear fluid in comparison with the untreated control eyes. Further at third hour the PMN count in tear fluid of eyes
following nanosuspension treatment was significantly lesser (p < 0.05) than that observed with aqueous solution treatment. Similar results have been previously reported for diclofenac loaded Eudragit S 100 nanosuspension [15].

Thus, significantly higher percentage (%) inhibition of PMN migration was observed in eyes treated with aceclofenac nanosuspension as compared to the eyes treated with aceclofenac aqueous solution. Small size, increased residence of the positively charged nanoparticles on ocular surface and cellular uptake could possibly account for the result.

3.7. Stability studies on optimized formulation
The optimized formulation made with 1:10 drug-polymer ratio showed around 95.21% aceclofenac content on storage under accelerated condition (i.e. 40 °C /75% RH) for 6 months, while following storage at room temperature for 12 months the drug content was around 96.08 %. On the basis of first order degradation rate constant (1.09 × 10⁻⁴ day⁻¹), the calculated t₉₀ of optimized formulation at room temperature was found to be 959.4 days indicating that the formulation would provide more than 2 years shelf life at room temperature.

4. Conclusions
Eudragit RL 100-based aceclofenac nanoparticles prepared by nanoprecipitation with 1:10 drug-polymer ratio showed the highest entrapment efficiency, low poly dispersity index and positive zeta potential. The positive zeta potential and fine particle size will help to prolong the corneal contact time. The PXRD and DSC indicated decrease of drug crystallinity in the nanoparticles. The nanoparticle was found to provide a biphasic release pattern: initial burst release followed by sustained release which fitted best into Higuchi-square-root release kinetics. The nanoformulation also showed higher anti-inflammatory activity compared to that of aqueous solution of drug. The developed formulation would provide more than 2 years shelf life at room temperature. The resulting nanoparticles are promising in reducing dose frequency and improving patient compliance for ocular delivery.
References


**Table 1** Effect of various drug-polymer ratios on particle size, zeta potential and entrapment efficiency of aceclofenac loaded Eudragit RL 100 polymeric nanoparticles.

<table>
<thead>
<tr>
<th>Formula code</th>
<th>Drug-to-polymer ratio</th>
<th>Particle size (nm ± SE)</th>
<th>PDI (± SE)</th>
<th>Zeta Potential (mV ± SE)</th>
<th>Entrapment efficiency (%) ± SE</th>
<th>Viscosity of organic phase* (cps ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>1:5</td>
<td>75.52 ± 6.7</td>
<td>0.380 ±0.004</td>
<td>22.5 ± 0.62</td>
<td>58.33 ± 0.93</td>
<td>0.415±0.009</td>
</tr>
<tr>
<td>A2</td>
<td>1:10</td>
<td>134.97±10.3†</td>
<td>0.186 ±0.01</td>
<td>30.5 ± 0.38</td>
<td>95.73 ± 0.28†</td>
<td>0.463±0.004</td>
</tr>
<tr>
<td>A3</td>
<td>1:20</td>
<td>184.36±20.2†</td>
<td>0.368 ±0.07</td>
<td>32.6 ± 0.6</td>
<td>87.77 ± 0.88†</td>
<td>0.565±0.001</td>
</tr>
</tbody>
</table>

* Viscosity of organic phase measured by Ostwald viscometer
†Statistically significant \( p < 0.05 \) compared with A1 (Drug to polymer ratio- 1:5), as determined by One-way ANOVA followed by Dunnett’s test. Values are mean ± S.E. \( n = 3 \)
**Table 2.** Transcorneal permeation characteristics of aceclofenac from Eudragit RL 100 based nanosuspension and aqueous solution through excised goat corneas (Paired)

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Amount permeated (mg)(120 min)</th>
<th>Permeation (%) (120 min)</th>
<th>Corneal Hydration (%)</th>
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</thead>
<tbody>
<tr>
<td>Aqueous solution</td>
<td>0.031±0.0009</td>
<td>3.08</td>
<td>76.31± 0.544</td>
</tr>
<tr>
<td>Nanosuspension formulation</td>
<td>0.061±0.0010†</td>
<td>6.13</td>
<td>78.33 ± 0.423</td>
</tr>
</tbody>
</table>

†Statistically significant ($p < 0.05$) compared with aqueous solution, as determined by paired t-test. Values are mean ± SE of 3 corneas in each group.
Table 3. Comparative effect of Eudragit RL 100 based nanosuspension and aqueous solution of aceclofenac on arachidonic acid-induced lid closure in the rabbit eye.

<table>
<thead>
<tr>
<th></th>
<th>Lid Closure Score</th>
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<tbody>
<tr>
<td></td>
<td>0.5 h</td>
</tr>
<tr>
<td>Control-Nano</td>
<td>2.66±0.51</td>
</tr>
<tr>
<td>Nano suspension</td>
<td>1.33±0.33</td>
</tr>
<tr>
<td>Control- Aqueous</td>
<td>2.66±0.51</td>
</tr>
<tr>
<td>Aqueous solution</td>
<td>2.33±0.33</td>
</tr>
</tbody>
</table>

Values are mean ± SE ($n = 3$)
Fig.1. Transmission electron micrograph (TEM) images of: (A) Aceclofenac:Eudragit RL 100 nanoparticles (1:5), (B) Aceclofenac:Eudragit RL 100 nanoparticles (1:10), (C) Aceclofenac:Eudragit RL 100 nanoparticles (1:20)
Fig. 2. PXRD of: (a) Aceclofenac, (b) Mannitol, (c) Eudragit RL 100, (d) Aceclofenac and Eudragit RL 100 physical mixture (1:10), (e) Eudragit RL 100 nanoparticles (1:10) without mannitol, (f) Eudragit RL 100 nanoparticles (1:10) with mannitol.
Fig. 3. DSC thermograms of (a) Aceclofenac, (b) Eudragit RL 100, (c) Aceclofenac and Eudragit RL 100 physical mixture (1:10), (d) Eudragit RL 100 nanoparticles (1:10) without mannitol, (e) Mannitol, (f) Eudragit RL 100 nanoparticles (1:10) with mannitol
Fig. 4. *In-vitro* release of aceclofenac from Eudragit RL 100 based nanosuspension formulations.

*Statistically significant (p< 0.05) compared with aqueous solution, as determined by One-way ANOVA followed by Dunnett’s test. Each bar represents the mean ± SE (n=3).
Fig. 5. Effect of Eudragit RL 100 based nanosuspension and aqueous solution of aceclofenac (0.1 % w/v) against arachidonic acid-induced PMN migration in tears of rabbits.

*Statistically significant (p < 0.05) compared with control as determined by paired t test
†Statistically significant (p < 0.05) compared with aqueous solution as determined by Student’s t-test.

Each bar represents the mean ± SE (n=3).