Research Article

Formulation and Characterization of Posaconazole Loaded Cubosomes For Ocular Application

Pragati Bailwal*, Jyoti Kalra, Ashutosh Badola

School of Pharmaceutical Sciences, Shri Guru Ram Rai University, Dehradun, Uttarakhand, 248001, India
*Corresponding author: Pragati Bailwal, School of Pharmaceutical Sciences, Shri Guru Ram Rai University, Dehradun, Uttarakhand, 248001, India; Email: Pragatibailwal90@gmail.com

ABSTRACT

Objective: Posaconazole is a BCS class II, broad spectrum anti-fungal agent, currently used off-label for the treatment of fungal keratitis. Any ocular formulation of Posaconazole is not available in market due its low aqueous solubility, poor corneal permeation and rapid elimination from the eye. To overcome these problems present work aimed the formulation and characterization of Posaconazole loaded cubosomes (PS cubosomes) for ocular application in the management of fungal keratitis. **Methods**: The PS loaded cubosomes was formulated by Top to Bottom Technique by using various concentrations of GMO as lipid and Poloxamer 407 as stabilizer. Total nine formulations were prepared and one final formulation was (CB6) optimized on the basis of particle size and entrapment efficiency of PS cubosomes. The optimized PS loaded cubosomal formulation (CB6) characterized by studying the surface appearance (SEM & TEM analysis), particle size analysis, entrapment efficiency, in-vitro drug release, ex-vivo corneal permeation through goat cornea, anti-fungal activity against Candida albicans and stability. **Results:** The optimized cubosomes (CB6) had smooth surface and cubic shape, exhibit a particle size 183 nm, -17.15 mV zeta potential and 88.01% entrapment efficiency. When compared with marketed PS suspension (40mg/ml), the CB6 demonstrate sustained diffusion-controlled drug release, 2.8 fold higher increase in corneal permeability and improved anti-fungal activity against Candida albicans. **Conclusion:** Posaconazole-loaded cubosomes are a potential nanocarrier system for topical ocular administration of posaconazole and could be very useful in treatment approach for fungal keratitis.

Key words: Cubosomes, Optimization, Entrapment efficiency, Fungal keratitis, ocular.

1.INTRODUCTION

Posaconazole (PS) is an extended spectrum triazole having structural similarity to Itraconazole [1], and has broad spectrum antifungal action currently used off-label for the treatment of fungal keratitis (FK) [2]. Fungal Keratitis is a severe and vision-threatening corneal infection characterized by ocular pain, redness, inflammation, and impaired vision. Fungal keratitis occurs due to several fungal species which attack the cornea and cause keratitis.[3]

Topical route is widely preferred route of drug administration into eye, the major drawback of conventional ocular drug delivery system is rapid and considerable loss of drug from the eye. [4] In order to deal with the obstacles associated with drug delivery in eye, recent advancements in nano drug delivery systems are garnering significant interest to facilitate the targeted and sustained drug delivery for both posterior and anterior segment eye diseases.[5] In the past few years, cubosomes have become very popular as ophthalmic nanocarriers because of their improved penetration, solubility, bio adhesive qualities and biocompatibility.[6]

Cubosomes are bicontinuous cubic phase structures formed by self-assembly of amphiphilic lipid molecules in aqueous media under appropriate condition of temperature and hydration [7]. These are nanostructures lies in size range of 100-500nm having high surface area and the existence of two continuous water channels separated by a contorted lipid bilayer.[8] Amphiphilic molecules having low molecular weight when combines with surfactant, self assembles in aqueous phase to form lipid bicontinuous phase.[9] Cubosomes consist of two continuous water channels with distinct symmetry (Primitive, gyroid and double diamond) and a single lipid bilayer, due to which these cubic nanostructures have higher loading capacity for hydrophobic as well as hydrophilic molecules, and have better chemical stability. [10]

Posaconazole is a broad spectrum anti-fungal agent that is effective against Candida species, Aspergillus, Mucorales, many Zygomycetes endemic fungi Cryptococcus neoformans, dermatophytes, shows superior efficacy against resistant fungal pathogens. Though posaconazole is not available as any marketed ocular formulation but its diluted oral suspension (Noxafil®) has been used as off label to treat severe Keratitis topically[11]. Posaconazole is available in oral dosage form but its application as ocular drug delivery is not exploited much due to poor aqueous solubility, poor corneal permeation and rapid pre-corneal elimination.

Nanocarriers can facilitate site-specific delivery by penetrating biological barriers such as the stratum corneum, mucosal epithelium, or ocular tissues, thereby achieving high local drug concentrations while minimizing systemic toxicity[12]. In the present work formulation and characterization of posaconazole loaded cubosomes was done for ocular administration of PS into the eye for management of fungal keratitis. Cubosomes encapsulates the drug, increase the precorneal retention and enhance the drug permeation and have shown effective anti-fungal activity.

2.MATERIALS AND METHODS

2. 1 Materials

Posaconazole was purchased from Yerrow chem Mumbai, While GMO, Poloxamer was purchased from Upasna Enterprise Gurugram Haryana. All analytical grade chemicals were used.

2.1.1 Formulation & optimization of PS cubosomes

PS cubosomes was prepared by Top to Bottom technique. Table 1 is showing the formulation table of PS cubosomal formulations. Glycerol monooleate (GMO) and Poloxamer (P407) were weighed, melted and stirred on magnetic stirrer. Posaconazole dissolved in ethanol and added in the beaker containing lipid mixture and stirred at raised temperature to evaporate the ethanol. On another beaker water was heated and the lipid mixture was added dropwise on aqueous solution with a continuous stirring on magnetic stirrer at room temperature. The cubic gel phase was established after 24 hours of equilibration. Calculated volume of water required for final formulation of cubosomes was added on cubic phase gel and then fragmentation of gel was done for 20 minutes by using intermittent probe sonicator. The resultant milky white dispersion of cubosomes was obtained and stored in well closed glass vial. [13-15] The PS cubosomes then optimized on the basis of particle size and entrapment efficiency.

Table 1: Formulation table of posaconazole loaded cubosomes

Formulation Code	GMO (% w/w)	P407 (%w/w)	Posaconazole (%w/w)
CB1	3.6	0.5	1
CB2	3.6	1.0	1
CB3	3.6	1.5	1
CB4	4.6	0.5	1
CB5	4.6	1.0	1
CB6	4.6	1.5	1
CB7	5.6	0.5	1
CB8	5.6	1.0	1
CB9	5.6	1.5	1

%w/w: Percent weight by weight

2.1.3 Particle size

Malvern Zetasizer was used for measurement of zetapotential and particle size analysis of PS cubosomes by using the dynamic light scattering (DLS) technique at 25 °C. [16]

2.1.4 Entrapment Efficiency

For determination of entrapment efficiency PS cubosomes was loaded in ultra centrifugation tube and centrifugated at 4500 rpm for 15-20 minutes to separate the free Posaconazole from PS Cubosomes. The supernatant obtained was separated and diluted. The amount of free Posaconazole was investigated by using UV-Visible Spectrophotometer at 260nm. The amount of free drug subtracted from the initially added total drug. The following formula was used for measurement of entrapment efficiency. [17]

$$\% \text{ Entrapment Efficiency} = \frac{\text{Total PS} - \text{FreePS}}{\text{Total PS}} * 100$$

2.1.5 Scanning Electron Microscopy (SEM)

Surface appearance and size of optimized PS cubosomes (CB6) was identified by SEM analysis. The PS cubosomal sample was fixed on copper grid, observed under scanning electron microscope (JSM-T330A, JEOL) and the photomicrographs were obtained. [19]

2.1.6 Transmission Electron Microscopy

One drop of diluted PS cubosomal sample was casted on TEM grid made of Cu, adsorbent filter paper used to remove extra fluid. Sodiumphospho-tungstate solution was used as staining solution. When the sample get dried the grid was placed inside the TEM instrument (JEOL, Japan). The images were taken on different magnification. [20]

2.1.7 In-Vitro release studies

Franz diffusion cell was utilized to carry out In-vitro release study of PS cubosomes and marketed posaconazole suspension. In donor compartment of the cell test sample was placed and the receptor compartment was filled with simulated tears fluid solution (STF). Previously STF soaked cellophane membrane was placed between both the compartments. The magnetic stirrer was set at 400 rpm and temperature was adjusted to 35 ^{0}C, sample was withdrawn at predetermined rate for 6 hours and sink condition was maintained. The withdrawn sample diluted with STF and drug concentrations were analysed from UV-visible Spectrophotometer (Shimadzu UV1800, Japan) at 260 nm. [21] For good understanding of kinetics of drug release the data achieved from In-vitro drug release study was put into kinetic models such as Zero and first order, Higuchi's and Korsmeyer–Peppas model. The obtained r^2 values compared for selection of best fit model.[22]

2.1.8 Ex-Vivo corneal permeation study

For Ex-vivo corneal permeability study of the optimized PS cubosomes and marketed posaconazole suspension, Franz diffusion cell was used. The goat eye balls were brought from a local meat shop and kept in STF. The cornea was separated, washed with STF and placed between acceptor and donor compartment of the diffusion cell. The test sample was placed in donor compartment and the acceptor compartment was filled with STF. The stirrer was set to 200 rpm at 34^{0}C temperature [23]. The samples were taken at a predetermined rate for 6 hours and sink condition was maintained by using STF. The sample collected was diluted with STF and analyzed under UV-visible Spectrophotometer (Shimadzu UV1800, Japan) at 260 nm. The data obtained was used for calculation of steady-state flux (Jss) and Apparent permeability coefficient (Papp) of the drug.

A graph of cumulative drug (Posaconazole) permeated per unit area against time was plotted. To calculate Steady state flux (Jss) from the regression line for permeation profile data was fitted and Jss was obtained from the slope of linear section of graph.

Following equation was used to calculate Apparent permeability coefficient :

$$\text{Papp} = \frac{\text{Jss}}{\text{A} * \text{C}_\text{o}}$$

Where, C_o is equals to initial concentration of Posaconazole, Jss is equals to Steady state flux and A is equals to diffusion area per cm^2 [24]

2.1.9 Anti-fungal activity

Agar well diffusion method was used to test the anti-fungal activity of optimized posaconazole loaded cubosomes and posaconazole suspension against Candida albicans. The negative control was sterile distilled water, while the positive control was fluconazole. The proper fungal culture media were used to sustain and revitalize the fungal cultures. Candida albicans was suspended in sterile potato dextrose broth to create the fungal inoculum. Sterile Petri dishes were filled with sterile Sabouraud dextrose agar (SDA) and left to harden. A Candida albicans sterile cotton swab was used to evenly inoculate the agar's surface with the prepared fungal suspension. 6mm diameter wells were punched aseptically into inoculated SDA plates. 20,50 and 100 mg/ml test formulations were added to the designated wells. Fluconazole was used as positive control, while sterile distilled water as negative control. The plates were incubated for 48 h at 30°C. The diameter of zone of inhibition was measured in millimetres. Zone of inhibition of test formulations and positive control was compared to assess the Anti-fungal activity of test formulations[25].

2.1.10 Stability Studies

To ensure the quality, efficacy and safety of optimized PS cubosomes stability study was performed. PS cubosomes was packed in tightly closed amber colored vials and subjected to room temperature (25±2°C / 60% Relative humidity), Refrigeration temperature (5°C ± 3°C) and (40°C ± 2°C / RH 75%) accelerated condition for six months in stability chamber, The sample was taken at 1st , 3rd and 6th month and analyzed for change in colour, entrapment efficiency and drug release. [26]

3.RESULTS & DISCUSSIONS

The cubosomes were formulated and then optimized on the basis of particle size and percent entrapment efficiency. Out of nine formulations (given in table 2) the formulation CB6 formulated by using 4.6% w/w GMO and 1.5 % P407 was selected as final optimized formulation. The formulation CB6 had particle size 183 nm and maximum entrapment efficiency 88.01%, hence selected as final optimized formulation and then further

evaluated for particle size, zetapotential, entrapment efficiency, surface morphology, drug release, drug permeation, anti-fungal activity and stability studies.

Table 2: Particle size and entrapment efficiency of posaconazole loaded cubosomes

Formulation Code	Particle size (nm)	Entrapment efficiency (%)
CB1	220	73.21
CB2	214	75.45
CB3	208	79.87
CB4	197	80.02
CB5	185	84.57
CB6	183	88.01
CB7	200	80.23
CB8	194	75.26
CB9	190	71.30

Nm = nanometer, %= percent

3.1.2 Particle Size analysis: For ocular drug delivery, particles size should not be over the limit, as larger particles can cause ocular irritation. The desired particle is smaller than 200nm.[27] The DLS analysis pointed in Fig 1 shows the optimized Cubosomes CB6 was found to have 183 nm Z-average diameter of particles and 0.2554 PDI. A moderately uniform particle distribution indicated by PDI value, shows the cubosomes were well dispersed and had a consistent size distribution. The zeta potential shows the optimized PS cubosomes CB6 had negative surface charge of -17.15 mV (shown in fig 2) which indicates formation of a stable formulation with small chance of particle aggregation[28]. The negative charge could be due to the presence of oleic acid in the structure of GMO.

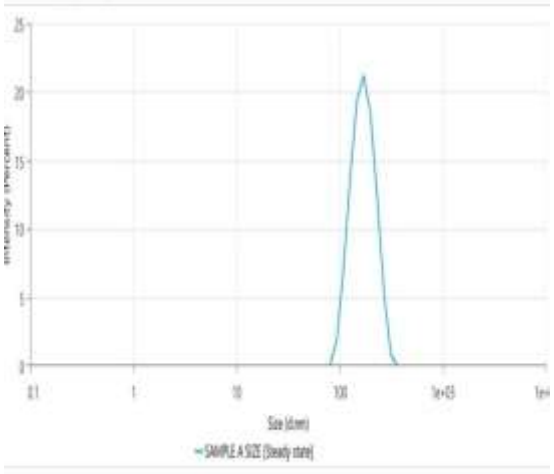

Fig.1Particle size of PS cubosomes (CB6)

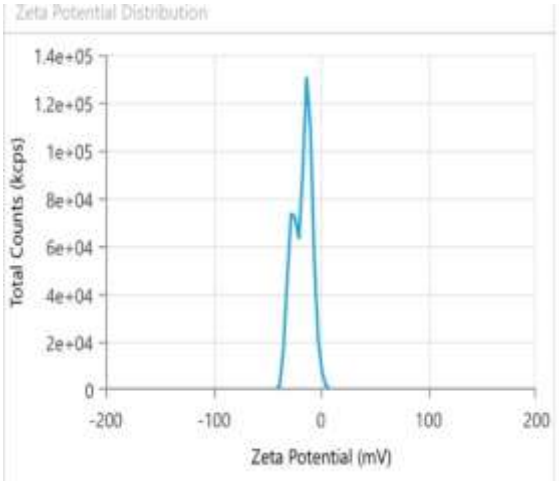

Fig.2 Zeta potential of PS cubosomes (CB6)

3.1.3 Entrapment Efficiency (EE): The % EE of optimized PS cubosomes was found to be 88.01 %, suggesting good drug loading capacity of optimized cubosomes. Higher lipophilicity of Posaconazole and greater internal area of cubosomal structure are the reasons for high value of entrapment efficiency.

3.1.4 Scanning Electron Microscopy (SEM): Surface morphology of Optimized cubosomes CB6 was analyzed by scanning electron microscopy (JSM-T330A, JEOL). Fig 3 shows that the cubosomes were smooth in surface and cubical in shape,

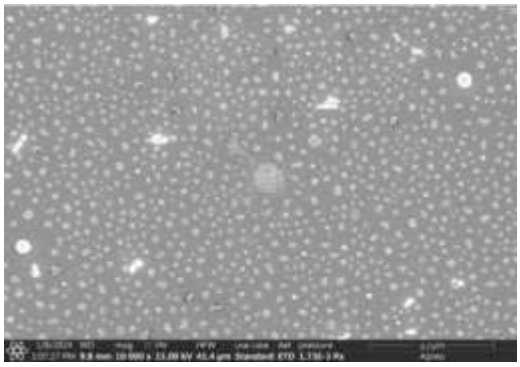

Fig 3. SEM of PS cubosomes (CB6)

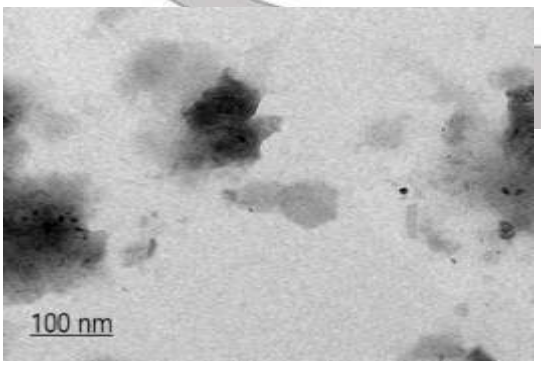

Fig 4. TEM image of PS cubosomes (CB6)

3.1.5 Transmission Electron Microscopy (TEM): TEM was utilized to identify the shape & surface morphology of cubosomes[29]. TEM image of optimized PS cubosomes CB6 (shown in Fig. 4) showed irregular cubic symmetry with embedded water channels. The diameter of cubosomal particles were 100 nm.

3.1.6 In-vitro Drug Release: In vitro drug release of PS cubosomes (CB6) were conducted in Franz diffusion cell and compared with drug release of marketed Posaconazole suspension (40mg/ml). The initial phase of PS cubosomes had shown the burst release which could be due to presence of adsorbed drug on the outer layer of cubosomes (Shown in Fig. 5). This burst release was good to attained sufficient quantity of posaconazole to show its anti-fungal action. After initial Phase the controlled release of drug from cubosomes was observed, on the other hand marketed PS suspension had release the 100% drug within 2 hours. Higuchi model gave the best linearity as it has $R^2 = 0.9964$. This shows that release of drug was diffusion controlled.[30]

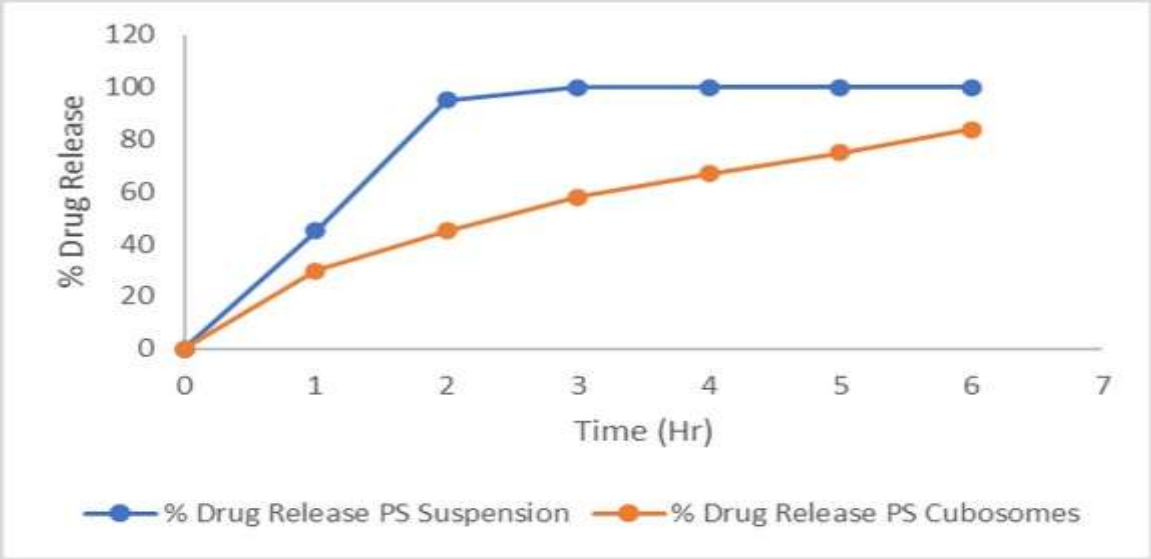

Fig. 5 In-vitro release of PS cubosomes (CB6) & PS suspension

3.1.7 Ex-Vivo Corneal Permeability

The Ex-vivo corneal permeability test of optimized PS cubosomes (CB6) and marketed PS suspension (40mg/ml) was performed by using goat cornea in Franz diffusion cell. Posaconazole is a BCS class II drug, it can permeate corneal epithelium and endothelium but stroma is hydrophilic which hinder the passage of drug.[31] The Jss and Papp value of PS suspension was found to be 12.20 µg/cm^2/hr and 0.0460 cm/hr, while the PS cubosomes had shown higher Jss and Papp values 34.04 µg/cm^2/hr and 0.1102 cm/hr. Jss values suggest that PS cubosomes had 2.8 fold higher corneal permeation of over PS suspension. Fig. 6 shows cumulative drug permeated vs time. GMO have an excellent permeation enhancer ability through cornea. The findings of this study agreed with earlier investigations that suggest cubosomes can improve drugs occular bioavailability and corneal permeability, enabling them to treat ocular disorders effectively. [32]

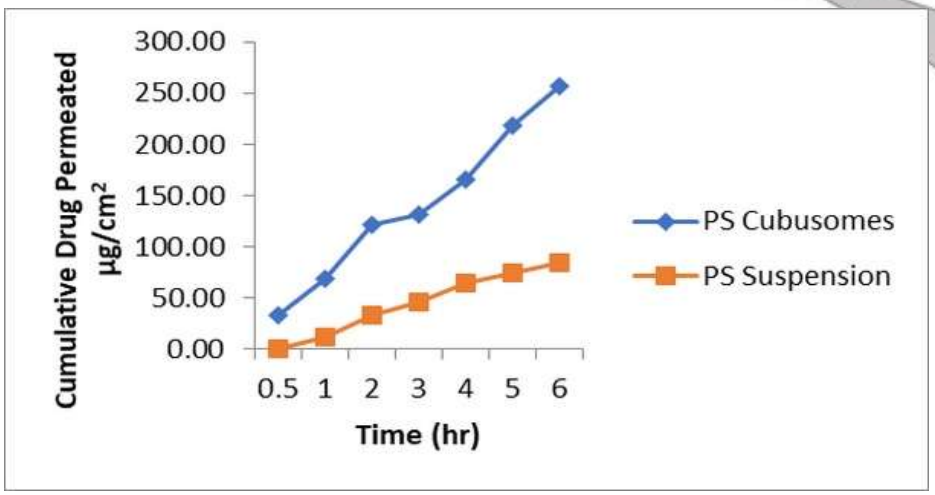

Fig.6 cumulative drug permeation of PS cubosomes (CB6) and PS suspension

3.1.8Anti-fungal Studies

The anti-fungal activity of PS cubosomes (CB6) and PS suspension against Candida albicans was evaluated by measuring the diameter of zone of inhibition. Both formulation samples showed detectable anti-fungal efficacy against fungal strain but the degree of inhibition varied depending upon formulation and dose (Shown in Fig. 7). PS suspension produced zones of 15mm and 11 mm at 100mg/ml and 50 mg/ml. No activity reported at lower tested concentration. The PS cubosomes produced zones of 20,12 and 9mm at 100mg/ml, 50mg/ml and 20 mg/ml, respectively (Shown in Table 3). 100mg/ml PS cubosomes produced largest inhibition zone of 20mm.

Table 3: Zone of inhibition (PS cubosomes and PS suspension) against Candida albicans

Test Samples	Concentration 100 mg/ml	Concentration 50 mg/ml	Concentration 20 mg/ml	Positive Control	Negative Control
PS Suspension	15 mm	11 mm	NA	20 mm	NA
PS Cubosomes	20 mm	12 mm	9 mm	25mm	NA

NA: No activity

Test Samples **(a)** PS suspension and **(b)** PS cubosomes against *Candida albicans*

Fig. 7 Zone inhibition of PS cubosomes(CB6) and PS suspension

3.1.9 Stability Studies

The Posaconazole loaded cubosomes were subjected at freezing, room temperature and accelerated temperature for six months and observed for any change in color, entrapment efficiency and drug release, but there was no significant change was observed and hence concluded that on storage PS cubosomes have good stability.

4. CONCLUSION

Posaconazole is an antifungal agent with broad spectrum of activity and is useful in treatment of Fungal infections of eye when other azoles are unable to do so[33]. Loading of Posaconazole into Cubosomes enhances the corneal permeability of drug possibly due to nano size and unique structure of Cubosomes which is capable of holding both hydrophilic and lipophilic drug moieties and helps them to penetrate the ocular barriers. The particle size of optimized PS cubosomal formulation CB6 was found to be 183 nm and 0.2554 PDI, the nano sized formulation may facilitate corneal surface interaction and contribute to enhanced corneal permeation. The CB6 had -17.15 zeta potential, showing stability of formulation. Posaconazole is a lipophilic drug and its entrapment efficiency 88.01% has shown that drug is incorporated inside the cubosomes which indicates efficient drug loading. Posaconazole loaded Cubosomes (CB6) has shown controlled drug release, higher corneal permeation and better anti-fungal action (against Candida albicans**)** than marketed posaconazole suspension, and good stability on storage. In this study we conclude that PS Cubosomes are suitable to used for ocular application in management of fungal keratitis.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Author's Contribution:

Pragati Bailwal: Conceptualization, methodology, formulation development, experimental investigation, data collection, data analysis, optimization, interpretation of results, and preparation of the original manuscript. **Dr. Jyoti Kalra:** Supervision, conceptualization, study design, scientific guidance, critical review, and revision of the manuscript. **Dr. Ashutosh Badola:** Co-supervision, scientific guidance, interpretation of results, critical review, and revision of the manuscript.

All authors reviewed and approved the final version of the manuscript.

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