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FORMULATION DEVELOPMENT AND CHARACTERIZATION OF FLUCINOLONE ACETONIDE NANOEMULSION FOR OCULAR DRUG DELIVERY SYSTEM

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ABSTRACT
Background: The purpose of this study is to enhance and demonstrate the effectiveness of the corticosteroid drug fluocinolone acetonide in the form of an ophthalmic nanoemulsion. This formulation is designed to improve targeted delivery and ocular penetration, making it suitable for the treatment of conditions such as age-related macular degeneration, diabetic macular edema, and posterior uveitis. **Methodology:** To formulate the nanoemulsion, we used polysorbate 20 and a castor oil derivative known as HCO-40. The continuous emulsification method was employed to prepare the formulation. Initial batches were tested for key properties, including pH, osmolality, drug content, globule size, and zeta potential. A factorial design approach was applied, in which polysorbate 20 and the castor oil derivative (Cremophor RH 40) were considered independent variables. The nanoemulsion was further evaluated for ocular irritancy using cell line analysis, in vitro scleral permeability, and the Hen's Egg Chorioallantoic Membrane (HET-CAM) test. **Results and Discussion:** The optimized batch of the nanoemulsion showed a penetration rate exceeding 80% and a small globule size of 19–20 nm. *In vitro* tests using human retinal pigment epithelial (ARPE-19) cells and the HET-CAM test indicated that the formulated nanoemulsion is non-toxic and non-irritating to the eye, confirming its cytocompatibility. **Conclusion:** The developed optimized nanoemulsion formulation of flucinolone acetonide provides improved targeting, non-invasive administration & enhanced patient compliance when used as a topical eye drop for treating ocular diseases such as age-related macular degeneration and posterior uveitis.

INTRODUCTION

The eye is a unique organ due to its anatomy and physiology, making it exceptionally resistant to outside chemicals, such as prescription drugs. The blood-retinal barrier, blinking, and tear production are among its defense systems that help protect it from external chemicals, but they also make it challenging to administer drugs effectively [1,2]. Numerous approaches, including nanoparticles, liposomes, in situ gels, and microneedles, have been thoroughly investigated to enhance ocular drug retention and overcome the limitations of traditional

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delivery methods [3]. These new techniques aim to reduce overall side effects, enhance drug absorption, and prolong its activity in the body. This is particularly important for long-term use of corticosteroids, which is common. Fluocinolone acetonide (FA), an anti-inflammatory corticosteroid, is used to treat posterior uveitis, diabetic macular edema (DME), and age-related macular degeneration [4]. DME, a complication of diabetes, causes fluid accumulation in the retinal region. This fluid buildup damages blood vessels, causes macular swelling, initially distorts vision, and can eventually lead to irreversible blindness. Research indicates that fluocinolone acetonide prevents ocular inflammation caused by edema, blood vessel dilation, fibrin deposition, cell or capillary growth, or collagen deposition [5-7]. The aqueous solubility of fluocinolone acetonide is poor (0.06 mg/mL), only 4% of that of dexamethasone [8,9]. The drug has a partition coefficient of 3.19 (n-octanol/water, log P).

The intravitreal drug Iluvien® (Alimera Sciences Inc., Alpharetta, GA, USA) is a micro-implant containing fluocinolone acetonide [11-13]. This implant is a non-biodegradable intravitreal device designed to release fluocinolone acetonide at an initial rate of 0.25 µg/day, maintaining this rate for 36 months. Iluvien is effective in managing individuals with long-term diabetic macular edema [14]. This implant delivers medication to the back of the eye for up to 36 months, resulting in significant improvement in vision [15]. However, a profound potential side effect of this dosage form is a rise in intraocular pressure, which patients receiving the fluocinolone acetonide implant are more likely to experience [16]. Current therapy involves surgical placement of an intravitreal fluocinolone acetonide implant. This therapy has several disadvantages, such as retinal detachment, redness, pain, and discomfort, all of which can lead to patient non-compliance, as well as increased treatment cost and hospitalization. Previous studies encapsulated FA with different types of cyclodextrins (α-, β-, and hydroxypropyl-β-CD), incorporated it with various lipids, and formulated liposomes. The optimized liposomes exhibited low drug encapsulation efficiency and rapid drug release within only 3 hours. Although intended for the treatment of ocular inflammatory disease, no pharmacological studies were performed [17].

Many systemic side effects are associated with oral corticosteroids, which can limit their use. In some cases, these side effects can be life-threatening [14,15]. Thus, the development of fluocinolone acetonide eye drops is urgently needed. To deliver fluocinolone acetonide in a controlled manner, non-invasive ocular administration methods with minimal side effects are recommended [16–21]. In addition to the well-known benefits of eye drops, properly encapsulated drug colloidal particles can provide longer retention and less frequent administration for ocular use [22]. Therefore, we attempted to develop a novel fluocinolone acetonide-loaded nano-micellar emulsion. A compound made from a castor oil derivative and a surfactant polymer was used to create the nanoemulsion. The developed formulation, being nanosized, may improve stability against dilution by tears in the eye.

MATERIALS AND METHODS

Materials

Maharshi Pharma, Ahmedabad, India, gifted flucinolone acetonide. Polysorbate 20 was obtained from Croda Inc., USA. Polyoxyl 40 Castor Oil was obtained from BASF, India. BKC (50%) and glycerol were procured from Merck KGaA, Germany. Povidone K90 and Sodium CMC were obtained from Ashland, India. All other reagents used were of analytical grade.

Solubility study

Flucinolone acetonide was administered in an excess quantity and added to vials containing 2 mL of each oil, surfactant, and co-surfactant to prepare a supersaturated solution, which was then kept aside for 72 hours in an automatic shaker at 30°C. The supersaturated solution was then filtered using Whatman filter paper to remove the excess Flucinolone acetonide. One milliliter of the filtrate was set aside for further dilutions with ethanol and tested using ultraviolet (UV) spectrophotometry to determine the solubility of Flucinolone acetonide in the respective oils, surfactants, and co-surfactants. Blank readings for each composition were prepared by diluting 1 mL of the respective component with ethanol to eliminate interferences in absorbance during UV-visible spectrophotometric analysis [2].

Formulation development and optimization of nanoemulsion

The nanoemulsion was prepared using the continuous emulsification method, producing a transparent, isotropic, and thermodynamically stable system that forms spontaneously without requiring high energy input. In this method, the oil and aqueous phases are combined in the presence of a high concentration of amphiphilic agents (surfactant alone or in

combination with a cosurfactant), which promotes the dispersion of one phase into the other by significantly reducing interfacial tension. The formulation was prepared under continuous stirring at 300 rpm, with the temperature maintained below 40°C. The oil phase was slowly added to the aqueous phase at a constant rate under uniform stirring for approximately 30 minutes to ensure the formation of a stable and homogenous nanoemulsion. The formulation was composed of the proper proportions of oil, water, surfactant, and co-surfactant, and the volume was made up to 100% using purified water. All dispensing of the API and excipients was performed on a four-digit analytical balance. The final product was filled into semipermeable LDPE containers at room temperature.

Nanoemulsions were optimized using a full factorial design approach with the statistical software Design-Expert (Stat-Ease Inc.) to evaluate the effects of different experimental components on the ophthalmic nanoemulsion. The experimental trials were conducted using two factors: oil concentration (X1) and surfactant concentration (X2). These factors were considered most influential on responses such as globule size (Y1) and light transmittance (Y2). The two factors, oil concentration and surfactant concentration, are presented in Table 1.

Table 1: Factors and levels for oil concentration and S-Mix concentration

Para-meter	Component	Units	Applied Levels		
			Low (-1)	Center (0)	High (+1)
X_1	Oil	%	0.5	1.0	1.5
X_2	Surfactant	%	0.06	0.12	0.18

Characterization of the Optimized Nanoemulsion

Drug content by UV spectroscopy

The content of flucinolone acetonide (FA) API was estimated using a UV spectrophotometric approach for both bulk drug and ophthalmic preparations. The drug content of the optimized nanoemulsions was determined using a clear and reliable UV spectrophotometric method (Mettler Toledo). Methanol was used to dilute flucinolone acetonide [16]. A precisely measured 1 mL of nanoemulsion or 0.25 mg of flucinolone acetonide was added to a 100 mL volumetric flask for analysis of the test formulations. The drug content of the prepared sample solution was determined using a UV spectrophotometer (Varian Cary 60, Agilent, USA) set at 237 nm for flucinolone acetonide. The method was validated for linearity, accuracy, specificity, and robustness. The assay results (%, w/v) were calculated using the simultaneous equation method [23].

Globule size

The Malvern Zetasizer Nano ZS (Malvern Instruments, United Kingdom) was used to determine the globule size of the optimized nanoemulsions. Approximately 1.0 mL of the test sample was mixed with 5 mL of Milli-Q water. The globule size was immediately measured using the resulting mixture.

Osmolality

The Osmomat 3000 equipment (Gonotec GmbH, Germany) was used to measure the osmolality of nanoemulsions using the freezing point depression method. For osmolality measurement, calibration of the osmometer was performed using the 3-point calibration technique with standard calibration solutions (zero point, 300 mOsmol/kg, and 850 mOsmol/kg). After calibration, 50 µL of the test sample was transferred using a micropipette into the measurement cuvette. The sample cuvette was inserted into the osmometer, and the "Start" button was pressed. The osmolality reading was displayed on the osmometer screen [24].

Viscosity

The viscosity of nanoemulsions was measured using a Brookfield viscometer (Model DV Next, Brookfield). The viscosity determination was done using a spindle (S-18) at 25.0 ± 0.5 °C [25].

In vitro drug release study

The in vitro dissolution of the formulated nanoemulsions was assessed using a Franz diffusion cell in in vitro drug release research. As the dissolving medium, freshly prepared simulated tear fluid (STF) (pH 7.4), which mimics the physiological conditions found on the corneal surface of the eyes, was maintained at 34 ± 0.5 °C. The cellulose membrane (MWCO 12,000 Da) used for dialysis was immersed in simulated tear fluid (STF) at pH 7.4. The donor and receptor compartments of the Franz diffusion cell were separated by the pre-soaked dialysis membrane and secured in position.

The prepared formulations (1 mL, equal to 0.25 mg of FA) were applied on the donor side of the membrane. One milliliter of the sample was withdrawn from the acceptor compartment at the following time points: 0.5, 1, 2, 4, 6, 8, 10, and 12 hours. One milliliter of fresh STF (pH 7.4) was added to the acceptor compartment after sampling at each time point. 0.1 M ethanol was used to further dilute the samples to 100 mL, and a UV-Vis spectrophotometer was used to measure flucinolone acetonide at

237 nm. After calculating the cumulative percent drug release at each time point, the percentage was plotted against time (h) [26].

Preservative efficacy study

The preservative efficacy test of BKC was performed using the agar diffusion test. Two general methods are usually employed. One is the cup-plate method (agar well diffusion method). The agar cup-plate method relies on the diffusion of the antibiotic from a vertical agar cylinder (well) through a solidified agar layer on a Petri dish. A measured volume of microbial suspension is spread evenly using sterile agar before solidification. A well with a diameter of 6 mm is punched aseptically using a stainless-steel borer. The antimicrobial solution, prepared at the desired concentration, is then added to the well. Based on the type of microorganism being tested, the agar plates are incubated under appropriate conditions.

The antimicrobial agent spreads through the agar medium, preventing the growth of the microbial strain and forming a clear zone around the cylinder that contains the test substance. The optimized nanoemulsion was added into the well in volumes of 100 µL and 200 µL. Each solution was filled into the wells bored into sterile nutrient agar seeded with test organisms (Escherichia coli and Staphylococcus aureus). The agar plates were incubated at 37 °C for 24 to 48 hours after the solutions had been allowed to diffuse for two hours. Each cylinder's (well) zone of inhibition was compared with the control zone. The complete experiment was conducted in a controlled area, i.e., a laminar airflow unit, except for the incubation step [27].

Ex vivo studies for the determination of ocular irritation

An ex vivo eye irritation study was performed using the hen's egg test on chorioallantoic membrane (HET-CAM) 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 while avoiding any damage to the delicate blood vessels. Three eggs were used in the pretesting stage to calculate the irritation score (IS) of the positive controls, and three eggs were used to test the nanoemulsion formulation [24]. For the pretesting stage, 0.1 N NaOH prepared in purified water was used as a positive control. The time needed in seconds for the advent of hemorrhage (H), lysis (L), or coagulation (C) was noted and applied in the following formula:

$$IS = \frac{(301 - tH) \times 5}{300} + \frac{(301 - tL) \times 7}{300} + \frac{(301 - tC) \times 9}{300}$$

For the nanoemulsion testing, 0.5 mL of the sample was applied onto the CAM. After 5 min of interaction, the membrane was cleaned with 5 mL of isotonic sodium chloride solution, and the reaction scores were recorded as follows:

- 0 = No reaction
- 1 = Slight/mild
- 2 = Moderate
- 3 = Severe

The result was considered the highest degree observed for any of the three reactions in the test samples [2].

Ex vivo studies for the determination of scleral permeation efficiency

Fresh goat corneas were procured from a local slaughterhouse. Using sterile scissors and forceps, the surrounding extracellular tissues were carefully removed to isolate the sclera. The excised scleral tissues were then mounted on a Franz diffusion cell, positioned between the donor and receptor chambers that were clamped. Special care was taken to maintain the natural convex curvature of the tissue, similar to that of the cornea, through proper alignment during mounting. Air bubbles were eliminated by gently inverting the diffusion cell and allowing them to escape through the sampling port. Simulated tear fluid (STF, pH 7.4) was added to the receptor chamber [23], and the receptor medium was maintained at 37 ± 0.5 °C using a water bath. A 1 mL aliquot of the test formulation was placed on the epithelial surface of each donor cornea and covered with a glass coverslip to prevent evaporation. The permeation study was conducted for 120 minutes, with samples collected at 0, 15, 30, 60, 90, and 120 minutes. At each interval, 1 mL of receptor fluid was withdrawn through the sampling port. The absorbance of each collected sample was measured using a UV–visible spectrophotometer to determine the drug permeation profile [32,33]. The permeation study was conducted for 120 minutes, with samples collected at 30, 60, 90, and 120 minutes. At each interval, 1 mL of receptor fluid was withdrawn through the sampling port and replaced with an equal volume of fresh STF to maintain sink conditions. The absorbance of each sample was measured using a UV–visible spectrophotometer to determine drug permeation.The cumulative amount of drug permeated per unit area was plotted against time, and the linear portion of the curve was used to

calculate the steady-state flux (Jss, µg/cm²/h) from the slope of the plot. The permeability coefficient (cm/h) was calculated using the equation:

$$Papp = \frac{Jss}{Cd}$$ ***, Where C_d is donor concentration***

In vitro cell line studies- MTT assay

Generally, in vitro assays on cell lines were performed with two primary objectives: 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 the passive diffusion of drugs to the posterior eye through a topical route is the corneal epithelium and the retinal pigment epithelium (RPE), as both possess tight junctions between cells, which limit paracellular transport. To mimic these in vitro cell line studies, ARPE-19 (adult RPE cells) was employed [31,34]. 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 × 10³ 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 [35]. The sample formula 1 (A1) was treated using DMEM high-glucose media supplemented with 1% FBS for 24 hours. Later, MTT reagent (5 mg/mL) was added, and incubation was carried out for 4 hours under culture conditions. Each well received 100 µL of isopropanol with 4 mM HCl after the MTT solution was removed, to dissolve the formazan crystals by gentle shaking for 20 minutes. The absorbance was then measured at 570 nm. Cell viability (%) was determined using the following formula:

$$\%\ \boldsymbol{Cell\ Viability} = \frac{\boldsymbol{OD\ (Treated)}}{\boldsymbol{OD\ (control)}} \times \mathbf{100}$$

RESULTS AND DISCUSSION

Solubility study

The solubility of flucinolone acetonide in screened oils and surfactants/co-surfactants is crucial for the formulation development of nanoemulsions, as it directly affects drug loading, mean globule size, and stability. In the current research, various oils, such as castor oil and Cremophor RH 40, and surfactants/co-surfactants, including polysorbate 80, Tween 20, Span 20, glycerol, and propylene glycol, were examined for use in eye drop formulations. The minimum amount of oil and surfactant required to solubilize flucinolone acetonide completely is the basis for the screening standard for oils and surfactants. At room temperature (20–25 °C), the solubility investigation was conducted. The maximum solubility of flucinolone acetonide was found in Cremophor RH 40 (16.12 ± 0.6 mg/mL), polysorbate 20 (25.1 ± 0.9 mg/mL), and glycerol (3.3 ± 0.10 mg/mL), as shown in Table 2.

Table 2: Solubility results of Flucinolone acetonide API in different oils, surfactants, and Co-surfactants

Excipients	Solubility (mg/mL)
Oils	
Cremophor RH 40	16.12 ± 0.6
Castor Oil	4.9 ± 1.10
Olive Oil	11.2 ± 0.7
Surfactants	
Polysorbate 20	25.10 ± 0.9
Polysorbate 80	33.1 ± 2.10
Polyethylene glycol 400	28.0 ± 2.13
Co-surfactant	
Glycerol	3.3 ± 0.10
Propylene glycol	1.9 ± 0.20

Formulation optimization studies of nanoemulsion

A general three-level factorial design was created to identify the interaction effect of the factors on the response. Experiments were designed on range-scaled factor levels of [−1, 0, +1]. Statistical analysis of the experiments, as listed in Table 3, was conducted using regression analysis in Design-Expert software. The experimental design and response inputs are displayed in Table 3. The applied experimental design represents the main terms and the two-factor interaction terms. The latter refers to two variables that interact with each other, 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 a three-level design, it was easy to identify any curvature effect, allowing for the selection of an appropriate model. The experimental data were then fitted to the following polynomial regression equations.

Regression equation for light transmittance

$$Y1 = 70.80 + 42.33X1 + 106.94X2 - 40.00X1X2 - 16.40X12 - 430.56X22 = 0.998, p\ value < 0.0001$$

Where X_1 (Cremophor RH 40) and X_2 (Polysorbate 20)

The correlation coefficient (R^2) of the regression model was 0.998. A p-value less than 0.05 indicates that the model is statistically significant and has predictive ability. The model was validated by interaction and contour plots of % transmittance versus Cremophor RH 40 and Polysorbate 20. An interaction & contour plot was made using the software, as shown in Figure 1.

Table 3: Outline of the experimental design and results

Nanoemulsion coding	Independent variables		Measured dependent variables*	
	Cremophor RH 40(X_1)	Polysorbate 20(X_2)	Light transmittance(Y_1)	Globule size(nm)(Y_2)
F1	0.5	0.06	91.4	117.0
F2	0.5	0.12	92.3	121.5
F3	0.5	0.18	89.5	137.4
F4	1.0	0.06	99.4	19.9
F5	1.0	0.12	98.3	20.1
F6	1.0	0.18	94.9	28.5
F7	1.5	0.06	98.6	37.5
F8	1.5	0.12	96.9	57.5
F9	1.5	0.18	91.9	63.8

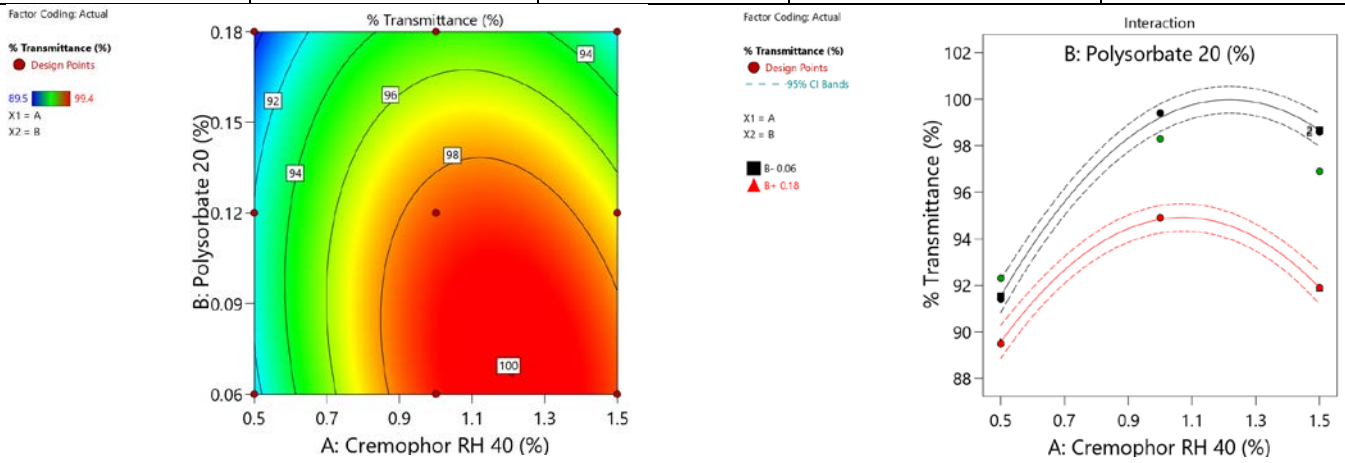

Figure 1: Contour plot & Interaction plot of % transmittance vs Surfactant concentration (%) & oil concentration (%)

As shown in the contour and interaction plots (Figure 1), increasing Cremophor RH 40 (X1) from 0.5% to ~1.0% led to a linear increase in % transmittance from approximately 91% to 99%, suggesting improved emulsification and reduced light scattering due to smaller and more uniform globules. Beyond 1.0% Cremophor RH 40, the effect plateaued, indicating that higher surfactant levels do not further improve clarity and may slightly reduce transmittance due to increased viscosity or micelle formation. Polysorbate 20 (X2) had a moderate positive effect on % transmittance, especially when interacting with Cremophor RH 40 in the range of 0.06–0.12%. The interaction plots indicate that optimal clarity is achieved at a combination of Cremophor RH 40 at ~1.0% and Polysorbate 20 at ~0.12%, corresponding to the formulation with the minimal globule size and maximum transparency. For % Transmittance, the factorial design effectively demonstrated that both main effects and interactions of oil and surfactant concentrations significantly influence the optical clarity of the nanoemulsion. Optimizing these factors ensures a transparent and homogeneous ophthalmic nanoemulsion, suitable for ocular administration.

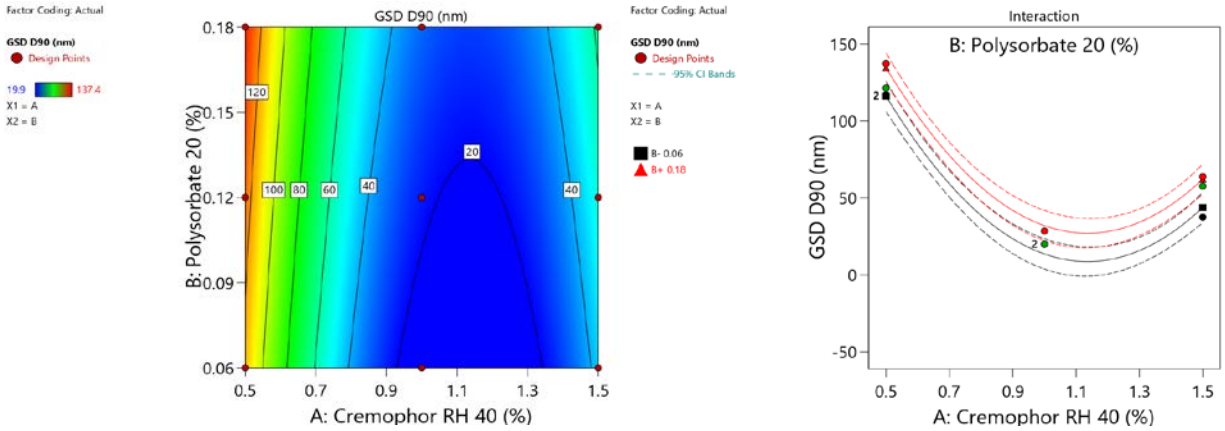

Figure 2: Contour plot & Interaction plot of globule size distribution (nm) vs Surfactant concentration (%) & oil concentration (%)

As observed in the contour and interaction plots (Figure 2), increasing Cremophor RH 40 (X1) from 0.5% to ~1.1% led to a decrease in globule size, reaching a minimum due to more effective emulsification and droplet stabilization. Above 1.1% concentration of Cremophor RH 40, further increases caused a slight increase in globule size, indicating a parabolic relationship likely due to micelle aggregation or viscosity effects that limit droplet breakup. Polysorbate 20 (X2) has a moderate impact on globule size, particularly when interacting with Cremophor RH 40. The plots show that the smallest globule size is achieved at Cremophor RH 40 concentrations of ~1.0–1.1% and Polysorbate 20 concentrations of ~0.12%, confirming the importance of the optimal surfactant-to-oil ratio. For globule size distribution, the factorial design demonstrated that both main effects and interactions of oil and surfactant significantly influence the globule size. Proper optimization ensures the formation of a stable, uniform nanoemulsion with minimal droplet size, enhancing ocular delivery and therapeutic performance.

The DOE was successfully utilized to understand the interactions between oil and surfactant concentration and the drug, thereby achieving an optimal formulation with high feasibility. Furthermore, the optimized nanoemulsion was subjected to characterization, including pH, viscosity, osmolality, in vitro drug release, ex vivo ocular irritation, cell line, and ex vivo scleral permeation.

Globule size distribution

The nanoemulsion size was determined using dynamic light scattering (DLS). The optimized nanoemulsion (F5) size was found to be around 20 nm with a narrow distribution. Such a small size allows it to effectively travel across all ocular tissues and easily permeate scleral pores. This property of the nanoemulsion may facilitate the delivery of the formulation to the back of the eye through the scleral route. The globule size distribution of the nanoemulsion is presented in Figure 3 below.

Osmolality

Osmolality is an important parameter to monitor in ocular formulations, as it can cause irritation to the cul-de-sac. The pH of the nanoemulsion was adjusted to 6.8 using a combination of sodium phosphate buffers, and the osmolality of the prepared nanoemulsion was 304 mOsmol/kg.

Viscosity

The viscosity of the formulation contributes to increased residence time in the conjunctival cul-de-sac, thereby enhancing therapeutic efficacy. The nanoemulsion exhibited a viscosity of approximately 32.3 cPs, which is within the acceptable range and does not significantly affect the ocular drainage rate. The inclusion of the mucoadhesive polymer (Povidone K90) further aids in prolonging the ocular residence time, thereby potentially improving drug retention and therapeutic performance.

In vitro drug release

The results of the in vitro drug release study are shown in Table 5. The in vitro drug release is extended medication release profile at all times is provided by the improved nanoemulsion formulation, with about 97.89% drug release in 12 hours. The in vitro drug release study demonstrated that the prepared nano-emulsion exhibited a controlled and sustained release profile of the drug over the study period. The graphical representation of the final optimized nanoemulsion is presented in Figure 4 below**.** The results below indicate that the optimized nanoemulsion can serve as an effective carrier for ocular drug delivery. Data on in vitro drug release is presented in Table 4 below.

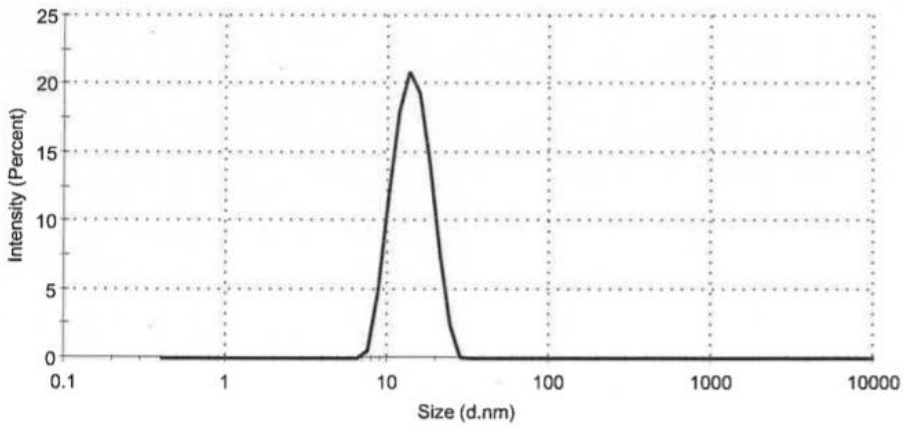

Figure 3: Globule size distribution of Optimized Nanoemulsion (F5)

Table 4: Results of In vitro drug release optimized batches

Time (hrs)	% Drug release of Nanoemulsion								
	F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**
2	14.32	19.65	12.35	18.65	14.65	24.32	25.36	14.65	20.6
4	33.25	36.65	38.95	34.56	39.65	40.35	30.32	37.65	31.65
6	49.56	58.65	50.65	55.62	51.65	64.56	48.65	49.62	45.35
8	64.65	66.52	64.62	69.25	69.01	75.65	64.23	68.56	64.65
10	72.65	81.26	79.65	79.65	81.56	84.65	75.62	84.65	77.65
12	83.9	86.9	94.78	93.89	97.89	96.47	93.78	88.76	84.89

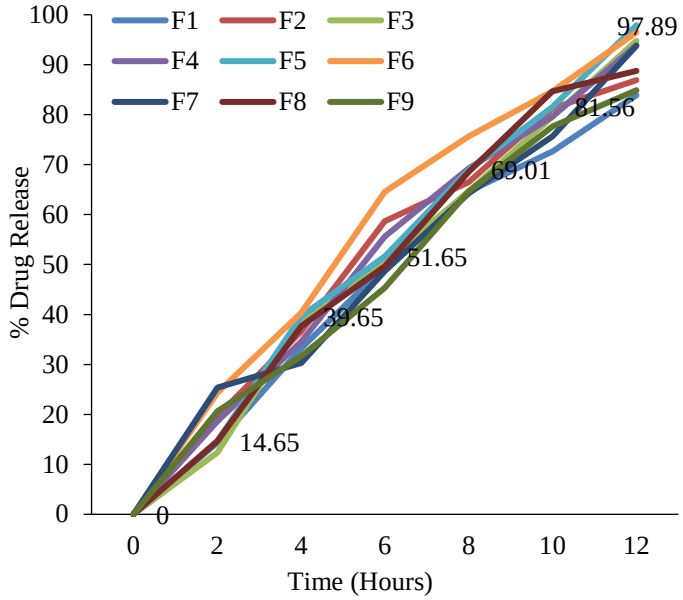

Figure 4: *In vitro* drug release of optimized nanoemulsion

Preservative efficacy study

In the present study, the optimized FA Nanoemulsion (F5) was developed as a multi-dose ocular formulation designed to remain sterile throughout its shelf life. The agar well diffusion method was used to evaluate the antimicrobial activity of the test sample (F5) against *Staphylococcus aureus* (a Gram-positive bacterium) and *Escherichia coli* (a Gram-negative bacterium). A control sample consisting of BKC solution (0.005% w/v, 100 µL) served as the standard, exhibiting significant antibacterial activity with inhibition zones of 10mm against *S. aureus* & 8mm against *E. coli*. The test nanoemulsion (F5) demonstrated measurable antibacterial potential, with inhibition zones of 8 mm (S. aureus) and 4 mm (E. coli) at a concentration of 200 µL, indicating a dose-dependent increase in activity. Although the antibacterial activity of the test formulation was lower than that of the standard BKC solution, it showed notable efficacy, particularly against *S. aureus*. The nanoemulsion was formulated with a reduced preservative Concentration(0.002% w/v BKC) to minimize the risk of ocular irritation commonly observed at higher preservative levels, while still providing adequate antimicrobial protection. According to USP <51> Category 1 (applicable to ophthalmic formulations), products should exhibit not less than a 1.0 log reduction in bacterial count within 14 days & no increase at 28 days. The observed antimicrobial performance of the FA Nanoemulsion (F5) indicates partial compliance with these criteria, confirming that the formulation possesses inherent antimicrobial potential along with preservative support. Therefore, it can be concluded that the optimized FA Nanoemulsion (F5), containing a reduced BKC concentration (0.002% w/v), provides adequate preservative efficacy while ensuring ocular safety and patient comfort. When maintained under sterile conditions, the formulation is expected to comply with USP <51> requirements for Type I (Ophthalmic) preparations, thereby ensuring microbial stability, sterility & extended shelf-life during multi-dose use. Images of the preservative efficacy test are presented in Figure 5, and the data are represented in Table 5.

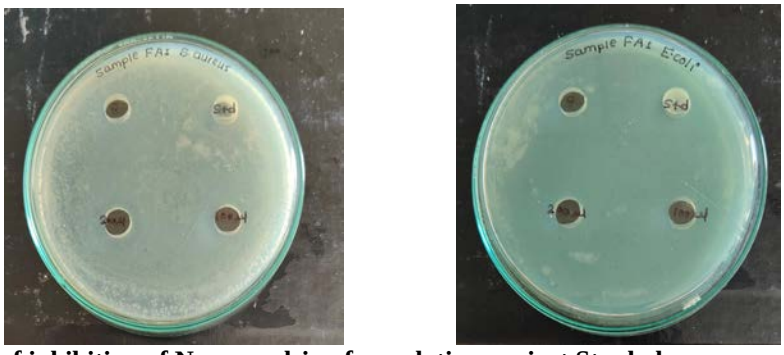

Figure 5: Zone of inhibition of Nanoemulsion formulation against Staphylococcus aureus and *E. Coli*

Table 5: Results of preservative efficacy test of optimized batches Vs the Standard sample of BKC solution (0.005%w/v)

Batch No.	Concentration	Zone of Inhibition *S. aureus* (mm)	Zone of Inhibition *E. Coli* (mm)
Standard sample	100 µl	10	8
FA Nanoemulsion (F5	100 µl	04	02
	200 µl	08	04

Ex vivo studies for determination of permeation efficiency

The optimized nanoemulsion (F5) formulation demonstrated higher permeation efficacy, as shown in Figure 6. The drug exhibited significant, time-dependent permeation through the scleral tissue, suggesting that this formulation could serve as a potential alternative for targeted delivery to the posterior segment of the eye. Data is presented in Table 6. The cumulative drug permeation through goat sclera increased progressively over 120 min, showing a nearly linear region between 30–120 min, indicative of steady-state diffusion The nanoemulsion exhibited a steady-state flux (Jss) of 8.71 µg/cm²/h and permeability coefficient (permeability coefficient (0.0871 cm/h) indicate a promising trans-scleral delivery profile) indicate a promising trans-scleral delivery profile and passive diffusion across the sclera. The increased scleral permeability after Nanoemulsion administration may have implications for enabling delivery of the formulation to the posterior segment of the eye.

Ex vivo studies for the determination of ocular irritation

According to the reported literature, the model is considered ideal when the IS of 0.1 N NaOH is between 10^ (–21) and 10^ (–21). Since the obtained result for 0.1 N NaOH was 20, the prepared model was ideal and can be used for testing the prepared nanoemulsion [30]. The results showed a significant difference between the positive control (0.1 N NaOH) and the test formulation (nanoemulsion). Sodium hydroxide inflicted significant damage on the vasculature of the chorioallantoic membrane (CAM). After applying 0.5 mL of 0.1 N NaOH solution, most parts of the membrane were affected by haemorrhages. Other visual damage, such as lysis of veins, was also observed, eventually leading to the death of the membrane after approximately 5 minutes of contact with the positive control. On the other hand, after the application of 0.5 mL of the test solution, there was no haemorrhage, lysis, or coagulation observed, and no death occurred even after 1 hour of observation, as shown in Figure 6. Under the HET-CAM assay, both the in-house formulations, i.e., nanoemulsion, exhibited scores of 0, indicating no vascular irritation. Consequently, both formulations are classified as non-irritant and suitable for ophthalmic application. Images of CAM after treatment are presented in Figure 7.

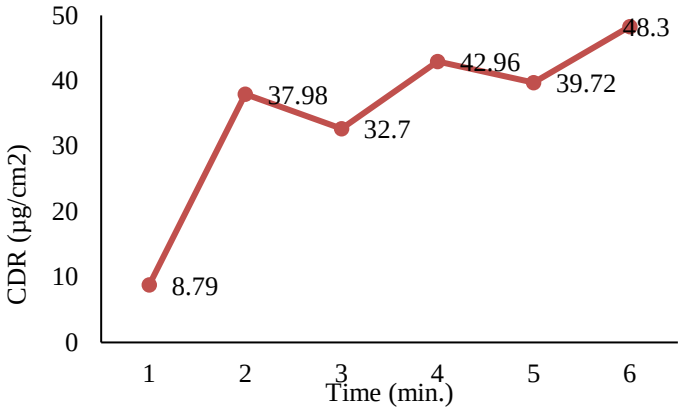

Figure 6: Graph of Cumulative drug permeated (µg/cm2) Vs Time for drug permeation across sclera (mean ±SD, n=3)

Table 6: Results of permeation efficiency test for optimized nanoemulsion formulation

Batch No.	Drug Permeation(mg)	Drug Permeation(%)
F5	0.193 mg	85.5%

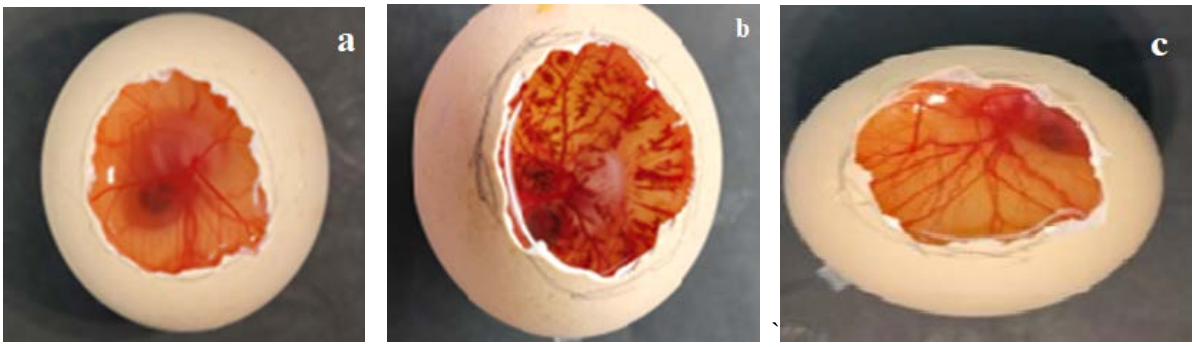

Figure 7: Chorioallantoic membrane (CAM): (a) Normal- 0.9% Sodium chloride, (b) CAM treated with 0.1N NaOH, (c) Treatment of CAM with FA nanoemulsion

Cell line Study - MTT assay

The nano-micellar formulation sample did not show any significant toxicity towards ARPE-19 cells after 24 hours of incubation at concentrations of 0.02 µg/mL and 0.2 µg/mL. However, at the remaining concentrations considered (2 µg/mL, 20 µg/mL, and 200 µg/mL), it showed significant toxicity when compared to the blank (only media) and placebo (i.e., nanoemulsion without the active pharmaceutical excipient). Data are presented in the graph below, and images of each studied concentration are presented in Figures 8 and 9.

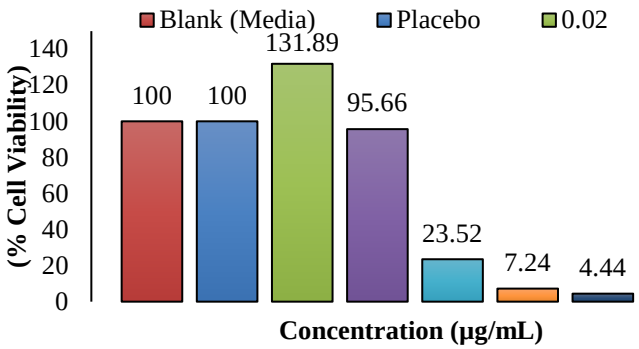

Figure 8: Graph of cell line study for blank, placebo, nanoemulsion concentration vs % cell viability

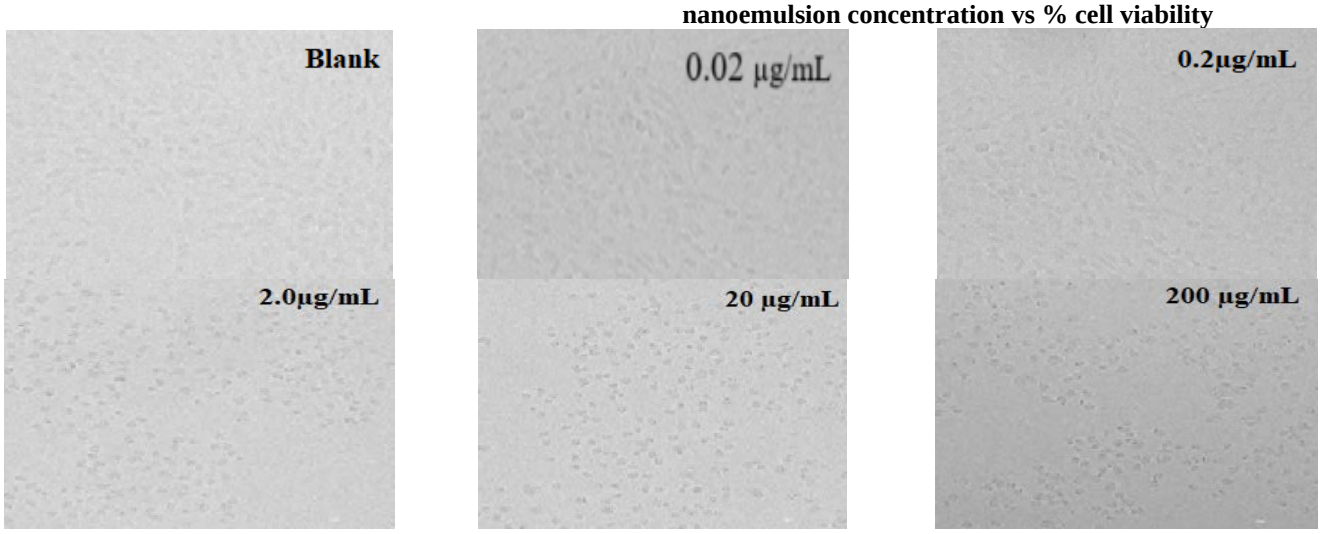

Figure 9: Images of cell viability at different concentrations

Based on the results of the MTT assay, it is suggested that the prepared nanoemulsion is safe, with no cytotoxic effects observed at concentrations up to 0.2 µg/mL. The optimized nanoemulsion also did not cause cell death or damage to the plasma membrane, and is safe and well-tolerated for further in vivo studies, including topical instillation into the eye. Therefore, the prepared nanoemulsion is safe for non-invasive ocular administration.

CONCLUSION

Formulating an ophthalmic nanoemulsion for fluocinolone acetonide is a pressing need, as the drug would be in a solubilized form in either the aqueous phase or the oil phase, allowing for an extended precorneal residence period and a reduction in dosage to once or twice a day. We employed spontaneous emulsification with Cremophor RH 40, polysorbate 20, and glycerol to produce a series of nanoemulsions. The optimized systems exhibited narrow globule-size distributions, remained physically stable, and enabled sustained drug release over an extended period. Ex vivo irritation testing confirmed that this nanoemulsion is non-irritating and safe for ocular application. In vitro scleral diffusion assays further indicated enhanced transscleral delivery. Additionally, cytotoxicity evaluation in ARPE-19 cells revealed excellent biocompatibility. Together, these findings highlight the promise of fluocinolone acetonide nanoemulsions as a non-invasive, patient-friendly therapy for posterior eye disorders such as posterior uveitis and age-related macular degeneration, offering improved compliance and reduced side effects compared to existing regimens.

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NIL

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

Chetan Amrutkar led the laboratory investigations, meticulously recorded experimental observations, and played a central role in drafting the manuscript and executing the research work. S. B. Patil critically reviewed the manuscript and provided insightful suggestions to enhance its technical precision and grammatical clarity.

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