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RESEARCH ARTICLE

Design, Development, and Characterization of Lyophilized Posaconazole-Loaded Mixed Micelles for Improved Fungal Treatment and Stability

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SUMMARY

Posaconazole (POS), a BCS class II drug with the low solubility of the drug prevents its absorption in the body leading to suboptimal drug levels and reduced treatment effectiveness. To address this issue, this research aimed to develop a lyophilized powder of Posaconazole-loaded mixed micelles (POS-MMs) using Pluronic F68 ® (PF68) and Soluplus ® to enhance fungal treatment and stability. A 32-factorial design optimized the POS-MMs formulation by varying PF68 and Soluplus® concentrations. Various characterization techniques were employed, including particle size analysis, zeta potential (ZP) measurement, FTIR, DSC, and XRPD. In vitro drug release studies indicated sustained release with 79.4% released within 24 hrs, while in vitro hemolysis studies confirmed safety and biocompatibility. Lyophilization with lactose as a cryoprotectant was successful, yielding a stable formulation over three months. The optimized POS-MMs exhibited a particle size (PS) of 66.30 ± 2.10 nm, a high % entrapment efficiency (%EE) of 94.88 ± 2.4%, and a desirable ZP of -51.1 ± 2.4 mV. These findings highlight the potential of POS-MMs to enhance the therapeutic outcomes of POS in the treatment of fungal infections. In addition, lyophilized powder is a promising technique for improving the stability of POS-MMs.

Key Words: *Fungal infection, lyophilization, mixed micelles, posaconazole, stability.*

Geliştirilmiş Mantar Tedavisi ve Stabilite için Liyofilize Posakonazol Yüklü Karışık Misellerin Tasarımı, Geliştirilmesi ve Karakterizasyonu

ÖZ

Posakonazol (POS), düşük çözünürlüğü nedeniyle ilacın vücutta absorbsiyonununu önleyerek optimal düzeyin altında ilaç seviyelerine neden olan ve tedavinin etkinliğini azaltan bir BCS Sınıf II ilaçtır. Bu sorunu çözmek için, bu araştırmada, Pluronic F68® (PF68) ve Soluplas® kullanarak hazırlanan posakonazol yüklü karışık misellerin (POS-MMs) liyofilize tozunun geliştirilmesi ile mantar tedavisinin iyileştirilmesi ve stabilitenin artırılması amaçlamıştır. POS-MMs formulasyonları, PF68 ve soluplas® konsantrasyonları değiştirilerek 32 faktöriyel tasarım ile optimize edilmiştir. Partikül boyutu analizi, zeta potansiyel (ZP) ölçümü, FTIR, DSC ve XRPD dahil olmak üzere çeşitli karakterizasyon teknikleri uygulanmıştır. İn vitro hemoliz çalışmaları biyouyumluluğu doğrularken in vitro ilaç salım çalışmaları 24 saatte %79,4 salım ile sürekli salımı göstermiştir. Kriyoprotektan olarak laktoz ile liyofilizasyon başarılı olmuştur ve üç ay boyunca stabil bir formülasyon elde edilmiştir. Optimize POS-MM'ler, 66,30±2,10 nm partikül boyutu (PS), %94,88±2,4 ile yüksek yükleme kapasitesi (%EE) ve istendiği gibi -51,1±2,4 mV ZP sergilemiştir. Bu bulgular, POS-MM'lerin mantar enfeksiyonlarının tedavisinde POS'un terapötik sonuçlarını geliştirme potansiyelini vurgulamaktadır. Ayrıca liyofilize toz, POS-MM'lerin stabilitesini arttırmak için umut verici bir tekniktir.

Anahtar Kelimeler: *Mantar enfeksiyonu, liyofilizasyon, karışık miseller, posakonazol, stabilite.*

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INTRODUCTION

Posaconazole (POS), a broad-spectrum antifungal agent, has shown great potential in treating various fungal infections. However, its inherent physicochemical instability, particularly in aqueous formulations, poses significant challenges to its storage and administration. To overcome this limitation, incorporating POS into mixed micelles (MMs) has emerged as a promising approach to enhance its stability and solubility (Ribeiro, 2022).

MMs are self-assembled structures composed of amphiphilic molecules, which form colloidal nanoparticles in aqueous media. These nanoparticles can encapsulate hydrophobic drugs within their hydrophobic core, providing an ideal environment for their protection. However, the long-term stability of such formulations remains a concern, especially during storage and transportation.

There are various ways by which stability can be achieved, such as lyophilization, spray drying, fluidized bed drying, coating, etc. Lyophilization, also known as freeze-drying, is widely used in pharmaceutical formulation and preservation (Kuperkar, 2022). It involves the removal of water from a sample by sublimation under reduced pressure and low temperatures, resulting in the formation of a dry, stable product (Trenkenschuh, 2021)

The previous literature revealed that POS micelles have been developed using Tween 80 as a surfactant. However, concerns arise due to the cytotoxic effects of Tween 80 at high concentrations or prolonged exposure, potentially affecting cell viability and function. Moreover, Tween 80 can lead to micelle aggregation, precipitation, and phase separation, impacting long-term stability (Thakral, 2021). Allergic reactions, including skin irritation and itching, can occur with Tween 80-containing micelles. Alternatively, researchers have utilized TPGS, but its limited solubility may hinder its effectiveness as a solubilizing agent, especially for poorly soluble drugs. Additionally, the thin-film hydration technique employed for micelle preparation can be time-consuming, labor-intensive, and prone to batch-to-batch variability, potentially affecting micelle properties and drug stability (Vinchurkar, 2021). These considerations highlight the need for exploring alternative surfactants and simplified micelle preparation methods to overcome these limitations. MMs exhibit multifunctionality by offering solubilization of hydrophobic drugs, improving drug bioavailability, and providing controlled drug release, making them versatile drug delivery systems (Jin, 2022).

Hence, this research aims to utilize the scalable solvent evaporation method to prepare Posaconazole loaded mixed micelles (POS-MMs) using novel Pluronic F68 (PF68) and Soluplus®. This will be achieved by employing a 3^2 full factorial design, followed by thorough characterization of the resulting MMs, to enhance the treatment of various fungal infections. The full factorial design is a prevalent and efficient approach used to understand the influence of multiple factors, and their interactions, and to prevent batch failure. It enables systematic analysis and process optimization. Further, research work is extended to lyophilize the liquid formulation to convert the stable solid POS-MMs form to improve its long-term storage.

MATERIAL AND METHODS

Materials

POS was kindly supplied by Lupin, Mumbai. PF68 and Soluplus® were procured from Sigma Aldrich and BASF, respectively. Analytical grade Methanol and distilled water were purchased from Fine Chemical, Mumbai. Lactose was purchased from Sigma-Aldrich.

Critical micelle concentration determination

The Critical Micelle Concentration (CMC) of PF68, Soluplus®, and their combination was determined using the iodine UV-visible spectrophotometric technique. KI/I_2 solution was added to different copolymer solutions, and absorbance was measured at 366 nm using a UV-visible spectrophotometer (Agilent 1800) (Suzuki, 2023).

Preparation of POS-MMs

The solvent evaporation technique was used for the preparation of POS-MMs. To prepare POS-MMs, 10 mg of POS, 10 mg of Soluplus®, and 95 mg of PF68 were combined with 2 mL of methanol and subjected to sonication for 5 min. The resulting mix was then gradually added to 10 mL of deionized distilled water and stirred for 6 hrs at room temperature, enabling methanol to evaporate. Afterward, the mixture was centrifuged at 2000 rpm for 5 min, and the supernatant was collected to analyze the % entrapment efficiency (%EE), particle size (PS), and Zeta potential (ZP) (Patil, 2022; Ugwu, 2022).

Optimization of the formulation

Optimization of formulation has a great influence on the composition and development method of MMs. A 3^2 factorial design was employed, using Design Expert® VR software, to examine how different formulations impact micelle properties. Nine unique combinations were investigated, with each formulation parameter set at three levels (-1, 0, +1) for PF68 concentration and Soluplus® concentration. The evaluation focused on two dependent variables: PS (Y_1) and %EE (Y_2). Preliminary studies were conducted before the implementation of the experimental design to select three levels of independent variables. During optimization using experimental design, the formulation components and other processing variables were kept constant. The coded levels translated to the experimental units, experimental runs, and their factor combinations considered in the present study are summarized in Table 1 (Hajare, 2021).

Table 1. Levels of independent variables in 3^2 factorial design

Coded Values Level	Independent Variables values	
	Factor 1 (X_1) PF68 Concentration (mg)	Factor 2 (X_2) Soluplus® Concentration (mg)
-1	0.8	0.06
0	1.1	0.08
+1	1.4	0.10

Characterization of POS-MMs

%Entrapment efficiency and %drug loading capacity

The micellar dispersion underwent a process to assess the drug %entrapment capability of the micelles. The amount of encapsulated POS was measured spectrophotometrically at 262 nm after dilution with methanol. The %EE and percent drug loading capacity (%DLC) were determined using the following formulas. (Patil, 2021):

$$\%EE = \frac{(Weight\ of\ encapsulated\ POS\ )}{Weight\ of\ POS\ used\ for\ micelle\ preparation} \times 100 \quad \text{Eq. (1)}$$

$$\%DLC = \frac{Weight\ of\ encapsulated\ POS}{Weight\ of\ POS\ used\ for\ micelle\ preparation + combined\ weight\ of\ both\ PF68\ and\ Soluplus®} \times 100 \quad \text{Eq. (2)}$$

Where,

Weight of feeding POS = Quantity of POS that is added to the formulation

Weight of total polymers = Combined weight of both PF68 and Soluplus®

Particle size and zeta potential measurements

The Horiba particle size analyzer was employed to assess the PS and ZP of the MMs. The PS was determined using dynamic light scattering (DLS). To perform PS analysis, 1 mL of the supernatant obtained from the POS-MMs was diluted with deionized dis-

tilled water to a final volume of 10 mL and then subjected to analysis using the particle size analyzer (Jain, 2019).

Drug-excipient compatibility study

Fourier transform infrared spectroscopy (FTIR)

The compatibility of POS, Soluplus®, and PF68 was investigated using FTIR spectroscopy. The FTIR spectra of both plain POS and final POS-MMs were recorded using an FTIR spectroscopy (Agilent, Alpha 100508) (Baviskar, 2022).

Differential scanning colorimetric study

The thermal behavior of plain POS and POS-MMs was analyzed using a differential scanning calorimeter (DSC; SDT Q600 V20.9 Build 20) with an intracooler under a dry inert nitrogen atmosphere, where powder samples were heated at 10 °C per min from 10 °C to 500 °C in hermetically sealed punctured aluminum pans (Patil, 2022).

Powder X-ray diffraction

An X-ray powder diffraction (XRPD) (Bruker D8 Advance) with Cu-K radiation (λ = 1.54) was employed for the crystallographic analysis of plain POS and POS-MMs. The diffractometer operated at a voltage of 40 kV and 50 mA, with scans conducted at 0.02º increments ranging from 5º to 100º diffraction angle (2θ) at a rate of 1 s/step. For accurate measurements, both plain POS and POS-MMs were scanned against a zero backdrop (Hajare, 2020).

***In vitro* drug release study**

Drug release study

The purpose of this study was to explore the drug release characteristics employing UV-visible spectrophotometry as the analytical technique. First, the calibration of the POS was carried out in pH 7.4 medium. Then calibration curve was meticulously constructed to derive an equation enabling the precise calculation of drug release percentages. To maintain the robustness and reproducibility of the findings, each experiment was replicated three times. Then, the release behavior of plain POS and POS-MMs was determined using the dialysis bag technique. A drug solution containing 10 mg of POS was prepared and placed in dialysis tubes with a molecular weight cut-off of 12000 Da, securely sealed to prevent leakage. The release study was conducted in a pH 7.4 medium with samples withdrawn at various time intervals (1, 2, 4, 8, and 24 hrs), and the collected samples were analyzed by measuring absorbance at 262 nm to estimate the percentage of drug release (Patil, 2023).

Drug release kinetics study:

The drug release profile of the optimized MMs batch was analyzed to calculate the coefficient of determination (R^2) and determine the appropriate model that describes its release pattern. Various models, including zero-order, first-order, Higuchi, and Korsmeyer-Peppas, were tested to assess the fitting of the release data and identify the most suitable model for the MMs formulation (Patil, 2023).

***In vitro* hemolysis study**

Hemolysis was evaluated by measuring the hemolysis percentage using human blood samples. The process included extracting fibrinogen from a 5 mL blood sample, combining it with the 0.9% NaCl solution, centrifuging, and collecting the supernatant. Red blood cell pellets were washed, dispersed, and diluted to prepare a 2% erythrocyte pellet solution. Samples containing POS, blank PF68 and Soluplus® MMs, and POS-MMs (A1), along with positive and negative controls, were incubated, and centrifuged, and their absorbance was measured at 420 nm to quantify hemolysis. The percentage of hemolysis was calculated using the following formula (Manjusha, 2023).

$$\%Hemolysis = \frac{Absorbance\ of\ samp - Absorbance\ of\ negative\ control}{Absorbance\ of\ positive\ control - Absorbance\ of\ negative\ control} \times 100 \quad \text{Eq. (3)}$$

Lyophilization of POS-MMs

Preparation of lyophilized POS-MMs

A precisely measured volume of 1 mL of the optimized batch composition of POS-MMs (A1) was combined with 15% w/v lactose and filled into glass vials with a capacity of 3 mL and these vials were subjected to pre-freezing at a temperature of -20 °C for 12 hrs. After pre-freezing, the vials were subjected to lyophilization using a Martin Christ lyophilizer. The lyophilization process involved setting the shelf temperature at -42 °C and maintaining the condenser temperature at -50 °C. A vacuum of 0.1 mBar was applied for 48 hrs, following the protocol from previous literature. Subsequently, the dried formulation underwent secondary drying. The vials were placed in a vacuum chamber with a pressure of 0.06 mBar for 12 hrs and then sealed under vacuum (Patil, 2022).

Characterization of lyophilized POS-MMs

After the process, the final product was obtained in the form of loose cakes. These cakes were further characterized to assess particle size, %EE, % drug loading capacity (%DLC), moisture content, SEM, and reconstitution time. The stability of the POS-MMs formulation was aimed to be proved by these characterization studies (Szente, 2021).

Scanning electron microscopy (SEM)

Morphological analysis was conducted using Scanning Electron Microscopy (JEOL JSM-6360, Japan). The samples underwent fixation on a brass stub with double-sided adhesive tape, and electrical conductivity was achieved by applying a platinum coating (6 nm/min) in a vacuum (6 Pa) using a Hitachi Ion Sputter (E-1030) for 120 s at 15 mA. The SEM images were analyzed using an image analysis system (Image-Inside Ver 2.32) (Singh, 2020).

Determination of moisture content

Moisture analysis of dried products was performed using a Karl Fischer Titrator (Vigo – Matic M.D.). Accurately, 20 mL of anhydrous methanol was transferred to the titration vessel and titrated to the endpoint. A sample of 10 µl of water, accurately measured, was used to standardize the Karl Fischer reagent. Accurately weighed samples were suspended in anhydrous methanol, and titration was carried out to the electromagnetic endpoint (Joseph, 2019).

Determination of reconstitution time

The Thiermann method was utilized to quantify the reconstitution times of lyophilized products. Each sample was reconstituted with 1 mL of sterile water for injection, and the process involved adding the solution onto the inner wall of the vial to ensure a smooth flow. Quantitative measurement determined the time required for the solution to achieve homogeneity without visible aggregates, thereby establishing the reconstitution time (Thiermann, 1998; Patil, 2023).

Stability study

Following the ICH Q1A R2 guideline for stability studies, the stability of the POS-MMs was assessed by storing them at a temperature range of 2–8 °C for 3 months (ICH Q1A(R2) guidelines, Hajare, 2021).

Statistical Analysis

Formulation and optimization data are averages of triplicates and expressed as means with ± SDs. The influence of formulation development variables on the response variables was statistically evaluated using ANOVA at a significance level of 0.05 with the Design-Expert® version (Stat-Ease Inc.) (Hajare, 2021). The polynomial equation, 3D response surface graphs, and contour plots to study the interaction of independent variables on dependent variables were established by applying ANOVA using Design-Expert® software. The data are presented as the mean ± standard deviation of three independent experiments. The obtained results were analyzed using one-way ANOVA and two-way ANOVA, and $p < 0.05$ indicated a statistically significant difference (Patil, 2022).

RESULTS AND DISCUSSION

In this research, POS-MMs were prepared by employing the solvent evaporation method and subsequently lyophilized to address the challenges related to stability and drug release.

Selection of formulation components

Soluplus® is a copolymer known for its exceptional solubilizing properties, which make it highly suitable for enhancing the solubility of poorly water-soluble drugs. By effectively solubilizing hydrophobic drugs, Soluplus® improves their dissolution rate and enhances their bioavailability, leading to better absorption and therapeutic efficacy. Moreover, Soluplus® demonstrates remarkable stability, safeguarding the encapsulated drug against degradation and physical changes. Acting as a protective barrier, it prevents drug precipitation and maintains the chemical integrity of the drug during formulation and storage (Attia, 2023). This ensures consistent and reliable drug delivery, minimizing the potential for variability in drug performance. On the other hand, PF68 is known for providing enhanced stability and protection, particularly for sensitive drugs, proteins, and nanoparticles. It acts as a shielding barrier, safeguarding the encapsulated payload from enzymatic degradation, pH changes, and physical stress. By preserving the stability and integrity of the encapsulated substances, PF68 ensures the efficacy and reliability of the drug delivery system (Fatima, 2022). Hence, by combining Soluplus® and PF68 the MMs of POS were prepared.

Critical micelle concentration

In a study by Patil et al., CMC of PF68, Soluplus®, and POS-MMs was determined using the iodine spectrophotometric method. The obtained CMC values were 0.037 mM, 0.0029 mM, and 0.033 mM, respectively. The POS-MMs exhibited a lower CMC than the other copolymers, suggesting enhanced stability when diluted in biological fluids. This indicates that MMs are less likely to disintegrate or experience drug leakage when exposed to biological environments (Patil, 2021).

Formulation optimization using a 3^2 factorial design

Initial experiments were conducted, and formulation optimization was performed by considering two factors: the concentration of PF68 (X_1) and the concentration of Soluplus® (X_2). The impact of these variables on the %EE and PS was investigated.

Table 2. 3^2 factorial design batches and the results of their characterization

Formulation code	Drug (mg)	Factor 1 (X_1) PF68 Concentration (mg)	Factor 2 (X_2) Soluplus® Concentration (mg)	Response 1 (Y_1) %EE (%)	Response 2 (Y_2) Particle size (nm)
A1	10	0.8	0.06	94.88±2.4	66.30±2.10
A2	10	0.8	0.08	91.60±2.1	77.31±1.18
A3	10	0.8	0.10	88.30±2.1	85.92±1.14
A4	10	1.1	0.06	92.46±1.6	75.56±2.10
A5	10	1.1	0.08	92.15±2.1	84.27±2.20
A6	10	1.1	0.10	90.67±1.9	92.69±2.24
A7	10	1.4	0.06	90.47±1.2	82.77±1.13
A8	10	1.4	0.08	91.82±2.2	85.70±2.41
A9	10	1.4	0.10	93.57±2.1	99.64±2.60

Values are mean ± SD (n = 3).

Among the nine batches of POS-MMs, the A1 batch was selected as the optimized formulation due to its high %EE (94.88±2.4%) and small particle size (66.3±2.1 nm) (Gaikwad, 2023).

%Entrapment efficiency and %drug loading capacity

The %EE of the optimized batch A1 was found to be 94.88±2.4%, and the %DLC was found to be 98.20±1.2%.

Effect of formulation variables on %EE

The %EE of the POS-MMs formulations ranged from 88.3 ± 2.1% to 94.88 ± 2.4%. It was observed that an increase in the concentration of PF68 and Soluplus® led to a decrease in %EE. Based on this observation, the optimum concentrations were determined to be 0.8 mg for PF68 and 0.06 mg for Soluplus® (Table 2). The factors and their interactions influencing %EE were represented using coded variables in the following equation:

$$\%EE = +91.75 - 0.1650A - 0.9017B + 2.40AB \quad \text{Eq. (4)}$$

The regression model equation (Eq. 1) suggests that the concentration of PF68 (X_1) and Soluplus® (X_2) exert a negative influence on the entrapment of the drug within the MMs. The significant model term is indicated by the model F-value of 191.49 and $p < 0.05$, implying the model's significance with X_1 and X_2. Moreover, the contour plot (Figure 1A.) and the 3D response plot (Figure 1B.) both illustrate the negative effects of X_1 and X_2 on the %EE (Li, 2023).

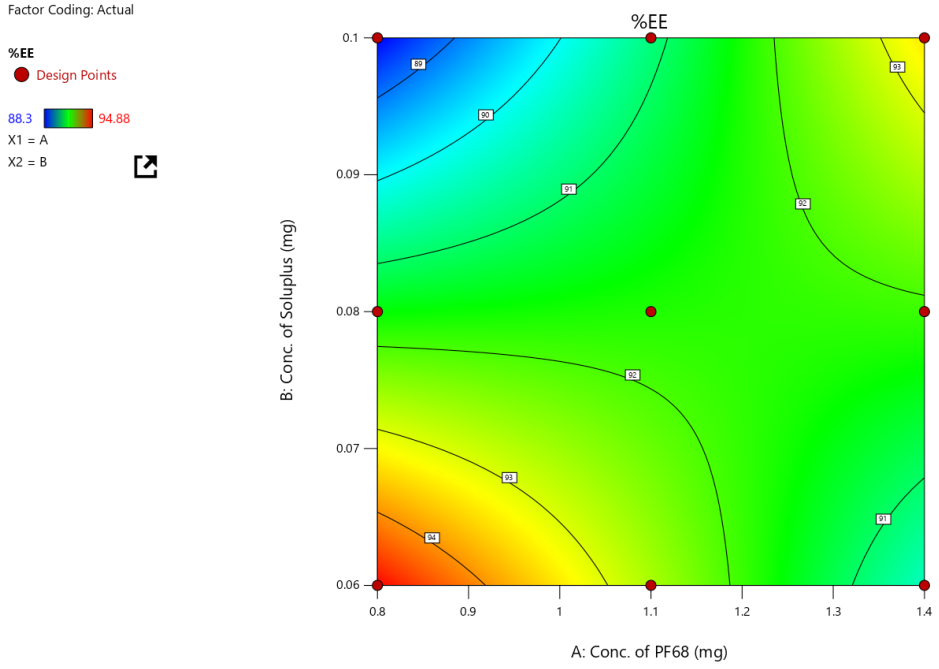

Figure 1A. Contour plot of %EE

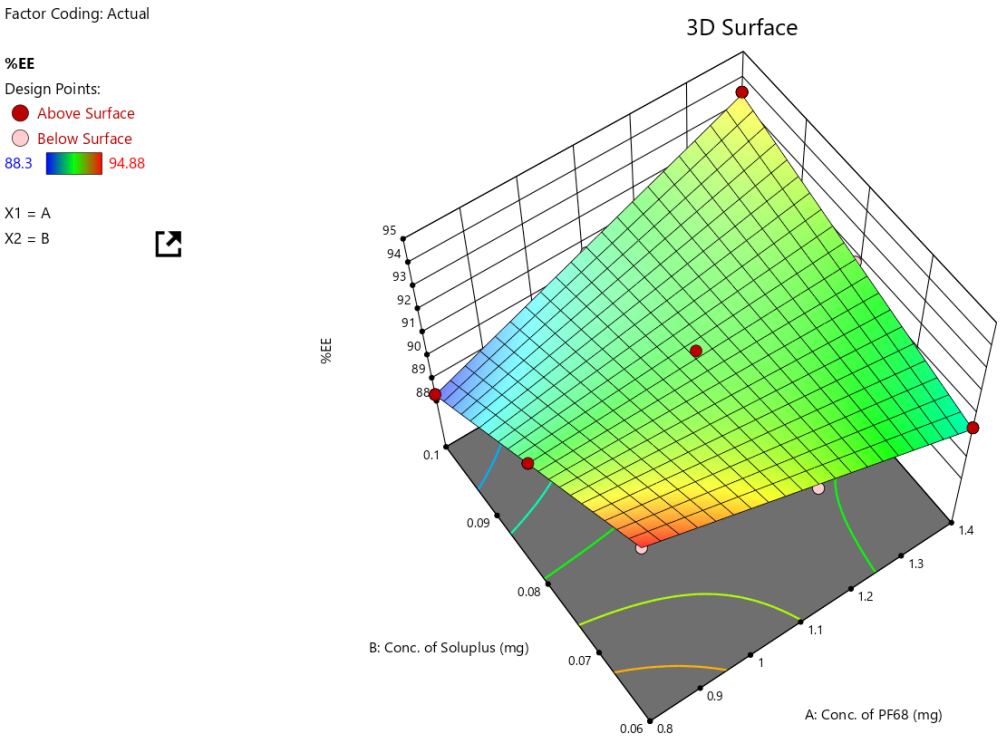

Figure 1B. 3D surface response plot of %EE

Effect of formulation variables on particle size

The particle size of the POS-MMs formulations varied between 66.3 ± 2.1 nm and 99.6 ± 2.6 nm. It was observed that the particle size increased with an increase in the concentration of PF68 and Soluplus®. Consequently, the optimal concentrations were determined to be 0.8 mg for PF68 and 0.06 mg for Soluplus®. The factors and their interactions influencing the particle size were represented in the following equation:

$$Particle\ size = +83.34 + 6.43A + 8.93B \quad \text{Eq. (5)}$$

The regression model equation (Eq. 2) suggests that the concentration of PF68 (X_1) and Soluplus® (X_2) have a positive impact on the particle size of the MMs. The significance of the model term is indicated by the model F-value of 82.77 and $p < 0.05$, indicating that the model is significant with X_1 and X_2. Furthermore, the contour plot (Figure 2A.) and the 3D response plot (Figure 2B.) both illustrate the positive effects of X_1 and X_2 on the particle size (Liu, 2022).

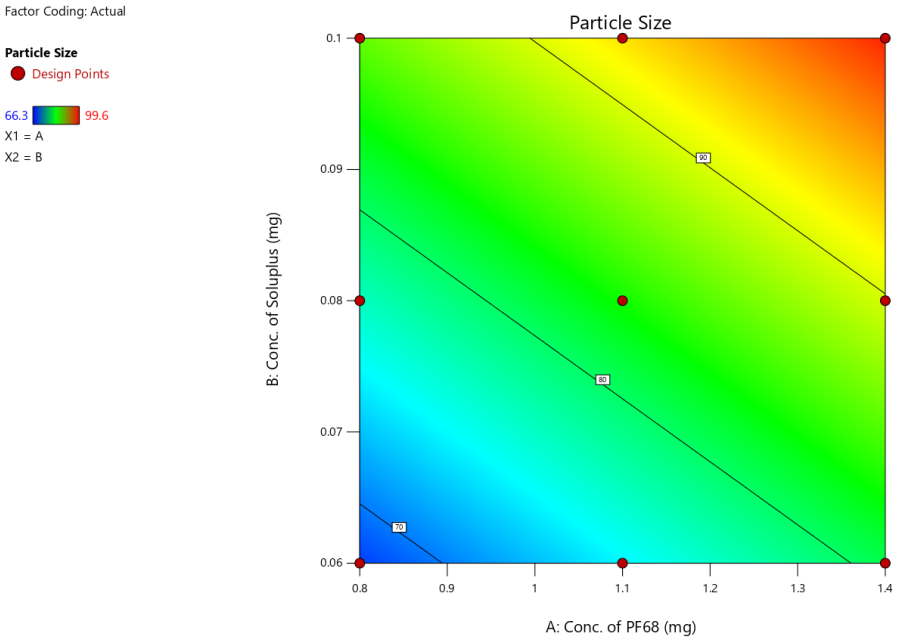

Figure 2A. Contour plot of particle size

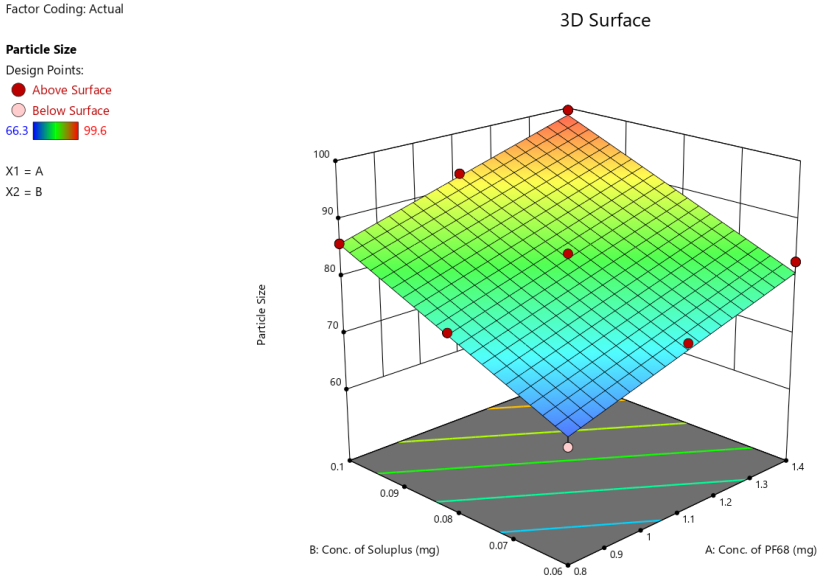

Figure 2B. 3D surface response plot of particle size

Particle size and zeta potential analysis

The optimized batch A1 displayed a PS of 66.30 ± 2.10 nm (with a PDI of 0.062) and a ZP of -51.1 ± 2.4 mV, as depicted in (Figures 3A and 3B). The PS was determined using dynamic light scattering (DLS). The nanoscale dimensions of the MMs are advantageous in fungal treatment, as they enable better penetration and more effective targeting of the infection site. The smaller PS offers an increased surface area for interaction, improved bioavailability, and enhanced cellular uptake, as fungal cells have specific mechanisms to internalize smaller particles more efficiently

(Zhang, 2023). Further, the high observed ZP of the MMs indicates excellent stability and the presence of strong electrostatic repulsion between micelles. This phenomenon plays a crucial role in preventing micelle aggregation or coalescence, which is particularly significant in applications such as drug delivery, where the stable dispersion of micelles is essential for the efficient transportation of active compounds. As reported by Feng et al. (2016), higher zeta potentials (usually > 30 mV) indicate stronger electrostatic repulsive forces, which are beneficial for maintaining a stable water dispersion formulation (Feng, 2016).

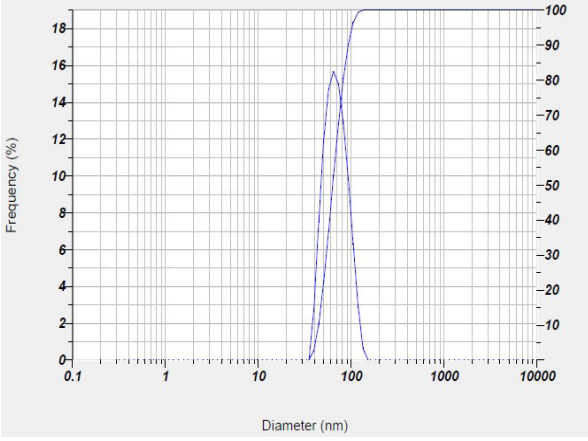

Figure 3A. PS of optimized batch

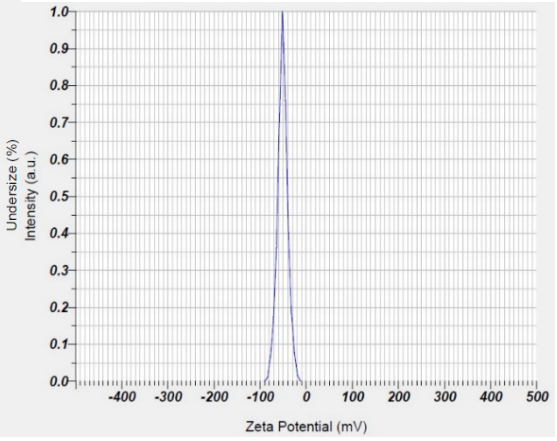

Figure 3B. ZP of optimized batch

Drug-excipient compatibility study

FTIR spectroscopy

Figure 4 shows the overlain FTIR spectrum of plain POS and the optimized formulation POS-MMs (A1). In the FTIR spectrum of POS, characteristic peaks were observed at 2966.73 cm^{-1}, corresponding to the stretching vibrations of C-H in the aromatic rings; 1685.72 cm^{-1}, representing the stretching vibration of the C=O bond; and 1509.46 cm^{-1}, indicating the stretching vibration of C=C in the aromatic rings of POS. IR spectroscopic studies were conducted on POS, PF68, and Soluplus® to evaluate potential drug-excipient interactions. The results indicate that the characteristic drug peaks remain unaltered, suggesting no significant chemical interactions between POS and the excipients. This confirms the stability of POS in its formulation (Hajare, 2021).

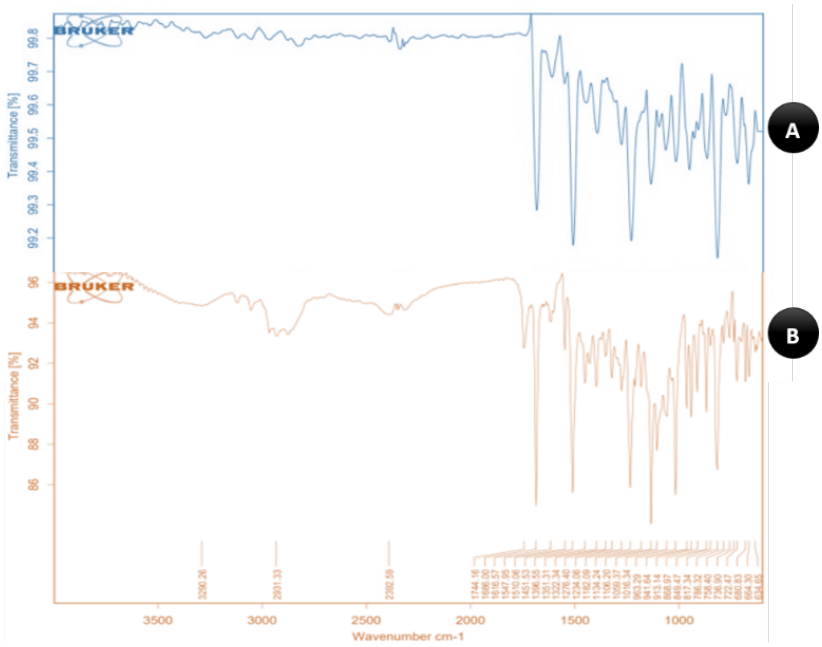

Figure 4. Overlain FTIR spectra of (A) plain POS and (B) POS-MMs A1 formulation

DSC analysis

Figure 5 displays the DSC thermograms obtained under a dry inert nitrogen atmosphere for plain POS and the POS-MMs (A1) formulation. The DSC thermogram of plain POS exhibited a broad peak at 169.98 °C, corresponding to its melting point. The DSC thermograms of the excipients in the formulation showed endothermic peaks at different temperatures, namely 138.58 °C, 155.17 °C, 215.00 °C, and 229.81 °C, representing their respective thermal events. In the POS-MMs (A1) formulation, the melting point peak of POS was no longer observed, suggesting amorphization within the MMs. This observation indicates that POS has been successfully entrapped and dispersed within the MMs (Hajare, 2020).

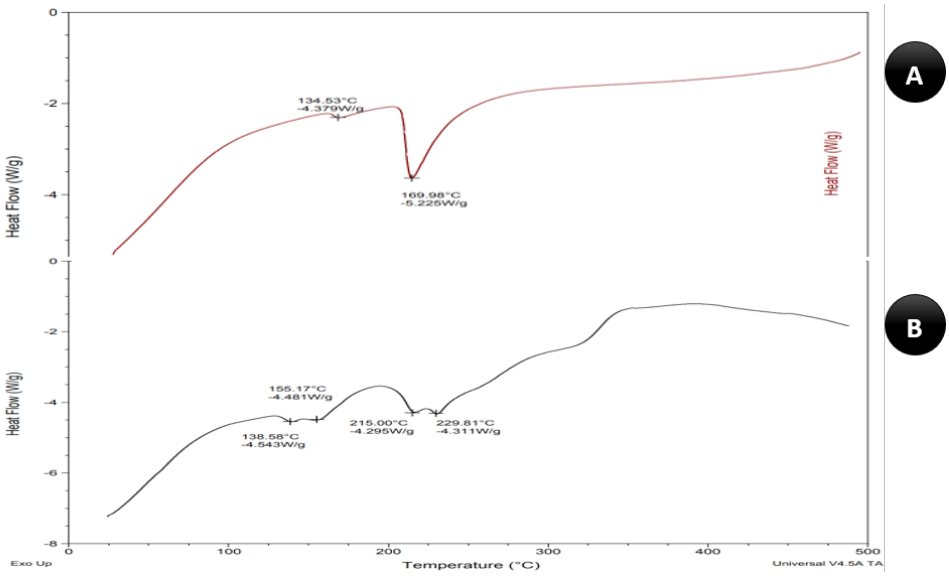

Figure 5. DSC thermograms of (A) plain POS and (B) POS-MMs A1 formulation

XRPD analysis

Figure 6 presents the XRPD patterns of plain POS and POS-MMs. The XRPD pattern of plain POS displayed distinct diffraction peaks, suggesting its crystalline nature. In contrast, the XRPD pattern of POS-MMs exhibited broadened peaks with decreased intensities, indicating partial amorphization of the drug within the micellar formulation (Patil, 2022).

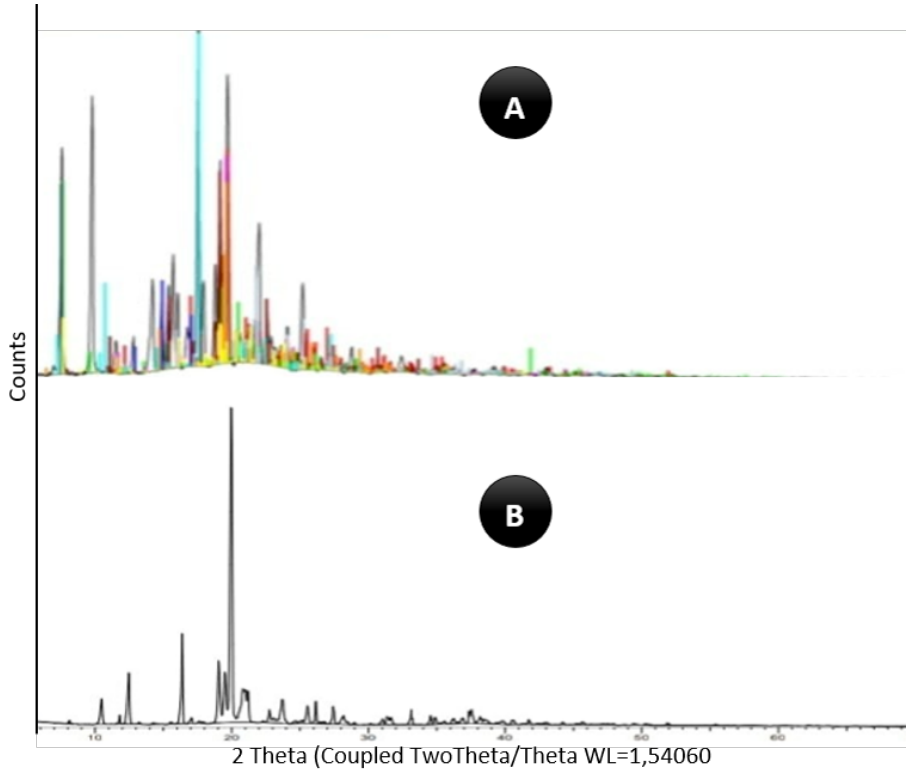

Figure 6. XRPD of (A) plain POS and (B) POS-MMs A1 formulation

In vitro drug release study

Drug release study

Figure 7 illustrates the release profiles of POS from both plain POS dispersion and POS-MMs (A1). In the PBS (pH 7.4) medium containing 0.5% w/v Tween 80, plain POS dispersion exhibited over 90% drug release within 12 hrs, indicating a rapid release profile. On the other hand, POS-MMs demonstrated sustained release behavior, with a release of 79.4% within 24 hrs ($p < 0.01$). The sustained release of POS from POS-MMs helps provide a continuous and controlled release of POS over an extended period, reducing the frequency of administration, enhancing its efficacy against fungal pathogens, and potentially improving patient compliance (Gupta, 2020).

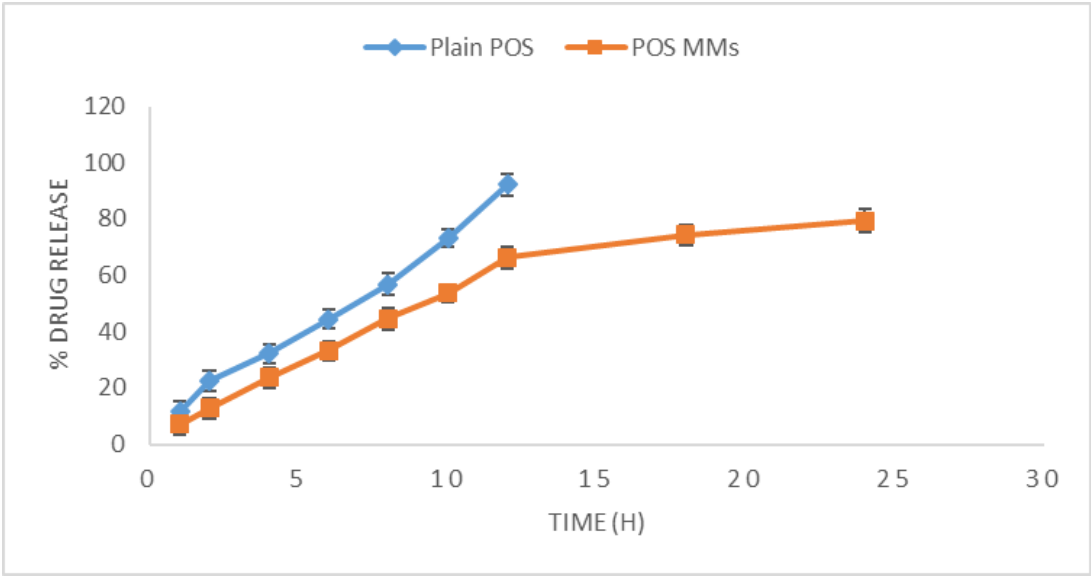

Figure 7. *In vitro* drug release study from (A) plain POS and (B) POS-MMs A1 formulation

Drug release kinetics study

To determine the mechanism of POS release from MMs, *in vitro* release data was fitted to various drug release kinetics models. The results indicated that the Korsemeyer-Peppas model (Figure 8) provided the best fit, suggesting that the release of POS from the MMs occurs through matrix diffusion. This implies that the drug molecules diffuse from the micellar core into the surrounding aqueous medium (Zlotnikov, 2023).

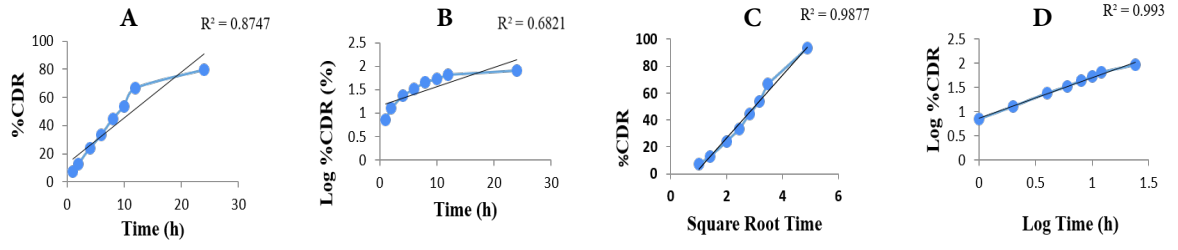

Figure 8. Comparative plots of (A) zero-order release kinetics, (B) first-order release kinetics, (C) Higuchi (SQRT) release kinetics, and (D) Korsemeyer-Peppas model for *in vitro* release profile of the optimized POS-MMS formulation

***In vitro* hemolysis study**

Devices and drug carriers derived from nanotechnology are becoming viable substitutes for traditional small-molecule drugs, and assessing their compatibility with blood components *in vitro* is an essential aspect of early preclinical development (Dobrovolskaia, 2008). In micelle-based drug delivery systems, *in vitro* hemolysis studies are of paramount importance, serving as an early assessment of biocompatibility and safety evaluation. These studies enable the detection and mitigation of hemolytic activity, contributing to safer drug delivery systems. They also provide predictive value for *in vivo* behavior, help meet regulatory requirements, and contribute to a deeper understanding of micelle interactions with biological components.

Figure 9 illustrates the results of the *in vitro* hemolysis study, which compared plain POS, blank PF68 and Soluplus® MMs, and POS-MMs. The positive control exhibited complete hemolysis, while the negative control showed minimal hemolysis. At various concentrations, regular POS caused more hemolysis at 50 µg/ml compared to both blank MMs and POS-MMs at the same concentration. Significantly, POS-MMs showed much lower hemolysis ($p < 0.01$) than plain POS at the same concentration. This finding highlights the favorable biocompatibility and safety of POS-MMs for intravenous administration (Khumaini, 2023).

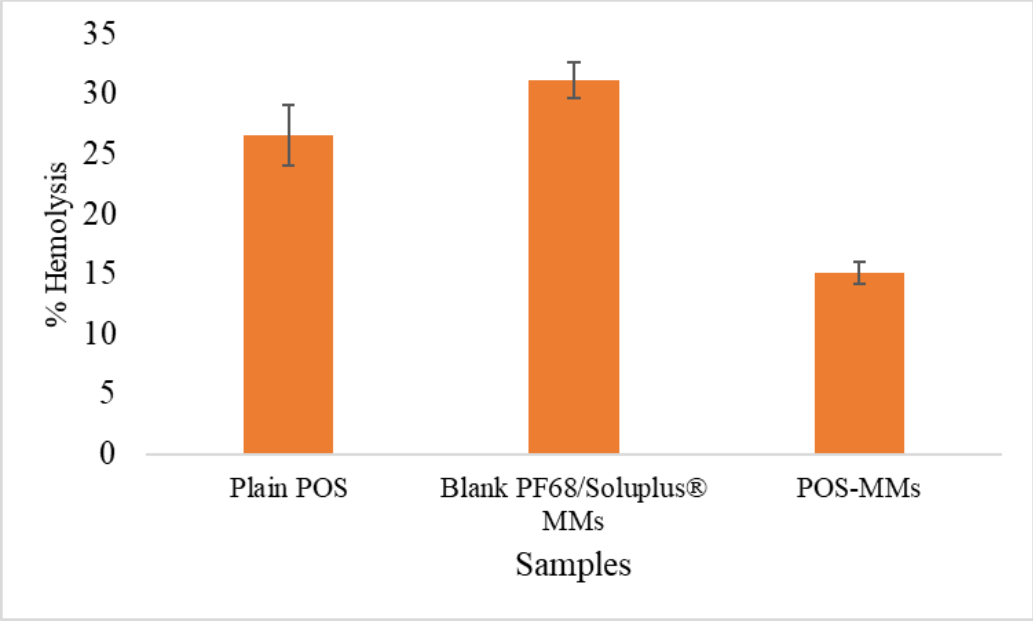

Figure 9. Results of hemolysis studies of plain POS, blank MMs, and optimized formulation (A1)

Characterization of lyophilized POS-MMs

The lyophilization of POS-MMs with 15% lactose as a cryoprotectant, as reported by Cheng (2020), has significantly enhanced the stability of the formulation. The increase in PS to 70.1±2.5 nm after lyophilization is due to the coating of the micelles by the cryoprotectant. When administered, these larger micelles can exhibit prolonged circulation times in the bloodstream and enhanced drug release at the target site, contributing to more effective and sustained treatment against fungal infections. The decrease in ZP of lyophilized POS-MMs to -25.3±2.8 mV demonstrates that lyophilization effectively prevented particle aggregation and maintained uniform particle dispersion. Furthermore, the high %EE of 96.46±4.1% and %DLC of 99.21±0.9% indicate that the active pharmaceutical ingredient (API) remains well-preserved within the

microspheres. The low moisture content of 2.49±0.5% confirms the absence of moisture-induced degradation. Additionally, the rapid RT of 46±4 sec highlights the ease with which the lyophilized POS-MMs can be redispersed, emphasizing preserving the microsphere integrity and drug release characteristics. Figure 11 represents the powdered formulation and the reconstitution product of the lyophilized POS-MMs. These collective findings underscore the critical role of lyophilization in maintaining the stability of POS-MMs, making them an attractive option for pharmaceutical applications where long-term stability and controlled drug release are essential (Bergonzi, 2020).

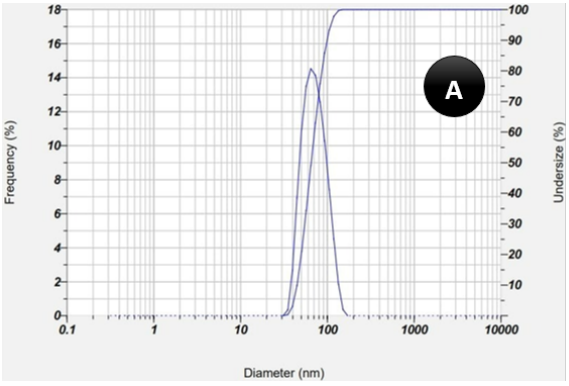

Figure 10A. PS lyophilized POS-MMs and

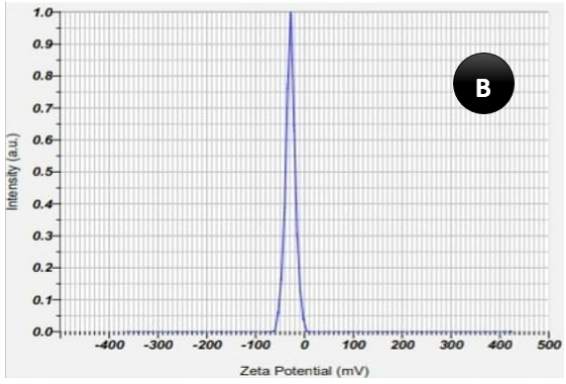

Figure 10B. ZP of lyophilized POS-MMs

Figure 11A. Lyophilized POS-MMs and

Figure 11B. Solution of POS-MMs after reconstitution

SEM was utilized to investigate the surface morphology of the POS-MMs, as depicted in Figure 12. The SEM images revealed that the POS-MMs exhibited irregularly folded, porous, and flocculated amorphous morphology, characterized by a smooth surface (Baviskar, 2022).

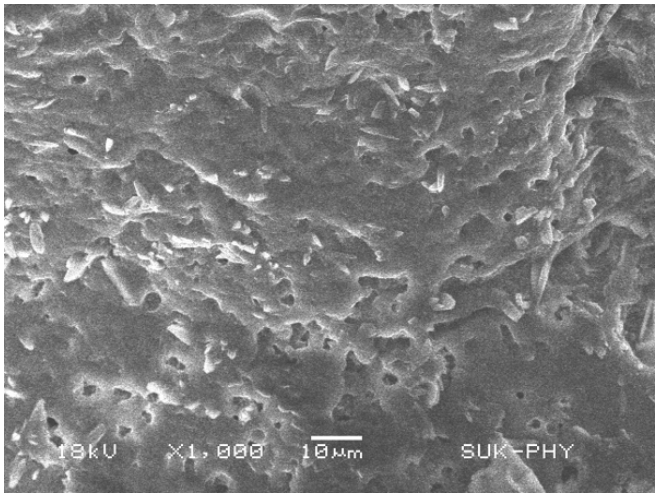

Figure 12. SEM images of POS-MMs

Stability study

Following the ICH Q1A R2 guideline for stability studies, the stability of the POS-MMs was assessed by storing them at a controlled temperature of 2–8 °C for three months. After this period, the %EE of the POS-MMs was determined to be 80.34%, as reported in Table 3 (Bhendale, 2023). A comparison between the stability of POS-MMs and lyophilized POS-MMs revealed that the PS of the liquid MMs increased rapidly, accompanied by a significant change in the %EE. In contrast, the lyophilized POS-MMs remained stable with minimal changes in %EE and particle size, demonstrating their superior stability compared to the liquid formulation (ICH Q1A(R2) guidelines).

Table 3. Stability study

Sampling Time	POS-MMs			Lyophilized POS-MMs		
	%EE (%)	Particle Size (nm)	%DLC	%EE (%)	Particle Size (nm)	%DLC
0 day	94.88±2.4	66.3±1.2	98.20±1.2	96.46±4.1	70.1±2.5	99.21±0.9
1 month	90.20±0.8	68.4±0.5	97.12±2.1	96.10±3.2	71.2±3.1	98.45±1.4
2 month	88.50±1.3	69.1±1.1	95.34±0.7	95.84±3.7	71.8±2.4	96.79±2.9
3 month	80.34±1.4	75.4±0.7	91.24±1.4	95.30±2.8	72.4±2.3	95.28±1.7

CONCLUSION

In conclusion, using PF68 and Soluplus® as excipients in the formulation of POS-MMs offers several advantages. The POS-MMs containing 0.08 mg PF68 and 0.06 mg Soluplus® exhibit a high %DLC, allowing for efficient encapsulation of POS. These excipients improve the stability of POS by protecting it from degradation and interactions with external factors. The micelles formed using PF68 and Soluplus® demonstrate controlled drug release, enabling sustained therapeutic effects.

The solvent evaporation method is simpler, less time-consuming, and more reproducible. It allows for the precise control of micelle composition and drug loading. This method ensures uniform drug distribution within the mixed micelles, resulting in consistent drug release profiles. Furthermore, the stability of the POS-MMs was further enhanced through the lyophilization process. Lyophilization effectively preserved the MMs structure, prevented drug degradation, and maintained drug loading efficiency. The resulting lyophilized powder exhibited improved sta-

bility during storage and handling, making it a more practical and reliable formulation. These findings contribute to the development of stable and efficient drug delivery systems, paving the way for improved therapeutic outcomes in treating fungal infections.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest

AUTHOR CONTRIBUTION STATEMENT

Concept (AK, KP), Design (AK, KP), Data Collection or Processing (AK, RC, VT, SP), Analysis and Interpretation (RC, KP, SP, JD, AH), Literature Search (AK, RC, VT), Writing (AK, RC, VT, KP, SP, JD, AH)

List of abbreviations

PF68: Pluronic F68®

POS-MMs: Posaconazole-loaded mixed micelles

FTIR: Fourier Transform Infrared spectroscopy

DSC: Differential scanning colorimetry

XRPD: X-ray powder diffraction

Hrs: hours

%EE: % entrapment efficiency

PS: Particle size

MMs: Mixed micelles

KI: Potassium iodide

I_2: Iodine

ZP: Zeta potential

%DLC: % drug loading capacity

POS: posaconazole

S: second

Da: Dalton

nm: Nanometer

DLS: Dynamic light scattering

CMC: Critical micelle concentration

SD: Standard deviation

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