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Cubosomes; An Approach to Sustain and Improve the Ocular Delivery for Glaucoma Treatment: Box Benhken Optimization, Formulation, In Vitro Characterization and Ex Vivo Permeation Study

Dr. Shubhangi Aher[1] and Bharati Nimase[2]

[1]Assistant Professor, Department of Pharmaceutics, Bombay College of Pharmacy, INDIA

[2]Masters, Department of Pharmaceutics, Bombay College of Pharmacy, INDIA

[1]Corresponding Author: shubhangi.aher@bcp.edu.in

ORCiD

https://orcid.org/0000-0002-0993-5203

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ABSTRACT

Introduction: In glaucoma, acetazolamide (ACZ) is used to lower intraocular pressure (IOP). Low aqueous solubility and decreased corneal permeation are two characteristics of ACZ. This research intends to enhance the ocular delivery of ACZ.

Materials and Methods: Using a Box-Behnken design, cubosomes loaded with acetazolamide were made using the Melt dispersion emulsification and sonication method. The independent variables included Glyceryl monoolein (GMO), polyvinyl alcohol (PVA) and Poloxamer 407 (P407. The prepared formulations underwent evaluation for polydispersity index (PDI), particle size and entrapment efficiency. Thermal analysis, *in vitro* characterisation and permeation were among the additional tests performed on the developed formulation.

Results: Optimized formulation showed PDI of 0.23 ± 0.03, mean particle size of 243 ± 4.2 nm, zeta potential of -26.1 ± 0.6 mV, entrapment efficiency of 73.99% and cubic structure under TEM. Drug penetration through goat cornea has increased, according to an ex vivo permeation study. The prepared cubosome safety, stability and ability to be delivered through the cornea were confirmed by the ocular irritation test.

Conclusion: The optimized cubosomal formulation has the potential to improve glaucoma treatment and be regarded as promising for ocular delivery of ACZ.

Keywords- Acetazolamide, Glaucoma, Intraocular pressure, Cubosomes, Box-Behnken design, Transmission electron microscopy.

I. INTRODUCTION

Globally, glaucoma is a major health issue. It causes permanent blindness through the damage of retinal ganglion cells and increased intraocular pressure (IOP) (1). The primary objective of treating glaucoma is to lower intraocular pressure by either increasing the amount of aqueous humor that flows out of the eye or decreasing its production (2).

Controlled release systems and bioavailability enhancement methods are two categories of approaches that have been looked into for improving ocular drug delivery. Viscosity enhancers, penetration enhancers and prodrugs are some strategies for increasing bioavailability. The goal of controlled release systems like ocuserts, collagen shields and in situ gel is to maintain and regulate drug delivery. However, there are problems with self-insertion and patient non-compliance.

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Mucoadhesive particulates, phospholipid vesicles and non-ionic surfactant vesicles are examples of colloidal drug delivery systems (3).

Acetazolamide Carbon anhydrase (CA) diuretic inhibitor has a half-life of approximately 2.4 to 5.7 hours after a single oral dose (4). A recognized carbonic anhydrase inhibitor, acetazolamide (ACZ) is normally taken orally for the decrease of glaucoma patients' intraocular pressure (IOP). However, substantial amounts of Acetazolamide are requisite to achieve the anticipated reduction in intraocular pressure, typically results in side effects like diuresis and metabolic acidosis (5). The majority of patients stop taking their medication because they can't handle the side effects. As a result, scientists attempted to a high-concentration topical formulation for the delivery of ACZ, PVA, complexing and viscolizing agents entrapped in drug delivery carriers (6).

Surprisingly, neither the severity of the reduction in IOP nor the improvement in side effects were significantly improved by the complex. The limited solubility and poor corneal penetration of acetazolamide limit the topical application (7). Due to their biocompatibility and bioadhesive properties, cubosomes have received increasing attention as ocular nanosystem in recent years. Additionally, cubosomes are distinguished by their high loading capacity for various hydrophilic drugs (8). In addition, they are prepared using straightforward and inexpensive methods and assist controlled and targeted drug release, increase transcorneal permeability, enhance drug stability and extend corneal retention (9, 10). The dispersion of amphiphilic lipid molecules into a liquid crystals results in the unique structure of cubosomes (11). Their size ranges from 100 to 500 nm and have the same structure as honeycomb structures. It was chosen as a result of the formerly stated properties of cubosomes as a way to deliver ACZ because it is anticipated to improve both the drug's solubility and the main issues with ACZ preparation, such as diffusion and topical eye drops (12, 13).

The goal of this study is to encase ACZ within cubosomes, a nanosystem, to improve ocular delivery and efficiency. The aim is to increase drug penetration into the eye, improve bioavailability and reduce undesirable gastrointestinal tract (GIT) side effects by optimizing the dosage form.

II. MATERIALS AND METHODS

ACZ was provided by Nakoda Chemicals Ltd., Hyderabad. Gattefose India Pvt and BASF India limited generously provided Poloxamer 407 (P407) and glyceryl monoolein (GMO). Polyvinyl alcohol was received from S.D. Fine Chem. Ltd, Mumbai, India. Propylene Glycol and Transcutol P were obtained from Gattefose India Pvt. Ltd. Tween 80 was obtained from Loba Chemie Pvt. Ltd, Mumbai.

Methods

Formulation of ACZ loaded cubosomes formulations:

The methods of melt dispersion, emulsification and sonication were used to fabricate ACZ-loaded cubosomes. Using this procedure to formulate the preparation at the lab size is simple and convenient. The lipid phase, which was made up of different ratios of Poloxamer 407 and Glyceryl monooleate (GMO), was melted at 60°C. After that, 1% w/v ACZ was added to the liquefied lipid phase, where it was mixed as it became dissolved. After that, miliQ-water was used to dissolve polyvinyl alcohol (PVA), which was then mixed into the molten mixture at 800 rpm and heated to the same temperature. The resulting dispersions were continuously swirled at room temperature for two hours and thirty minutes. After that, the size of the coarse dispersions was reduced by five minutes of probe sonication. In glass vials, the formulation was kept at room temperature after cooling (14).

Experimental Design

Box-Behnken design was carried out with the help of Design-Expert® software (Version 13, Stat-Ease Inc., MN, and USA) to study how various variables of formulation affected the cubosome formulations that were produced. The main independent variables are Glyceryl monoolein (GMO) (with a range of 2.5 to 7.5% w/w), Poloxamer 407 (P407) (with a range of 0.25 to 0.75% w/w), and Polyvinyl alcohol (PVA) (with a range of 30 to 90 mg). The dependent variables, on the other hand, are Particle size (PS) (nm), polydispersity index and entrapment efficiency (15).

Evaluation of ACZ-loaded cubosomes

Particle size, polydispersity index:

Particle analyzer was used to measure particle size and polydispersity index after dilution with miliQ water. These characteristics reveal details about the vesicles' size, homogeneity and stability (16).

Entrapment efficiency:

The ultra-centrifuge filtering method, an indirect method, was used to measure entrapment efficiency. The formulation of ACZ-loaded cubosomes was made and centrifuged for 45 minutes at 4°C and 80000 rpm (Optima TM MAX-XP, Beckmann Coulter, and USA). The supernatant was then subjected to spectrophotometric analysis at 264 nm, using a UV spectrophotometer from Shimadzu, USA, to measure the quantity of free (non-entrapped) ACZ (17). The following formula used to calculate the entrapment efficiency:

$$\textbf{Entrapment efficiency} = \frac{\textbf{Total drug} - \textbf{free dug}}{\textbf{Total drug}} \textbf{X100}$$

Characterization of optimized ACZ loaded cubosomes

Zeta potential analysis:

At 25°C, the zeta potential of cubosomes was evaluated with a zeta-sizer called Nano ZS. Samples were diluted and placed in 1.5 milliliters of filtered miliQ water in a capillary cell for the zeta potential measurements.

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After that, the capillary cell was put into a chamber with a thermostat.

Evaluation of isotonicity:

Because it prevents eye tissue damage or irritation, isotonicity is an essential component of ophthalmic formulations.

The optimized formulations were examined under a microscope at a magnification of less than 45 to check their isotonicity after being mixed with a small amount of blood. Control solutions including hypertonic NaCl 2 percent, isotonic NaCl 0.9 percent and hypotonic NaCl 0.2 percent were compared to the shape of blood cells. The integrity of blood cells is maintained by isotonic solutions, whereas hypertonic solutions cause cell shrinkage and hypotonic solutions cause cell bulging (18).

Fourier transform infrared (FTIR) spectroscopy analysis:

Cubosomes containing ACZ, GMO, Poloxamer 407, PVA, and GMO were loaded onto an FTIR spectrophotometer (Shimadzu 43000, Kyoto, Japan) and their FTIR spectra were recorded. The FTIR spectra scans for each sample were recorded at a resolution of two centimeters, and they covered the range of 450–4000 cm1 after each sample was placed in the sample holder (19).

Differential scanning calorimetry (DSC):

The DSC STARE system, manufactured by Mettler Toledo in Switzerland, was used to measure the temperature of ACZ and ACZ-loaded cubosomes. Samples in precisely calibrated amounts (10 mg) were put into aluminium containers and sealed tightly. Every sample was exposed to a temperature increase of 10°C per minute, all while being in a nitrogen environment, and falling between 30 and 300°C (20).

Transmission electron microscopy (TEM):

The shape and surface characteristics of ACZ-loaded cubosomes were examined with a high-resolution transmission electron microscope (Jeol, Jem 2100 plus). A 300 mesh carbon-coated grid with a small amount of sample (2-10 l) was used for the analysis and allowed to air dry for ten minutes. After the grid had completely dried, negative staining with 2% phosphotungstic acid was applied to it for two minutes and filter paper was used to get rid of any excess solution. The sample was then examined, and images were taken at various magnifications (21).

In-vitro release study:

Using a dialysis membrane, the in vitro release study of cubosomes was carried out at temperatures ranging from 32 to 34 °C. Before use, the dialysis membrane was hydrated for twelve hours in STF (simulated tear fluid). A 50 mL beaker containing a release medium was and STF at a pH of 7.4 used for the release study. 1 mL samples were taken at predetermined intervals and to ensure sink conditions, same volume fresh medium were replace the release medium, and 0.45-mm pore filter was used to filter the samples. After being diluted appropriately, analysed for drug content using a UV visible spectrophotometer at 269 nm (22).

Ex-vivo corneal permeation study:

The permeability of ACZ was measured with membranes from goat corneas. The corneas were removed from the eyeballs, leaving behind approximately 3–4 millimeters of scleral tissue. For preservation, the corneas were immersed in freshly prepared simulated tear fluid (STF) at pH 7.4 after being rinsed with cold saline solution. The experimental setup made use of Franz diffusion cells' donor and receptor compartments. The release medium temperature was maintained by the receptor compartment, which was encased in a water jacket. To guarantee direct contact between the cornea and the donor medium, a section of corneal tissue was placed between the compartments. Freshly prepared STF filled the receiver compartment in the lower chamber. A water jacket was used to keep the release medium at 32-34 °C and constantly stirred at 25 rpm. To maintain sink conditions, 1 mL aliquots were removed at intervals of up to 8 hours and replaced with an equal volume of fresh STF at pH 7.4 containing 0.5% sodium lauryl sulfate (SLS). The samples were then examined with UV spectroscopy at 269 nm wavelength. (23).

Study of corneal retention:

Similar to the previously documented method, acetazolamide corneal (goat cornea) retention from produced formulation and commercialized eye drops was carried out (24). The cornea was first placed between compartments and it was given 15 minutes to stabilize with artificial tear fluid that had been preheated to 32 degrees Celsius. In place of the donor chamber's STF, 100 milliliters of commercial eye drops and NPs suspension in STF were used. After eight hours, the exposed cornea of the study was taken out and thoroughly cleaned with STF. Before being extracted in methanol, the same volume of STF was used to homogenize this cornea as well. The homogenate was centrifuged for ten minutes at 10,000 rpm after being obtained. The filtered supernatant was quantitatively analyzed at absorption maxima (max) using the membrane filter (0.45).

Gamma sterilization's effect:

Gamma radiation from a 60Co irradiator (A V PROCESSORS (P) LTD) was used to terminally sterilize the improved formula. The radiation dose was 10 kGy. The drug content, particle size, PDI, zeta potential and entrapment efficiency were compared before and after the sterilization procedure (25).

IP sterility test:

The aseptic collection of samples from the containers is the first step in the sterility testing procedure for ocular eye drop formulations. Direct inoculation is used to collect samples for suspensions, whereas membrane filtration is used to collect samples for liquid formulations. After that, these samples are cultured for at least 14 days on appropriate sterile media. During the incubation period, microbial growth observations are recorded. The sample is deemed to have passed the test if no microbial growth is observed within this time frame (26).

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The Chorioallantoic Membrane (HET-CAM) Hen's Egg Test:

In place of the in vivo Draize rabbit eye test, the HET-CAM test provides in vitro method for determining formulations' ocular irritancy. The chorioallantoic membrane (CAM) of fertilized hen eggs is examined for negative changes following exposure to the test substances in this test. Freshly procured fertilized hen eggs weighing between 50 and 60 g are incubated for nine days at 37 0.5 °C, rotating manually every 12 hours. After removing the inner membrane, a portion of the eggshell above the air cell is carefully removed on day ten, exposing the vascular CAM. After that, the test formulation, 1N NaOH as the positive control, 0.2 mL of 0.9% NaCl as the negative control, and 0.2 mL of 0.9% NaCl as the positive control are directly injected into each egg's CAM membrane, where they remain in contact for five minutes. After that, the membrane is checked for signs of coagulation and haemorrhage, and irritancy is checked at specific intervals. The irritancy of positive and negative controls is compared to that of formulation-treated eggs to conclude the irritancy test (27).

Study of stability:

The newly developed formulation underwent stability studies in accordance with ICH Q1A (R2) to evaluate its long-term drug stability, potential drug leakage, formulation integrity, and interactions with excipients. The Acetazolamide (ACZ)-loaded Cubosomes were kept for three months in tightly sealed glass vials at four degrees Celsius and 75% relative humidity (RH), twenty-two degrees Celsius and 65% RH, and forty-two degrees Celsius and 75% RH, respectively. Changes in the formulation's particle size, zeta potential, polydispersity index (PDI) and drug content over the course of the three-month study were used to evaluate its stability and integrity (28).

III. RESULTS AND DISCUSSION

Statistical evaluation of the experimental design:

The ACZ-loaded cubosomal formulation was improved using the Box-Behnken design (BBD), and the entrapment efficiency concentrations of glyceryl monooleate (GMO), lipid phase stabilizer, and aqueous phase stabilizer were used to evaluate the impact of independent factors on the dependent responses. The experimental design resulted in the production of 15 batches, each with three center points. As shown in Table No. 2, these experiments yielded measured values for entrapment efficiency ranging from 46.28 to 73.99%, PDI ranging from 0.102 to 0.272 and particle size ranging from 195.68 to 244.4 nm. The impact of independent factors on dependent responses was then investigated through statistical analysis using ANOVA and response surface analysis. The fit statistics results, presented in Table 3, demonstrated satisfactory R^2, adjusted R^2, and predicted R^2 values. 3D surface plots and Polynomial equations were utilized to understand the relation between dependent responses and independent factors (29).

Effect of independent factors on response particle size:

The particle size was falling in the range of 195.68 to 244.4 nm in all 15 batches.

Mean particle size = +175.24-14.94A+2.45B+20.67C+1.78AB-24.85AC+21.7BC (1)

According to the polynomial equation (Eq. 1) and the 3D response plot (Figure 1), it was evident that polyvinyl alcohol and Poloxamer 407 had a positive impact, while the cubosome vesicle's particle size was reduced by Glyceryl monooleate. Specifically, the results indicated that an increase in polyvinyl alcohol concentration could result in nanoparticle aggregation and increase in particle size. Conversely, increasing concentrations of GMO and P407 was associated with a decrease in cubosome accumulation and a reduction in cubosome size (30).

Effect of independent factors on response Y_2 (polydispersity index (PDI)):

The polydispersity index in all 15 formulations was falling in range of 0.102 to 0.272.

Polydispersity index = 0.343-0.012A+0.02B+0.048C-0.06AB+0.044AC-6.042BC (2)

According to polynomial equation (2), both Poloxamer 407 and polyvinyl alcohol concentrations had a positive effect. Increased concentrations of these components led to higher polydispersity index values due to the formation of varied structures and self-aggregation within dispersions, resulting in heterogeneity. Conversely, higher concentrations of Glyceryl monooleate resulted in decreased polydispersity index values by forming a lipid coat around polymeric particles. This inhibited aggregation and promoted uniformity in particle size distribution (31).

Effect of independent factors on response Y_3 (entrapment efficiency):

The entrapment efficiency was found to be between 46.28 and 73.99% in all 15 experimental runs.

Entrapment efficiency = +843.41+298.15A-8403.B+321.45C-251.58AB-501.9AC+106.41B (3)

The 3D response plot (Figure 3) and the polynomial equation (3) revealed that the concentrations of PVA and GMO had a beneficial effect. Glyceryl monooleate contributed to the formation of a denser lipid coat around the polymeric core, thereby hindering drug leakage. Meanwhile, polyvinyl alcohol increased dispersion viscosity, enhancing nanoparticle rigidity and reducing drug leakage during ultracentrifugation. Conversely, increasing Poloxamer 407 concentration had a negative impact on entrapment efficiency. The decreased capacity of the cubic structure to retain the drug as a result of increased hydrophilicity may be to blame, allowing the drug to escape into the surrounding aqueous environment (32).

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Table 2: Variables used and observed responses from randomized runs in the BBD.

Run	Factor1	Factor2	Factor3	Response1 nm	Response2	Response3 (%)
1	-1	-1	0	223.2	0.165	53.42
2	+1	0	-1	234.7	0.244	71.19
3	0	0	0	217.12	0.184	62.68
4	-1	0	+1	207.04	0.137	46.28
5	-1	0	-1	198.31	0.126	50.75
6	0	0	0	218.11	0.165	60.74
7	+1	+1	0	213.5	0.203	70.21
8	+1	-1	0	244.4	0.231	73.99
9	+1	0	+1	226.5	0.272	69.53
10	-1	+1	0	195.68	0.102	47.66
11	0	-1	-1	233.8	0.208	65.30
12	0	+1	-1	211.5	0.138	61.43
13	0	-1	+1	238.2	0.222	58.27
14	0	+1	+1	206.8	0.156	56.29
15	0	0	0	220.5	0.187	62.25

Table 3. Regression analysis summary for the response.

Response	Model	p-value	Lack of fit	Adjusted R^2	Predicted R^2	Adequate precision
Particle size	Quadratic	Significant	Non-significant	0.9759	0.9676	19.34
PDI	Linear	Significant	Non-significant	0.8901	0.8275	11.032
%EE	Linear	Significant	Non-significant	0.9756	0.9641	14.82

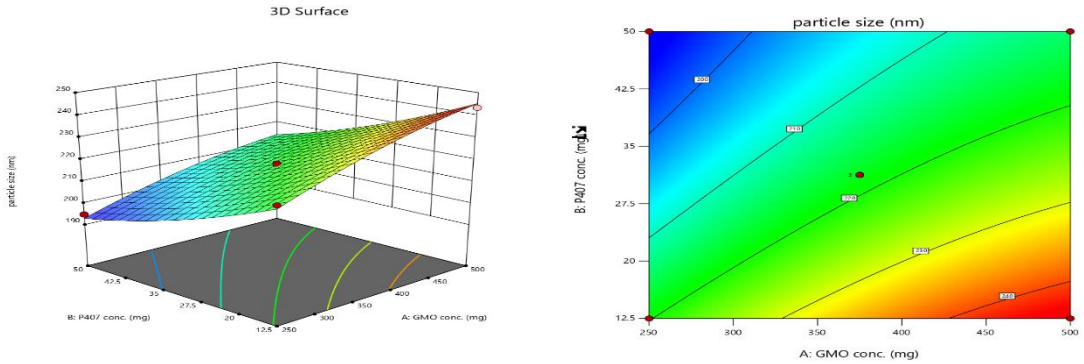

Figure 1: Response plots of particle size

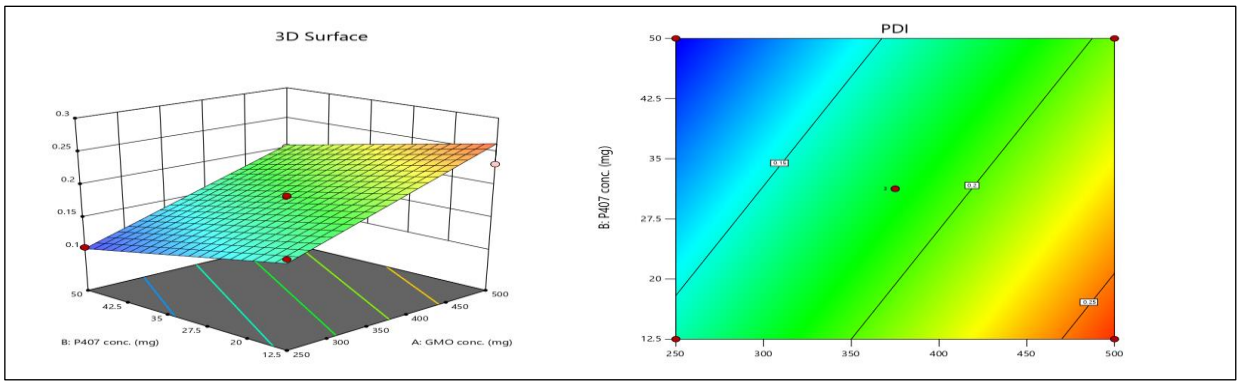

Figure 2: Response plots of PDI

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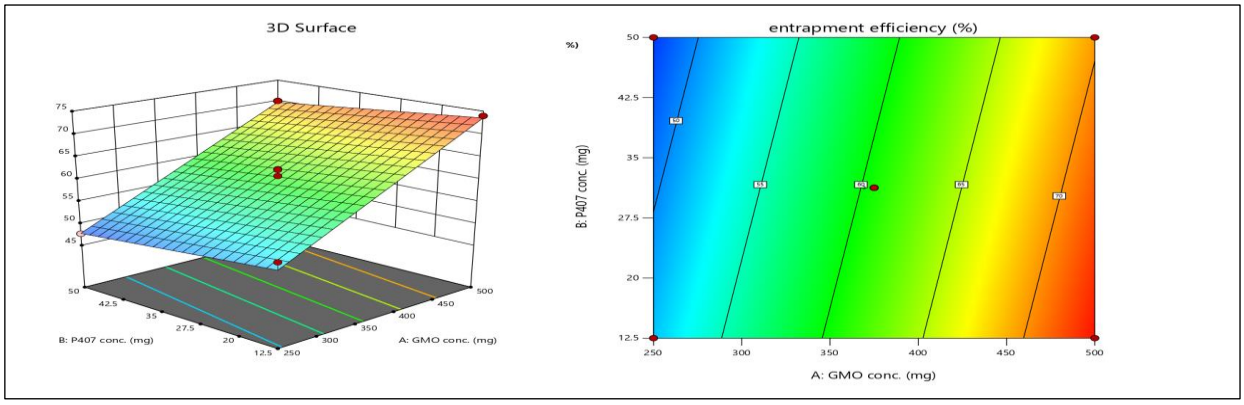

Figure 3: Response plots of entrapment efficiency

The ACZ-loaded cubosomal formulation was optimized using the point prediction approach of the Box-Behnken design within the response surface methodology in this study. The formulation composed of 500 mg glyceryl monooleate, 12.5 mg Poloxamer 407 and 60 mg polyvinyl alcohol met the criteria for an optimal formulation. The optimized formulation exhibited a particle size of 243.4 nm, a polydispersity index of 0.232 and an entrapment efficiency of 73.99%, with a desirability value of 0.982. Table 4 displays the outcomes of the optimized formulation, demonstrating that all the responses were in close agreement with the values that the Design Expert had predicted.

Table 4 Validation of Optimized cubosomal preparation

	Independent variables			Responses			Desirability
	GMO (mg)	P407 (mg)	PVA (mg)	PS (nm)	PDI	EE (%)	
Solution	500	12.5	60	240.9	0.230	71.24	0.982
Practically performed	5001	12.5	60	243.4	0.232	73.99	

***Characterization of ACZ loaded cubosomal formulation*:**

Zeta potential measurement:

The ACZ-loaded cubosomal formulation zeta potential was measured at -26.1 ± 3.94 mV. This negative charge on the cubosomes' surface is anticipated to create a shielding effect, which prevents particle aggregation and ensures the stability of the formulation (33).

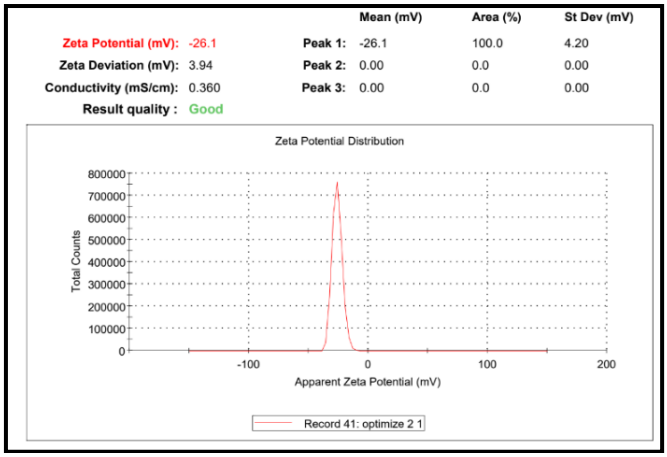

Figure 4: Zeta potential of ACZ loaded cubosomes

Isotonicity test:

It was determined by the use of a microscope to observe isotonicity that the aforementioned formulation is isotonic with both lachrymal fluid and blood lachrymal fluid. Since the presence of the ACZ-loaded cubosome formulation does not change the shape or size of blood cell, the isotonicity test demonstrated that the created formulation isotonic for human use (34).

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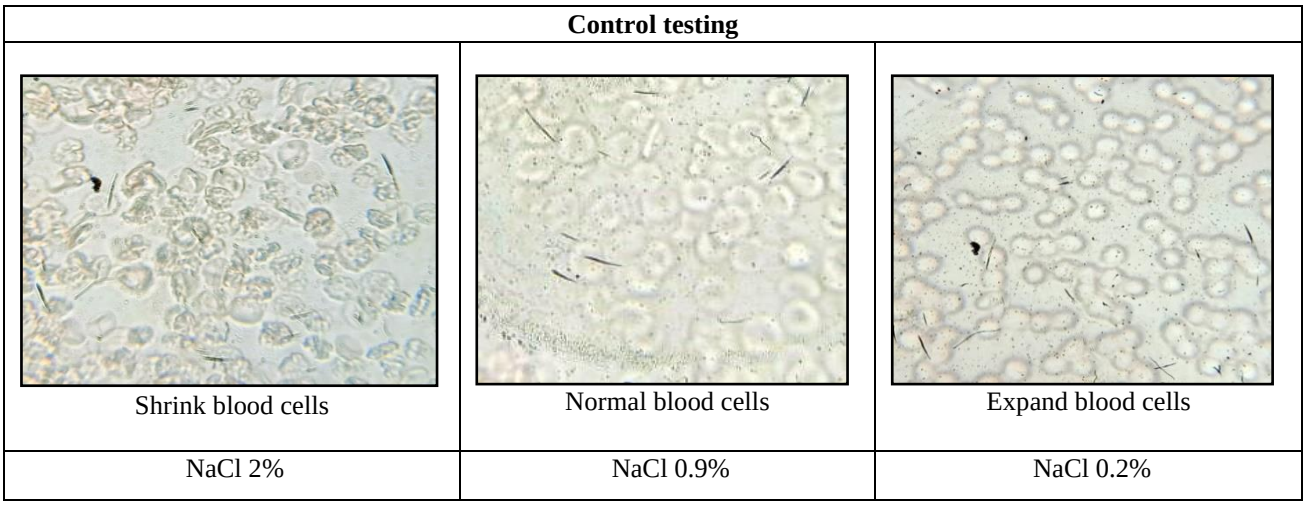

Figure 5: Observation of the isotonicity test results on the control solution

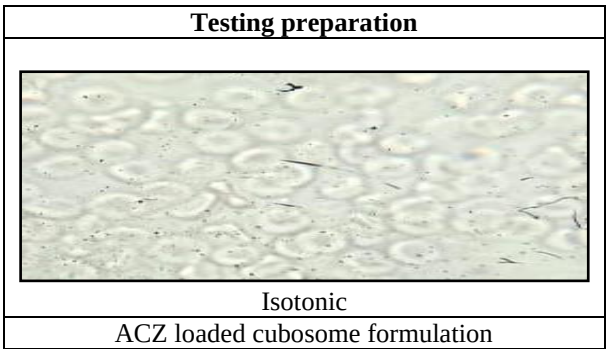

Figure 6: Outcomes of the observational isotonicity test on the ACZ-loaded cubosome formulation.

Transmission electron microscopy

Due to the TEM's high magnification capability, the optimized formulation sample has visible cubic nanostructures. Figure 7 depicts the typical cubosome structure of the cubic nanoparticles. The size indicated by the studied formula is nearly identical to that found by the Malvern particle analyzer. It demonstrated how the nanoparticles are kept apart from one another to ensure stability for human use (35).

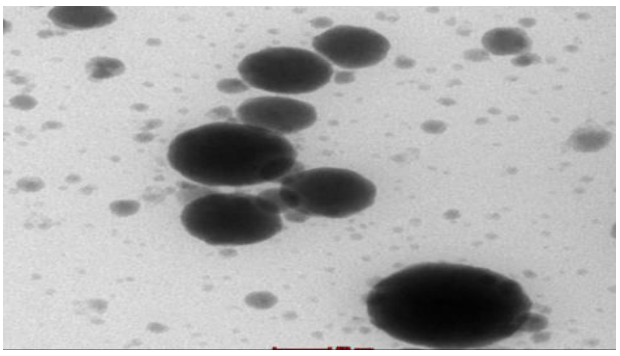

Figure 7: Transmission electron microscopy image of ACZ loaded cubosome

Fourier Transforms Infrared (FT-IR) Spectroscopy analysis

The IR spectra of ACZ showed the following values: 3288.63 and 3172.90cm^{-1} for the secondary amine's N-H stretching. The distinctive peak, caused by the S=O stretch of sulfonyl groups, was located at 1168.86cm^{-1}. 902.69cm^{-1} was the observed S-N stretching. Distinct peals at 1969.3cm^{-1} for the ester bond and 2875.86 cm^{-1} for the C-H aliphatic stretch were visible in the FTIR GMO spectra. P407's infrared spectra revealed vibrations associated with OH stretching and C-H aliphatic stretch at 2918.30cm^{-1} and 3305.99cm^{-1}. The FTIR spectra of the improved formulation showed distinct peaks for ACZ and various excipients, indicating that ACZ and the excipients did not interact chemically for human use (36).

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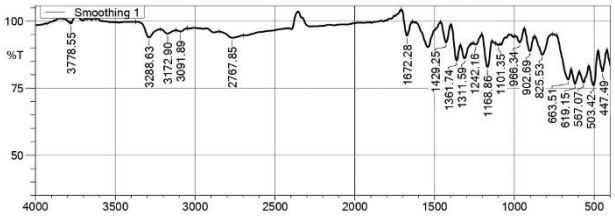
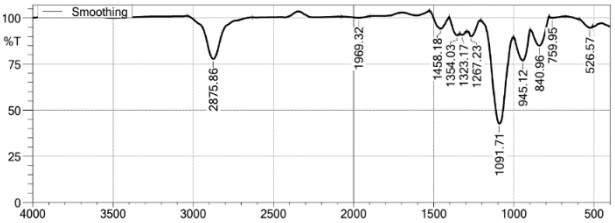
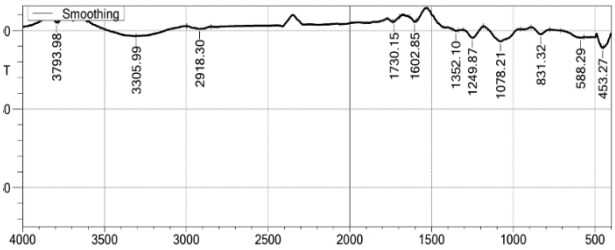


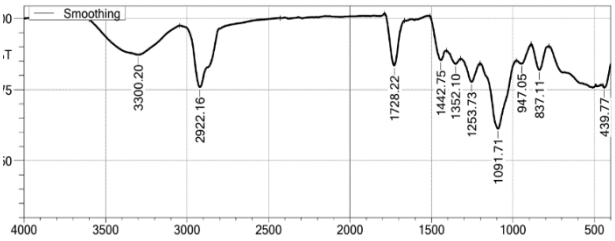
(a)ACZ



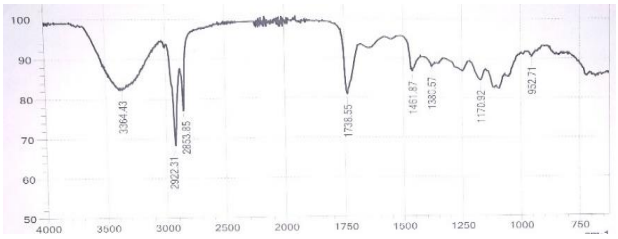
(b)GMO



(c) P407



(d) PVA



(e) ACZ loaded cubosome formulation

Figure 8: FTIR spectra of drug, excipients and ACZ loaded cubsome formulation

Differential Scanning Calorimetry (DSC) Analysis:

Figure 9 illustrates the peaks of ACZ, glyceryl monooleate, P407, and the optimized formulation. The peak at 273.96°C on the ACZ DSC thermogram hints at its crystallinity, the DSC thermogram of glyceryl monooleate shows peak at 42.92°C. P407's melting transition is represented by a peak at 55.90°C. Interestingly, the optimized cubosomal formulation's DSC thermogram shows a peak at 101.63°C. Notably, the endothermic peak of ACZ disappears in the formulation's thermogram. This suggests the successful encapsulation of ACZ within the cubosomal vesicles, leading to its dispersion in an amorphous state within the nanovesicles. Consequently, this results in an increase in the phase transition temperature for human use (37).

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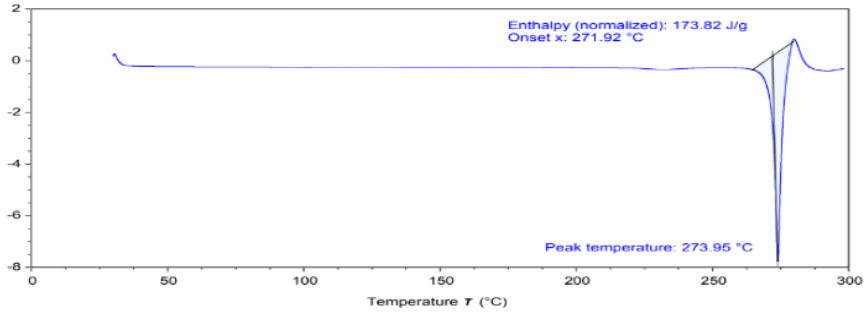
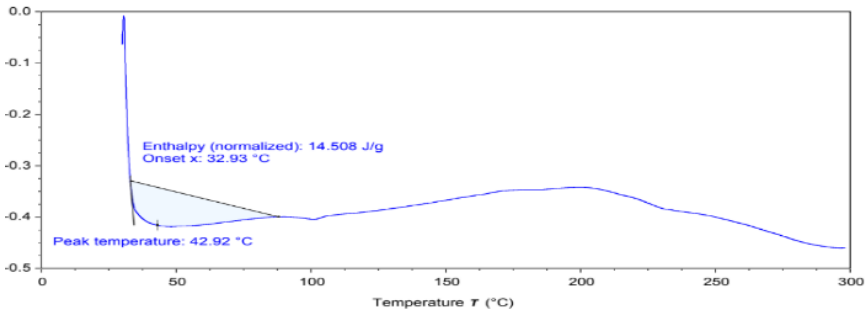
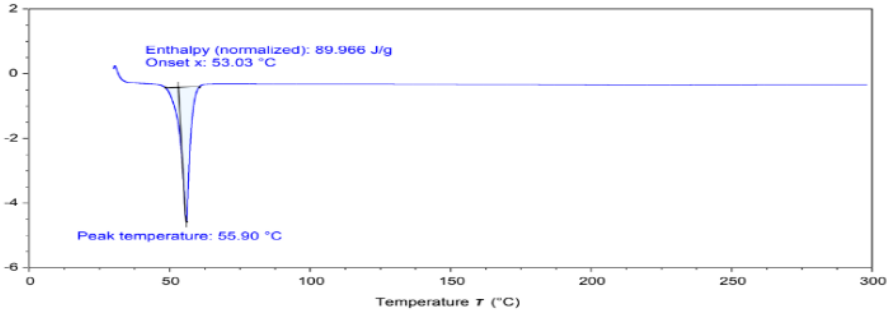


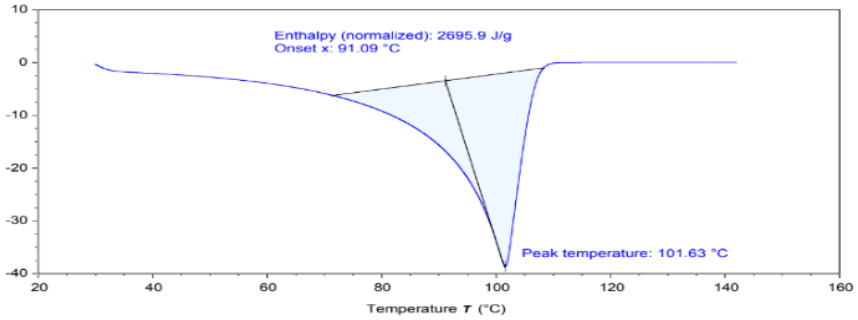
(a) ACZ



(b)GMO



(c)P407



(d) ACZ loaded cubosomes

Figure 9: DSC thermograms of ACZ, GMO, P407 and ACZ loaded cubosomes

Study of *in vitro* drug release:

The *in vitro* release profiles of an ACZ-loaded cubosome formulation, a generic formulation, standard drug were markedly different from one another. The cubosomal formulation released ACZ at a rate of 69.789% over the course of eight hours, while the marketed formulation and plain drug solution released 99.99% and 99.2863 percent, respectively. The plain drug may have a higher release rate than the ACZ-loaded cubosomes formulation due to the combination of the ACZ release pattern from the cubosome vesicles and diffusion through the Poloxamer polymer's cross-linked structure. Moawad et al. found that free drug was released significantly faster than cubosome dispersion for human use, which is consistent with these findings (38).

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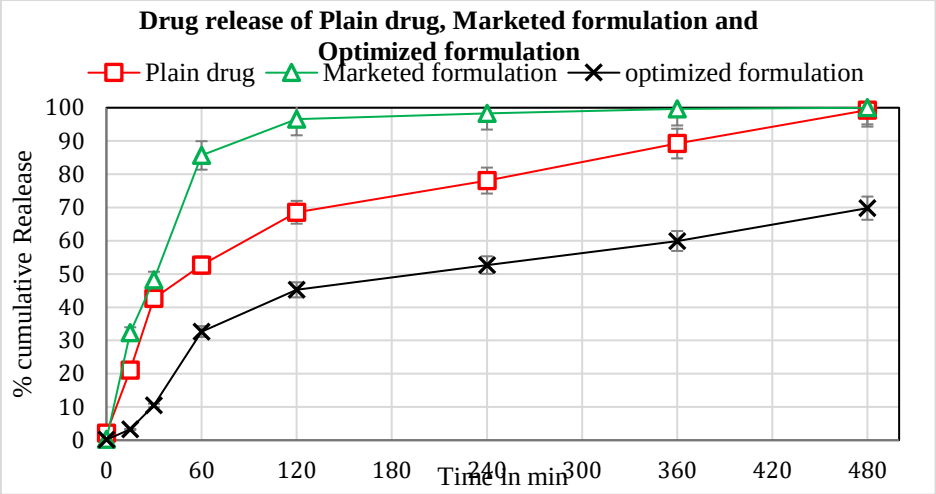

Figure 10: *In vitro* release study of ACZ

These outcomes demonstrate that the ACZ-loaded, cubosome-optimized formulation prolongs drug release, allowing for longer contact time with the cornea.

Table 5: Release kinetics

Release kinetics	R^2
First order	-0.9545
zero order	0.9746
Higuchi	0.9858
Hixson	0.9770
Korsmeyer pappas	0.9703

Study of corneal permeation in *ex vivo*:

In the ex vivo permeation, the drug solution's cumulative percentage of drug permeation through the goat cornea was 26.36 percent, the marketed formulation's percentage was 49.59 percent, and the optimized formulation's percentage was 53.24 percent. As depicted in Figure 11 the optimized cubosomes had significantly higher drug retention in the eye and better corneal penetration when compared to the drug solution and commercial formulation.

The optimized cubosome formulation's strong bioadhesive properties and cubic shape aid in controlled drug release and corneal penetration. The small particle size makes it easier to transport to the cornea, and the lipid phase (GMO) makes it easier for the ACZ-loaded cubosome formulation to stick to the cornea's mucosa. Cubosomes have been shown in a number of studies to increase corneal permeability and drug bioavailability, making useful for treating ocular diseases (39).

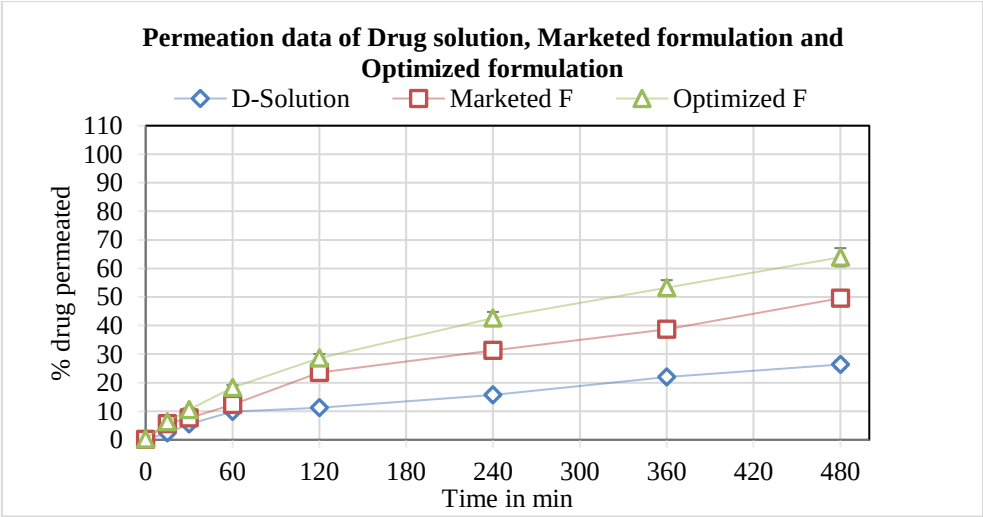

Figure 11: Ex vivo corneal permeation study

Corneal retention study:

The estimation of ACZ concentration in the cornea following treatment with ACZ solution and ACZ-loaded cubosomes revealed noteworthy findings. Specifically, the cornea treated with ACZ-loaded cubosomes exhibited a significantly higher drug content (0.367 µg/mg) compared to that treated with ACZ solution (0.124 µg/mg). The mucoadhesive properties of

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the ACZ-loaded cubosomes, which facilitate enhanced penetration and prolonged retention within the cornea, may be to blame for the increase in drug concentration in the cornea following treatment for human use (40).

Effect of gamma sterilization:

To avoid eye infections caused by harmful microorganisms in the formulation, ocular drug delivery systems must be sterilized. Because it ensures that the final product does not contain any microorganisms, terminal sterilization has fewer adverse effects than aseptic sterilization for human use (41).

The drug content (DC) (percent), polydispersity index (PDI), particle size (nm) and zeta potential (Zp) (mV) of the ACZ-loaded cubosomes formulation did not differ significantly, as shown in Table No. 6. These values were different before and after gamma sterilization, respectively.

Table 6: ACZ loaded cubosome formulation results before and after sterilization

Parameters	Before sterilization	After sterilization
Drug content (%)	98.972	97.85
Particle size (nm)	243.72	242.02
Polydispersity index (PDI)	0.224	0.245
Zeta potential (mV)	-26.83	-23.13

IP sterility study:

The cubosomes that were loaded with acetazolamide (ACZ) in the optimized formulation were able to pass the sterility test. Turbidity was not evident during the incubation period of at least 14 days at 30-35°C, indicating that there was no microbial growth for human use (42).

Study of ocular irritation:

Ophthalmic formulations' potential to cause irritation can be assessed quickly, sensitively, and inexpensively using this method of irritation study**.** This assay mimics the ocular condition in vivo using the functional vasculature of the chicken placenta. The ACZ stacked cubosomes upgraded plan was compared to standard saline and 0.1 N NaOH for the purpose of testing it for visual bothering. The score was measured for up to five minutes and the results showed that normal saline (0.9% NaCl) and ACZ-loaded cubosomes in an optimized formulation were nearly non-irritating. The images demonstrated that there was no apparent harm to the cornea, further showing the system's safety. The fact that the 0.1%N NaOH solution coagulated indicates that it caused irritation. The study found the ACZ-loaded cubosomes is safe and well-tolerated for ocular administration for human use (40).

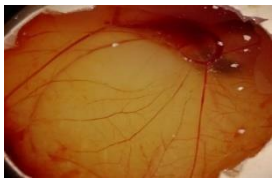
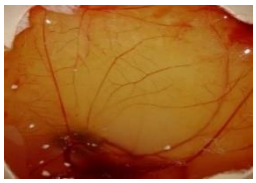
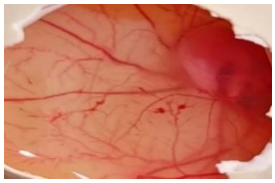
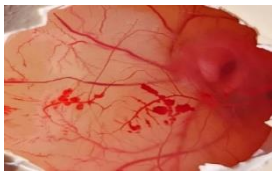
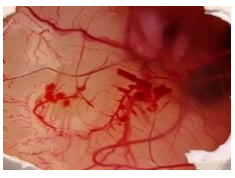
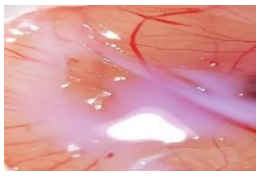
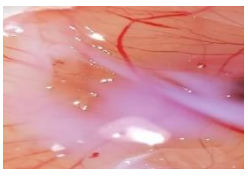
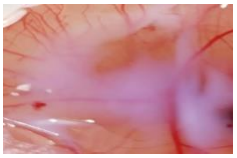
Time	0.5 min	2.5 min	5 min
(a)Negative control			
(b)Positive control			
(c) Test			

Figure 12: HET CAM ocular irritation study images of (a) Negative control, (b) Positive control and (c) Test (ACZ loaded cubosome formulation)

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Study of stability:

Until the end of the storage period, the ACZ-loaded cubosome formulation retained milky appearance without phase separation or aggregate formation. Particle size and polydispersity index decreased only slightly and zeta potential and drug content decreased slightly. The high structural stability of ACZ-loaded cubosome formulation for human use may be attributable to the intricate arrangement of the two stabilizers (P407 and PVA) within the cubosome matrix, which maintained the cubic nanostructure and resisted its alteration (43).

Table 7: Results of stability studies of ACZ loaded cubosome formulation

Parameters	ACZ loaded cubosome formulation			
	Initial	**After 3 months at 4±2°C with 75±5%RH**	**After 3 months at 25±2°C with 60±5%RH**	**After 3 months at 40±2°C with 75±5%RH**
Physical appearance	Clear milky white solution without any phase separation and aggregation of particles	No change	No change	No change
Particle size (nm)	243.45	245.86	249.06	250.22
PDI	0.226	0.234	0.22	0.250
Zeta potential (mV)	-26.32	-25.65	-24.82	-24.42
Drug content (%)	98.97	97.88	97.69	97.52

IV. CONCLUSION

Using a melt dispersion-emulsification method, acetazolamide-loaded cubosome formulations were successfully prepared and further optimized using a Box–Behnken design (BBD). The range and shape of the optimized formulation were found to be nanometric and cubical. The in vitro results demonstrated that cubosomes had promising mucoadhesive properties and could withstand gamma irradiation sterilization and that they were stable in storage. The optimal formulation significantly increased ACZ's transcorneal permeability, as demonstrated by the ex vivo corneal permeation study. In addition, it was able to maintain a high corneal retention rate, and an ocular irritation test proved that it was safe to administer to the eyes. Cubosomes loaded with Acetazolamide are considered to be a potentially useful platform for enhancing Acetazolamide ocular delivery.

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