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Development and Characterization of Fluocinolone Acetonide Loaded Nanosized Lipid Carriers for Ocular Delivery via Subconjunctival Route: Synergistic Effect of Vitamin a Palmitate

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DEVELOPMENT AND CHARACTERIZATION OF FLUOCINOLONE ACETONIDE LOADED NANOSIZED LIPID CARRIERS FOR OCULAR DELIVERY VIA SUB-CONJUNCTIVAL ROUTE: SYNERGISTIC EFFECT OF VITAMIN A PALMITATE

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ABSTRACT

Though nanotechnology which exploits the use of nanoparticles for therapeutic application have embarked as an emerging tool for drug delivery applications, the delivery of therapeutic molecules into deep ocular compartments has always been a challenging task. In this regard, the main objective of our current investigation was to develop and evaluate the fluocinolone acetonide (FA) loaded nanostructure lipid carrier (FA-NLC) *in situ* gel for improved ocular bioavailability by subconjunctival injection. FA, a corticosteroid administered for inhibiting the ocular inflammation either as intravitreal injection or as intravitreal implants. The FA-NLCs were developed by using vitamin A palmitate as one of the lipid components, to get an additional advantage in dry eye reduction which is caused by vitamin A deficiency. FA-NLCs were evaluated for physicochemical characteristics (size, polydispersity index and charge), release and stability studies. Further, *in situ* gel of FA-NLC was developed using gellan gum and hydroxypropyl methylcellulose as gelling agents. *In situ* gels were characterized for rheological behavior, *in vitro* release and permeability studies using corneal and scleral tissues. Furthermore, the ocular pharmacokinetic studies of *in situ* gel were conducted in New Zealand albino rabbits after subconjunctival injection, and compared with control formulation. Differential scanning calorimetry and Fourier-Transform Infrared Spectroscopy (FTIR) studies revealed no interaction between the drug and lipid phase and also reduction of the FA crystallinity in the NLC. FA-NLC (FN2b) showed 159.1 ± 3.8 nm of size, 0.150 ± 0.05 of polydispersity index, -8.4 ± 0.4 of surface charge, 96.53 ± 2.4 of assay and 95.43 ± 2.89 % as entrapment efficiency, respectively. *In situ* gel prepared with gellan gum showed excellent rheological properties, sustained drug release and significant permeability compared with control formulation. FA-NLC and *in situ* gel was found to be stable for 30 days at room

and 4^{o}C temperature. The *in vivo* studies revealed high drug concentration in the posterior section of the eye owing to high bioavailability of the drug to ocular tissues.

LIST OF ABBREVIATIONS AND SYMBOLS

ACN	Acetonitrile
AH	Aqueous humor
BSS	Balanced salt solution
DME	Diabetic macular edema
DPBS	Dulbecco's phosphate buffer saline
EE	Entrapment Efficiency
FA	Fluocinolone acetonide
HPMC	Hydroxypropyl methylcellulose
IC	Iris-ciliary
IPBS	Isotonic phosphate buffer saline
NLCs	Nanosized lipid carriers
PDI	Polydispersity index
RC	Retinal choroid
THF	Tetrahydrofuran
VH	Vitreous humor
ZP	Zeta potential

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CHAPTER I

INTRODUCTION

The eye consists of complex anatomical and physiological barriers thereby making it a complicated organ for the delivery of therapeutic molecules across the ocular compartments. On this regard, lipid nanocarriers due to their diverse physical and structural properties help in the delivery of therapeutics across several ocular compartments. The complex ocular microenvironment makes eye resistant to various extraneous entities.[1] Cornea which forms the outermost part of the eye functions as one of the primary obstacles for drug permeability into ocular milieu. Apart from the physiological structure and reduced permeability, poor pre-corneal volume, and residence time contribute to the diminished bioavailability (less than 5%) of the ocular therapeutics.[2]–[4]

Topical delivery is the most accessed methods for the treatment of ocular diseases and nanotechnology, which exploits the use of nanoparticles has shown a great promise in transporting molecules to various tissue compartments. Delivering drug molecules via nanoparticulate systems like liposomes, lipid nanoparticles and nanoemulsions in the form of eye drops, solution or suspension, gels, lotions or ointments are the most opted ways to administer both hydrophilic and lipophilic drugs.[3], [5]–[8] Lipid nanoparticles are drug nanospheres that are shelled in biodegradable lipids. With the size ranging from 40 to 1000 nanometers, lipid nanoparticles demonstrate a promising vehicle in drug permeability into the ocular tissues. Along with the nanoparticulate system, inclusion of permeability enhancer,

viscosity enhancer, or *in situ* gelling agent are considered to be the prime approach to subdue the low precorneal residence time.

Vitamin A is a fat-soluble retinoid, plays a key role in the vision. An aldehyde derivative of vitamin A is the precursor for rhodopsin, a glycoprotein pigment component of rod cells presents in the retina. Deficiency in vitamin A causes ocular disorders such as dry eyes and keratoconjunctivitis and investigations have shown that 0.05% of vitamin A palmitate in the form of eye drops are efficacious for the treatment of dry eye disorder and this was the motivation behind the use of Vitamin A palmitate as one of the lipids to formulate our NLC system.[9]–[11]

In situ gels are viscous fluids which forms pliant translucent gels by the influence of body pH, temperature, the presence of electrolytes or by photolytic interactions. As the formulation gels only after its application, *in situ* gel provide enhanced drug delivery similar to ointments or creams.[5], [12], [13] Gellan gum an anionic polysaccharide obtained from Pseudomonas elodea, transforms from solution to gel in the presence of cations. Tear which comprises of cations like sodium and potassium, facilitates the formation of double helix cross-linking in gellan gum, thus increasing viscosity to form the gel.[13], [14] Hydroxypropyl methylcellulose (HPMC) is a thermos-sensitive nonionic polysaccharide, that forms a transparent viscous gel in contact with the tear fluid. The gelling of the polymer is due to the formation of the hydrogen bonding with mucin. Therefore, addition of HPMC to the formulation not only provides bio-adhesion but also increases the viscosity of the formulation.[13], [15], [16] However, due to limitations of topical formulations like tear turnover, constant drug dilation and impediment endowed by the corneas results in the sub therapeutic ocular drug levels.

Subconjunctival injections are introduced into the conjunctival layer surpassing the lipid bulbar layer which hinders the drug penetration via the corneal route. After injecting, drug penetration follows the trans scleral route, thus releasing a higher amount of the drug to the posterior section of the eye. Due to depot formation in the conjunctival layer, this route delivers a greater edge for the drug concentration in the ocular tissues compared to topical formulations. Thus, subconjunctival injection extend to provide advantage over local drug delivery with limited intricacy compared to intravitreal administration.[1], [3]

Diabetic macular edema (DME) which is one of the adverse effects of diabetes, induces the expansion of the fluid in the retinal section. This fluid buildup causes damage to the blood vessels and causes the macula to swell which initially deforms the vision and can eventually lead to irreversible blindness. Studies have shown the use fluocinolone acetonide (FA), a corticosteroid for the inhibiting the ocular inflammation caused by edema, blood vessel dilation, fibrin disposition, cell or capillary proliferation or by collagen accumulation. Currently, FA is administered as the intravitreal injection or as intravitreal implants for the treatment of such inflammations.[17]–[19]

Hence, in the current study we attempted to develop a novel fluocinolone acetonide loaded nanostructured lipid carriers (FA-NLC) *in situ* gels using vitamin A palmitate. The physicochemical characteristics like particle size, charge, polydispersity index, assay and entrapment were studied. Furthermore, the gelling characteristics like viscosity with and without inclusion of simulated tear fluid, gelling time and gel intact time, firmness, and work of adhesion were investigated for *in situ* gel formulation. The optimized formulation was evaluated for the drug release, *in vitro* tissue permeation, tear kinetics and drug content in the ocular tissues by administering the *in situ* gel formulation via subconjunctival route in the New Zealand male albino rabbits compared with control formulation.

CHAPTER II
METHODOLOGY

Materials

Fluocinolone acetonide, vitamin A palmitate, sesame oil, capmul PG-8, tween 80, and hydroxypropyl methylcellulose (HPMC) were acquired from Fischer Scientific (Hampton, NH, USA). Compritol 888 ATO (glyceryl behenate) was procured from Gattefossé (Paramus, NJ, USA). Gellan gum was purchased from MP Biomedicals, LLC (Santa Ana, CA, USA). Other entities required for the study like amicon ultra centrifugal filters (lined with 100 kDa cellulose membrane), high-performance liquid chromatography (HPLC) grade, and liquid chromatography mass spectroscopy (LCMS) optima grade solvents, and other analytical grade chemicals were obtained from Fisher Scientific (Hampton, NH, USA).

Animal and animal tissues

Complete eye globules of male albino New Zealand rabbits were acquired from Pel-Freez Biologicals (Rogers, AR, USA). Male albino New Zealand rabbits, weighing 2 to 3 kg were procured from Envigo (Indianapolis, IN, USA). All the *in vivo* studies were carried out in accordance with the University of Mississippi Institutional Animal Care and Use committee approved protocols (Protocol # 17-018).

Preparation of FA-NLC and *in situ* gels

Selection of lipids

Lipids were screened on the basis of the relative drug affinity towards them. About 5 mg of drug was added to 10 mL of water in glass vials. To these approx., 1 g of lipid was added, and the vials were kept in shaking water bath at 75ºC for 24 h. The solubility of the drug was later measured by precipitating out the lipid in methanol, followed by centrifugation for 15 minutes (AccuSpin 17R centrifuge, Fisher Scientific, USA) at 13,000 rpm and analyzing the drug in methanol using HPLC method.[20]

Selection of surfactants

Drug solubility was carried out in the categories of surfactants based on their HLB values. For this purpose, about 5mg of FA was added to 10 mL of different 5% surfactant solutions and was kept at 34ºC with continuous shaking. Later the samples were centrifuged with AccuSpin 17R centrifuge (Fisher Scientific, USA) at 13,000 rpm for 15 min and the supernatant was diluted with methanol and analyzed using HPLC method. The surfactant screening was performed on the basis of the solubility of the drug on various surfactants.[20]

Preparation of FA-NLC

Hot melt homogenization coupled with probe sonication technique was utilized to prepare FA-NLCs.[21] The proportion of the drug and all the excipients for different trials were provided in Table 1. The quantity of lipids and surfactants for the formulation were determined based on different ratios taking one factor at a time. The lipid phase which constitutes of Compritol 888 ATO and vitamin A palmitate as solid lipids and sesame oil as liquid lipid, were melted together at 80oC. FA was added to this liquefied lipid with steady

magnetic stirring until a homogenous mixture was obtained. Later, capmul PG-8 was added to this homogenous mixture. In a separate vial, aqueous phase comprising of deionized water and tween 80 was heated to 80^{o}C. On attaining the constant temperature, the aqueous phase was added to the apparent lipid with constant stirring to get pre-emulsion. This mixture was homogenized at high speed with T25 digital Ultra-Turrax for 5 min at 12000 rpm. The homogenized mixture was then imposed to probe sonicator for 5 min at 40% amplitude with 15 sec pulse and 15 sec resting time to get desired nanoparticles. The pH of the formulations was analyzed using pH meter (Mettler Toledo, USA).

Table 1: Composition of fluocinolone acetonide loaded nanostructure lipid carriers

Formulation	Lipid : Surfactant	Solid : Liquid Lipid	Compritol (mg)	Retinol Palmitate (mg)	Sesame Oil (mg)	Capmul PG 8 (mg)	Tween 80 (mg)
FN1	1 to 1	70 to 30	63	7	30	33	66
FN2	1 to 1	80 to 20	72	8	20	33	66
FN3	1 to 1	90 to 10	81	9	10	33	66
FN4	2 to 1	70 to 30	126	14	60	33	66
FN5	2 to 1	80 to 20	144	16	40	33	66
FN6	2 to 1	90 to 10	162	18	20	33	66
FN7	2 to 1.5	70 to 30	126	14	60	50	100
FN8	2 to 1.5	80 to 20	144	16	40	50	100
FN9	2 to 1.5	90 to 10	162	18	20	50	100

F and N indicate fluocinolone acetonide and NLC respectively. Each formulation contains 10 mg of drug and were prepared for 10 mL.

Fluocinolone acetonide *in situ* gels were prepared in the similar fashion as specified above (using optimized FA-NLC formulation). The gelling agents, gellan gum, and HPMC were added to aqueous phase and homogenizing the two phases.

HPLC Chromatographic conditions

The drug content in the samples was analyzed by means of High-Performance Liquid Chromatography (HPLC) using Waters 717 plus autosampler, 600 Waters controller pump, Waters 2487 ultraviolet (UV) dual absorbance detector and Agilent 3395 integrator. The column used for analysis was C18 Luna ® 4.6mm x 250 mm and mobile phase comprised of acetonitrile (ACN), water and tetrahydrofuran (THF) in the ratio of 77:13:10, respectively. The detection wavelength (λ_{max}) was set at 238 nm with the flow rate of mobile phase at 0.8 mL/min. All the samples were analyzed in triplicates for each study.[22]

Characterization of NLC

Measurement of particle size, polydispersity index (PDI) and zeta potential (ZP)

All the prepared NLC formulations were evaluated for particle size, zeta potential (ZP) and PDI in triplicate, using a Zetasizer Nano ZS Zen3600 (Malvern Instruments, Inc., MA, USA) by photon correlation spectroscopy at 25°C and with 173° backscatter detection in clear, disposable folded capillary cells. The results for the particle size and PDI were acquired based on volume displacement at 633 nm using a helium-neon laser. The samples were diluted with doubled distilled 0.2µ filtered water in the ratio of 1:500.

Assay and entrapment efficiency

The assay of the FA-NLC and *in situ* formulation was quantified by lipid precipitation method. About 100 µL of the formulation was added to 900 µL of mobile phase (77:13:10 of ACN: Water: THF) and vortexed to precipitate out the lipid. Later, the samples were centrifuged for 15 min at 13,000 rpm and the supernatant was analyzed for the drug content using HPLC method.

Entrapment efficiency (EE) of the FA-NLC formulations was determined by analyzing the free drug in the filtrate collected after centrifuging the formulation through amicon ultra centrifugal filters for 15 min at 5000 rpm. Drug analysis was done by HPLC method as discussed earlier. The entrapment efficiency (%) of FA was calculated using the equation mentioned below.

$$\%\boldsymbol{EE} = \frac{(\boldsymbol{Wf} - \boldsymbol{Wi}) * \mathbf{100}}{\boldsymbol{Wi}}$$

Where, W_i is the theoretical weight of the drug in formulation and

W_f is the total free drug in the filtrate.

Differential scanning calorimetry (DSC)

The purity and interaction of the drug with other lipid components was determined by subjecting to DSC analysis. DSC of pure FA and the lipid phase of the formulation including drug were obtained by DSC 250 (TA instruments, USA). Approx. 5 mg of solid samples were hermetically sealed in the T0 aluminium pans and scanned from 0 to 320 °C with the heating rate of 10°C/min with constant nitrogen purging.

Fourier transform infrared spectroscopy (FTIR)

The FT-IR spectra of pure drug (FA), excipients and the physical mixture of the lipid phase was evaluated utilizing Cary 660 series FTIR (Agilent Technologies, Place, Country) and MIRacle ATR (attenuated total reflectance) systems to investigate the interactions of the excipient on the drug.

Characterization of FA-NLC *in situ* gel

Rheological and *in vitro* gelling characteristics

In situ gelling agents in the formulation includes gellan gum alone and combination of gellan gum with HPMC. The viscosity of the FA-NLC formulations and *in situ* gels was determined using Brookfield cone and plate viscometer (LV-DV-II+ Pro Viscometer, Middleboro, USA). The distance between the cone spindle and sampler cup was maintained to the minimum and the temperature of the system was set to 34 ± 0.2 °C. Later approximately 500 µL of sample was added to the cup and viscosity of the sample was evaluated at different shear stress using CPE 52 spindle. The viscosity of the gel formulations was also determined with simulated tear fluid (STF) with the ratio of 50:7 of the formulation to STF so as to mimic the viscosity of the formulation after application into the eye.

Simulated tear fluid with pH of 7.0 ± 0.2 consists 0.678 g of NaCl, 0.0084 g of CaCl2·2H2O, 0.138 g of KCl, and 0.218 g of NaHCO3 in 100 mL of deionized water.[23] The viscosities were registered from the Rheocalc software.

Gelling formation and gel residence time

Gel formation time of the *in situ* formulation was determined by placing approx. 50 µL of the formulation in a vial containing 10 mL of freshly prepared STF and visibly checking the time taken by the formulation to gel in the fluid. Gel residence time was the total time of the formulation resides intact within the STF. Approximately 50 µL the formulation was added to the 10 mL of freshly prepared STF and the vial was kept at 34 ± 0.2°C with constant stirring. Time was recorded when the gel was breaking down or no longer remained intact.

Gel firmness and adhesion

TA XT2i texture analyzer (Texture Technologies Corp. NY. USA) was utilized to measure the gel firmness and work of adhesion for the optimized *in situ* gel formulation. At the room temperature, the gel formulation was mixed with freshly prepared STF in ratio 50:7 was held on soft matter holder (TA-275). The measurement was done by compression mode where 1-inch cylinder acrylic probe was embedded to the distance of 1 mm into the gel formed with the speed of 0.5 mm/sec and 5 g of the triggering force.

Measurement of turbidimetry

FA-NLCs and *in situ* gels were examined for the percent transmittance (%T) at the wavelength (λmax) of 520 nm using UV Genesys 6 (Thermo Fischer Scientific, USA) to check visible transparency.[13] The turbidity of the formulations was measured over a period

of 6 h after placing the formulation in STF in the ratio of 50:7 of formulation to tear fluid. %T was calculated using the following formula:

$$\boldsymbol{A = 2 - log\ (\%T)}$$

Where, A is absorbance of the sample when compared with deionized water.

In vitro release study

The release kinetics for the FA-NLCs and *in situ* gels were evaluated by adding 200 µL of the formulation to 10 mL of isotonic phosphate buffer saline (IPBS) (pH 7.4) with 5% methyl β cyclodextrin. The temperature of the vials was maintained 34 ± 0.2 °C with continuous magnetic stirring for 4 h. Approx. 1 mL of the aliquot was withdrawn at predetermined time points and was replaced with equal volume receiver medium. The samples were analyzed for the amount of drug by HPLC method.

Stability studies

The formulations were placed at 4^{o}C and 25^{o}C for weeks to assess their stability during storage. Samples from the formulations were withdrawn at predetermined time point and were evaluated for their particle size, PDI, surface charge, EE and assay as specified earlier. About, 3 mL of the formulations, both NLCs and *in situ* gels were taken in the glass vials and placed in the autoclave (AMSCO Scientific Model SI-120, USA) for pressure controlled sterilization at 121°C for the time period of 60 min at 15 psi pressure. The formulations were examined for their physical characteristics compared to the formulation of before sterilization.

In vitro permeation studies

Post-development of the FA-NLC and corresponding *in situ* gel, the formulations were examined for their permeation release in the ocular tissues. The *in vitro* permeation studies were carried out on corneal and scleral tissues. Whole rabbit eyes were bought overnight from Pel-Freez Biologicals in Hanks balanced salt solution. The permeation study was performed immediately after receiving the eyes. The tissues were separated and were washed within ice-cold Dulbecco's phosphate buffer saline (DPBS) solution with pH 7.4 and later were mounted in between the donor and receiver on valia-chein diffusion cells (PermeGear, Inc®, USA) with the epithelial tissue section towards the donor compartment of the cell.

The receiver medium constituted of balanced salt solution (BSS) (pH 7.4) with 5% methyl-β- cyclodextrin. The temperature of the cells was maintained at 34 ± 0.2°C and the receiver was under continuous magnetic stirring throughout the experiment of 3 h. Approx. 200 µL of the formulation along with 50 µL of DPBS was added to the donor compartment for the formation of sol-gel. The control used for the study was fluocinolone acetonide (0.1%) suspended in deionized water containing gellan gum (0.3%) and HPMC (0.5%). About 600 µL of aliquots were pipetted out of the receiver cells and was replaced with equal volume receiver medium. The samples were analyzed for the concentration of drug by HPLC method.

From the concentration; cumulative concentration, steady-state flux, and tissue permeability were estimated. The equations for calculating cumulative concentration was

$$\boldsymbol{M_n = V_r C_{r(n)} + \sum_{x=1}^{x=n} V_{s(x-1)} C_{r(x-1)}}$$

Where, n is the number of sampling time point; V_r (mL) is the volume in the receiver cell and V_S (mL) is the volume of the sample collected on time points. $C_r(n)$ (µg/mL) is the drug concentration in the receiver chamber medium at the nth time point.

Flux is the rate of drug permeated through the tissue membrane per area of the tissue. The calculation of the flux was determined from the slope of the cumulative drug permeation versus time plot. The equation for calculating flux was:

$$\boldsymbol{Flux(J) = (dM/dt)/A}$$

Where, M is the cumulative amount of drug transported and A is the surface area of the tissue (0.636 cm2)

The equation to determine the tissue permeability was:

$$\boldsymbol{Permeability\ (Papp) = \frac{Steady\ state\ flux\ (J)}{Donor\ concentration}}$$

In vivo tear kinetics and distribution studies

In vivo studies were conducted in New Zealand male albino rabbits. Before the study, the animals were kept for acclimatization up to 7 days with absolute access to food and water. The formulations used for drug distribution studies were *in situ* gel and control, which were administered via topical and subconjunctival route. The animals were divided into 4 groups and the formulations were administered to the left eye of each animal and keeping the other eye untreated. The study was conducted for 3 h on the animals treated with topical drops and 6 h for animals treated with subconjunctival injection. Topically, 50 µL of the formulations were instilled into the cul-de-sac of left eye while for the subconjunctival route, 50 µL of the formulations were injected accurately into the conjunctival membrane with tuberculin syringe and 27-gauge needle. Around 15 minutes prior to injection, the rabbits were anaesthetized

with combination of ketamine and xylazine via intramuscular route. Approximately 5 µL of tear fluid was pipetted out from the cul-de-sac of the test eyes using micro pipette at every 30 minutes for animals treated topically and at 60 minutes for animals treated with subconjunctival injections. Approximately 10 minutes prior to the end time of the study, all the animals were anaesthetized with same combination and were euthanized with pentobarbital overdose via marginal ear vein. Both the test and untreated eyes were excised, and washed with ice cold IPBS solution. The tissues were carefully isolated and were stored at -80 °C until the drug extraction and quantification.

Extraction procedure of drug from ocular tissues

Drug extraction from the ocular tissues were carried out by protein precipitation technique. Analysis for the drug content was performed on plasma, aqueous humor (AH), vitreous humor (VH), cornea, iris-ciliary (IC), retinal choroid (RC), sclera and conjunctiva. Individual weights of all the solid tissues were measured and later each tissues were minced into small fragments and kept in separate vials. To all the tissues, the relevant amount of triamcinolone acetonide with the concentration of 1 µg/mL (as internal standard) was added. The vials were then vortexed thrice for 30 seconds each with the gap of 10 minutes between each vortexing. Later, 1000 µL ice-cold methanol with 0.1% formic acid was added to each vial and the process of vortexing was repeated. The samples were then centrifuged for 15 minutes at 13,000 rpm to separate out the precipitate, and the supernatant was then collected, diluted if required and spiked in UPLC-triple quadrupole (TQ)-MS system for analysing the concentration.

For liquid samples, 100 µL of plasma, 100 µL of AH and 500 µL of VH samples were collected and similar extraction procedure was followed with 100 µL, 100 µL and 500 µL of

ice-cold methanol was utilized for plasma, AH and VH respectively. After centrifuging for 15 minutes the samples were analyzed in UPLC-triple quadrupole (TQ)-MS system. The drug content from the tear samples collected during the *in vivo* study was extracted using ice cold methanol and analyzed in the same system.

For analysis of FA, Ultra-Performance Liquid Chromatography system coupled with a triple quadrupole mass spectrometer (UPLC-TQ-MS) (Waters, USA), was utilized. The quantification peaks of both the drug and the internal standard were obtained on the basis of specific mass to charge (m/z) values of same.[24] 1 µL of the sample was eluted through BEH C18 1.7 µm Acquity UPLC® column and separated using isocratic mode of ACN with 0.1% formic acid and water with 0.1% formic acid 1 µL of the sample was eluted at the ratio of 98:2 respectively. The extraction efficiency, limit of detection and limit of quantification of the drug was analyzed prior to drug concentration analysis.

For tear kinetics, from the concentration, the slope for the graph of time v/s concentration was calculated which was further used to calculate time at which the concentration of drug is half the initial concentration (t1/2) using the formula

$$\boldsymbol{t1/2 = \frac{0.693}{slope * -2.303}}$$

Area under curve (AUC) for the plot was calculated using the formula

$$\boldsymbol{AUC = \sum[\frac{(C1 + C2)}{2} * (t2 - t1)]}$$

Where C1 and C2 are drug concentration in tear at time t1 and t2 respectively.

Statistical analysis

Results were presented in their mean values ± standard deviation (SD). Statistical analysis for *in vitro* permeation study and *in vivo* drug distribution study in ocular tissues was done by One-way ANOVA (analysis of variance test) with Tukey's post hoc test (R Studio software, USA). The confidence interval set for the statistical analysis was 95% and hence the p-value for the significant difference was less than 0.05 ($p < 0.05$).

CHAPTER III
RESULTS AND DISCUSSION

Formulation development and optimization of FA-NLCs

Fluocinolone acetonide NLCs were formulated by hot homogenization along with probe sonication. For formulating the NLCs, varied ratios of lipid to surfactant, and solid lipid to liquid lipid and primary surfactant to secondary surfactants were considered. Optimization of the formulation was determined based on the effect of these ratios on particle size, ZP and drug content. All the ingredients utilized in the formulations are naturally occurring and thus are biodegradable and the extent to which they were used in the formulations were below the safety limits mentioned by FDA inactive ingredient limit.[25]

Selection of lipids whether solid or liquid and surfactant was based on the partitioning behavior of the drug in respective component. Better the affinity, higher quantity of the drug could be withholding within the formulation, thereby increasing both the percent drug content and entrapped into the formulation. The selection of the lipid study shows that the drug had a higher solubility towards didodecyldimethylammonium bromide, lauric acid, and compritol 888 ATO (Figure 1). Since compritol 888 ATO had shown the highest solubility i.e. 4.37 mg/g, hence, compritol 888 ATO was preferred over other as solid lipid for the development of FA-NLC formulation.[20] Retiene, an aldehyde derivative of vitamin A has a fundamental effect on the vision. Inadequacy in the content for vitamin A in the retina may progressively lead to night blindness. Also, due to its low melting point (<30^{o}C), vitamin A palmitate provides more flexibility and imperfect crystallinity to the NLC structure, thus decreasing the

drug leaching and also act as a permeation enhancer. Hence, vitamin A palmitate was selected as solid lipid in combination with compritol 888 ATO for the formulation. Similarly, in case of liquid lipids FA showed a higher affinity towards isopropyl myristate, miglyol and sesame oil (Figure 1). However, because of its high physical compatibility and also due to its natural origin, sesame oil was chosen over others as the liquid lipid component for the preparation of FA-NLCs. The total lipid content in the formulation ranged from 1 to 2% where higher lipid content contributes to high drug solubility.

Further, the selection of the surfactants was done based on the highest solubility of FA in the surfactant solution. In general, a combination of the surfactants with the HLB range from 4 to 7 (W/O emulsifiers) and with the HLB range from 8 to 18 (O/W emulsifiers) was adopted for the preparation of NLCs were showed better stability.[26] From the solubility results, a higher amount of FA solubility was observed with capmul PG 8, capryol 90, tween 80 and propylene glycol (Figure 1). Similarly, O/W surfactant that shows low solubility was preferred as secondary surfactant for the formulation preparation. From the figure 1 we can see that tween 80 and poly ethylene glycol 4000 had lowest solubility from all O/W surfactants. Therefore, on the basis of their physical compatibility and formulation stability, capmul PG 8 and tween 80 were selected as W/O and O/W emulsifiers for the preparation of FA-NLC respectively.[12]

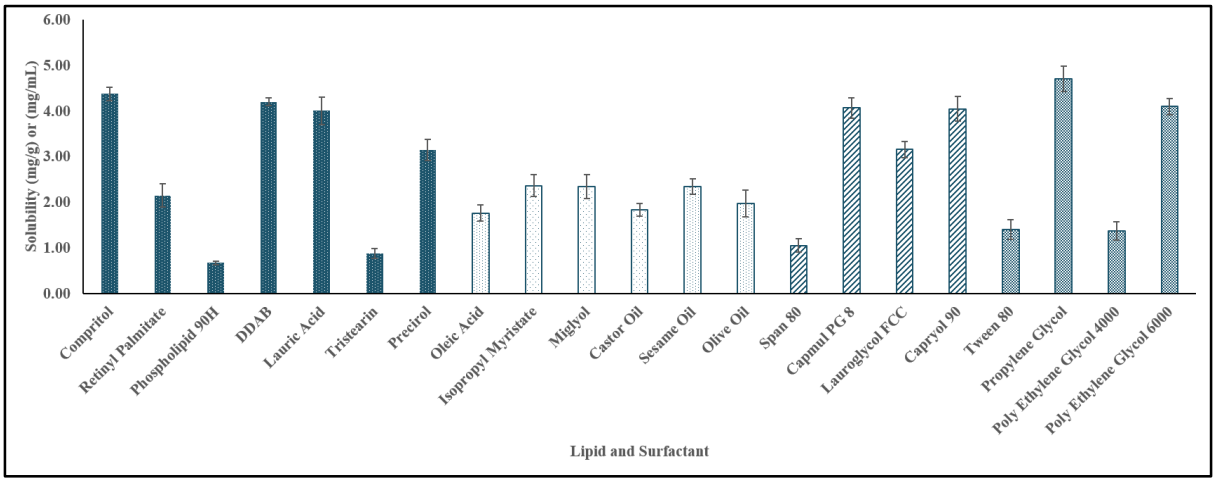


Figure 1: Solubility of drug in various lipids and surfactants (mean±SD, n=3)

The proportion of the excipients utilized to formulate FA-NLCs were mentioned in Table 1. For the development and optimization of the NLCs, various variables along with their impact on characteristic features and stability of NLC were taken into consideration.[20] These variables include different ratios of lipid to surfactant, solid to liquid lipid as well as the ratio of W/O and O/W emulsifiers. Percent FA entrapped in all the formulation was in the ranging of 60 to 97, with size and PDI in the range of approx. 160 to 270 nm and 0.15 to 0.52, respectively. The EE of the drug in all the formulations was greater than 91%, while the charge for the most of the formulations falls in the range of -8 to -10 mV (Table 2).

Table 2: Effect of lipid to surfactant and solid to liquid lipid ratio on size, PDI, ZP, EE and assay of fluocinolone acetonide NLC formulations (mean±SD, n=3)

Formulation	Lip:Sur	Sol:Liq	Size (nm)	PDI	ZP (mV)	Assay (%)	EE (%)
FN1	1 to 1	70 to 30	212.1 ± 0.4	0.43 ± 0.037	-9.4 ± 0.2	82.47 ± 2.12	92.89 ± 4.30
FN2	1 to 1	80 to 20	159.1 ± 3.8	0.15 ± 0.009	-8.4 ± 0.4	96.53 ± 2.43	95.43 ± 2.89
FN3	1 to 1	90 to 10	159.9 ± 4.3	0.263 ± 0.018	-9.7 ± 0.3	92.87 ± 6.12	95.29 ± 4.50
FN4	2 to 1	70 to 30	192.0 ± 4.2	0.402 ± 0.027	-8.7 ± 0.5	86.66 ± 4.02	94.06 ± 1.79
FN5	2 to 1	80 to 20	204.5 ± 6.7	0.329 ± 0.026	-4.2 ± 0.1	75.44 ± 3.29	94.45 ± 1.92
FN6	2 to 1	90 to 10	271.3 ± 6.5	0.473 ± 0.022	-3.4 ± 0.1	92.77 ± 3.74	94.8 ± 2.04
FN7	2 to 1.5	70 to 30	175.9 ± 5.9	0.317 ± 0.017	-7.6 ± 0.4	70.76 ± 7.31	94.05 ± 2.71
FN8	2 to 1.5	80 to 20	165.3 ± 3.5	0.298 ± 0.016	-8.5 ± 0.3	61.17 ± 8.92	91.85 ± 1.75
FN9	2 to 1.5	90 to 10	216.5 ± 8.4	0.522 ± 0.061	-4.6 ± 0.2	87.84 ± 2.55	95.58 ± 1.35

In the early stages of formulation development, the ratio of lipid to surfactant and solid to liquid lipid was premeditated keeping the surfactant concentration constant and their corresponding effect on particle size, PDI, and the assay were studied (Table 2). If the lipid to surfactant ratio was 1:1, the particle size and PDI of the nanoparticles was found to be considerably low in the range of 160 to 210 nm and responsible for better NLC formulation. These results were observed in the formulations FN1, FN2, and FN3. However, increase in the lipid content compared to surfactant concentration i.e., the ratio of 2:1 and 2:1.5, gave a good particle size but distribution of the particles was more. These results exhibit that if the amount of surfactant, when compared to lipid was less, distribution of the particles in the

formulation increases. Similarly, the overall percent drug content of the FA-NLC scales down to less than 90%, when the difference between the lipid and surfactant was higher. These results were observed in FN4, FN5, FN7, FN8 and FN9 NLC formulations. The overall reduction in PDI and drug content could be due to the measure of surfactant which was adequate enough to form nanoparticulate emulsion, but inadequate to stabilize the same. This can also be attributed to the surface charge of the formulations. Higher the difference between lipids and surfactant features the surface charge more towards 0 which in turn leads to more of instability. The ratio of solid to liquid lipid was determined on the basis of higher drug solubility in lipid along with the suitable blend of solid and semi solid consistency of the phase at room temperature. Since the solubility of FA was observed higher for solid lipids, a higher proportion of solid lipid was favored in total lipid content. The preferable ratio selected for formulation trials were from 70:30 to 90:10.[27] The evaluation results with respect to different ratios of solid to liquid lipid ratio were mentioned in Table 2. It can be inferred that relatively lower amount of sesame oil in the total lipid combination gave lower assay values and when the ratio was other than 80:20, the PDI of the formulation was also high. Therefore, in accordance with above assumption, the ratio of lipid to surfactant and solid to liquid lipid was set as 1:1 and 80:20 respectively.

Table 3: Effect of O/W to W/O surfactant ratio on size, PDI, ZP, EE and assay of fluocinolone acetonide NLC formulations (mean±SD, n=3)

Formulation	O/W:W/O ratio	Capmul PG 8 (mg)	Tween 80 (mg)	Size (nm)	PDI	ZP (mV)	Assay (%)	EE (%)
FN2a	1 to 1	50	50	220.1 ± 6.1	0.281 ± 0.012	-9.4 ± 0.5	91.83 ± 2.26	85.89 ± 4.24
FN2b	1 to 2	33	66	159.1 ± 3.8	0.150 ± 0.009	-8.4 ± 0.4	96.53 ± 2.43	95.43 ± 2.89
FN2c	1 to 3	25	75	195.7 ± 5.5	0.350 ± 0.023	-10.2 ± 0.4	93.16 ± 2.78	94.78 ± 2.11

Both lipid to surfactant (1:1) and solid to liquid lipid (8:2) ratio are constant for all the formulations. Formulation FN2b is same as formulation FN2 from table 2. Change in name of formulation was done to have different representation in accordance with table 3.

After finalizing the quantity of lipids and total surfactant, formulation FN2 was further evaluated for its characteristic features with various fraction of O/W and W/O emulsifiers. From the Table 3, it can be presumed that size and PDI of nanoparticles with 1:1 ratio for capmul PG 8 and tween 80 was high and % entrapment was low compared to other ratios. On the other hand, when the ratio of two surfactants was 1:3, the polydispersity of formulation was high. When the ratio of O/W and W/O emulsifiers was taken as 1:2, the average size, polydispersity index and assay of FA NLC was found to be 159.1 ± 3.8 nm, 0.150 ± 0.05, -8.4 ± 0.4, 96.53 ± 2.4 % and 95.43 ± 2.89 % respectively. The combination of capmul PG 8 and tween 80 surfactants i.e. with the HLB value 6 and 15 exhibited excellent NLC formulation possibly because capmul PG 8 (W/O surfactant) forms a layer around the formed particles and tween 80 (O/W surfactant) stabilizes the formed particles with surrounding aqueous phase by lowering the surface tension.[26] Therefore, formulation FN2b was considered as optimized and selected for the preparation of *in situ* gel formulation.

Formulation development and optimization of FA-NLC *in situ* gels

One of the critical quality attributes for *in situ* ophthalmic gel formulation is its flow properties, as it helps to administer the formulation without any complication while instillation into the ocular milieu. FA *in situ* gels was prepared by using various concentration of gellan gum alone and combination with HPMC to provide ideal gelation along with formulation's rheological characteristic. Gellan gum is an anionic polysaccharide, which forms a clear gel due to the presence of mono or divalent cations. An ideal viscosity for ophthalmic solution ranges from 25-50 cP.[28] The concentration of gellan gum used and their corresponding results on viscosity and other rheological characteristics of *in situ* gel presented in Table 4. Since the viscosity of the formulation FN2G3 (0.3% of gellan gum) was 40.3 ± 1.8, gels immediately when in contact with STF and reside as gel for up to 6 h. Hence, 0.3% of gellan gum was selected as the optimum concentration for *in situ* gel formulation and was used for the further analysis.

Methocel E5 is a neutral polymer which forms viscous solution by hydration of hydrogen bonding in the polymer chains. Methocel E5 is a low molecular weight, temperature sensitive polymer and increases the viscosity at around 35°C. The rheological characteristic results of the formulation with varied concentrations of HPMC was presented in Table 4. The viscosity of the formulation has been increased with increased concentration of HPMC. The viscosity of the formulation holds in the ideal range for ophthalmic solution even after addition of 0.5% concentration of HPMC to FN2G3 formulation owing to form ideal *in situ* gelling system with higher viscosity value after addition to STF.[15], [16]

One of the major fall back for ophthalmic formulation is low inhabitancy in the cul de sac. The gelling time of the formulation was determined so as to compute the time required by *in situ* gelling formulation to form gel on contact with tear fluid. All the concentration of gellan gum formulations (with and without HMPC) were observed to gel as soon as they were added to the simulated fluid. The results of gelling time and gel residence time of *in situ*

gels were specified in the Table 4. Firmness of the formulation was tested to analyze the resistance of the gel to the external deformation and work of adhesion for the formulations was done to analyze the adhesiveness of the gels when in contact with the tear fluid.[14], [29] Firmness and work of adhesion for the formulation with 0.3% and 0.5% of gellan gum and HPMC respectively with respect to time was showed in Figure 2. From the results, the values for firmness and work of adhesion do not show significant variation even after 6 h, suggesting the intactness of the gel for extended period of time (6 h). Therefore, in accordance with the results and inference discussed in above sections, formulation FN2b and FN2GH05 were considered as optimized FA-NLC and *in situ* gel formulation and were chosen for the further studies.

Table 4: Rheological evaluation of fluocinolone acetonide NLC *in situ* gel formulations (mean±SD, n=3)

Formulation	Gellan Gum (% w/v)	HPMC (%w/v)	Viscosity (cP)	Gel formation (sec)	Gel residence time (h)	Viscosity with STF (cP)	Firmness (g)	Work of adhesion (g.sec)
FN2	-	-	15.7 ± 2.0	-	-	-	-	-
FN2G1	0.1	-	17.6 ± 1.4	< 5	< 1	93.5 ± 19.0	170.46 ± 7.85	-0.64 ± 0.37
FN2G2	0.2	-	26.4 ± 1.3	< 5	3 – 4	186.9 ± 27.4	180.91 ± 4.28	-2.81 ± 0.30
FN2G3	0.3	-	40.3 ± 1.8	< 5	6 – 8	530.2 ± 43.1	185.90 ± 4.56	-18.73 ± 0.38
FN2G4	0.4	-	61.6 ± 4.7	< 5	> 24	1820 ± 69.3	193.97 ± 1.25	-23.81 ± 0.29
FN2G5	0.5	-	90.4 ± 6.8	< 5	> 24	2524 ± 101.2	221.03 ± 6.76	-26.26 ± 0.56
FN2G6	0.6	-	159.7 ± 11.9	< 5	> 24	3348 ± 123.2	224.90 ± 5.08	-31.89 ± 1.37
FN2GH05	0.3	0.5	45.5 ± 2.5	< 5	6 – 8	1202 ± 51.1	189.49 ± 4.26	-19.90 ± 1.52
FN2GH1	0.3	1	52.4 ± 3.4	< 5	7 – 9	1526 ± 59.2	193.78 ± 3.31	-20.71 ± 2.39
FN2GH15	0.3	1.5	57.7 ± 2.9	< 5	9 – 10	1892 ± 73.4	199.78 ± 5.99	-23.67 ± 0.27

G and H indicates gellan gum and HPMC containing *in situ* gels respectively.

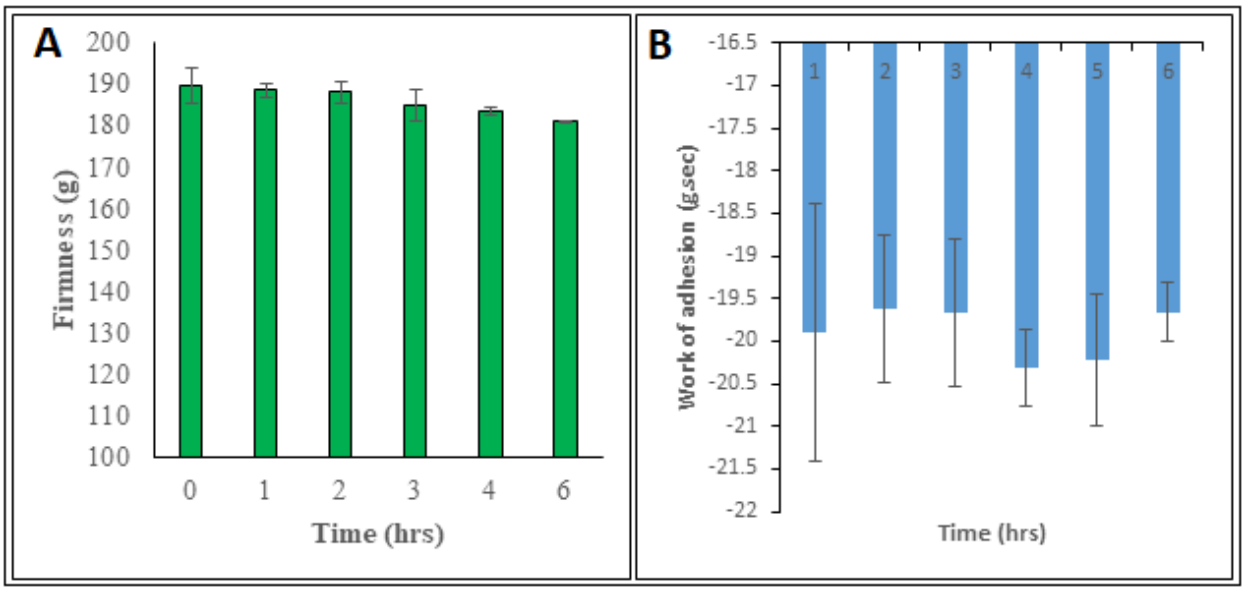


Figure 2: Firmness (A) and work of adhesion (B) for *in situ* gels with respect to time (mean±SD, n=3)

Stability studies of FA-NLC

The stability of FA-NLC formulation upon storage was evaluated by studying their physical appearance and characteristics once a week for up to 4 weeks. Until 30 days, the formulation did not show any sign of physical separation or breaking. The results for particle size, PDI, assay and EE were shown in Figure 3. Further, the formulation was also tested for postproduction sterility by moist heat sterilization. As per stability studies, the physical aspects of the formulation did not exhibit any breaking or phase separation. It can be determined that there was no significant change in any of the parameters tested. This could be stating that the formulation was still intact after sterilization and also post 28 days of storage without any phase separation or particle aggregation or drug oozing out of NLC gel.

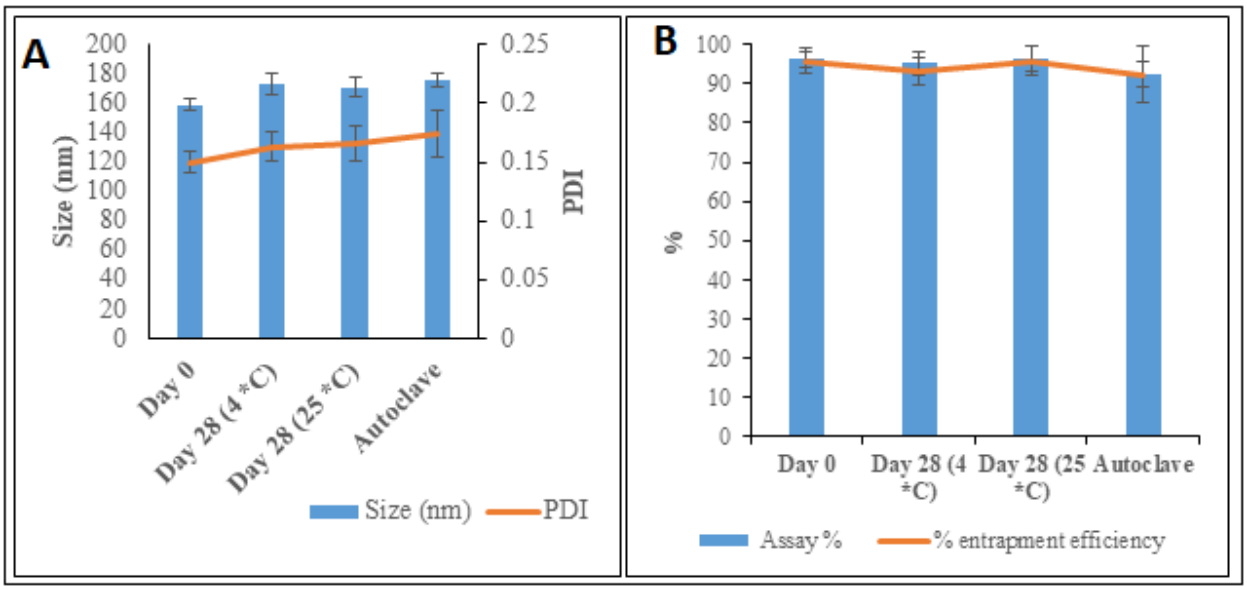


Figure 3: Stability studies of optimized FA-NLC and *in situ* gel at 4°C and 25 °C for 28 days, by autoclave on size and PDI (A), assay and entrapment efficiency (B) (mean±SD, n=3)

Compatibility studies - DSC and FTIR

The DSC thermograms of the FA and the physical mixture of the drug with lipid phase is portrayed in Figure 4. The melting point of pure FA was found to be at around 270^{o}C as shown by evolution of a sharp endothermic peak corresponding to its reported melting point. In case of lipid phase (including drug) DSC thermogram, there was a slight concavity at around 55 to 60^{o}C of compritol 888 ATO followed by constant heat flow up to 300oC. This verifies the complete solubility and/or conversion of the drug in to amorphous form in the lipid phase, as no separate endothermic peak was observed for the drug in NLC-lipid phase thermogram.

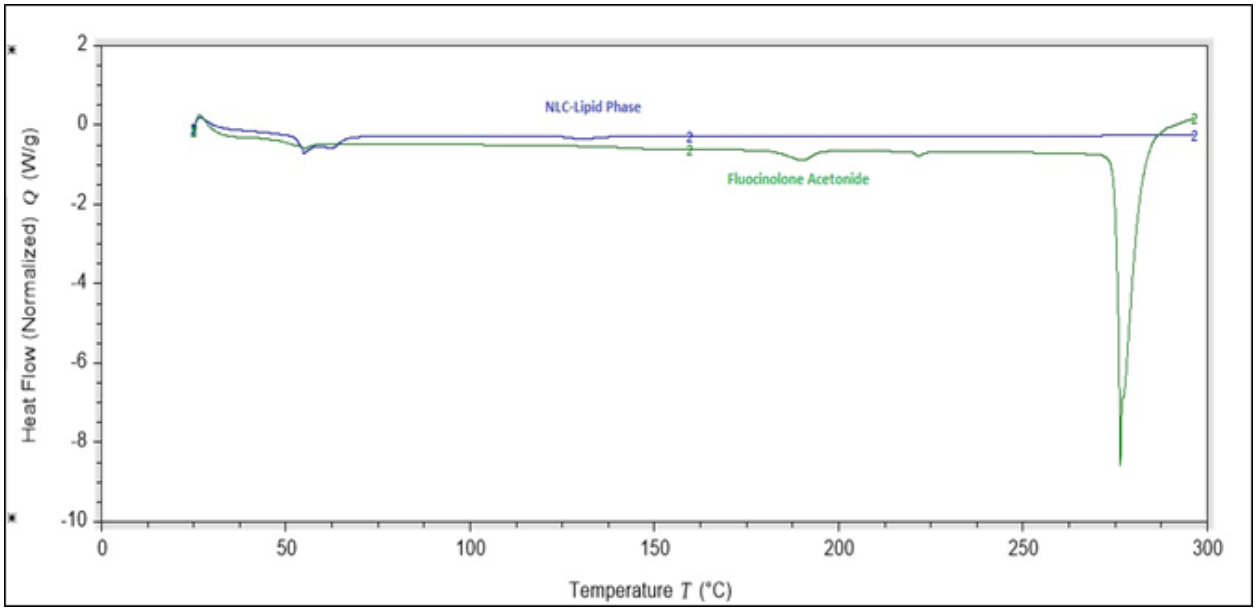


Figure 4: DSC thermograms of drug and NLC-Lipid phase

FTIR spectroscopy was performed for the drug and NLC to determine the potential interaction between FA and lipid matrix throughout the formulation procedure. The FTIR spectra for drug, lipid excipients and FA-NLC formulation is showed in Figure 5. For all the spectras of lipid excipients and drug, the major bands were observed at around 1640 cm^{-} assigned to C=C, and around band 1728 cm^{-} was assigned to C=O group. The bands from 2995 to 2952 cm^{-} were assigned to C-H group. The NLC spectra exhibited the peak at 2916 cm^{-} for C-H group and 1665 cm^{-} for C=C. There was a slight spectral variation for NLC, which might be due to overlapping of the similar functional group for various components. However, there was no change in the band peaks for pure excipients and peaks in the NLC spectra even in the presence of FA, indicating no substantial interaction between the drug and excipients.

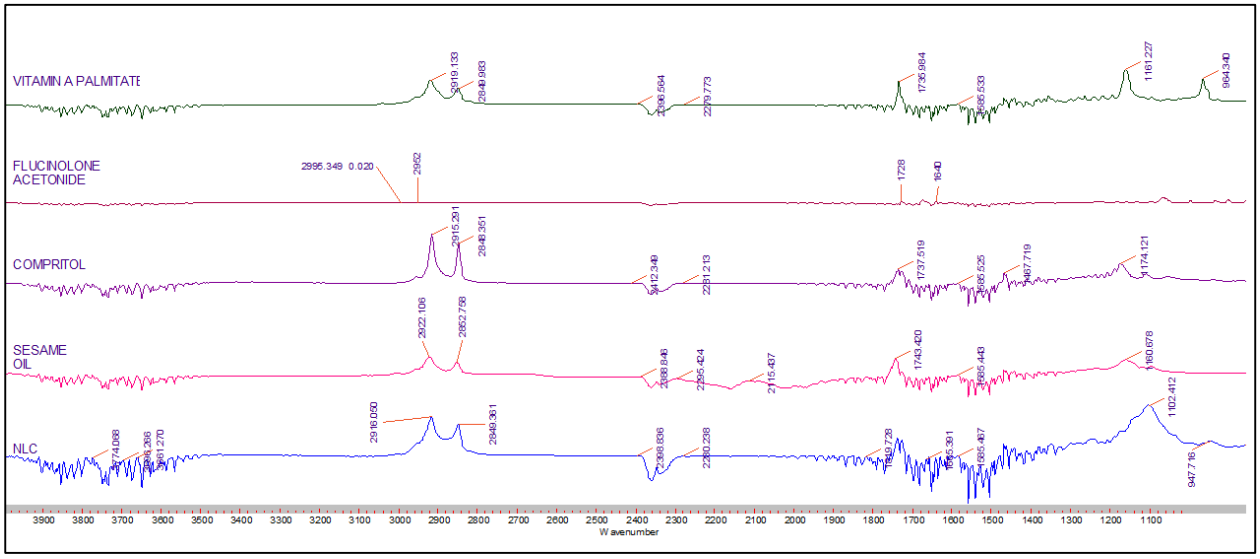


Figure 5: FTIR spectra of the drug and NLC-Lipid phase and excipients

In vitro release study

Since the saturation solubility of FA was found to be maximum in BSS (pH 7.4) with 5% methyl- β- cyclodextrin. BSS solution was preferred as the receiver medium for *in vitro* release and *in vitro* permeation studies.[30] The *in vitro* release profile of FA from control, NLC, and *in situ* gel are presented in Figure 6. The cumulative release of the drug from control, NLC, and *in situ* gel was found to be 102.34 ± 8.08, 46.62 ± 4.65, and 35.44 ± 4.39 % respectively within the period of 4 h. It could be due to the slow diffusion release from the shell layer of lipid nanoparticles during the period of 4 h.

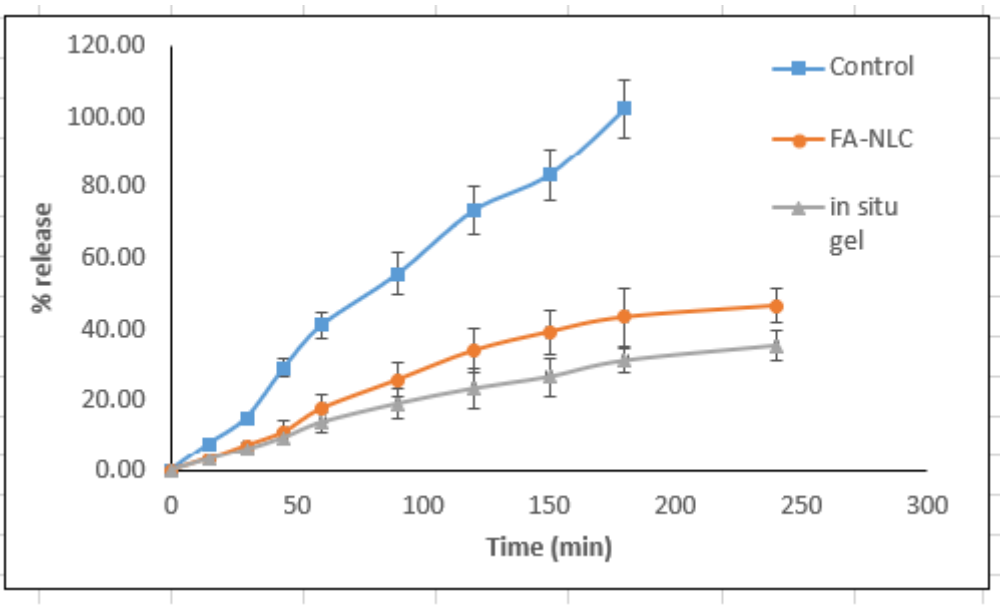


Figure 6: *In vitro* drug release profiles of fluocinolone acetonide from control gel, FA-NLC and *in situ* gel (mean±SD, n=3)

Turbidimetry measurement

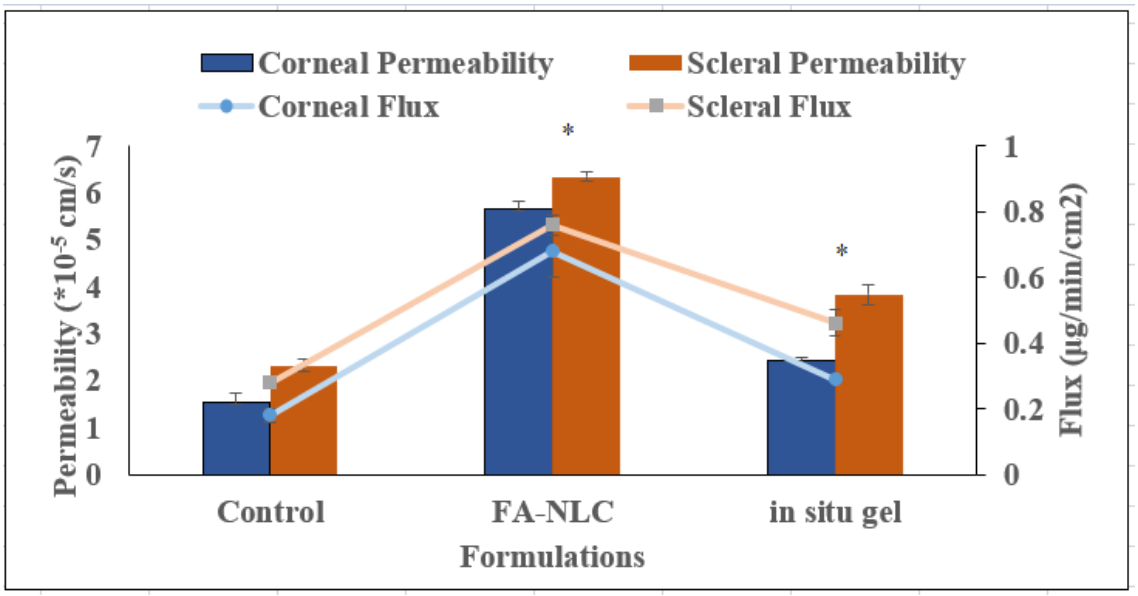
The percent transmittance (%T), provide the extent of turbidity which could be the extent of light obstruction during post injecting the formulation into the eye.[13] The transmittance results for formulations over the period of 6 h are mentioned in Table 5. The % transmittance for the *in situ* gel formulation was low due to cross-link formed by the gelling agent in contact with STF. This indicates that, the gel link breaks with time with increase in transmittance and thus releasing the formulation at the controlled rate.

Table 5: Transmittance (%) of fluocinolone acetonide NLC and *in situ* gel formulation (mean±SD, n=3)

Formulation	Transmittance (%)		
	0 h	**3 h**	**6 h**
FA-NLC	35.08 ± 2.18	35.73 ± 3.21	38.11 ± 2.54
***In situ* gel**	3.35 ± 0.71	15.35 ± 1.84	36.48 ± 4.47

In vitro permeability studies

The permeability of FA-NLC, *in situ* gel and control formulation were performed in corneal and scleral tissues and the results presented in Figure 7. The tissue permeability of PA form control, FA-NLC and *in situ* gel formulations were found to be 1.52 ± 0.2 x10-5 cm/s, 5.66 ± 0.18 x10-5 cm/s and 2.41 ± 0.08 x10-5 cm/s, respectively from corneal tissue and 2.32 ± 0.14 x10-5 cm/s, 6.34 ± 0.1x10-5 cm/s, and 3.83 ± 0.21x10-5 cm/s, respectively from scleral tissue. The flux values for control, FA-NLC and *in situ* gel formulations were found to be 0.18 ± 0.02 µg/min/cm2, 0.68 ± 0.08 µg/min/cm2 and 0.29 ± 0.01 µg/min/cm2, respectively for corneal tissue and 0.28 ± 0.02 µg/min/cm2, 0.76 ± 0.03 µg/min/cm2, and 0.46 ± 0.04 µg/min/cm2, respectively for scleral tissue. A significant increment in the tissue permeability and flux was observed form both FA-NLC and *in situ* gel formulation compared to the control formulation. The enhancement in permeability of FA form NLC and *in situ* gel formulations in comparison to control might be due to lipophilicity of the formulation and which imparts higher penetration through the tissues. Further, the lower flux of the drug from *in situ* gel formulation compared to NLC formulation indicates the slow release of the drug from *in situ* gel owing for controlled release. Furthermore, the drug permeability was higher through the sclera compared to cornea, suggesting that non-corneal route may show better drug penetration compared to the corneal route[1], [7], [12].



* indicates the data for formulations is significant compared to control

Figure 7: *In vitro* permeability studies of fluocinolone acetonide from control suspension, FA-NLC and *in situ* gel through ocular tissues (mean±SD, n=3)

In vivo tear kinetics and distribution study

In vivo distribution studies of FA from control and FA-NLC *in situ* gel formulations was carried out in New Zealand albino rabbits treated by topical and subconjunctival administration. The drug content was observed in rabbits treated with topical and subconjunctival injection after 3h and 6h, respectively. The drug contents were estimated in the tear after every 30 min for topically and every 60 min for subconjunctivally administered formulations and were presented in the Table 6. From the results, it was observed that the

highest concentration of the drug was observed at first 30 min (3.37 ± 2.10 µg/mL) with topical and first 60 min (3.10 ± 0.20 µg/mL) with subconjunctivally administered formulation. The half-life of FA from *in situ* formulation was found to be 3.22 h and 1.64 h by subconjunctival injection and topical formulation. In case of ocular bioavailability, about 2.32 and 2.29-folds enhancement was observed with subconjunctival and topical administration when compared with control gel. This could be due to the diffusion release of the drug from the lipid nanoparticles into the tear and then its permeation through the cornea. As evident from the Table 5, the rate of drug expulsion from NLC *in situ* gel was predicted to be a sustained release, as the concentration of the drug in tear decreased gradually compared to the control formulation. Thus, the tear kinetic analysis clearly explains the release pattern of the drug for different formulations.[31]

Table 6: *In vivo* tear kinetics for fluocinolone acetonide from *in situ* gel formulation and control in New Zealand albino rabbits (mean±SD, n=3).

Formulation	0	1	2	3	4	5	6	AUC (ug/mL.h)	$T_{1/2}$ (h)
Control subconjunctival	0	1.19 ± 1.48	1.17 ± 0.04	0.93 ± 0.91	0.40 ± 0.23	0.22 ± 0.06	0.11 ± 0.00	3.82	1.01
***in situ* subconjunctival**	0	3.10 ± 0.20	2.01 ± 0.39	1.60 ± 1.31	1.10 ± 0.32	0.76 ± 0.51	0.21 ± 0.07	8.86	3.22
Control topical	0	0.94 ± 0.69	0.73 ± 0.16	0.59 ± 0.16	0.32 ± 0.11	0.31 ± 0.12	0.25 ± 0.13	1.51	1.20
***in situ* topical**	0	3.37 ± 2.10	1.31 ± 0.82	0.90 ± 0.72	0.68 ± 0.15	0.59 ± 0.08	0.36 ± 0.11	3.47	1.64

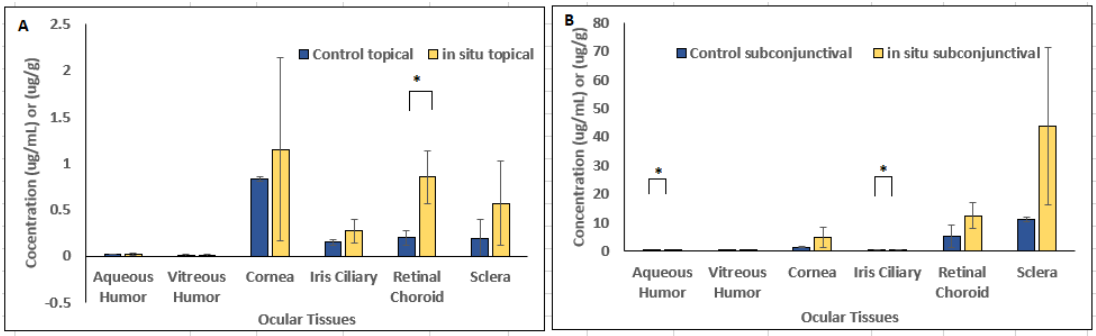
For subconjunctival formulations 0, 1, 2, 3, 4, 5 and 6 represents 0 min, 60 min, 120 min, 180 min, 240 min, 300 min and 360 min respectively and for topical formulations 0, 1, 2, 3, 4, 5 and 6 represents 0 min, 30 min, 60 min, 90 min, 120 min, 150 min and 180 min respectively

The concentration levels of FA from *in situ* gel and control formulation in aqueous humor (AH), vitreous humor (VH), cornea, iris ciliary (IC), retinal choroid (RC) and scleral tissues after topical and subconjunctival administration are showed in Table 7 and Figure 8. From the results, the drug concentration in the aqueous humor and vitreous humor were determined to be 0.016 ± 0.00 µg/mL and 0.005 ± 0.01 µg/mL for control formulation and 0.021 ± 0.01 µg/mL and 0.008 ± 0.01 µg/mL respectively for *in situ* gel by topical administration (Figure 8a). The high concentration of drug was observed in cornea and IC after 3 h post administration and thereby explaining the effectiveness of the *in situ* gel system in ocular tissues. Additionally, significantly high concentration of the drug was observed in scleral and RC tissues (p value < 0.05), suggesting high drug permeability from our novel *in situ* gel system in the posterior ocular compartment. Hence, enhancing the release kinetics of the drug in ocular tissue.[5], [32]

Table 7: *In vivo* fluocinolone acetonide distribution in ocular tissues from *in situ* gel formulation in New Zealand albino rabbits (mean±SD, n=3).

Formulation	Time (h)	Aqueous Humor (µg/mL)	Vitreous Humor (µg/mL)	Cornea (µg/g)	Iris Ciliary (µg/g)	Retinal Choroid (µg/g)	Sclera (µg/g)
Control subconjunctival	6	0.003 ± 0.00	0.005 ± 0.00	1.42 ± 0.15	0.28 ± 0.13	5.24 ± 3.78	11.12 ± 0.65
in situ subconjunctival	6	0.021 ± 0.01*	0.024 ± 0.02	4.87 ± 3.64	0.51 ± 0.03*	12.44 ± 4.60	43.81 ± 27.71
Control topical	3	0.016 ± 0.00	0.004 ± 0.01	0.83 ± 0.02	0.15 ± 0.03	0.20 ± 0.08	0.19 ± 0.20
in situ topical	3	0.019 ± 0.01	0.008 ± 0.01	1.15 ± 0.99	0.27 ± 0.13	0.85 ± 0.29*	0.57 ± 0.45

* indicates the value is statistically significant compared to control for same route.



* indicates the value is statistically significant compared to control for same route.

Figure 8: *in vivo* fluocinolone acetonide distribution in ocular tissues (A) Topical formulation (B) Subconjunctival formulation (mean±SD, n=3).

For subconjunctival injection, considerably high concentration of the drug was observed in sclera and RC when treated with *in situ* gel compared to control formulation. The concentration of drug in AH, cornea, IC, sclera and RC were 0.021 ± 0.01 µg/mL, 4.87 ± 3.64 µg/g, 0.51 ± 0.03 µg/g, 43.81 ± 27.71 µg/g, 12.44 ± 4.60 µg/g and 0.003 ± 0.00 µg/mL, 1.42 ± 0.15 µg/g, 0.28 ± 0.13 µg/g, 11.12 ± 0.65 µg/g and 5.24 ± 3.78 µg/g, respectively from *in situ* gel and control gel, which was significantly higher. Thus, it can be presumed that the drug permeation into the ocular tissue follows both corneal and non-corneal route. Due to the depot formation in conjunctiva, there is controlled and simultaneous drug release from the NLC into cornea and sclera since the tissue is in direct contact with cornea and sclera. The drug concentration in sclera and RC were calculated to be approximately 3.93 and 2.37-folds compared to control subconjunctival injection. Compared to the topical *in situ* gel, drug concentration in the ocular tissues for subconjunctival formulations were 3.0, 4.23, 1.88, 14.63 and 76.87 folds for VH, cornea, IC, RC and sclera respectively. The superlative levels of drug content in all the tissues shows the high-caliber for subconjunctival administration compared to topical administration. However, compared to posterior section, the amount of drug in the anterior section of the eye was considerably low. This was suggesting that the

drug flow route is notably towards the posterior end of the eye. Drug concentration were also analyzed in the conjunctival tissue post 6 h of study. The amount of the drug in conjunctiva was found to be 31 µg/mL (with the average weight of tissue of 107 mg). Thus, a higher drug level in the posterior section of the ocular tissues even after 6 h post injection suggest the extended release rates of the drug from the *in situ* gel system there by proving the translational ability of these formulation to clinics.[1], [19]

CHAPTER IV
CONCLUSION

In this study, fluocinolone acetonide NLC *in situ* gels were successfully formulated with vitamin A as one of the lipid by hot homogenization coupled with probe sonication method. The formulation had excellent particle size distribution (159) and PDI (0.150). The assay and drug entrapment were more than 94% with excellent stability for the period of up to four weeks. The stable *in situ* gel formulated with gellan gum and HPMC showed higher *in vitro* tissue permeation and high drug concentration at posterior end of the ocular tissues when administered as subconjunctival injection. Thus exhibiting suitability of these carrier systems for prolong ocular pharmacotherapy.

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