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Original Article

Cubosome-based Corticosteroidal Drug Delivery System for Sustained Ocular Delivery: A Pharmacokinetic Investigation

Snehal Shashikant Chakorkar[1,2], Jameel Ahmed Sayed Gous Mulla[1,*]

[1]Department of Pharmaceutics, Shree Santkrupa College of Pharmacy, Ghogaon, Karad, Maharashtra, INDIA.
[2]Department of Pharmacology, Dr. D. Y. Patil Institute of Pharmaceutical Sciences and Research, Pimpri, Pune, Maharashtra, INDIA.

ABSTRACT

Background: Fluorometholone is an anti-inflammatory glucocorticoid. It has been used in various ocular inflammatory as well as infectious conditions. Opting for sustained release in ocular drug delivery is a favorable option for managing ocular diseases. To improve efficacy and to overcome side effects, fluorometholone was encapsulated in cubosomal vesicles. **Aim:** In this study, fluorometholone-loaded cubosomal vesicles were prepared using top-down techniques and applying the QbD approach. The optimized formulation releases the drug in a sustained release manner. **Materials and Methods:** The optimization of cubosomal vesicles was conducted using a 3^2-CDD. The independent parameter was selected: Concentration of both polymers Glyceryl monooleate (GMO) and Poloxamer 407 (P407), sonication time. The desired property for five important critical attributes of fluorometholone-loaded cubosome vesicles, namely % entrapment efficiency, Cumulative drug release, particle size, polydispersity index and Viscosity. **Results and Discussion:** The optimized formulation suggested by the central composite design was the concentration of GMO and P407; sonication times were 0.36 g, 0.46 g and 8 min, respectively. The optimized formulation exhibited % entrapment efficiency, % Cumulative drug release, particle size, polydispersity index and Viscosity were 82.89%, 88.33%, 137.7 µn, 0.22 and 169.3 m.Pas. The results confirm that implementing a QbD approach in cubosomal design leads to demonstrably improved formulation outcomes. The optimized batch was used for further evaluation like pH and Refractive index, Morphological feature evaluation, Release kinetics study, Test for sterility and stability and *in vivo* pharmacokinetic study. **Conclusion:** The present work confirms the improved ocular bioavailability of fluorometholone-loaded cubosomes.

Keywords: Fluorometholone, Cubosomes, Drug delivery system, Ocular delivery, Quality by Design, Central composite design.

Correspondence:
Dr. Jameel Ahmed S. Mulla
Professor and Head, Department of Pharmaceutics, Shree Santkrupa College of Pharmacy, Ghogaon, Karad, Satara-415111, Maharashtra, INDIA.
Email: jameelahmed5@gmail.com

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INTRODUCTION

Fluorometholone, a glucocorticoid, is frequently prescribed as an anti-inflammatory drug[1] for conditions like conjunctivitis and dry eye condition and post-operative ocular healing.[2] The glucocorticoid receptor mediates the fluorometholone action.

The glucocorticoid receptor belongs to the nuclear receptor subfamily and functions as a ligand-activated transcription factor.[3-5] Long-term usage of fluorometholone induces ocular hypertension. Elevated ocular pressure poses challenges in ocular outflow and causes alterations in the microstructure of the trabecular meshwork. This will increase visual outflow resistance and pressure. The commercial products of fluorometholone include ocular suspension five due to its limited aqueous solubility.[6]

Additionally, frequent administration of fluorometholone eye drops results in multiple adverse effects, including ocular irritation, low bioavailability,[6] inconvenience of administration[7] and long-term usage results in systemic absorption causing elevated intraocular pressure and hypercorticism.[8] At the same time, low aqueous solubility is a challenge in the development of formulation.[9] Nevertheless, due to the high potency and efficacy of fluorometholone, there is an ongoing necessity to investigate alternative dosage forms for widely underutilized glucocorticoids.Nano-formulated corticosteroids could provide discrete advantages, including improved penetration and retention,[10] reduced toxicity and prolonged release.[11] Over the past two decades, pharmaceutical scientists have shown significant interest in exploring vesicular carriers for the topical delivery of fluorometholone. Among the various colloidal carrier-based formulations, lipid-based formulations have gained widespread acceptance and approval from the FDA and other regulatory agencies globally for ocular drug delivery.[12,13]

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Cubosomes are considered liquid crystalline nanoparticles.[14] The penetration of cubosomes into the skin and mucosa is facilitated by the structural similarity between the cell membrane and lipid bilayer.[15,16] The biocompatibility and bioadhesive properties of cubosomes improve the sustainability and bioavailability of the incorporated drug.[17] Due to the entrapment of corticosteroids in cubosomes, ocular irritation is overcome and improves patient compliance.[18] Cubosomes can also protect the drugs from chemical and enzymatic degradation at an ocular site.[19]

Additionally, the polymers used for the formulation of cubosomes form twisted 3D structures with continuous hydrophilic and hydrophobic regions.[20] Consequently, cubosomes can increase the encapsulation efficiency of hydrophilic and lipophilic drugs.[21] Typically, while formulating cubosomes, a hydrophilic and lipophilic drug added in the lipid phase (Glyceryl monooleate) contains a surfactant (Poloxamer 407). Then, a mixture was added drop by drop with constant homogenization in the aqueous phase. This novel preparation method of cubosomes formulation is called the top-down method.[22] In our previous work, we reported the Effect of the preparative method on the physicochemical properties and bioavailability of optimized formulation. Moreover, we have observed the excellent therapeutic potential of cubosomes formulated by the top-down method.

Furthermore, there are reports detailing the ocular administration of nanoparticles loaded with fluorometholone. This paper delves into examining various critical process parameters and critical quality attributes utilizing the Quality by Design (QbD) approach. This will help to identify how these factors influence the optimization property of fluorometholone-loaded cubosomes. The fluorometholone-loaded cubosomal formulation was prepared using the top-down method. The cubosomal compositions were obtained by 3^2 Central Composite Designs (CCD). This approach is laborious and needs a clear cause-and-effect relationship between formulation variables and attributes. The present study uses a two-step statistical approach to develop fluorometholone-loaded cubosomes for ocular delivery systematically. In the initial step, crucial factors were identified from the process and compositional variables were also called the factor screening step.[23] The second step comprehensively examines predefined factors, including % Entrapment Efficiency, % Cumulative drug diffusion, particle size, polydispersity index and Viscosity.Moreover, employing the 'Design of Experiments' (DoE) as a statistical optimization approach offers a valuable opportunity to comprehend the impact and interactions of individual factors.This study can be performed with limited experiments without compromising the resulting quality. Lastly, the optimized formulation is further characterized for additional evaluation parameters, such as desired pH, Refractive index, structural evaluation and sterility. Additionally, *in vivo*, pharmacokinetic and *in vivo* corneal tolerance studies were performed on rat models. This helps to compare the Effect of plain fluorometholone solution with fluorometholone-loaded cubosomal vesicles on ocular bioavailability.

MATERIALS AND METHODS

Fluorometholone was procured from Festiva Pharma in Gujarat. Glyceryl Monoolein and Poloxamer 407 were graciously provided as gift samples by Mohini Organics and BASF, Mumbai, respectively. Porcine stomach mucin, ethanol, sodium bicarbonate, sodium chloride and other reagents were acquired from Sigma-Aldrich® Inc. in the USA. Dialysis membranes with molecular weight cut-offs of 12,000-14,000 were purchased from Sigma Aldrich in Darmstadt, Germany. All other chemicals and solvents were of analytical grade and used without additional purification. Deionized, distilled water was utilized for all purposes and animal organs were obtained from the slaughterhouse.

Screening Study of Cubosomal Dispersion

The screening study was conducted to check the compatibility of polymers and methods with the selected drug moiety. The literature review was conducted to determine the appropriate polymers for the formulation of cubosomes. Here, GMO is chosen as a lipid polymer and P407 as a surfactant. The pure drug (Fluorometholone), individual excipients (GMO and P407) and a mixture of the formulation components in a ratio of 1:1:1 (Drug: GMO: P407) were kept in a glass vial and stored at a temperature of 50°C and 60°C with 75% RH as per ICH guidelines for stress testing. A physical evaluation like appearance, FTIR and DSC studies are carried out on the 0^{th} and 15^{th} day to check the Effect of GMOs and P407 on the significant peak of the fluorometholone. The sufficient ratio of excipients affects the properties of cubosomal formulation. Based on the literature survey, the minimum and maximum polymer concentrations were selected and the QbD approach was applied. The formulation showing the optimum result was chosen for further evaluation.

Method of Fluorometholone loaded cubosome formulation

The method employed for the formulation of cubosome was the top-down method.[24] It is a very simple emulsification technique prescribed by Esposito *et al*. In this method, GMO and P407 were heated in the water bath at 60°C. Once the P407 is fully dissolved in GMO, a clear liquid gets formed and then fluorometholone and is stirred properly. This solution is called a lipoidal solution. On the other side, maintain water in ice-cold condition with a probe sonicator. Add a lipoidal solution drop by drop with a syringe in this aqueous solution and keep it for 15 min. Immediately after sonication, homogenizes the solution at 15,000 rev min at 60°C for up to 5 min. Sonication helps to form the vesicles, while homogenization helps to avoid the fusion of the formulated vesicles.The once-obtained solution looks white opaque without any aggregates stopping homogenization. Cool the cubosomal solution and store it in glass vials for further evaluation.

Optimization of fluorometholone-loaded cudosomal dispersion by DoE technique and Effect of variables for selection of best formulation.

Design-Expert® version 13 was used to generate the batches according to our goal. This software considers all responses at the same time.

To investigate the impact of specific variables on the formulation of fluorometholone-loaded cubosomes, a 3^2-Central Composite Design (CCD) was employed. The software proposed 20 formulations comprising eight factorial points, six axial points and six replicated center points.

The independent variables were examined at three levels and coded as (-1, 0, +1) while keeping all other formulation variables constant. The optimized formulation is strategically designed to exhibit minimal Particle Size (PS) and Polydispersity Index (PDI), maximum Entrapment Efficiency (%EE) and Cumulative Drug Diffusion (%CDD) (Table 1).

In vitro evaluation and responses for the dependent variables of the fluorometholone Cubosomes

After the optimization of formulation, the next step is its evaluation.The optimized formulation was evaluated for various evaluation parameters like % Entrapment Efficiency (%EE), *In vitro* drug diffusion or % Cumulative Drug Diffused (%CDD), Particle Size (PS), Polydispersity Index (PDI) and Viscosity. Based on the required parameters, as discussed earlier, the formulation was selected, prepared and used for further evaluation.

Determination of percentage Drug Content (%DC)

Formulated cubosomes taken 1 mL and diluted with methanol. Sonicate the solution for the breakdown of the vesicle for 30 min. Filter the solution and calculate the amount of fluorometholone present in methanol with the help of UV spectrophotometry. λ_{max} (nm) at 242 by using methanol as blank using the following formula;

% Drug content=Actual drug content/Theoretical drug content x100

Encapsulation Efficiency

Entrapment efficiency defines the amount of fluorometholone entrapped inside the vesicle.

Before the evaluation, rupturing the vesicle and removing the drug outside is necessary. To rupture the vesicles, centrifugation is carried out at 15000 rpm for 30 min. The supernatant liquid is collected, diluted with methanol and spectroscopically evaluated at 242°C. % EE is calculated using the following formula: All the determinations were carried out in triplicate.

$$EE\% = \frac{[Ct]-[Cf]}{[Ct]} \times 100$$

Where;

Ct=The UV absorbance at total concentration/Formulation drug content.

Cf =UV absorbance of filtrate after centrifuge was labeled as (namely, filtrate concentration).

Ex vivo drug diffusion/release study

The diffusion study of fluorometholone-loaded cubosomal vesicles was carried out using a bi-chambered Franz diffusion cell apparatus (Electrolab Diffusion Cell apparatus EDC-07). The freshly excised goat cornea was used as a diffusion membrane and simulated ocular fluid was used as the medium. The sample withdrawal at a predetermined time interval, i.e., 30, 60, 120, 180, 240, 300, 360, 420, 480, 540 and 600 min; sink condition is maintained. The graph plotted % CDD vs time of drug diffusion.

Determination of Particle Size (PS), Polydispersity Index (PDI), Zeta Potential (ZP) and viscosity

cubosomal dispersions were appropriately diluted with distilled water for the assessment of particle size, Polydispersity Index (PDI) and zeta potential of the prepared cubosomes. This analysis used advanced instrumentation like Malvern Zetasizer and particle size analyzer (Model ZEN3600, Malvern Instruments Ltd.,). The formulation's Viscosity was evaluated using Brookfield Viscometer (model DV2T Gel-Timer GT-2000).

Response surface analysis

The Design Expert® used for experimental design while 3^2 central composite designs were followed to generate batches. It helps to check design fitting. Several evaluation tests, including Analysis of Variance (ANOVA), F tests and correlation coefficients at a 95% significance level ($p<0.05$), were employed to confirm statistical significance and validate the results. The software compares the results and suggests the batches according to our inputs. We require batches with maximum entrapment efficiency, minimum particle size, zeta potential and PDI to fulfill all requirements of ocular drug delivery, according to the suggestion that the batch be prepared and further evaluated to confirm the predicted response.

Characterization of optimized cubosomal formulation

The optimized batch was evaluated for further parameters to achieve the parameters for ocular drug delivery of fluorometholone-loaded cubosomal vesicles. Various evaluation tests like pH, Refractive index, Mucoadhesive strength, TEM, sterility, Effect of terminal sterilization and short-term stability, *In vivo* pharmacokinetic study and *in vivo* corneal tolerance were carried out to check the *in vivo* compatibility of the optimized formulation.

Evaluation of pH and Refractive Index

The evaluation of pH was carried out with the help of a pH meter (Labindia PICO+). From each batch, 10 mL were collected and measured in the pH meter following the manufacturer's instructions.[25] The refractive index of the optimized formulation was determined at 25°C using Hilger and Watts refractometer (model-46.17/63707, Hilger and Watts Ltd.,). All the evaluations are carried out in triplicate.

Morphological feature evaluation

To reveal the morphological characteristics of fluorometholone-loaded cubosomes, they underwent analysis using Transmission Electron Microscopy (TEM). A small formulation volume was suitably diluted and the solution was adsorbed onto a carbon-coated copper grid. Excess dispersion was gently removed using filter paper and the sample was left to dry for 10 min at room temperature before examination under the Joel JEM 216 1400 TEM (Tokyo, Japan).

Release kinetics study

KinetDS Copyright (C) 2010 Aleksander Mendyk software was used to determine the release kinetics. The best-fit model was evaluated for all formulations by uploading all drug release data in Kinet DS software.

Test for sterility and stability

The optimized cubosomal formulation sterilized by gamma rays was further studied for sterility. As per the procedure mentioned in Indian pharmacopeia, the optimized formulation was kept for sterility testing for 14 days. The formulation was evaluated for turbidity or the presence of any clumps. Transfer a sample using aseptic precautions to a tube containing a suitable culture medium to immerse it completely. Incubate and carry out the Test.

In vivo pharmacokinetic study

Approval of protocol

The protocol in prescribed Proforma B for animal studies entitled 'Design, development and characterization of Corticosteroids loaded cubosomal vesicles for ocular delivery' was submitted on 15 December 2021 to IACE of Tatyasaheb Kore College of Pharmacy, Warananagar. IAEC approved the protocol in the presence of the CPCSEA nominee with Approval no. 1090/PO/Re/S/07/CPCSEA dated 29 January 2022 at Tatyasaheb Kore College of Pharmacy, Warananagar. The association for research in vision and ophthalmology generated guidelines that were followed while using animals for eye research. CPCSEA and institutional guidelines were followed while conducting all experimental procedures.

Selection and preparation of experimental Animals

The 24 male Wister rats (weighing 180 g-200 g) were used for pharmacokinetic evaluation of cubosomes and pure fluorometholone. Divide the animals into groups (A and B) containing 12 animals. Group A is administered with a plain fluorometholone solution called a standard group. Fluorometholone-loaded cubosomal vesicle was administered to the B group, called the test group-single dose-response design conducted for pharmacokinetic study. The route was selected in the inferior conjunctival sac of the right eye, where the left eye served as control and was treated with saline solution. After drug installation, the eye was held open for 20 seconds. This will provide adequate formulation- ocular surface contact. Due to excessive eye blinking, formulation loss takes place. After specified time intervals like 1, 2, 5, 10, 16 and 24 hr, two rats from each group were anesthetized by inhalation anesthesia and sacrificed by thoracic opening. By cardiac puncture of 3 mL, blood samples were withdrawn in a collection tube. The cornea was separated and kept for further evaluation. All collected samples were centrifuged for 10 min at 10,000 rpm. After centrifugation, the separated plasma was kept at 8°C for further analysis. Drug concentrations in plasma were evaluated with the help of HPLC analysis.[26,27]

Estimation of Pharmacokinetic Parameters

The non-compartmental method was followed for analysis and Pharmacokinetics (PK) evaluation. From the plasma concentration-time profile (AUC), estimation of C_{max}, T_{max}, biological half-life and elimination rate constant (Ke) in plasma and ocular tissue were evaluated.

In vivo corneal tolerance

A histopathological study was performed to verify whether the obtained optimized formulation produced any damage to corneal tissues. The usage of the control eye was implied for the blank cubosomal solution. At the end of the pharmacokinetic evaluation, the experiment means after 24 hr collecting the eye parts. Rinse those parts with a standard saline solution.

Following washing, the specimens were dehydrated with alcohol and preserved in a 10% v/v formaldehyde solution for 24 hr. Subsequently, the eye parts were immersed in melted paraffin wax and solid blocks were created after cooling. These blocks were sliced into cross-sections of 3-4 µm thickness and stained with hematoxylin and eosin. The stained sections were observed using a digital microscope (DMS1000 B; Leica, Cambridge, UK) for visualization.

RESULTS AND DISCUSSION

Screening Study of Cubosomal Dispersion by Top-down Approach

Many literature surveys were conducted to select the polymers and methods for preparing fluorometholone-loaded cubosomes. The minimum and maximum concentrations of polymer ratio were found in the literature survey. This data was added to the software and several batches were generated.

Optimization of fluorometholone-loaded cubosomal dispersion by DoE technique and Effect of variables for selection of best formulation.

The selected variables are lipid phase (GMO) concentration (X1), Poloxamer 407 concentration (X2) and Sonication time (X3) for optimization of fluorometholone-loaded cubosomes. These variables show various effects on each other: main effects, quadratic effects and interaction effects by 3^2 Central Composite Designs (CCD). By application of statistical parameters, the significance model is decided.

The experimental design matrix was structured according to 3^2 Central Composite Designs (CCD); 17 experiments were built to pre-screen the influence of three parameters at their lowest and highest factor levels. According to the above inputs, batches are suggested by evaluated software and their values are further added to the software. After adding values in the software, it will show various interactions and polynomial equations.

In vitro evaluation and responses for the dependent variablesof the fluorometholone Cubosomes

After the evaluation software generates an equation, an obtained equation explains the interaction of independent variables. The negative sign of any value from the equation says that decreased polymer concentration produces an inverse effect on a dependent variable.

The generated counterplots and surface response plots show a diagrammatic relationship between defined variables (Table 2). As shown in Figure 2, % EE, %CDD, Particle size, PDF and Viscosity are sharply influenced by a change in X1, X2 and X3.

Determination of drug content

Before the drug content, the calibration curve of fluorometholone was calculated. The calibration curve and UV spectrum of fluorometholone are mentioned in Figure 1. The drug content of fluorometholone-loaded cubosomes was evaluated by the UV-spectroscopic method at 242 nm. The lower drug content is due to adherence of the drug to the container or flask during the formulation. The lower % yield observed in the formulations may be attributed to a significant portion of the drug being lost on the flask's wall rather than being adsorbed on the surface of cubosomes.[28]

Model analysis of % Entrapment Efficiency

The % EE of fluorometholone-loaded cubosomes ranged from 98.80 (Formulation 9) to 68.35 (Formulation 11). The interacting equation of % entrapment efficiency was as below,

$$Y1\ (\text{Entrapment efficiency}) = 68.70 + 0.1843\ X_1 + 1.35\ X_2 - 5.87\ X_3 + 3.60\ X_1X_2 + 0.6487\ X_1X_3 - 2.93\ X_2X_3 + 10.17\ X12 + 5.19\ X_2^2 + 4.38\ X_3^2$$

Factors with a *p*-value less than 0.05 were deemed significant, while those with $p \geq 0.05$ were considered insignificant. From the above equation, it is clear that increasing polymer concentration leads to increased % entrapment.[29,30] Increased concentration of GMO improves bilayer characteristics of cubosomes and improves cubosomal stability. The good entrapment efficiency of fluorometholone is due to the low amount of fluorometholone, resulting in proper entrapment of the drug.[16]

Model analysis of cumulative drug release/Diffusion

As such, fluorometholone is a lipophilic derivative, so the problem of drug release is not a big deal. CDR (%) of fluorometholone-loaded cubosomes ranged from 98.94% (Formulation 9) to 71.22% (Formulation 11). Smaller particle size improves drug release from the membrane; hence, nano-sized cubosomal drug delivery has better permeability.

The generated equation is below;

$$Y_2\ (\%\ \text{Cumulative Drug Diffused}) = +99.25 - 14.43\ X_1 - 17.96\ X_2 - 0.75\ X_3 + 4.17\ X_1X_2 + 0.045\ X_1X_3 - 0.33\ X_2X_3 + 3.77\ X_1^2 - +18.39\ X_2^2 + 0.016\ X_3^2$$

From the equation, it is clear that an increased concentration of GMO resulting a decreased %CDD due to the larger particle size of cubosomes, resulting in a slower drug to be diffused.[31] The cubic phase is stable in contact with excess water; due to this property, we can encapsulate a large amount of drug in cubosomes. This will result in sustained drug action from the cubosomal system.[16] The drug release rate could be raised as the drug loading increases (% EE).

Model analysis of particle size

As we are designing nanoformulation, the particle size of the drug delivery system plays a crucial role. In the case of ocular drug delivery, if the particle size increases above the limit, it results in ocular irritation. The desired particle size of the formulation to penetrate the ocular membrane must be smaller than 200 nm.[16] The particle size of fluorometholone-loaded cubosomes ranged from 111.04 nm to 199 nm. All formulations are within the nanometric range. This technique is known to minimize ocular irritation when applied to the eye.

The software-generated equation is as follows;

$$Y_3 \text{ (Particle Size)} = 144.56 + 3.406\ X_1 + 9.333\ X_2 - 2.438\ X_3 + 0.278\ X_1X_2 + 0.0078\ X_1X_3 - 0.5492\ X_2X_3 + 0.3896\ X_1^2 - 3.481\ X_2^2 + 0.0489\ X_3^2$$

From the results, it is observed that increased concentration of both polymers resulted in increased particle size. An elevation in the percentage of GMOs from 5% to 10% results in a notable increase in the particle size of the cubic crystal nanoparticles.[31] This is due to the increased drug loading capacity of the formulation.[24] It has increased sonication time during the formulation of cubosomes, resulting in reduced particle size. High poloxamer 407 led to decreases in particle size[29] andparticles transition into the cubic state, accompanied by the formation of smaller particles.[17]

Model analysis of Polydispersity Index (PDI)

The PDI value provides information about the particle size distribution within the formulation. PDI ranges from 0 (indicating completely monodisperse particles) to 1 (suggesting highly polydispersed particles).[32] The obtained equation is below;

$$Y_4 \text{ (Entrapment efficiency)} = 0.239 + 0.020\ X_1 + 0.0428\ X_2 - 0.004\ X_3 - 0.005\ X_1X_2 - 0.0008 X_1X_3 + 0.0001\ X_2X_3 - 0.003\ X_1^2 - 0.0175\ X_2^2 + 0.0001\ X_3^2$$

The equation shows that high lipid concentration leads to cubosomal aggregation, which increases PDI.[33] The addition of P407 leads to decreased aggregation.[33] This will result in a stable cubosomal formulation. Increased sonication time resulting in uniform distribution.[34] So, optimum ratios of both polymers promote the stability of the vesicles.[34]

Model Analysis of Viscosity

The Viscosity of the formulation plays a crucial role in ocular drug delivery. Highly viscous formulations produce ocular irritation, while low viscous formulations are drained through tear fluid. The Viscosity of fluorometholone-loaded cubosomes ranged from 98 (Formulation 5) to 391(Formulation 20). After statistical calculations were generated, the equation is as follows;

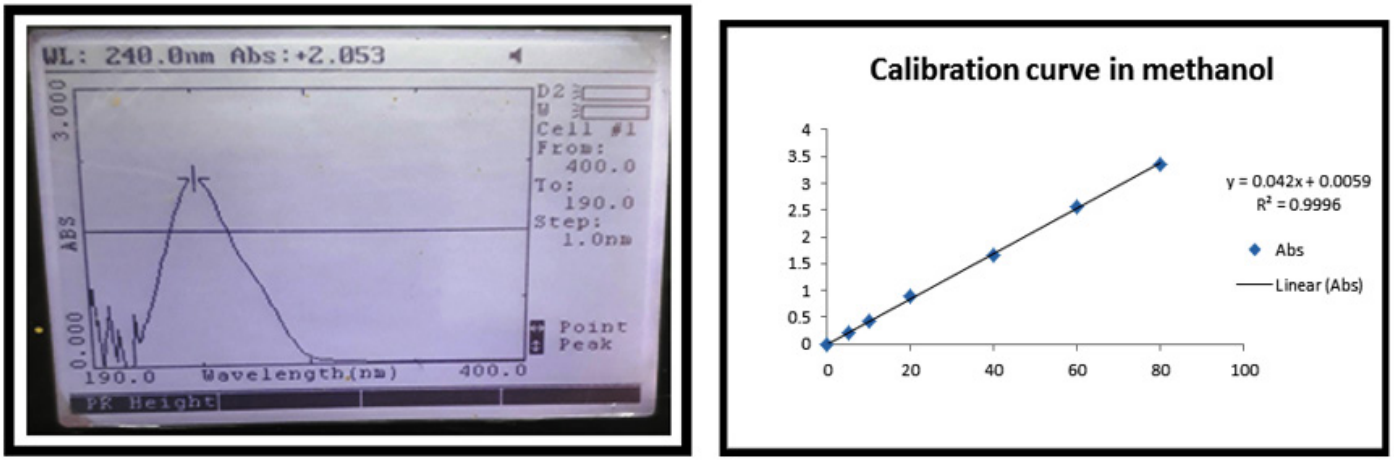

Figure 1: UV Visible spectra of fluorometholone showing λ_{max} at 240 nm and Calibration curve of Fluorometholone in methanol.

Table 1: Independent variables with their respective levels in the 3^2 Central Composite Designs (CCD) for fluorometholone-loaded cubosome preparation.

Factors (independent variables)	Levels		
	Low (-1)	Medium (0)	High (+1)
Lipid phase (GMO) concentration (X1) (%w/w).	0.1	0.65	3
Poloxamer 407 concentration (X2) (%w/w).	0.01	0.27	1
Sonication time (X3) (Min).	1	18.2	30
Independent variables	Dependent variables		
lipid phase (GMO) concentration (X1).	% Entrapment Efficiency (%EE) (Y1)		Maximize
	In vitro drug diffusion or % Cumulative Drug Diffused (%CDD) (Y2)		
Poloxamer 407 concentration (X2).	particle size (PS) (Y3)		Minimize
Sonication time (X3).	The polydispersity index (PDI) (Y4)		
	Viscosity (Y5)		

Table 2: Batches were generated in the 3^2-Central Composite Design (CCD) with coded and observed response values.

Formulation code	Coded Value			Actual Value			Observed response values				
	(X1) (g)	(X2) (g)	(X3) (Min)	(X1) (g)	(X2) (g)	(X3) (Min)	1 (Y1) %EE	2 (Y2) %CDD	3 (Y3) PS	4 (Y4) PDI	5 (Y5) Viscosity
F1	0	0	-1	1.55	0.505	0	92.65	95.17	199	0.39	-
F2	0	0	0	1.55	0.505	15.5	69.31	72.95	128.44	0.23	177
F3	-1	0	0	0	0.505	15.5	98.58	98.21	113.2	0.21	-
F4	-1	1	-1	0.1	1	1	93.74	96.75	144	0.25	143
F5	-1	-1	-1	0.1	0.01	1	93.47	96.3	134.7	0.22	98
F6	-1	1	1	0.1	1	30	73.87	78.43	112.33	0.33	122
F7	0	0	0	1.55	0.505	15.5	69.02	72.74	119.2	0.23	177
F8	1	0	0	3.98	0.505	15.5	98.62	98.35	143.2	0.22	102
F9	1	1	-1	3	1	1	98.8	98.94	153.8	0.25	158
F10	0	0	0	1.55	0.505	15.5	68.37	71.31	125.03	0.23	177
F11	0	0	0	1.55	0.505	15.5	68.35	71.22	125.03	0.23	177
F12	1	-1	0	1.55	0	15.5	98.58	98.21	113.2	0.21	-
F13	-1	-1	1	0.1	0.01	30	79.21	84.06	128.04	0.23	-
F14	0	-1	1	3	0.01	30	88.23	92.14	122.4	0.27	314
F15	1	1	1	3	1	30	81.47	86.16	132.06	0.21	191
F16	1	0	1	1.55	0.505	39.88	84.45	88.92	126.39	0.23	131
F17	0	1	0	1.55	1.337	15.5	71.75	75.91	111.04	0.27	115
F18	0	0	0	1.55	0.505	15.5	89.78	93.07	118.9	0.22	162
F19	0	0	0	1.55	0.505	15.5	68.37	93.07	125.03	0.23	177
F20	0	-1	-1	3	0.01	1	68.37	71.31	125.03	0.23	177

Table 3: Comparative analysis between the model constant and the obtained mean experimental outcomes in the center of the domain (*n*=5) with Checkpoint analysis of all responses.

Analysis	β0	Mean results	Predicted Mean	Predicted Median	Observed	StdDev	n	SE Pred	95% PI low	95% PI high
%EE	+68.70	+96.90193	80.76	80.76	82.89	2.24	1	2.45	75.27	86.24
%CDR	+75.45	+99.25332	85.47	85.47	88.33	6.49	1	7.11	69.62	101.31
Ps	+124.69	+144.56537	132.71	132.71	137.7	9.49	1	10.39	109.54	155.88
PDI	+0.2304	+0.239518	0.23	0.23	0.22	0.03	1	0.038	0.15	0.32
Viscosity	+180.88	+144.45984	163.58	163.58	169.3	62.88	1	69.63	8.42	318.74

$$Y_5 \text{ (viscosity)} = +144.459 + 55.635\ X_1 - 31.323\ X_2 + 3.542\ X_3 - 25.426\ X_1X_2 - -2.508\ X_1X_3 - 1.114\ X_2X_3$$

When we slightly change X1, X2 and X3, the Viscosity of the formulation is greatly affected. So, it was clear that the concentration of polymers and sonication time play a major role in the Viscosity of the formulation. Increased concentration of both polymers will increase the formulation's Viscosity.[35] Increasing the speed and time of the sonication decreases the Viscosity of the formulation.

Response surface analysis

While designing ocular drug delivery, several parameters must be considered. All critical parameters like particle size, PDI, Viscosity, drug release and % entrapment efficiency are set as the dependent variable. After evaluation and the addition of observation, we need to optimize the formulation. Here, we must design fluorometholone-loaded cubosomal vesicles to possess the smallest particle size, PDI and Viscosity with maximum % CDD and %EE. Design expert® software suggested several formulations with different combinations to get an optimized formulation, i.e.,

Table 4: Pharmacokinetic data for fluorometholone-loaded cubosomes in various tissues and plasma.

Sl. No.	Pharmacokinetic parameters	Fluorometholone-loaded cubosomal formulation	Pure Fluorometholone solution	Pharmacokinetic parameters	Fluorometholone-loaded cubosomal formulation
		Plasma	Cornea	Plasma	Cornea
1.	C_{max} (ng/ mL)	1	4	2	1
2.	T_{max} (h)	3	1	5	2
3.	AUC (ng/mL×hr) (0 -10 h)	18771	164241	42851	13536
4.	AUC (ng/mL×hr) (0 -∞ hr)	6887	326952	32260	8158
5.	Ke	5579	31	578	7498
6.	$t_{1/2}$ (hr)	26.63	6.97	12.92	4.23
7.	Clearance	1.45	3	3.1	1.23

*Calculated on AUC (0-∞ hr) with Pure Fluorometholone solution as a reference.**Calculated on AUC (0-∞ hr) with Fluorometholone Cubosomal formulation as standard.***Significant differences: $p<0.001$.

X1: Concentration of GMO, X2: Concentration of P407 and X3: Sonication time. The suggested formulation having maximum desirability (0.905) was selected for actual optimization purposes. The desired batch was formulated and evaluated the same as previous evaluation parameters like % EE, %CDD, Particle size, PDF and Viscosity. All actual responses of the optimized formulation compared with the predicted response confirm the validity of the optimized formulation. All final models exhibited sub-5% prediction error across dependent variables.

Additionally, the model achieved high precision and close agreement between adjusted and predicted R^2 values, supporting the validity and generalizability of its predictions within the inherent uncertainty of the data.[33] The desirability of the optimized model was achieved by placing qualitative and quantitative relationships between dependent and independent parameters. All factors are considered equally important for the calculation of each response. The desirability value D is selected equal to 1.00 to develop an optimized formulation. The coded values were obtained for each independent variable X1, X_2 and X_3 at 0.36 g, 0. 46 g and 8 min, respectively. This optimized batch was prepared and evaluated for further validation.

Predicted values for % Entrapment Efficiency, % Cumulative drug diffused, particle size, polydispersity index and Viscosity of optimized formulation and actual values obtained after checkpoint analysis are reported in Table 3. For each optimized formulation, observed and predicted values are close to each other. This will lower the percentage of bias and prove the excellent correlation between variables. The low bias confirms the validity and precision of the generated model.

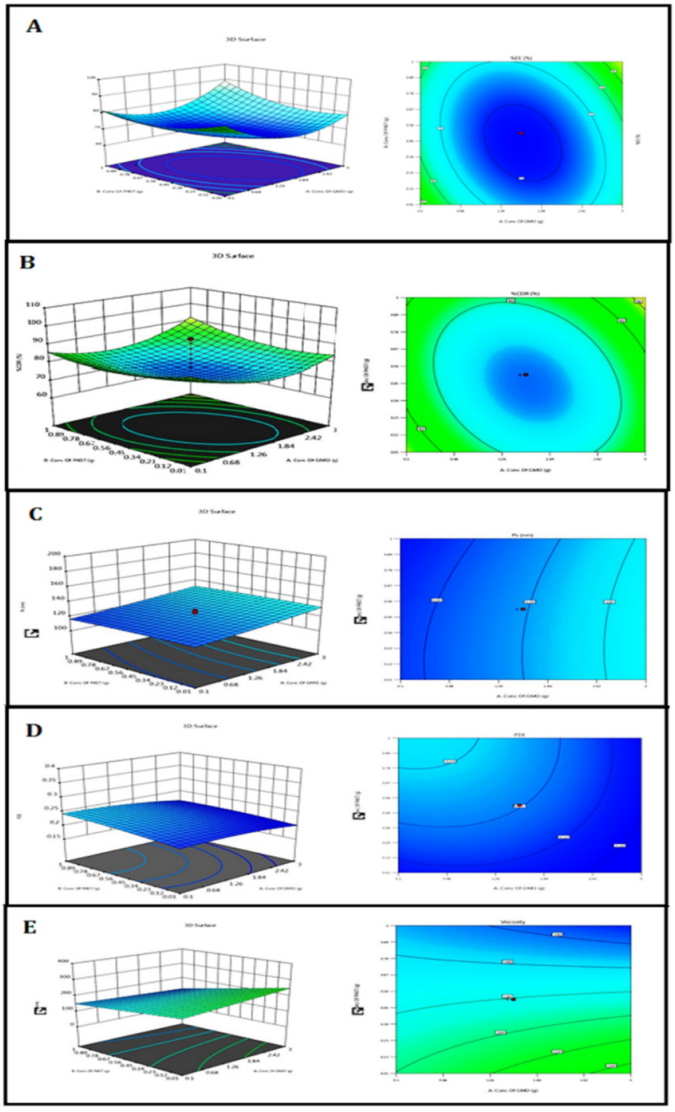

Figure 2: 3D and response plots showing effects of variables on A) % Entrapment Efficiency, B)% Cumulative drug diffused, C) particle size, D) polydispersity index and E) Viscosity.

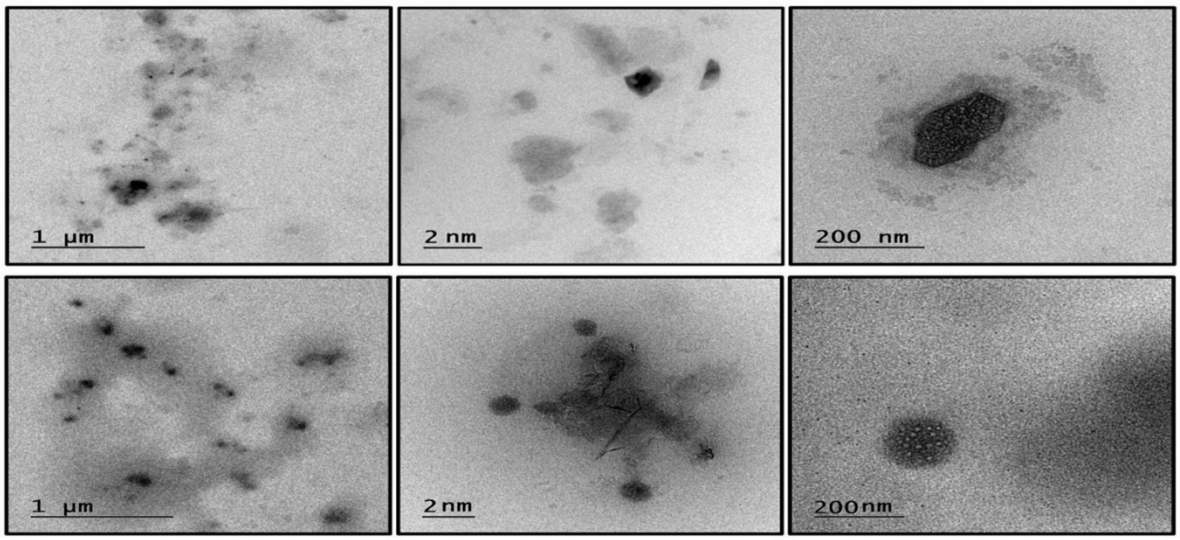

Figure 3: TEM image of optimized formulation.

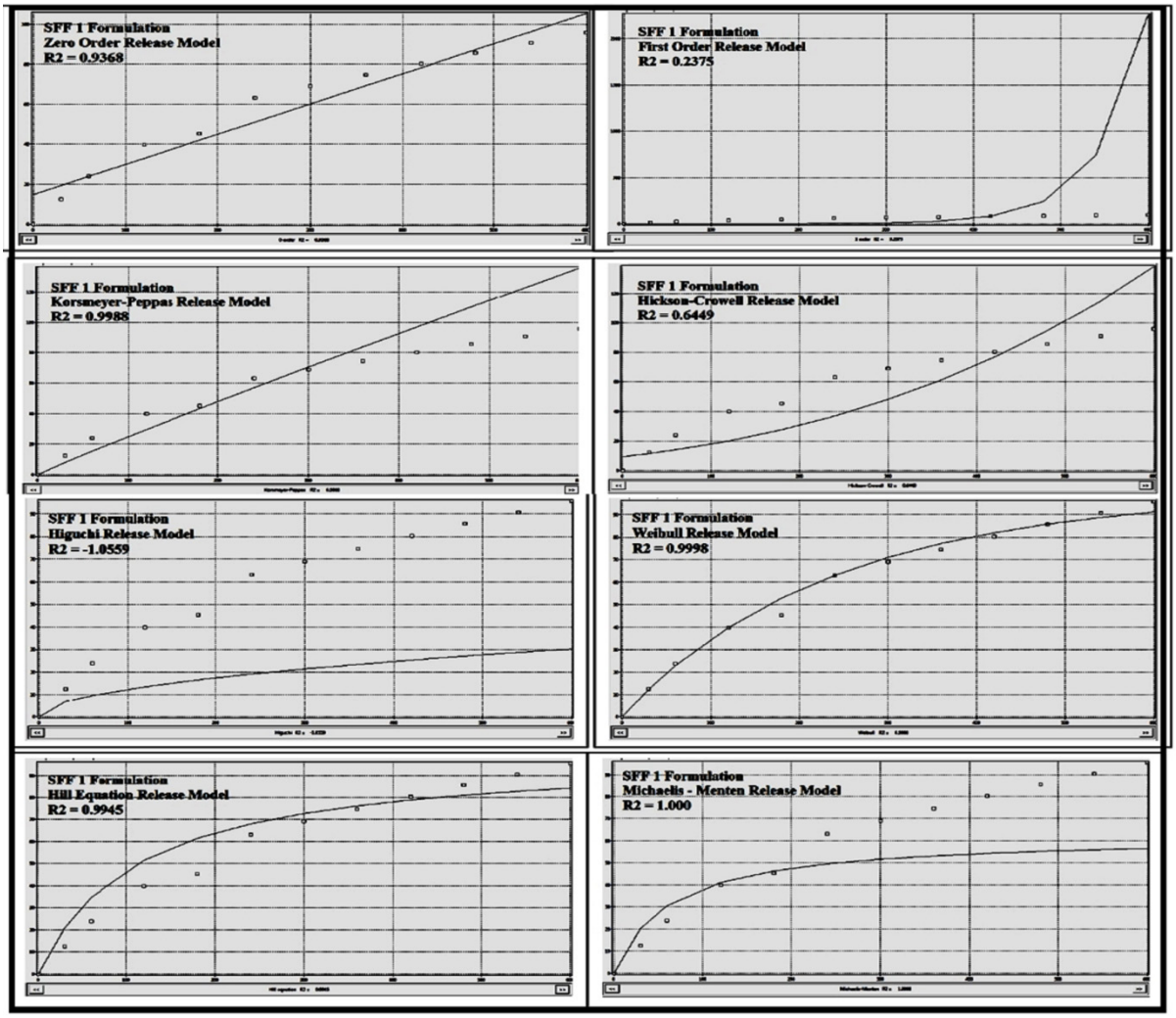

Figure 4: Release kinetic models of optimized formulation generated from KinetDS Copyright (C) 2010 Aleksander Mendyk software.

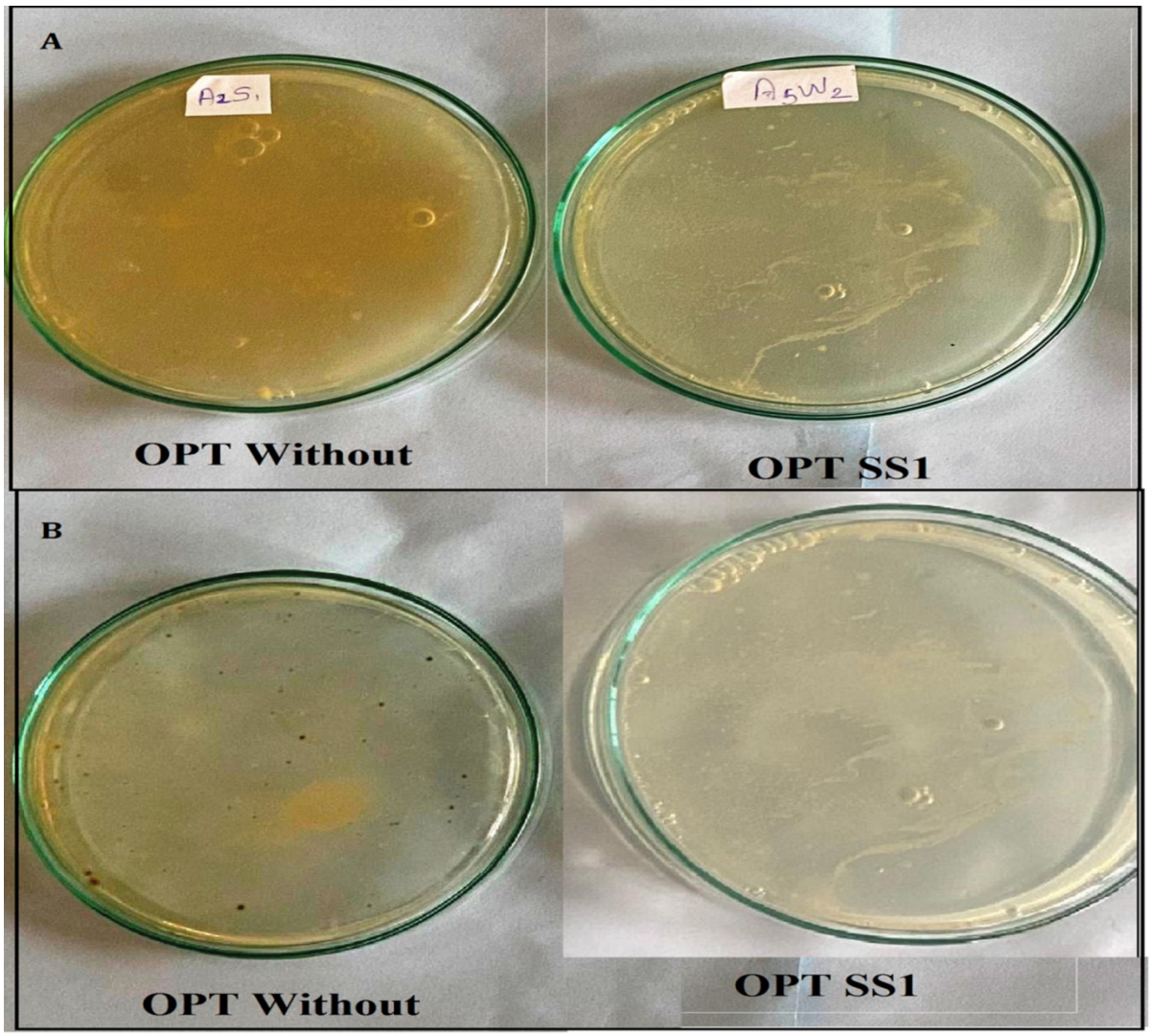

Figure 5: A: Formulations Before incubation for sterility testing. B: After 14 days incubation for sterility testing (OPT without Formulation without sterilization) OPT SS1: Optimized formulation after gamma irradiation sterilization).

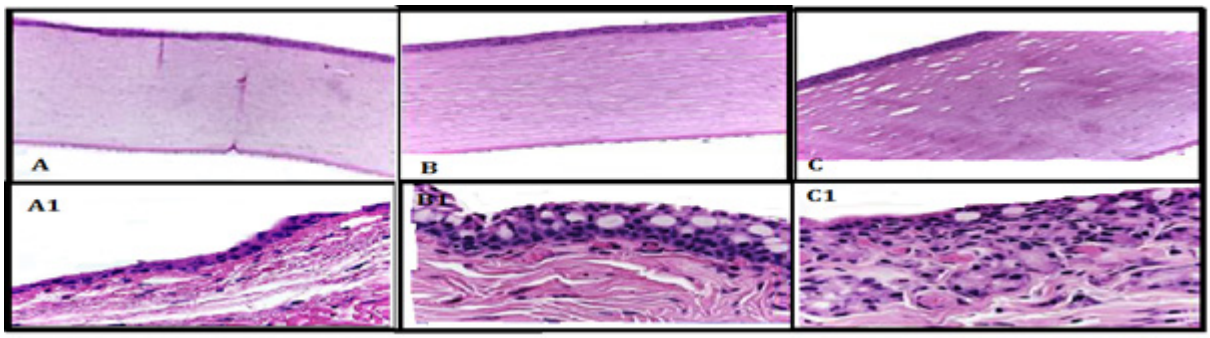

Figure 6: Histopathological examination at various sections A represents control ocular tissue; B: Fluorometholone loaded cubosomal formulation; C: Pain Fluorometholone solution.

Characterization of optimized cubosomal formulation

The fluorometholone-loaded optimized cubosomal formulations batch was evaluated for additional parameters like desired pH, Refractive index, sterility, *In vivo* pharmacokinetic study and *In vivo* corneal tolerance.

Evaluation of pH

The pH of the fluorometholone-loaded cubosome formulation was between 6±0.5. The ocular site can tolerate pH between 3.5-8.5. The formulation having a pH range above or above the specified range shows irritation in the ocular cavity. All of the polymers used to make cubosomes have a pH range of 5.5-7, resulting in the ocular stability of the cubosomal formulation.

Refractive index

The Refractive Index (RI) is essential in ocular dosage forms because its high values correlate with ocular irritation and blurred vision.[36] The RI of the optimized formulation was found to be 1.34±0.02, which is within an acceptable range. Any ocular formulation's standard range for RI is less than 1.5.[31]

Morphological feature evaluation

TEM evaluation was employed to examine the structure of optimized cubosomes. The obtained result is mentioned in Figure 3. TheTEM results confirm the uniform cubic structure of cubosomes. The cubosome's polyangular shape this result was in supports with Ali.[17]

Release kinetics study

The *in vitro* drug release of the optimized formulation was compared with various kinetic models to identify the most fitting model (Figure 4). The release kinetics pattern varies depending on the drug delivery system.[37] After comparing models, the model shows a good correlation coefficient ($R^2 \geq 0.9915$), which could be selected as the most suitable model. Comparing all formulations revealed that the optimized formulation showed the best Weibull release profile with $R^2=0.9998$. This result follows previously reported findings.[38,39] The variations in the release kinetics of nanoparticles are attributed to the physicochemical properties of the drug-polymer. These properties encompass the shape and size of the nanoparticle, its water absorption capability (swelling) and the extent and rate of degradation. Additionally, factors such as chemical composition, molecular weight, solubility and crystallinity of the material forming the nanoparticles, along with similar parameters for potential degradation products, contribute to these variations.[40]

The Weibull model, commonly employed in drug dissolution experiments, effectively captures the complexity of drug release from nanoparticles. It considers the multi-step process, encompassing Diffusion through the polymer matrix and mechanisms triggered by solvent action.[41] The model's "shape parameter" reflects the factors influencing the release profile.[38]

Test for sterility and stability

For the stability test, it was observed that after 14 days of incubation, no turbidity or clumps were observed. The formulation does not show any microorganism growth in the culture medium (Figure 5). Hence, gamma radiation is an effective method for nanoparticle sterilization.[42]

In vivo pharmacokinetic study

Estimation of Pharmacokinetic parameters of fluorometholone

The pharmacokinetic parameters of pure fluorometholone (standard solution) drug solution are compared with the fluorometholone-loaded cubosomal vesicle (test solution) (Table 4). The estimation of the drug in the plasma and corneal samples was done by the HPLC evaluation method. It was observed that group A, administered with plain fluorometholone solution called a standard group, shows the maximum amount of drug present in plasma. In contrast, a negligible amount of drug was observed in the corneal region.The case of group B, which receivesfluorometholone-loaded cubosomal vesicle (test solution), showed the absence of the drug in plasma.

In contrast, the maximum drug was present in the corneal region. It may happen due to cubosomes having mucoadhesive and sustained release properties, which result in adherence of the vesicle in the corneal region. This will result in the slow release of the drug at the site and local action. In the case of pure drug solution, fluorometholone has high permeation, resulting in the drug entering the plasma and showing less drug in the ocular region. However, the detailed mechanism of corneal penetration of cubosomal molecules needs further investigation.

This complete process resulted in precise results.

Histopathological examination

The histopathological process was performed to study formulation influence on corneal cell structure and tissue integrity. According to histopathological results, after 24 hr (Group A), the cross-section of the cornea shows continued and intact epithelial membrane (Figure 6). This will confirm the non-inflammation and non-irrational properties of the formulation. On the other hand, when we compare the histopaths of the control group and Group B, it was observed that the histopathology of Group B animals shows swollen epithelial cells. Some localized areas offer loss of the epithelial layer and ocular irritation.

According to the results of the histopathological report, the rats of group A instilled with fluorometholone-loaded cubosomal vesicles showed no inflammation and irritation, which confirms their ocular compatibility.

CONCLUSION

In the current study, we applied the quality by design, i.e., experimental design, to improve the quality of optimized formulation. The fluorometholone-loaded cubosomal vesicles were prepared using the QbD approach. The cubosomes were prepared using a top-down approach.The critical processing parameters for the preparation of cubosomes were evaluated. The % entrapment efficiency, % Cumulative drug release, particle size, polydispersity index and Viscosity were investigated to assess the Effect of critical process parameters. The risk assessment was carried out to identify several high-risk factors that influence various properties of the formulation. These findings will help to understand the interactions among all variables for getting appropriate experimental data. The optimized formulation was further evaluated for various parameters like pH and Refractive index, Morphological feature evaluation, Release kinetics study, Test for sterility and stability and *In vivo* pharmacokinetic study. The optimized batch shows cellular compatibility in the histopathological analysis.

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CONFLICT OF INTEREST

There authors declare that there is no conflict of interest.

ABBREVIATIONS

GMO: Glyceryl monooleate; **P407:** Poloxamer 407; **CCD:** Central composite design; **%EE:** % Entrapment Efficiency; **%CDD:** % Cumulative drug diffused; **PS:** Particle size; **PDI:** Polydispersity index; **ZP:** Zeta Potential; **DoE:** Design of experiments; **ANOVA:** Analysis of variance.

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