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Original Article

Optimization of Liposomal Eye Drops Co-Loaded with Triamcinolone Acetonide and Folic Acid for Retinal Vascular Disorders

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ABSTRACT

Background: Diabetic Retinopathy (DR) and Diabetic Macular Edema (DME) require effective posterior segment drug delivery, challenged by ocular anatomical barriers. Triamcinolone Acetonide (TA) despite its intravitreal efficacy carries risks of elevated intraocular pressure and cataract formation. Folic Acid (FA) exhibits anti-angiogenic and anti-oxidant potential but suffers poor corneal permeability. Co-loaded liposomal systems offer a non-invasive strategy by encapsulating both hydrophilic and lipophilic drugs enhancing therapeutic outcomes. **Materials and Methods:** Liposomes were prepared by thin-film hydration method and optimized by 2^3 full factorial design, by varying L-α Phosphatidylcholine, cholesterol, and dicetyl phosphate concentrations with vesicle size, Zeta potential and Entrapment Efficiency (EE) as responses. The optimized formulation was characterized for physicochemical properties (pH, viscosity, Density, Surface tension and osmolarity), SEM morphology, *in vitro* release and its kinetics, anti-inflammatory activity, *ex vivo* permeation and 90-day stability assessment. **Results:** The optimized formulation F9 exhibited 206 nm vesicle size, PDI of 0.145, zeta potential of -32.35 mV, and EE of 69.21±3.2% for TA and 78.6±1.4% for FA. Physicochemical evaluation confirmed stability. *In vitro* Release demonstrated enhanced release of TA (98.5±2.5%) and FA (96.4±1.3%) over 8 hr compared to plain TAFA via zero-order, non-Fickian kinetics. *Ex vivo* Permeation was significantly greater than plain TAFA solutions. Anti-inflammatory inhibition reached 90.1±2.1% with strong IVEVC correlations (R^2>0.997). **Conclusion:** This novel liposomal formulation of TA and FA represents a clinically viable, non-invasive topical alternative to intravitreal injections for DR and DME management.

Keywords: Anti-inflammatory, Folic acid, Liposomes, Ocular Drug delivery, Retinal diseases, Triamcinolone acetonide.

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INTRODUCTION

The International Diabetes Federation (IDF) predicted that the global population with Diabetes Mellitus will reach 700 million by 2045 (Teo *et al*., 2021). Among the various complications of the disease, eye disorders are among the most disabling. It is challenging to treat disorders that affect the posterior part of the eye when compared to anterior segment disorders. Diabetic Retinopathy (DR), Diabetic Macular Edema (DME), and Age-related Macular Degeneration (AMD) are the most common conditions affecting the posterior segment of the eye (Patel *et al*., 2013).

DR is a diabetes-related retinal disorder that can lead to vision loss due to microvascular damage (Fangueiro *et al*., 2015), whereas DME results from fluid leakage into the macula, causing retinal swelling. Treatment mainly involves inhibition of Vascular Endothelial Growth Factor (VEGF) to reduce vascular leakage and edema. Despite advances in understanding the pathophysiology of retinal vascular disorders, effective drug delivery to the posterior ocular segment remains a challenge. Conventional routes of administration are limited by the complex anatomical barriers of the eye. Various methods have been employed for drug delivery to the posterior segment of the eye, including topical, systemic, intraocular and periocular (subtenons, retrobulbar).

Triamcinolone Acetonide (TA), a synthetic corticosteroid exerts potent anti-inflammatory effects, is one of the most widely used steroids for managing a range of retinal problems. Intravitreal TA injections are commonly used to treat several conditions affecting the posterior segment of the eye, including retinal vein occlusions, DME and DR (Aceves-Franco *et al*., 2023; Navarro-Partida *et al*., 2020).

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One of the main drawbacks of intravitreal TA injection is the rapid elevation of intraocular pressure (Ciulla *et al.*, 2001) and certain other serious complications like glaucoma, cataract, endophthalmitis and pseudoendophthalmitis (Vedantham and Kim, 2006).

Folic Acid (FA), a water-soluble B Vitamin has attracted growing attention as a retinal protective agent (Lei *et al.*, 2019). FA and its metabolite homocysteine are implicated in vascular endothelial dysfunction, oxidative stress and inflammatory signalling pathways that underlie retinal neurodegeneration and vascular leakage in DR. Supplementation with FA has been shown to attenuate angiogenic signalling via suppression of Vascular Endothelial Growth Factor (VEGF) expression, reduce oxidative damage to retinal pigment epithelial cells and mitigate inflammation, thereby preserving retinal structure and function in preclinical models of DR (Gu *et al.*, 2023; Wang *et al.*, 2018).

Topical administration is the most common route for treating eye disorders. However, achieving therapeutic drug levels in ocular tissues is difficult due to barriers such as the tear film and corneal layers (Agarwal *et al.*, 2016).

Nanoparticulate drug delivery systems including nanoparticles, niosomes, pharmacosomes, discosomes and liposomes have been extensively explored as vehicles to surmount ocular barriers and improve intraocular drug bioavailability (Wadhwa *et al.*, 2009).

Among these, liposomes are particularly attractive owing to their structural versatility, biocompatibility and capacity to co-encapsulate both hydrophilic and lipophilic therapeutic agents. Lipophilic drugs such as TA partition into phospholipid bilayer, while hydrophilic agent such as FA are entrapped within the aqueous core, enabling simultaneous delivery from a single nano system (Mishra *et al.*, 2011).

Liposomes of appropriate size (100-300 nm) have been shown to enhance corneal epithelial uptake, prolong precorneal residence time and facilitate drug transport to deeper ocular tissues including the vitreous and retina. Chitosan coated liposomes incorporating TA have previously demonstrated prolonged drug retention and improved retinal delivery in topical applications further supporting the rationale for a liposomal approach (Cheng *et al.*, 2019).

The combination of TA and FA in a single liposomal system is rationally justified on the basis of complementary pharmacological mechanisms. TA addresses the inflammatory and oedematous components of DME via glucocorticoid receptor activation and suppression of pro-inflammatory cytokines, while FA simultaneously mitigates the oxidative and angiogenic pathways that drive retinal vascular dysfunction in DR.

Co-delivery of these agents within a liposomal carrier may achieve synergistic therapeutic effects, reducing the risk of steroid-associated adverse effects such as raised intracocular pressure while affording the patient the convenience of a single formulation.

Formulation optimization is critical to achieving the desired vesicle size, charge and entrapment efficiency that determine pharmacokinetic performance. Factorial experimental design is a well-established statistical approach enabling systematic evaluation of multiple independent variables thereby efficiently identifying optimal formulation.

The present study therefore aimed to design, optimize by 2^3 full factorial design and evaluate co-loaded liposomal ocular formulation of TA and FA with objectives of achieving a nanosized vesicle suitable for corneal permeation; controlled drug release with favourable kinetics; establishing enhanced transcorneal permeability and acceptable physicochemical and anti-inflammatory properties appropriate for ophthalmic application as a potential non-invasive alternative to intravitreal therapy in DR and DME.

MATERIALS AND METHODS

L-α-phosphatidylcholine was purchased from Sigma life sciences. Cholesterol was obtained from S.D Fine Chem Ltd., (Mumbai), Folic acid was sourced from Otto Chemie Pvt. Ltd., (Mumbai) and Triamcinolone acetonide was received as a gift sample from Auro Labs Pvt. Ltd., (Madurai, India). Dicetyl phosphate was acquired from Avanti Polar Lipids in Alabester, USA. Diclofenac sodium was purchased from Yarrow chem products (Mumbai), and Potassium dihydrogen phosphate was supplied from nice chemicals Pvt. Ltd., India. All reagents and chemicals used were of analytical grade.

Statistical Experimental Design

A 2^3 randomized full factorial design was employed to systematically investigate the influence of formulation variables on the physicochemical characteristics of TAFA liposomes (Triamcinolone acetonide-Folic acid loaded liposomes). This design evaluates three independent factors, each at two levels, generating a total eight distinct experimental runs representing all possible factorial combinations and implemented using Design-Expert® software, version 10.0 (Stat-Ease Inc., Minneapolis, USA). The concentrations of L-α-Phosphatidylcholine(X1), Cholesterol (X2), and dicetyl phosphate (X3) were selected as independent variables. The low (-1) and high (+1) coded levels for each factor were set based on preliminary screening experiments and the established literature on liposome formulation. The low (-1) and high (+1) levels for (X1) were 125 and 250, for (X2) were 50 and 100, and for (X3) were 12.5 and 25 respectively. Phospholipid concentration was selected as a primary variable because it is the primary structural component of the bilayer and directly governs vesicle size and encapsulation capacity. Cholesterol was included owing to its role in modulating bilayer fluidity and stability as well as its

well-documented interaction with lipophilic drugs such as TA. Dicetyl phosphate was incorporated as a negatively charged lipid to confer surface charge and electrostatic stability to the vesicular system. Vesicle size (VS, nm, Y1), Zeta potential (ZP, mV, Y2) and entrapment efficiency (EE, %, Y3) were selected as dependent variables. Polynomial equations were generated by multiple linear regression analysis to describe the quantitative relationship between the formulation variables and each response. Statistical significance was evaluated using ANOVA with p-value of <0.05 considered significant. Optimization criteria - formulations were ranked by numerical optimization targeting minimum vesicle size, maximum Entrapment Efficiency and zeta potential

Preparation of Drug-Loaded Liposomes

TAFA liposomes were prepared by the thin-film hydration technique (Khalil *et al*., 2020). Accurately weighed quantities of L-α-Phosphatidyl choline, Cholesterol and Dicetyl Phosphate and TA were dissolved in 30 mL of 2:1 carbinol: chloroform mixture in a round-bottom flask. The organic solvent was removed by rotary evaporation at 70°C under reduced pressure at 100 rpm until a thin, uniform lipid film was deposited on the inner wall of the flask. FA, as the hydrophilic drug was dissolved in Phosphate-Buffered Saline (PBS, pH 7.4) to prepare the hydrating medium. The dried lipid film was hydrated for 1 hr above the liquid crystal transition temperature of soy lecithin phospholipid, which is approximately 55-65°C with gentle rotation in rotary evaporator without applied vacuum. The resulting Multilamellar Vesicles (MLV) suspension was then allowed to equilibrate at 4°C overnight. The MLV suspension was subjected to Ultrasonication using Bath sonicator (RP 120 Ralsonics, Mumbai) for 15 min to reduce the vesicle size. The liposomal suspension was subsequently stored at 4°C for further analysis.

Characterization of Drug-Loaded Liposomes

Fourier Transform Infrared spectroscopy (FT-IR) analysis

FT-IR was used to assess the compatibility of the drug and excipients using KBr pellet technique. FTIR spectra of pure TA, pure FA, TA-FA (drug mixture) and TA-FA-excipients were analysed using FT-IR 8400 Shimadzu 240V, Japan. The medium infrared range of 4000 cm^{-1} - 400 cm^{-1}, with a resolution of 1 cm^{-1}, was typically used to get FTIR spectra.

Dynamic light scattering: Vesicle size, PDI and Zeta Potential

The mean vesicle size, PDI, and zeta potential of all liposomal formulations were determined by Dynamic light scattering (DLS) using Zeta sizer Nano ZS 90 (Malvern instruments, UK) at 25°C (Ansar and Mudalige, 2020). Each sample was appropriately diluted with deionised water (1:100) prior to analysis. The zeta potential of the liposomes was determined from electrophoretic mobility measurements (Cheng *et al*., 2019).

Entrapment Efficiency

The Entrapment Efficiency (EE%) of the liposomal dispersion loaded with TAFA was measured using centrifugation (Eppendorf centrifuge 5810 R) at 4000 rpm for 20 min at 4°C (Parsa *et al*., 2023). The amount of free drug was determined by measuring the supernatant's absorbance using UV-Vis spectroscopy at 241 nm for TA and 281 nm for FA against blank.

$$EE\ (\%) = [(\text{Total drug added} - \text{Free drug in supernatant}) / \text{Total drug added}] \times 100$$

Standard calibration curves for each drug were established over the linear range (r^2>0.999) before sample quantification.

Selection of Optimized Formulation

The optimal TAFA liposomal formulation was identified based on the desirability factor determined by Design Expert software version 10 (Stat-Ease, Minneapolis, USA). It was chosen based on its small vesicle size, high entrapment efficiency and stable zeta potential.

Surface Morphology Characterization

The sample of the optimized drug-loaded liposomal formation (F9) was placed on a glass slide and allowed to air dry. After the samples were encased in the gold, the surface morphology of the liposomes was evaluated using an electron microscope (SEM) operating at 2kV at magnifications ranging from 1000x to 10,000x (Karpuz *et al*., 2021). Scale bar was included on SEM micrograph for dimensional reference.

Physicochemical evaluation of the optimized liposomal eye drops

The physicochemical suitability of optimized formulation F9 was assessed by measuring pH, viscosity, density, osmolarity and surface tension, all conducted in triplicate (n=3) and expressed as mean ± SD.

pH of Optimized formulation

The pH was measured using a calibrated digital pH meter. (Kumar and Singh, 2018).

Viscosity of the Optimized formulation

The viscosity was determined using Brookfield viscometer (Model DV-E) with spindle 31 operating at 10 rpm and room temperature (25°C) (Kumar and Singh, 2018).

Density of the Optimized formulation

Density was measured using a calibrated pycnometer of known volume (Kumar and Kumar, 2023; Matušová *et al*., 2023).

Surface tension of Optimized formulation

Surface tension was evaluated using stalagmometer based on the drop count method (Kumar and Kumar, 2023).

Osmolarity of Optimized formulation

A theoretical calculation method was used to determine the osmolarity of the formulation (F9). This method calculates osmolarity based on the known concentrations of solute and their dissociation or ionization properties. Osmolarity was calculated using the following formula (Naplekov *et al*., 2020).

$$C_{osm} = \Sigma\ (mi \times ni / Mi) \times 100$$

C_{osm} is the solution's osmolarity (mOsm/L); m_i is the material content(g/L); n_i is the total number of ions that are generated when a single solute molecule dissociates; M_i is the Molar mass of the substance (g/mol).

In vitro drug release and kinetics

In vitro drug release was conducted using an open-ended cylinder method with 200 mL of PBS (pH 7.4) as the release medium maintained at 37±0.5°C. 1 mL of the liposomal formulation was placed in a cellophane membrane (MWCO 12-14 kDa) to study the release characteristics. At set of intervals (0.5, 0.45, 1, 2, 3, 4, 5, 6, 7 and 8 hr). 5 mL aliquots were taken and replaced with the equal volume of buffer to maintain sink conditions. The release of TA and FA was measured at 241 nm and 281 nm respectively; TA and FA were individually analysed from the co-loaded TAFA liposomes and calibration curve was used to calculate cumulative drug release. The release kinetics of the liposomal formulation (F9) and TAFA solution were fitted to various kinetic models, including Zero order, first-order, Higuchi, and Korsmeyer-Peppas models using DD solver software.

In vitro anti-inflammatory assessment

The *in vitro* anti-inflammatory activity of formulation (F9) was assessed using protein denaturation assay (Rahman *et al*., 2023). Different volumes (5-50 µL) of Formulation F9 (equivalent to 15-75 µg of drug) were mixed with 0.2 mL bovine serum albumin (200 µg) and diluted to 5 mL with simulated tear fluid (pH 7.4). Pure TA drug solutions were prepared at the same concentrations for comparison. Diclofenac sodium (10 mg) in distilled water was used as reference standard and blank liposomal dispersions (placebo) were used as controls. All mixtures were incubated at 37±0.2°C for 15 min, followed by heating for 5 min at 70°C to induce protein denaturation. After cooling, absorbance was measured at 660 nm using UV-vis spectrophotometer. The percentage reduction in protein denaturation was calculated to evaluate the anti-inflammatory effect of the liposomal formulation relative to the pure drug and reference standard.

$$\% \text{ Inhibition} = [(\text{Absorbance Control} - \text{Absorbance sample}) / \text{Absorbance control}] \times 100$$

Ex vivo permeation study

Freshly excised goat cornea obtained from a local slaughterhouse was rinsed with normal saline (0.9% NaCl) to remove blood and mucus, and visually inspected for any signs of physical damage. The cornea was mounted between donor and receptor compartments of Franz diffusion cell.1 mL of Formulation F9 were added to the donor compartment, and their permeability was compared with pure drug solutions which were tested separately. Simulated tear fluid (pH 7.4) at 37±0.2°C with continuous stirring at 100 rpm was used as the receptor medium. Samples were collected at intervals from 0.5 to 8 hr, and replaced with equal volume to maintain sink conditions. TA and FA concentrations was quantified using UV Spectroscopy at 241 nm and 281 nm respectively. The permeated drug concentration was calculated at various time points using the formula (Anbarasan *et al*., 2019):

$$\% \text{ Permeation} = [\text{Amount of drug permeated in Receptor compartment} / \text{Initial amount of drug in Donor compartment}] \times 100$$

The apparent permeability coefficient P_{app} was calculated using the equation:

$$P_{app} = (\Delta Q / \Delta t) / (A \times C_0) \times 60$$

where A is the diffusion surface area (cm^2), $\Delta Q / \Delta t$ (µg/min) is the rate of diffusion across the corneal tissue, and C_0 is the drug's initial concentration in the donor compartment. The conversion factor from minute to sec is 60 (Dave and Paliwal, 2014).

In vitro ex vivo correlation (IVEVC)

Logarithmic models were used to analyse the relationship between the *in vitro* drug release rate and the *ex vivo* transcorneal flow. The correlation between the two studies was determined by plotting the *ex vivo* permeation rate against the *in vitro* release rate (Bao *et al*., 2018).

Stability studies

The physicochemical stability of the optimized formulation F9 was evaluated under two storage conditions; ambient temperature 25±2°C and refrigerated conditions (2-8°C). At predetermined time points (0, 30, 60 and 90 days) samples were withdrawn and analysed in triplicate (*n*=3) for vesicle size, PDI and zeta potential.

RESULTS

FT-IR Spectroscopic Analysis

FT-IR studies confirmed the presence of characteristic functional groups of TA and FA. The drug-drug and drug-excipients mixtures retained principal peaks indicating absence of any interactions. This suggests that the drugs remain stable in presence of each other and the selected excipients.

Factorial Design Optimization: Statistical Analysis

A 2^3 full factorial design was used for the formulation optimization. To assess the effect of variables on responses, polynomial equation was generated using multiple linear regression. The final equations are:

- **Vesicle size (Y1)** = 200.50 +25.50*A
- **Entrapment efficiency (Y2)** =49.54+10.04*A-9.64*B
- **Zeta potential (Y3)** = -24.48 -7.87*C
- **A: Phospholipid B: Cholesterol C: Dicetyl phosphate**

The positive coefficient for phospholipid (A) for vesicle size indicates that higher phospholipid concentrations result in larger vesicles. For entrapment efficiency, the positive coefficient of (A) reflects increasing phospholipid concentration enhances drug entrapment whereas negative coefficient of cholesterol (B) indicates that at higher concentrations it hinders the entrapment of lipophilic drugs by filling the space within the bilayer, increasing its rigidity thereby reducing entrapment. The negative coefficient of dicetyl phosphate (C) for zeta potential indicates that increasing its concentration imparts a greater negative surface charge, improving colloidal stability.

The solution with the highest desirability score (0.911) by numerical optimization algorithm was selected.

Vesicle size, PDI, Zeta potential and Entrapment Efficiency

The vesicle size and Polydispersity Index (PDI) of formulations F1-F8 ranged from 156 to 241 nm with corresponding PDI values of 0.112 to 0.732. The Optimized formulation F9 exhibited size of 206 nm which is optimal for transcorneal drug delivery and PDI of 0.145, indicative of a monodisperse nanosized formulation. The zeta potential ranged from -10.5 to -32.8 mV and the formulation F9 exhibited a zeta potential of -32.35 mV with adequate colloidal stability as shown in Table 1.

The entrapment efficiency (%) ranged from 29.7±0.9% to 71.5±1.2% for TA and 39.7±1.5% to 86.5±3.3% for FA. Formulation F9 had an entrapment efficiency of 69.21±3.2% for TA and 78.6±1.4% for FA representing a satisfactory balance of encapsulation for both TA and FA.

Surface morphology

SEM analysis of the optimized liposomal formulation F9 (Figure 1) confirmed the successful formation of discrete particles with predominantly spherical morphology and relatively smooth surfaces.

pH

pH of the optimized formulation (F9) was found to be 6.33±1.3.The pH of ocular solutions should ideally range from 6.5 to 7.8 to ensure maximum comfort, matching the natural pH of lacrimal fluid. A pH that appears more acidic or alkaline might cause tearing,pain and discomfort (Garcia *et al.*, 2004). Tears have a strong buffering ability,they can quickly neutralize the pH of unbuffered eye drops, even if the pH ranges from 3.5 to 10.5 (Cooper and Gunn, 2008).

Viscosity

The optimized formulation (F9) had a viscosity of 98.2±0.9 $mPas^{-1}$ within the accepted ophthalmic viscosity range of 15-150 $mPas^{-1}$, Appropriate viscosity prolongs precorneal residence time (Baranowski *et al.*, 2014).

Density

The optimized Formulation (F9) had a density of 1.004 g/mL confirms that the formulation components do not substantially increase the density above that of water, a desirable characteristic for ophthalmic drops.

Table 1: Results of Vesicle size, PDI, Zeta potential, Entrapment Efficiency of TAFA loaded liposomes optimized by 2^3 Factorial design.

Formulation code	Vesicle Size (nm)	PDI	Zeta Potential (mV)	Entrapment Efficiency (%)	
				TA	FA
F1	241	0.732	-32.8	40.8±1.5	86.5±3.3
F2	217	0.133	-10.5	71.5±1.2	79.1±2.8
F3	208	0.124	-32.2	68.5±2.1	77.2±3.2
F4	186	0.112	-31.9	29.7±0.9	42.4±2.1
F5	156	0.137	-12.7	47.8±2.6	39.7±1.5
F6	238	0.162	-11.4	57.5±2.9	74.5±1.6
F7	175	0.56	-35.5	48.9±2.5	46.4±2.1
F8	183	0.254	-11.8	31.6±2.3	42.1±1.3
F9	206	0.145	-32.35	69.21±3.2	78.6±1.4

Data are expressed as mean ± SD (n=3).

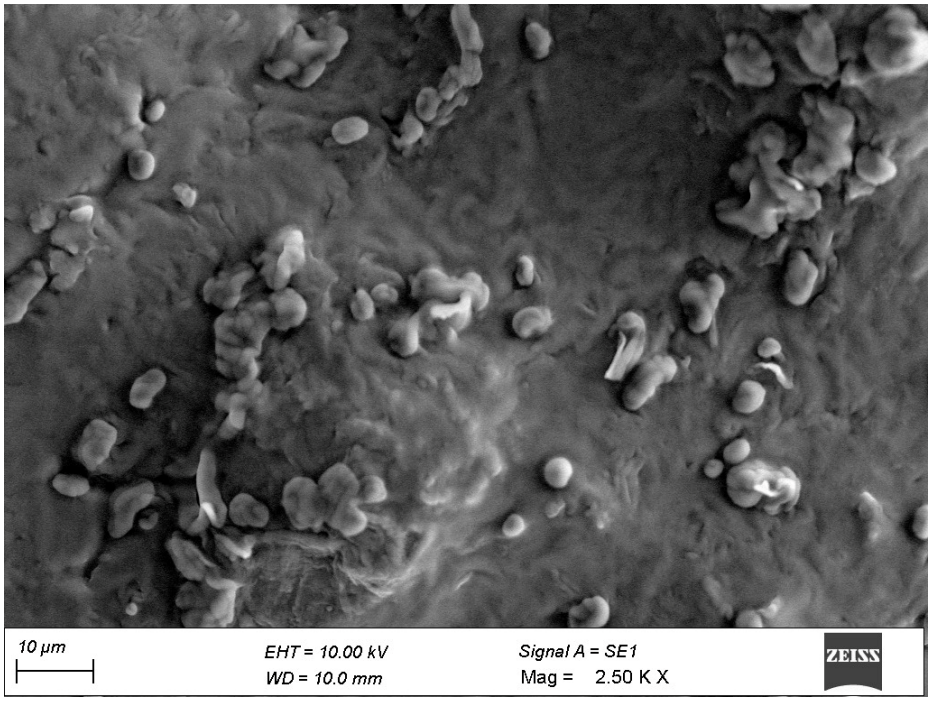


Figure 1: Representative Scanning Electron Micrograph (SEM) of the optimized liposomal formulation F9.

Table 2: Stability studies of optimised formulation F9.

Sl. No.	Duration	Vesicle Size (nm)		PDI		Zeta Potential (mV)		Entrapment Efficiency%			
								TA		FA	
		25°C	4°C	25°C	4°C	25°C	4°C	25°C	4°C	25°C	4°C
1.	0thday	206	-	0.145	-	-32.35	-	69.21	-	78.6	-
2.	30thday	225	208.3	0.235	1.47	-30.6	-32.1	45.6	68.4	64.5	78.2
3.	60thday	273.6	210	0.32	1.54	-22.3	-31.11	41.9	65.8	63.1	76.1
4.	90thday	289.9	214	0.332	1.75	-18.6	-29.8	41.5	65.3	58.5	74.3

Surface tension

The optimized formulation (F9) has a surface tension of 44.6±2 mN m^{-1} The surface tension of tear fluid in a healthy eye range from 43-46 mNm^{-1} at room temperature. Eye drops that have a surface tension of less than 35 mN m^{-1} cause pain and irritation. Moreover, the ophthalmic solution's surface tension is a critical element in determining the size of the eye drop (Van and Ludwig, 2004).

Osmolarity

Tear fluid osmolarity in healthy people is reported to be between 275-308 mOsm/L (Sollanek *et al.*, 2012). The optimized Formulation (F9) has an osmolarity of 282±2 mOsm/L, confirming isotonicity with tear fluid.

In vitro drug release and kinetics

The *in vitro* release of TA from the plain TAFA solution was as low as 8.1±2.4% at 8h. Conversely, FA from TAFA solution exhibited a burst release with over 93.60±2.3% released in the 1st 2 hr. In contrast, the liposomal Formulation (F9) showed significant improvement, with 98.5±2.5% of the TA released by the 8 hr. Liposomal FA showed a release of about 27.4±0.94% in the 1st 2 hr and 96.4±1.3% by the 8 hr.

TA liposomes (R^2=0.975) and FA liposomes (R^2=0.998) followed zero order kinetics indicating a steady and controlled release over time. By contrast, plain TA and FA solutions followed first-order kinetics (R^2=0.916 and 0.992) respectively.

The moderate Higuchi model fit (R^2 of 0.895 and 0.901 for TA and FA liposomes) supports role of diffusion through lipid matrix. The Korsmeyer-Peppas exponent values of n=0.701 for TA and n=0.888 for FA in liposomes indicates non-Fickian (anomalous) transport mechamism.

In vitro anti-inflammatory assay

The results of the anti-inflammatory study are shown in Figure 2. The anti-inflammatory potential of TAFA liposomes was assessed by measuring the percentage inhibition of protein denaturation. The TAFA liposomal formulation exhibited an inhibition range of 22.47±0.9% to 90.1±2.1%. This significantly exceeded the inhibition achieved by the plain TAFA solution (4.80±0.3% to 29.68±1.1%), and was also superior to the standard reference drug, Diclofenac sodium (14.42±1.3% to 76.5±1.9%).

Ex vivo Permeation study

The apparent permeability coefficients for the TAFA liposomal Formulation F9 were significantly greater than those of the plain drug solutions; liposomal TA were 0.126 cm/hr and 0.631 cm/hr for FA, compared to 0.0067 cm/hr for TA solution and 0.067 cm/hr for FA solution. These findings demonstrate that liposomal encapsulation promotes the transcorneal permeation of both TA and FA.

In vitro ex vivo correlation (IVEVC)

The drug penetration rate in the *ex vivo* model was slightly slower than the *in vitro* release rate due to the ocular barrier. Regression analysis of *in vitro* release vs *ex vivo* permeation yielded R^2 values of 0.981 for TA solution,0.883 for FA solution,0.998 for TA liposomes and 0.997 for FA liposomes.

Stability studies

The liposomes exhibited decreasing stability over 90 days at room temperature (25ºC) as indicated by increase in vesicle size and PDI, along with decrease in zeta potential and entrapment efficiency. In contrast, storage at 4ºC markedly attenuated physicochemical deterioration and stabilises liposomes (Table 2).

DISCUSSION

FT-IR analysis confirms no significant interactions between the drug and excipients, ensuring the stability of TA and FA in the formulation. Since the polynomial equations generated by multiple linear regression are statistically significant ($p<0.05$) the model effectively describes the relationship between the responses (vesicle size, entrapment efficiency and zeta potential) and the variables (phospholipid, cholesterol and dicetyl phosphate).

Optimized Formulation F9 has a vesicle size of 206 nm, ideal for penetrating the human corneal epithelial membrane and improving drug transport to the retina. It shows zeta potential of -32.35 mV, indicating good stability. Enhanced entrapment efficiency in F9 results from optimizing cholesterol and phospholipid levels. SEM Analysis confirmed the formation of vesicular structures in the optimized formulation.

The Formulation F9 demonstrated favourable physicochemical property, including appropriate pH, viscosity, density, surface tension and osmolarity all within the optimal range for ocular comfort and stability.

The *in vitro* release profile shows that nearly 40% of TA liposomes was released within the 1st 2 hr, followed by prolonged release. This extended release is recommended to minimize adverse effects. In contrast to the burst release of FA from the TAFA solution, liposomal FA demonstrates controlled release, maintaining steady drug levels over time. Overall, liposome encapsulation improves the release profiles of TA and FA compared to their free forms. The strong fit of TAFA liposomes to zero order kinetics indicates controlled release, maintaining stable drug levels and reduces dosing frequency. The moderate diffusion-based release in the Higuchi and Korsmeyer-Peppas models suggests a non-Fickian

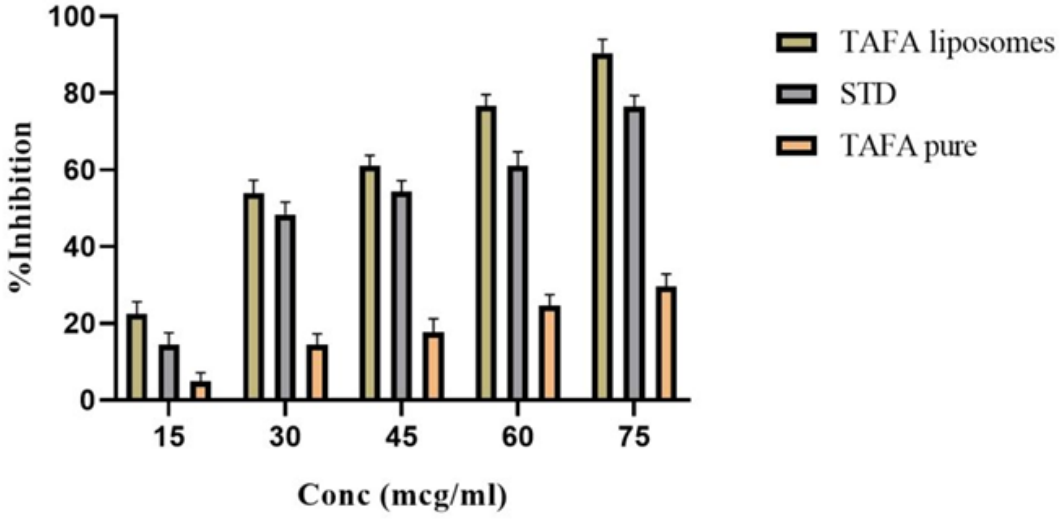


Figure 2: *In vitro* Protein denaturation Studies.

(anomalous) transport mechanism, indicating that drug release from liposomal formulations is governed by a combination of diffusion and polymer relaxation (erosion).

TAFA liposomes significantly reduce protein denaturation compared to pure TAFA solution or standard Diclofenac sodium. This suggests that the liposomal formulation may provide a safer and more effective anti-inflammatory treatment and does not facilitate further protein denaturation.

Ex vivo results confirmed that liposomes significantly enhance the transcorneal permeability of TA and FA. The IVEVC study showed no significant difference between *in vitro* release and *ex vivo* permeation for TA solution and Formulation F9. Storing liposomes at 4ºC improved stability, maintaining vesicle size, zeta potential and entrapment efficiency.

CONCLUSION

A novel co-loaded liposomal ocular formulation incorporating Triamcinolone Acetonide (TA) and Folic Acid (FA) was successfully developed and optimized using 2^3 full factorial design. The optimized Formulation (F9) exhibited an appropriate vesicle size, PDI, zeta potential and adequate entrapment efficiency, indicating that it is stable enough for ocular use. Surface tension, osmolarity, density, pH, and viscosity confirmed the formulation's suitability for ocular application. *In vitro* release studies indicated controlled release of both TA and FA, while *ex vivo* permeation studies confirmed enhanced ocular drug penetration, improving the formulation's therapeutic potential. Additionally, the formulation exhibited strong anti-inflammatory effects, positioning it as a promising option for improved outcomes in retinal diseases. The formulation retained acceptable physicochemical integrity over 90 days of storage at 4ºC. The principal novelty of this work lies in the simultaneous liposomal co-delivery of lipophilic corticosteroid (TA) and hydrophilic neuroprotective vitamin (FA) within a single topical platform as a potential non-invasive alternative to intravitreal injections in DR and DME. Further *in vivo* and clinical studies are required to confirm its efficacy and safety for practical application.

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ABBREVIATIONS

DR: Diabetic Retinopathy; **DME:** Diabetic Macular Eedema; **TA:** Triamcinolone Acetonide; **FA:** Folic Acid; **FT-IR:** Fourier Transform Infra-Red Spectroscopy; **EE:** Entrapment Efficiency; **TAFA:** Triamcinolone Acetonide with Folic Acid (co-loaded liposomes); **PBS:** Phosphate Buffer Saline; **PDI:** Poly Dispersity Index; **IVEVC:** *In vitro-Ex vivo* Correlation; **EE:** Entrapment Efficiency; **SEM:** Scanning Electron Microscopy.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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