RESEARCH ARTICLE

Formulation Development and Characterization of Cyclodextrin Based Nepafenac Eye Drops for Ocular Drug Delivery System

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ABSTRACT

The current investigation work was to design and evaluate cyclodextrin (CD)-based complex formulations to adeptly deliver NSAID drug topically to the eye cavity, to serve inflammation associated with the anterior and enhanced levels to posterior part of the eye. The physicochemical properties of formulations containing nepafenac-CD complex formulations were screened for viscosity, pH, Osmolality, mucoadhesion, *In-vitro* drug release, ocular irritancy by HET CAM's study, scleral permeability, anti-inflammatory activity and *In-vitro* cell line study on human retinal pigmental epithelium (ARPE-19) cell lines were investigated. The results were collated with a commercially marketed nepafenac suspension, Nevanac® 1 mg/mL. All formulations showed neutral pH, isotonic, non-irritating and nontoxic and showed high permeation observed through sclera and thus suggesting cytocompatibility. Formulations containing combinations of polymer like carboxymethyl cellulose (CMC) and PVP K-90 showed greater anti-inflammatory activity, even higher than the commercial formulation, Nevanac® 0.1%. The optimized formulations represent a moment for topical instillation of drugs with improved patient compliance to treat anterior segment of eye and opportunity for the posterior segment of the eye.

Keywords: Nepafenac, Age related macular edema, Eye Inflammation, hen's egg chorioallantois membrane test, *In-vitro* diffusion study, Ex-vivo Permeation, Cell line study.

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INTRODUCTION

The eye is an organ for visual perception of the environment. Its unique purpose is reflected in its specific anatomical and physiological characteristics. Inflammation of the eye and besides tissues are the main cause for the pathogenesis in Ocular diseases. Anterior part of the eye includes various symptoms of inflammation like redness, eye pain, discomfort, blurred vision, swelling, cataract, glaucoma, pterygium, conjunctivitis[1] etc. and disease related to posterior or back of the eye are vitreoretinal diseases like Retinopathy, diabetic macular degeneration, age related macular degeneration, retinitis, choroidal neovascularization etc.[2-5]

To treat front part of the eye i.e. anterior segment, physician generally recommends eye drop formulations. Eye drops are highly patient compliant, but the bioavailability of these topical eye drops is about 5% and for this low bioavailability reason behind that is due to the excessive tear generation and blinking[6] followed by administration of eye drops which serve to a loss of eye drop formulation and thus loss of therapeutic medicaments. Another cause of low bioavailability is the low solubility of NSAID's in water and due to low solubility, which is further formulated as Ocular suspension. Nepafenac is having very low to moderate solubility in aqueous therefore it is commercially available eye drops of Nepafenac (Nevanac 0.1% and Ilevro 0.3%) having strength of 1mg/mL and 3 mg/mL. Suspension formulation are better w.r.t. extended release or for providing long action of medicament for longer duration. Major drawback of suspension formulation after administration or instilling into the eye, it often causes discomfort, foreign particle feeling or irritation and due to that it creates hinderance in normal vision of the patient[7-9]. Due to all these reasons fast elimination of drug medicament from the eye surface.

In 2005, the US Food and Drug Administration (FDA) granted approval for Nevanac® 0.1% (1 mg/mL) nepafenac suspension eye drops for the treatment of pain and inflammation following cataract surgery and preventing cystoid macular oedema since it proved to be more effective in vivo than other non-steroidal anti-inflammatory drugs (NSAIDs) used in ophthalmic formulations like diclofenac 0.1 %, ketorolac 0.4 % and bromfenac 0.09 %. In 2012, a 0.3 % NEP containing ophthalmic suspension (ILEVRO®) was also approved by the FDA which can reduce the frequency of instillation of NEP eye drops from 3 times a day to only one daily dose, meanwhile having the same efficacy.[10]

Therefore, to avoid problems associated with current available suspension of Nepafenac there is a need to have better ocular medicament and to improve bioavailability of ocular suspension. The bioavailability [11-13] of an ophthalmic

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suspension can be improved by either increasing the amount of solubilized API by using complex formation, Nanoparticles, or increasing the ocular retention time on the corneal surface by adding viscosity modifying agents or by in-situ gelling systems. Cyclodextrins (CDs) are oligosaccharides shaped like cones that can create inclusion complexes with lipophilic drugs due to their hydrophobic cavity, while the hydrophilic outer surface of the cone ensures excellent solubility in water.

In ophthalmic formulations Cyclodextrins not only increase the solubility of poorly soluble by forming complex with poorly water-soluble compound in the aqueous solution but also improve stability and reduce the tissue irritating effects of the drug and thus improves the bioavailability of the ocular eye drops.

In Ocular formulation cyclodextrins are approved for the use i.e. hydroxypropyl-ß-CD (HP-ß-CD), HPβCD and sulfobutylether-β-CD (SBE CD). In that hydroxypropyl-ß-CD (HP-ß-CD) is the commonly used CDs by making the most stable 1:1 complex with the drug. There is usually no risk in using Cyclodextrins as they are nontoxic, non-irritant and well tolerated[15-19] by the eye and also, they enhance drug permeation in ocular tissues when use along with the polymers.

Aim of this study, to formulate a novel Nepafenac ophthalmic solution containing eye drops were prepared by solubility enhancing by forming a complex of HP-ß-CD and Nepafenac. In this study all excipients used are already approved for the ocular used and the concentration of excipients is as per IID data base. Prepared formulation was tested for Drug content, pH, osmolality, *In-vitro* drug diffusion, Anti-inflammatory study, Ocular Irritation study (HET CAM study) and Ex-vivo Cell line study.

MATERIALS AND METHODS

Materials

Materials Nepafenac from Analtec private limited, Ambernath, Maharashtra, India; Hydroxy propyl β cyclodextrin from roquette and Polyvinyl pyrrolidone K90 and Sodium CMC from Ashland, India. BKC (50%) from

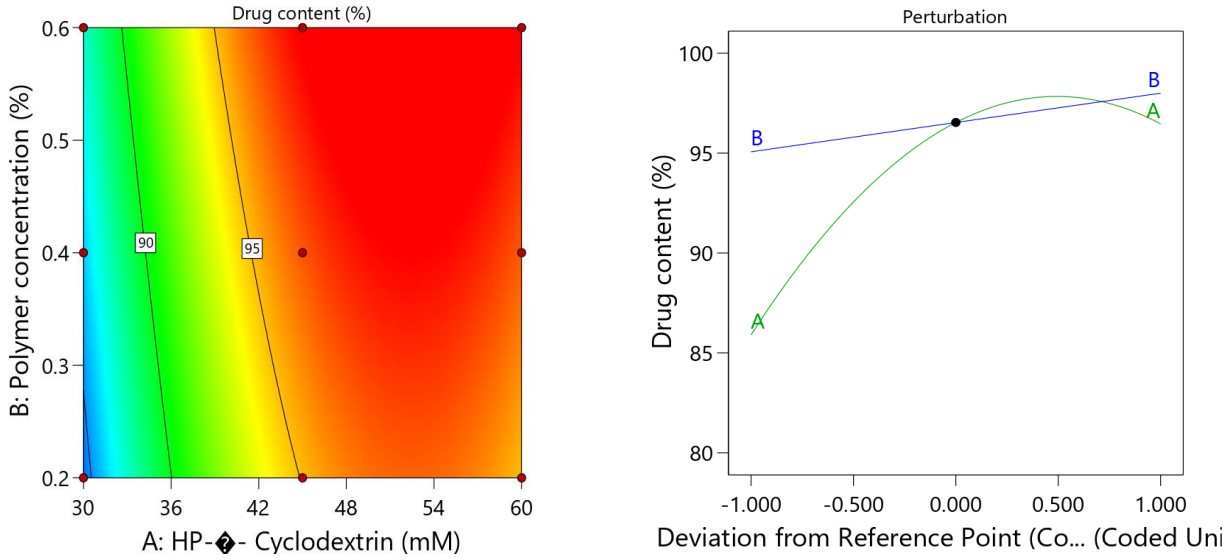


Figure 1: Contour plot and Interaction plot of Drug content versus HP-β-Cyclodextrin (%) and Polymer concentration (%)

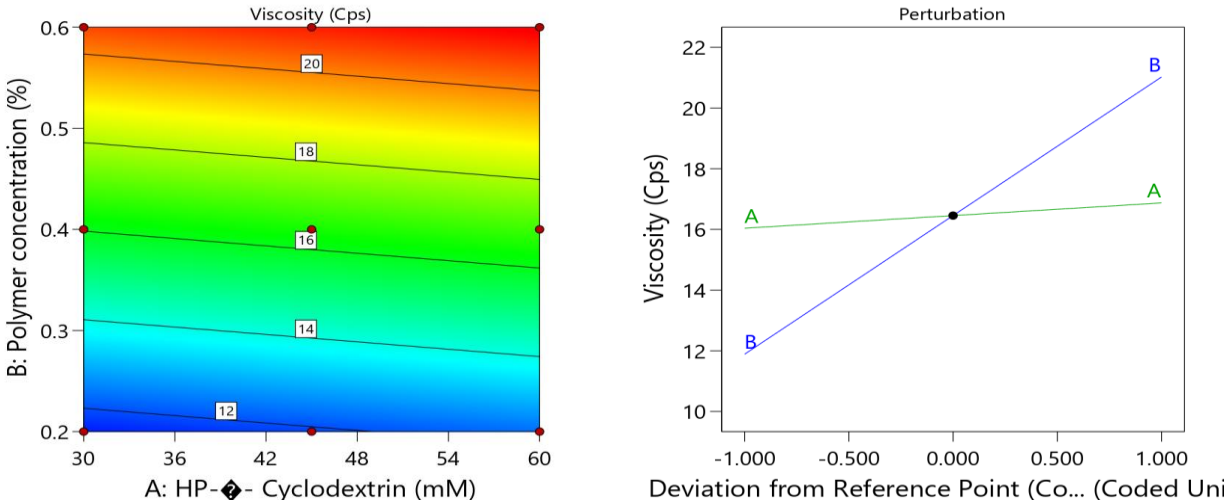


Figure 2: Contour plot and Interaction plot of viscosity (nm) versus Polymer concentration (%) and HP-β-Cyclodextrin concentration (%)

Merck KGaA, Germany; Various reagents were used as analytical grade.

Solubility Study

Nepafenac was taken in excess quantity and added to vials containing 2 mL trial of individual oils, surfactants, and different solubiliser to make a supersaturated solution and kept aside for 72 h in an automatic shaker at 35°C. The supersaturated solution was filtered using a Whatman filter paper to remove the excess Nepafenac and 1 mL of that filtrate was kept aside to perform further dilutions with Ethanol and tested using Ultraviolet (UV) spectrophotometric technique to check the solubility of Nepafenac API in the respective solubiliser. Blank readings of every composition were prepared using 1 mL of respective component and diluting it with Ethanol solution to eliminate the interferences in the absorbance while conducting the UV visible spectrophotometric analysis.[7-9]

Formulation Development and Optimization of Nepafenac Solution

Nepafenac ocular solution is a light-yellow color, homogenous that is formed by mixing the dissolving the API in cyclodextrin containing solution and then addition of buffers, tonicity adjusting agent, viscosity[21] modifiers and pH adjusting agent and finally volume was made up to 100% using purified water. All the dispensing of the API and excipients were done on a four-digit analytical balance. The final product was filled in semipermeable LDPE containers at room temperature. Final prepared solutions of Nepafenac were optimized by full factorial design approach using the Design Expert (V.13 Stat-Ease) to find the effect of different experimental components on the ophthalmic solution. The experimental trials were executed using two factors: HP-β-Cyclodextrin concentration (A) and Polymer concentration (combination of Sodium CMC and PVP K-90) (B). These factors were considered most effective on responses like Drug content (Y1) and Viscosity (Y2). The two factors were HP-β-Cyclodextrin concentration and Polymer concentration as displayed in Table 1.

Characterization of the Optimized Nepafenac Solution

Drug Content by UV Spectroscopy

UV spectrophotometric methods have been used for the estimation of Nepafenac API in bulk and in ophthalmic formulations. For estimation of drug content of optimized formulations, a clear and robust UV spectrophotometric method (Shimadzu UV 1800) was used. Nepafenac is diluted in methanol. For analysis of test formulations, an accurately measured amount of Solution (1 mL) equivalent to 1 mg of Nepafenac was transferred into 100 mL volumetric flask,

The drug content of prepared sample solution was assessed by UV spectrophotometric (Shimadzu UV 1800) at 237 nm for Nepafenac. The method was validated for linearity, precision, specificity, and robustness test. The assay (%, w/v) values were determined by simultaneous equation method.[22]

Osmolality

Osmolality of solutions was performed using Osmomat 3000 instrument (Gonotec GmbH, Germany) that applies freezing point depression method to performs osmolality of solutions. For osmolality measurement, calibration of the osmometer using the 3-point calibration technique with standard calibration solutions (Zero point, 300 mOsmol/kg and 850 mOsmol/kg) was performed. Sample cuvette was inserted into the osmometer and the "Start" button was pressed. The osmolality reading was displayed on the osmometer screen.[23]

Viscosity

Viscosity of Nepafenac solution was using Brookfield viscometer (Model DV3, Brookfield Engineering Labs Inc.) related with Rheocalc T 1.2.19 programming software. The viscosity measurement was done using an S- 18 spindle at 25.0 ± 0.5°C.[24]

Mucoadhesive Study

Mucoadhesive study was performed on TA. XT plus Texture analyser (Stable microsystems Products) To study nepafenac solution, formulation was taken in glass vial and place vial with simulated surface on texture analyser stage[25]. Apply sample to cylindrical probe and then probe moves down to make contact with surface of the solution. Maintain controlled contact for 10 min at 35± 0.5°C. After end of time probe withdraw and moves upward and force vs distance data recorded. Mucoadhesive force is determined as the detachment force is needed to separate the formulation from the cylindrical probe after applying a force of 0.5 N For 10 minutes.

***In-vitro* Drug Release Study**

Franz diffusion cell apparatus was used for evaluating *In-vitro* dissolution of prepared formulated solution. Freshly prepared phosphate buffered saline (PBS) (pH 7.4) was kept at 35 ± 0.5°C, which simulate physiological conditions present on corneal surface of eyes was picked as the dissolution media. Dialysis membrane (cellophane membrane,) was soaked in simulated tear fluid (STF) (pH 7.4). The cellophane membrane was positioned between donor and receptor compartment of Franz diffusion cell and hed in place. The prepared formulations (1 ml equal to 1 mg of Nepafenac) were applied on the donor side of the membrane. At 1, 2, 3, 4, 5 and 6 h time points, and a 1 mL sample was removed from the acceptor compartment. After sampling at each time point, the acceptor compartment was replenished with 1 mL of new STF (pH 7.4). The samples were further diluted to 100 mL using 0.1 M Ethanol and analysed UV-Vis spectrophotometer for Nepafenac 237nm. The percentage of drug released at each time point was calculated as cumulative percent drug release and were plotted as function of time (h).[26-28]

***In-vitro* Anti-inflammatory Activity by Protein Denaturation Method**

Protein coagulation, or denaturation, is a key marker in inflammation. When proteins like albumin are exposed to heat or chemicals, they unfold and lose their structure mimicking inflammatory conditions. Substances that prevent this denaturation may have anti-inflammatory properties. The denaturation of protein can induce inflammation. The reaction mixture (10 mL) consisted of 0.4 mL of egg albumin (from fresh hen's egg), 5.6 mL of phosphate buffered saline (PBS, pH 6.4) and 500 µL of Sample Similar volume of double-distilled water served as control. Then the mixtures were incubated at (370c ±2) in a incubator for 15 min and then heated at 70oc for 5 min.

Table 1: Factors and levels for HP-β-CD's concentration and Polymer concentration

Parameter	Component	Units	Applied Levels		
			Low Level (-1)	Center Level (0)	High Level (+1)
X1	HP B cyclodextrin	%	30mM	45mM	60mM
X2	Polymer combination	%	0.2	0.4	0.6

Table 2: Outline of the experimental design and result

Solution coding	Independent variables		Measured dependent variables*	
	HP-β-Cyclodextrin (A)	Polymer conc. (B)	Drug Content (Y_1)	Viscosity (Cps) (Y_2)
A1	30	0.2	82.9	11.2
A2	30	0.4	86.4	16.2
A3	30	0.6	88.4	20.4
A4	45	0.2	95.1	11.9
A5	45	0.4	97.8	17.2
A6	45	0.6	96.7	20.9
A7	60	0.2	95.4	12.1
A8	60	0.4	96.9	16.9
A9	60	0.6	97.1	21.3

After cooling, their absorbance was measured at 660 nm by using vehicle as blank. Diclofenac sodium at concentration 1000 µg/ml) was used as reference drug and treated similarly for determination of absorbance [29]. The percentage inhibition of protein denaturation was calculated by using the following formula,

% inhibition = absorbance of control - absorbance of test / absorbance of control x 100

***Ex vivo* Studies for Determination of Ocular Irritation**

Ex vivo eye irritation study was done by using hen's egg test on chorioallantoic membrane (HET-CAM) test method. Samples of 9-day-old fertilized eggs from white Leghorn chickens were incubated for 24 h at 37.5°C and 55% relative humidity. The eggshell was opened along the edge of the air chamber, and the egg white film was removed out while avoiding any damage to the fine blood vessels. Three eggs were utilized in pretesting stage for the purpose of the irritation score (IS) calculation of the positive controls, and 3 eggs were used to test the solution formulation.[30-36] For pre-testing stage, 0.1 N NaOH prepared in purified water were used as a positive control. The time needed in seconds for the advent of hemorrhage (H), lysis (L), or coagulation (C) was noted and put up in the below formulae:

1. $IS = (301 - tH) \times 5 + (301 - tL) \times 7 + (301 - tC) \times 9$
2. 300 300 300

For testing of Nepafenac solution, 0.5 mL sample was applied on to the CAM. After 5 min of interaction, the membrane was cleaned with 5 mL of isotonic sodium chloride solution and the score of the reactions was recorded by the following:

0 = No reactions,
1 = Slight/mild,
2 = Moderate, and
3 = Severe.

Table 3: Results of mucoadhesive study

Formulation	Work of adhesion (N.sec)
A5	33.92
Marketed Formulation	44.38

Table 4: Results of *In-vitro* anti-inflammatory activity by protein denaturation

Sample	Concentration	% Inhibition of Concentration
Control sample	-	4.2 ± 0.1
Test Formulation (A5)-Nepafenac Solution	5000µL	66.67 ± 0.8
Marketed Formulation (Nevanac)	5000µL	63.58 ± 1.1

Table 5: *In- vitro* drug release studies of optimized batches of Nepa-CD solutions

Time (h)	% Drug release of Nepa-CD solutions								
	A1	A2	A3	A4	A5	A6	A7	A8	A9
0	0	0	0	0	0	0	0	0	0
1	11.1	10.1	8.1	12.8	12.1	8.1	11.1	9.5	6.9
2	23.7	21.7	19.4	24.7	29.7	19.5	23.7	23.7	18.7
3	38.4	36.1	32.1	40.1	41.4	33.2	38.4	38.4	31.8
4	57.1	55.2	51.2	57.1	59.1	51.2	57.1	60.2	49.7
5	71.1	69.1	64.2	73.1	74.1	71.5	71.1	77.8	66.1
6	82.8	79.2	81.5	90.1	93.2	85.8	91.1 4	92.2	84.2

The result was the highest degree found for any of the 3 reactions in the test samples. [2]

***Ex vivo* Studies for Determination of Scleral Permeation Efficiency**

Fresh goat corneas were gained from a local slaughterhouse, by using sterile scissors and forceps, remove the extracellular tissues and separate the sclera, and then placed on the Franz diffusion cell by placing the scleral tissues between the clamped donor and the accepter chamber. Care was taken to sustain the convex surface shape of the cornea by appropriate arrangement. Phosphate buffered saline (PBS) pH 7.4 was filled in receiver chamber after expelling all the air bubbles by inverting the diffusion cell and then allowing the bubbles to travel through the sampling port.[23] The receiver fluid was monitored at 37 ± 0.5°C with the help of water bath. A 1 mL portion of the test solution was deposited onto the epithelial side of each donor cornea and overlaid with a glass coverslip to forestall moisture loss. The permeation assay ran for 180 minutes, with sampling at 30, 60, 90, 120 and at 180 minutes.[31-35] At each interval, 1 mL was withdrawn from the receptor compartment via the sampling port. Absorbance of each aliquot was determined using a UV spectrophotometer.

***In-vitro* Cell Line Studies- MTT Assay**

Generally *In-vitro* assays on cell lines were performed with two main objectives i.e. To assess the cytotoxic potential of the newly developed formulation and to examine its effect on the integrity of tight junctions in ocular epithelial cells. A major membranous barrier for passive diffusion of drug to the posterior eye through a topical route is the corneal epithelium and retinal pigmental epithelium (RPE) because both possess tight junctions between cells marring

paracellular transport. To mimic these *In-vitro* cell line studies ARPE-19 (adult RPE cell) was employed.[36-37]

An MTT-based assay was used to evaluate the cytotoxic effects of the test sample on ARPE-19 cells, a human retinal pigment epithelial cell line. Cells were plated at a density of 5 × 10^3 per well in a 96-well plate containing high-glucose DMEM (25 mM) supplemented with 10% fetal bovine serum and incubated for 48 hours at 37 °C in a humidified atmosphere with 5% CO_2. The sample formula 1 (A1) was treated using DMEM high glucose media supplemented with 1% FBS for 24 hrs. Later, MTT reagent (5mg/mL) was added and incubation was done for 4 hr under culture conditions. The MTT solution was removed, and 100 µl of isopropanol with 4mM HCL, was added to each well to dissolve the formazan crystals by gentle shaking for 20 minutes, and absorbance was measured at 570 nm. Cell viability (%) was cacuated by using the following formula:
% Cell Viability = [OD (Treated)/ OD (control)] * 100

RESULTS AND DISCUSSION

Solubility Study

Solubility of Nepafenac in screened solubiizers plays an important role in formulation development of solution, since it directly impacts drug loading and stability of solution. In the current research, various oils such as, castor oil, Cremophor RH 40, PEG 400, surfactants/co-surfactants such as, polysorbate 80, Tween 20, Span 20, glycerol and propylene glycol also solubilizers like different cyclodextrins (HP-β-Cyclodextrins, HP- cyclodextrins and sulfobutylether cyclodextrins etc. are generally used for ophthalmic use were screened. The standard of screening of oils, surfactant and solubiizers depends on usage of minimum proportion of oils, surfactant and solubiizers for complete solubilization of Nepafenac. The solubility study was done at temperature (30–35°C), and maximum solubility of Nepafenac was found in HP-β-CDs and sulfobutylether cyclodextrins and 43.2 mM concentration of HP-β-CDs and 3.0 mM concentration of sulfobutylether cyclodextrins and in other screened oils and surfactants solubility found very less and not suitable for formulation of Nepafenac solution. Based on the observation and clarity of the formulation HP-β-Cyclodextrins is selected at 45 mM concentrations.

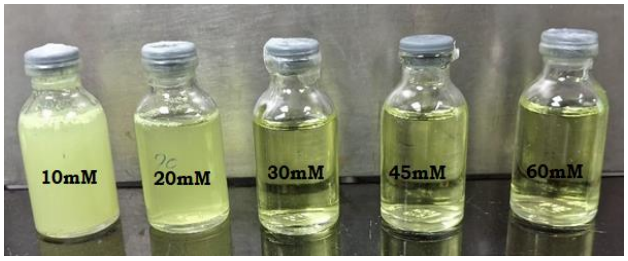


Figure 3: Nepafenac formulation in different concentrations of Hp-B -cyclodextrins

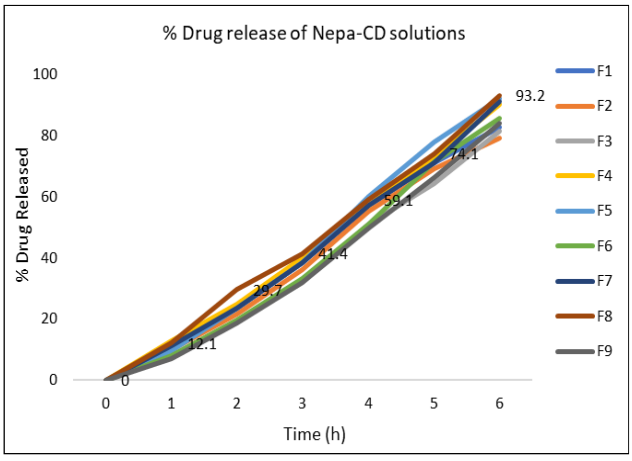


Figure 4: Results of *In-vitro* drug release of Nepa-CD solutions

Table 6: Results of permeation efficiency test for optimized formulation

Formulation	Drug Permeation (mg)	Drug Permeation (%)
Nepafenac solution_A5	891.3 mg	89.13%
Marketed Formulation	714.3 mg	71.43%

Formulation Optimization Studies of Nep-CD Solution

General three level factorial design was created with objective to identify the interaction effect of factors on response. Experiments were designed on range-scaled factor upsides of [−1, 1, +1]. Statistical analysis of experiments mentioned in table 3 performed using regression analysis in design expert software. The experimental design and response inputs were added as displayed in Table 2.

The applied experimental design represents main terms and two-factor interaction terms. The latter refers to two different variables that interact together, creating a joint effect on the response that would not occur if each acted independently. As such, the regression model utilized both the principal effects and the two-way interaction terms. with three level design it was easy to identify any curvature effect and model can be selected accordingly. The experimental data were then fitted to the below polynomial regression.

Regression equation for Drug Content

$Y_1 = 29.60 + 2.492\ A + 7.333\ B - 0.023778A^2$

$= 0.9674$, p value < 0.0001

Where A (HP-β-Cyclodextrin) and B (Polymer concentration)

The corelation coefficient (R^2) the regression model was 0.9674 and a p value is less than 0.05 suggests that the model is significant and could predict Drug Content. This model also validated by plotting Interaction and contour plot for percent transmittance as a function of HP-β-Cyclodextrin and Polymer concentration. Interaction and contour plot was made using the software as shown below (Figure 1).

From the above contour plot the change in concentration of HP-β-Cyclodextrin from 35mM to 60mM, Drug content is increasing linearly from 82 to 97.8%. This change is completely governed by HP-β-Cyclodextrin concentration irrespective of change in concentration of Polymer concentration. From above perturbation (Interaction) and Contour plot, it can be understood that factor A (level of HP-β-cyclodextrin) is impacting significantly (non-linear),

while factor B (level of Polymer concentration) is not impacting significantly on % drug content.

Regression equation for Viscosity

$Y_2 = +6.07222A+0.027778B+22.83333B$

$= 0.993$, p value < 0.0001

The corelation coefficient (R^2) the regression model was 0.993 and a p value is less than 0.05 suggests that the model is significant and could predict viscosity. This model also validated by plotting Interaction and contour plot for viscosity as a function of HP-β-Cyclodextrin and Polymer concentration. Interaction and contour plot was made using the software as shown below (Figure 2).

With the change in concentration of HP-β-Cyclodextrin from 30mM to 45mM and Polymer concentration from 0.2% to 0.6 %, Drug content is increasing linearly from 82 to 97.8%, this change is completely governed by HP-β-Cyclodextrin concentration irrespective of change in concentration of Polymers. However, significant impact on Drug content is seen due to interaction of concentration of HP-β-Cyclodextrin (in range of 30mM-60mM) and

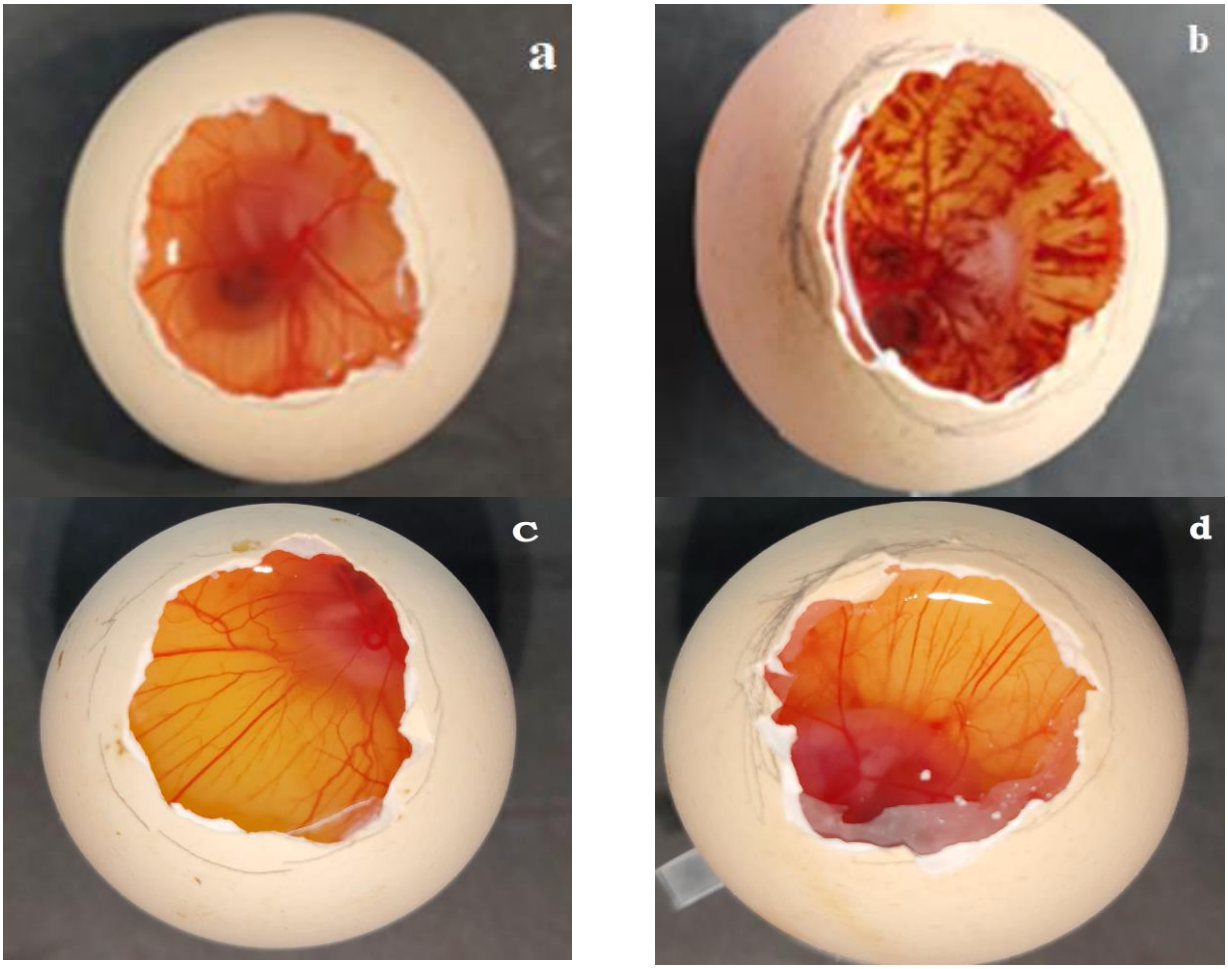
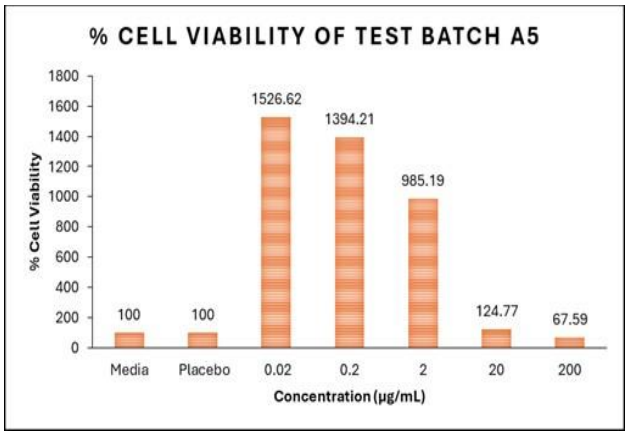


Figure 5: Chorioallantoic membrane (CAM): (a) Normal- 0.9% Sodium chloride; (b) CAM treated with 0.1N NaOH; (c) Treatment of CAM with Nepafenac Solution; (d) Marketed formulation



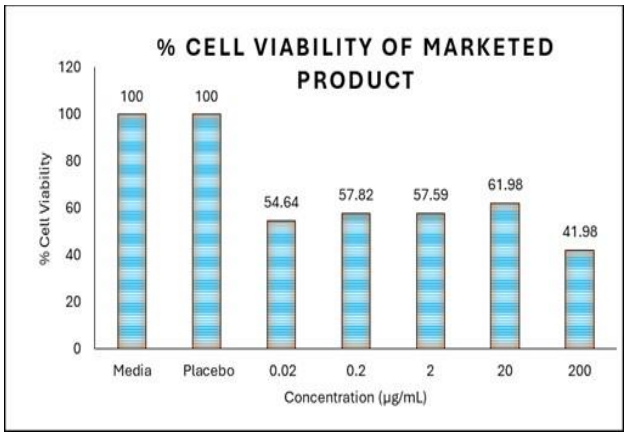


Figure 6 and 7: Graph of cell line study for blank, placebo, Test formulation and marketed formulation

significant impact on Polymer concentrations (in range of 0.2-0.6%) is seen on viscosity of the formulation.

The DOE is was successfully utilised to understand the interactions between the HP-β-Cyclodextrin and Polymer concentration w.r.t drug and thus achieve the optimal formulation with high feasibility. Further, optimized s solution was subjected to characterization such as pH, viscosity, osmolality and *In-vitro* drug release, ex-vivo Ocular Irritation, cell line and ex-vivo scleral permeation. Solution formulation images is shown in (Figure 3).

Osmolality

Osmolality is an important parameter to be monitored in ocular formulations as it causes irritation to cul-de-sac and pH of Nep Solution is adjusted to 7.2 by using pH adjusting agents i.e. sodium hydroxide, osmolality of prepared solution was 307 mOsmol/kg.

Viscosity

High viscosity of formulation leads to an increase in residence time in the cul-de-sac which exhibits an enhanced therapeutic effect. Formulation having viscosity having viscosity around 10 to 24 centipoise (cPs) which can not affect drainage rate. Solution formulation viscosity provides longer residence time which may increase ocular absorption.

Mucoadhesive Study

As it is known that less than 5% bioavailability of the topical administration of ocular eye drop and it is due to rapid elimination due to precorneal factors and reflex mechanism. So to overcome this mucoadhesive polymeric system along with drug strategy has been developed. Polymer and mucin bonds can be used to adhere formulation on the surface of the eye, thus increasing the residence time of the eye drop and prevent the elimination of medicament from the eye. Various techniques are used to study mucoadhesive strength. *In-vitro* tensile test is widely used to determine the mucoadhesion with respect to detachment force needed to separate two layers. Results of mucoadhesive study are presented in below table 3.

***In-vitro* Anti-inflammatory Activity by Protein Denaturation Method**

The anti-inflammatory activity was evaluated by measuring their ability to inhibit heat-induced denaturation of egg albumin. The results of *In-vitro* anti-inflammatory activity of control sample, Test sample and Marketed samples are shown in table 4. No significant difference between the marketed drug and test sample at 500 µg/mL, suggesting comparable efficacy at concentration 500µL.

***In-vitro* Drug Release**

The drug release from optimized Nepa-CD solutions formulation gives an extended drug release profile at all-time points shows almost 90% in 6 h The drug release from solutions was found to be more than 90% in 6 h. The results of *In-vitro* drug release study are summarised in below Table 5 and in Figure 4.

***Ex-vivo* Studies for Determination of Scleral Permeation Efficiency**

The results of the permeation efficacy from the optimized formulation batch were greater as indicated in Table 6. The drug displays significant and time-dependent permeation across scleral tissue. Therefore, the formulated solutions may offer an alternative method for targeting drugs to the back of the eye.

The increased scleral permeability after Nep-CD solution conversance may have inference for enabling delivery of formulation to the posterior segment of the eye.

***Ex-vivo* Studies for Determination of Ocular Irritation**

As per the reported literature, the model is considered as ideal in case IS of 0.1 N NaOH is between 10-21. Since the obtained results for 0.1 N NaOH was 20, the prepared model was ideal, and it can be used for the testing of prepared Nep-CD soution.[33]

The results showed a significant difference between the positive control (0.1N NaOH) and the test formulation (Namo emulsion). Sodium hydroxide inflicted significant damage on the vasculature of the chorioallantoic membrane (CAM). After applying 0.5 mL of 0.1N NaOH solutions, most of the parts of the membrane were affected by haemorrhages. Other visual damage like lysis of veins was furthermore seen. Eventually that leads to the death of the membrane after approximately 5 min of contact with the positive controls. On the other hand, after the application of 0.5 mL of test solutions. There was no haemorrhage, lysis, or coagulation observed and also no death observed even after 1 h of observation (Figure **5**). Under the HET-CAM assay, both the in-house formulations i.e., Nep-CD solution exhibited scores of 0, indicating no vascular irritation. Consequently, both formulations are classified as non-irritant and suitable for ophthalmic application.

***In-vitro* Cell line Study - MTT Assay**

Test solution formulation sample did not show any significant toxicity towards ARPE-19 cells after 24 hrs of incubation at concentration 0.02 µg/mL, 0.2 µg/mL and at 2.0 µg/mL. However, at the rest of the concentrations considered (20 µg/mL and 200 µg/mL), it showed significant toxicity when compared to blank (only media) and placebo (without API). Data is presented in below graph and images of each studied concentration presented in Figure 6 and Figure 7.

From the above results of mtt assay suggest that prepared test formulation was found to be safe without any cytotoxic effects at concentration from 0.02 µg/mL to 2.0 µg/mL. On the contrary, marketed sample showed marginal cytotoxicity between 0.02 and 20 ug/mL concentrations. Also optimized test formulation does not cause cell death or any damage to plasma membrane and are safe and can be well tolerated for further *in vivo* studies with topical instillation into the eye. Therefore, this prepared test formulation is safe for non-invasive ocular administration.

CONCLUSION

This study was first to used combination of hydrophilic polymers to Nepafenac-cyclodextrin complexes to exhibit the solubility of drug and formation cyclodextrin based solution. Prepared formulation can deliver the drug to posterior part of the eye via scleral route to urge inflammation. Optimized solution of nepafenac were found to be non-toxic or biocompatible and non-irritant to eye and it is confirmed by performing HET-CAM assay and *Ex-vivo* cell line study in ARPE-19 cells. Optimized eye drop formulation containing combination of Polymers i.e., PVP

and CMC, showed highest diffusion through cellophane membrane, high scleral permeability and shows higher anti-inflammatory response compared to marketed formulation. This new approach of formulation as a topical, Nove nepafenac-CD based solution provided an alternative to treat ocular inflammation associated at the back of the eye and also offering improved compliance and reduced side-effects relative to existing available medicaments.

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