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Original Article

QUALITY BY DESIGN-DRIVEN STATISTICAL SCREENING APPROACH TO SELECT EFFECTIVE VARIABLES FOR THE FORMULATION DEVELOPMENT OF PEGYLATED BILOSOMES FOR AN ANTIFUNGAL DRUG

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ABSTRACT

Objective: The study aimed to use a quality-by-design approach to screen out the most suitable process and formulation parameters for developing antifungal drug-loaded pegylated bilosomes.

Methods: Thin film hydration technique was used to prepare the formulations. A design experiment [Design Expert® software; Design of Experiments (DOE)] employing two levels at three factors was used to conduct eight runs to select and screen formulation and process variables. It was assessed for different response variables, such as Particle Size (PS), Polydispersity Index (PDI), Zeta Potential (ZP), and Entrapment Efficiency (%EE). The screened formulation was evaluated for *in vitro* drug release and kinetic model evaluation.

Results: The significance of each term in the model was evaluated using an Analysis of Variance (ANOVA). Statistical model terms with a significant P-value of less than 0.05 and graphical analysis (Interaction plot, Pareto chart, and 3D plots) generated by DOE version 13 demonstrated that Span 60, Brij C2, and amplitude of 30% were effective variables for formulating pegylated bilosomes with a desirability value of 0.965. The validated formulation showed a PS of 299.1±5.12 nm, PDI of 0.481±0.07, ZP of-36.6±0.55 mV, and %EE of 79.25±2.75. The *in vitro* release showed a sustained drug release of 55.53±6.75% over 24 h.

Conclusion: Statistical screening approach using a full factorial design can serve as a valuable tool in identifying and screening significant variables for developing antifungal-encapsulated pegylated bilosomes formulations.

Keywords: *Candida albicans*, Pegylated bilosomes, Screening, Design of experiments, Full factorial design

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INTRODUCTION

Yearly, over 150 million severe cases of fungal infections occur worldwide, resulting in 1.7 million deaths [1, 2]. Yeast and dermatophytes are potential pathogenic fungi that cause fungal infections. Superficial and cutaneous fungal infections are more frequent and common. The prevalence rate of Superficial Fungal Infections (SFI) has been estimated to be 20-25% globally, as per the World Health Organization. It is more frequent in Asian countries such as India, where the temperature and humidity are high for most of the year [3]. SFI is caused primarily by yeasts from the genus Candida, mainly *Candida albicans*. According to the Centers for Disease Control and Prevention, more than 150 species of Candida exist. *Candida albicans* species is responsible for approximately 70-80% of all Candida infections [4].

Ketoconazole is the most prescribed antifungal drug to treat superficial and systemic Candida fungal infections. It shows its action by inhibiting the synthesis of Ergosterol, a key fungal constituent of the cell wall. It is classified under the Biopharmaceutical Classification System class II (low solubility and high permeability) [5]. The available commercial cream and lotion formulations suffer from poor skin permeation and retention on the affected skin. Topical therapy in a vesicular delivery carrier is a safe and promising approach [6]. However, the conventional vesicular drug delivery systems remain confined to the upper layers of the stratum corneum, with poor permeation and early release of the encapsulated drug leading to the instability of the formulation [7]. So, a novel vesicular system, bilosomes, will be used for drug delivery via skin. Bilosomes are elastic, ultra-deformable, and flexible nano-vesicular carriers stabilized by bile salt, and the addition of a pegylated edge activator results in the formation of pegylated bilosomes. This innovative strategic approach can potentially provide a safe, stable, and effective treatment for fungal infections with improved drug permeation by formulating vesicles having lesser Particle Size (PS), Polydispersity Index (PDI), and higher Entrapment Efficiency (%EE) and Zeta Potential (ZP) values [8, 9].

In order to attain the above Quality Target Product Profile (QTPP), a methodological QbD (Quality by Design) strategy was used [10]. It is a methodical process of product growth that starts with well-defined goals and prioritizes comprehension of the product and its production process while ensuring process control by applying scientific principles and effective quality risk management. In QbD, Critical Material Attributes (CMA) and Critical Process Parameters (CPP) that affect predefined Critical Quality Attributes (CQA) are identified and assessed. This results in design space formation by the intricate interplay and amalgamation of input factors, such as materials attributes and process parameters, that are proven to ensure product quality [11, 12]. The selection of CPP and CMAs that can substantially impact the QTPPs is based on scientific knowledge from earlier published literature [13].

Screening studies are used to determine and identify the final product's desired characteristics and confirm the quality of the product. It is a systematic process that uses the statistical Design of Experiments (DOE) and modeling to identify the input variables or controllable factors that significantly affect the output or response, which can be observed from a physical process or calculated from a numerical model [14]. An experimental design was employed for this particular purpose. The factorial design is a statistical research approach that accounts for the interdependent effects of multiple variables in each set of experiments. A full factorial test is a statistical design encompassing multiple factors with discrete levels. This design involves testing every potential combination of the factor levels and experimental units comprising all possible combinations across the factors [15]. Such a design can be used for screening and/or optimization to examine the interaction and main effects [16]. It is a cost-effective technique that requires less time

and fewer experimental runs. So, a QbD method involving the DOE approach was used in this research work [17].

This work aimed to identify, screen, and investigate the effect of important formulation and processing independent parameters (CPP and CMAs) on the dependent responses (CQAs), such as PS, ZP, PDI, and %EE of ketoconazole-loaded pegylated bilosome formulations by utilizing a DOE approach. The screened and validated ketoconazole-loaded pegylated formulation was studied for *in vitro* drug release and kinetic model evaluation.

MATERIALS AND METHODS

Materials

Ketoconazole (Hi-Media Laboratories Pvt. Ltd., Mumbai), Sodium Deoxycholate (SDC) (Sisco Research Laboratories, Mumbai), Span 60, Span 20, Brij C2, and BrijO20 were obtained as a gift sample from Croda Ltd., Mumbai, and Methanol and Chloroform (Finar Ltd, Ahmedabad).

Formulation of ketoconazole-loaded pegylated bilosomes

The preparation of ketoconazole-loaded pegylated bilosomes was carried out using a thin-film hydration technique. An accurately weighed amount of drug (20 mg), 150 mg of surfactant (Span 60 or Span 20) and cholesterol in (5:1) ratio, 5 mg of bile salt (SDC), and 15 mg of Brij(Brij C2 or Brij O20) was dissolved in 10 ml of the chloroform-methanol mixture (7:3). The organic phase was evaporated using a rota evaporator under reduced pressure at 60 °C in a water bath and vacuumed for 30 min at 120 rpm. The thin film was hydrated at 60 °C for 1h using distilled water at 150 rpm under normal pressure. The resultant suspension was probe-sonicated by setting the parameters at different amplitude levels to reduce the large multilamellar vesicles into small unilamellar vesicles [18, 19].

Risk assessment and screening study by full factorial design

Based on existing literature reviews and initial investigations, a risk evaluation was conducted to detect and prioritize high-risk material traits and process factors that could potentially impact the formulation of ketoconazole-encapsulated pegylated bilosomes. To graphically emphasize the elements affecting the CQAs of formulation, a fishbone (Ishikawa or cause-and-effect) diagram was created as a graphical aid [13]. The literature documented various variables encompassing many categories, such as materials, process, environment, probe sonication, and personnel, presented within the framework of the Ishikawa diagram [9, 10, 18, 20–24]. Based on the outcomes from the Ishikawa diagram, the two categoric factors [surfactant type, pegylated edge activator (Brij) type)] and a numeric factor (amplitude) were considered as the crucial aspects influencing and impacting the characteristics of the final antifungal-loaded pegylated bilosomes formulation. These factors were systematically screened using a full factorial design (DOE). Three independent variables, each having two levels, were included in the study. Eight experimental runs were generated through a 2-level, 3-factor full factorial design to perform the study (table 1). The results of the prepared batch were then employed to screen the suitable variable for developing a formulation of pegylated bilosomes for an antifungal drug.

Table 1: 2^3 Full factorial design for ketoconazole encapsulated pegylated bilosome formulations

Run	Type of surfactant	Type of pegylated edge activator	Amplitude (%)
1	Span 60	Brij O20	40
2	Span 60	Brij C2	40
3	Span 20	Brij C2	40
4	Span 20	Brij C2	30
5	Span 20	Brij O20	40
6	Span 20	Brij O20	30
7	Span 60	Brij O20	30
8	Span 60	Brij C2	30
Independent variables	Levels		Dependent responses
	Low	High	Particle size
Surfactant Type	Span 20	Span 60	Polydispersity Index
Pegylated Edge activator Type	Brij O20	Brij C2	Zeta potential
Amplitude (%)	30	40	Entrapment Efficiency

Validation of the model and verification of the software-derived optimal solution

The response and factor relationships were graphically analyzed and developed using model graphs. The model was validated with numerical optimization corresponding to the formulation of optimal pegylated bilosomes. Based on the highest desirability value, a solution was selected and carried out under the recommended parameters. The data obtained was further validated using the software's predicted values [25].

Characterization studies

Particle size, zeta potential, and polydispersity index

The average PS, ZP, and PDI of ketoconazole-loaded pegylated bilosome formulations were analyzed using a Malvern zeta sizer by a dynamic light scattering technique at 25±2 °C. Before taking measurements, the formulations were diluted (10 times) with distilled water. All the characterization studies were performed thrice, and results were expressed as the mean±standard deviation (SD) [8].

Entrapment efficiency

The EE% for ketoconazole in pegylated bilosomes was assessed employing the indirect method. 1 ml of the formulation was placed in an Eppendorf tube and centrifuged at 15000rpm through a cooling centrifuge for 20 min at 4 °C. The resulting supernatant was then diluted with distilled water. The same procedure was repeated for the blank pegylated bilosomal dispersion. The concentration of the free ketoconazole was then analyzed via a UV-visible spectrophotometer against a blank supernatant at 225.5 nm. The EE% was estimated using the following subsequent equation: 1 [8, 26].

$$EE\% = \frac{\text{Amount of total drug} - \text{Amount of free drug}}{\text{Amount of total drug}} (\times 100) \ldots\ldots \text{Eq. 1}$$

In vitro drug release study

An in vitro drug release study was conducted using a modified Franz diffusion cell apparatus. A cellophane membrane with a molecular weight cutoff of 12,000 Daltons was presoaked overnight in a phosphate buffer solution (pH 5.5). The membrane was then placed in the donor compartment, which contained 50 ml of the release medium, maintained at 37±0.5 °C. A 1 ml aliquot of the validated ketoconazole-loaded pegylated bilosome formulation containing 2 mg of the drug was added to the donor compartment. The system was stirred at 50 rpm using a thermostat-controlled magnetic stirrer. At predetermined intervals, 2 ml samples were withdrawn from the receptor compartment and replaced with fresh buffer to maintain sink conditions. The absorbance of the collected samples was measured using a UV-visible spectrophotometer at 225.5 nm [27].

Release kinetics

Different mathematical models were applied to determine the drug release mechanism, including first-order kinetics, zero-order kinetics, Korsmeyer-Peppas, and the Higuchi model.

Zero-order kinetics describes systems where the drug release rate remains constant and is not influenced by the drug concentration. First-order kinetics pertains to systems where the drug release rate is directly proportional to its concentration. The Higuchi model characterizes drug release from an insoluble matrix as a process dependent on the square root of time, based on the principles of Fickian diffusion. The Korsmeyer-Peppas model provides a relationship for understanding mechanisms of drug release from polymeric systems [28].

RESULTS

Risk assessment by ishikawa diagram

Risk assessment serves as a valuable, science-driven methodology within quality risk management. It is instrumental in pinpointing process parameters and material attributes that could impact the CQAs of the product, as exhibited by the fishbone Ishikawa diagram (fig. 1) [25].

Screening study of ketoconazole-loaded pegylated bilosomes

The obtained values for PS, PDI, ZP, and EE% are represented in table 2.

The impact of independent factors on the dependent response was evaluated through Pareto charts (fig. 2), interaction plots of PS and PDI (fig. 3), 3D response plots (fig. 4), and interaction plots of ZP and %EE (fig. 5). These are useful for visually representing the impact of multiple variables on a single response simultaneously. The effect of the variables was also quantified using mathematical polynomial equations. The Analysis of Variance (ANOVA) for each response indicated a statistically significant value at a $P<0.05$ [29]. It was utilized to assess the model's statistical significance concerning PS, PDI, ZP, and EE% (table 3).

Table 2: Factors and responses of ketoconazole-loaded pegylated bilosomes

S. No.	Type of surfactant	Type of pegylated edge activator	Amplitude (%)	PS (nm)	PDI	ZP(mV)	%EE
1	Span 60	Brij O20	40	177.93±1.53	0.536±0.084	-32.6±0.556	75.3±1.29
2	Span 60	Brij C2	40	236.53±5.63	0.496±0.088	-42.7±0.458	76.3±0.950
3	Span 20	Brij C2	40	125.1±1.248	0.51±0.010	-57.23±0.208	52.25±1.078
4	Span 20	Brij C2	30	187.16±6.621	0.632±0.0267	-57.3±0.557	32±0.818
5	Span 20	Brij O20	40	130.46±2.250	0.6±0.045	-48±1.929	16.14±1.008
6	Span 20	Brij O20	30	102.87±5.216	0.483±0.020	-51.3±1.501	44.65±1.618
7	Span 60	Brij O20	30	203.1±0.945	0.543±0.092	-38.16±0.529	78.2±1.709
8	Span 60	Brij C2	30	294.23±3.745	0.548±0.548	-38.6±0.378	83.7±1.925

PS: Particle size, PDI: Polydispersity index, ZP: Zeta potential, EE%: Entrapment efficiency. The data is presented as mean±SD (n = 3), where n represents the total number of observations

Table 3: ANOVA for factorial model-particle size, polydispersity index, zeta potential, and entrapment efficiency

Particle size	Source	Squared sum	df	Mean square	F-value	p-value		R^2	Predicted R^2	Adjusted R^2	Adeq precision
	Model	26885.56	4	6721.39	15.19	0.0248	significant	0.9530	0.6655	0.8902	10.7803
	A	16762.80	1	16762.80	37.89	0.0086					
	B	6535.67	1	6535.67	14.77	0.0311					
	C	1721.08	1	1721.08	3.89	0.1431					
	BC	1865.99	1	1865.99	4.22	0.1323					
	Residual	1327.24	3	442.41							
	Cor Total	28212.79	7								
PDI	Model	0.0172	4	0.0043	13.59	0.0290	significant	0.9477	0.6282	0.8780	10.1727
	A	0.0013	1	0.0013	4.11	0.1356					
	AB	0.0011	1	0.0011	3.49	0.1584					
	BC	0.0101	1	0.0101	31.89	0.0110					
	ABC	0.0047	1	0.0047	14.88	0.0308					
	Residual	0.0009	3	0.0003							
	Cor Total	0.0181	7								
Zeta Potential	Model	588.81	5	117.76	73.47	0.0135	significant	0.9946	0.9134	0.9810	22.9001
	A	476.94	1	476.94	297.57	0.0033					
	B	83.01	1	83.01	51.79	0.0188					
	C	2.92	1	2.92	1.82	0.3098					
	BC	20.77	1	20.77	12.96	0.0693					
	ABC	5.17	1	5.17	3.22	0.2144					
	Residual	3.21	2	1.60							
	Cor Total	592.01	7								
	Model	4338.01	6	723.00	1389.85	0.0205	significant	0.9999	0.9923	0.9992	100.1384
Entrapment efficiency	A	3547.35	1	3547.35	6819.20	0.0077					
	B	112.20	1	112.20	215.69	0.0433					
	C	43.06	1	43.06	82.77	0.0697					
	AB	35.96	1	35.96	69.12	0.0762					
	BC	244.87	1	244.87	470.72	0.0293					
	ABC	354.58	1	354.58	681.62	0.0244					
	Residual	0.5202	1	0.5202							
	Cor Total	4338.53	7								

ANOVA: Analysis of variance, A: Surfactant, B: Type of edge activator, C: Amplitude, PDI: Polydispersity index, df: degree of freedom

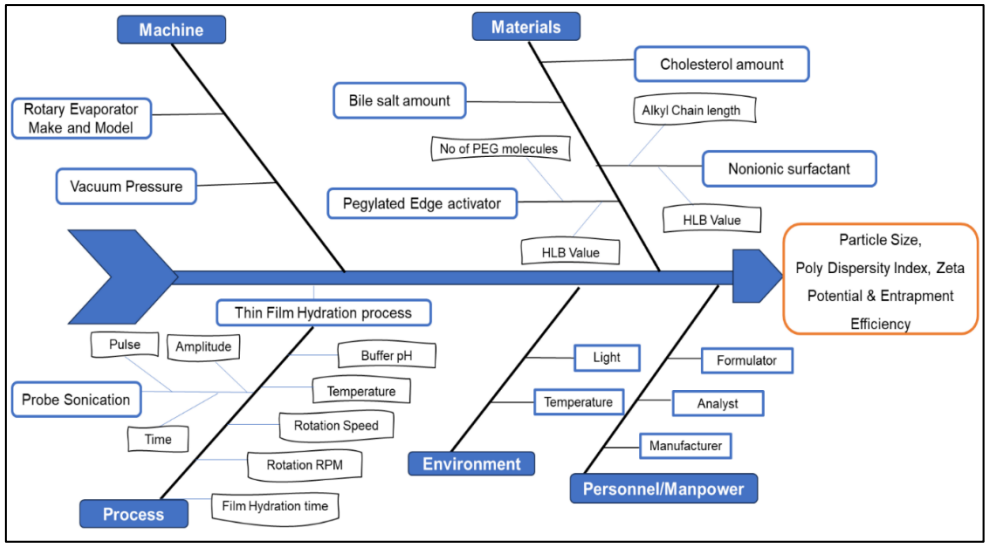

Fig. 1: Fishbone ishikawa diagram, PEG: Polyethylene glycol, HLB: Hydrophilic-lipophilic balance, RPM: Revolutions per minute

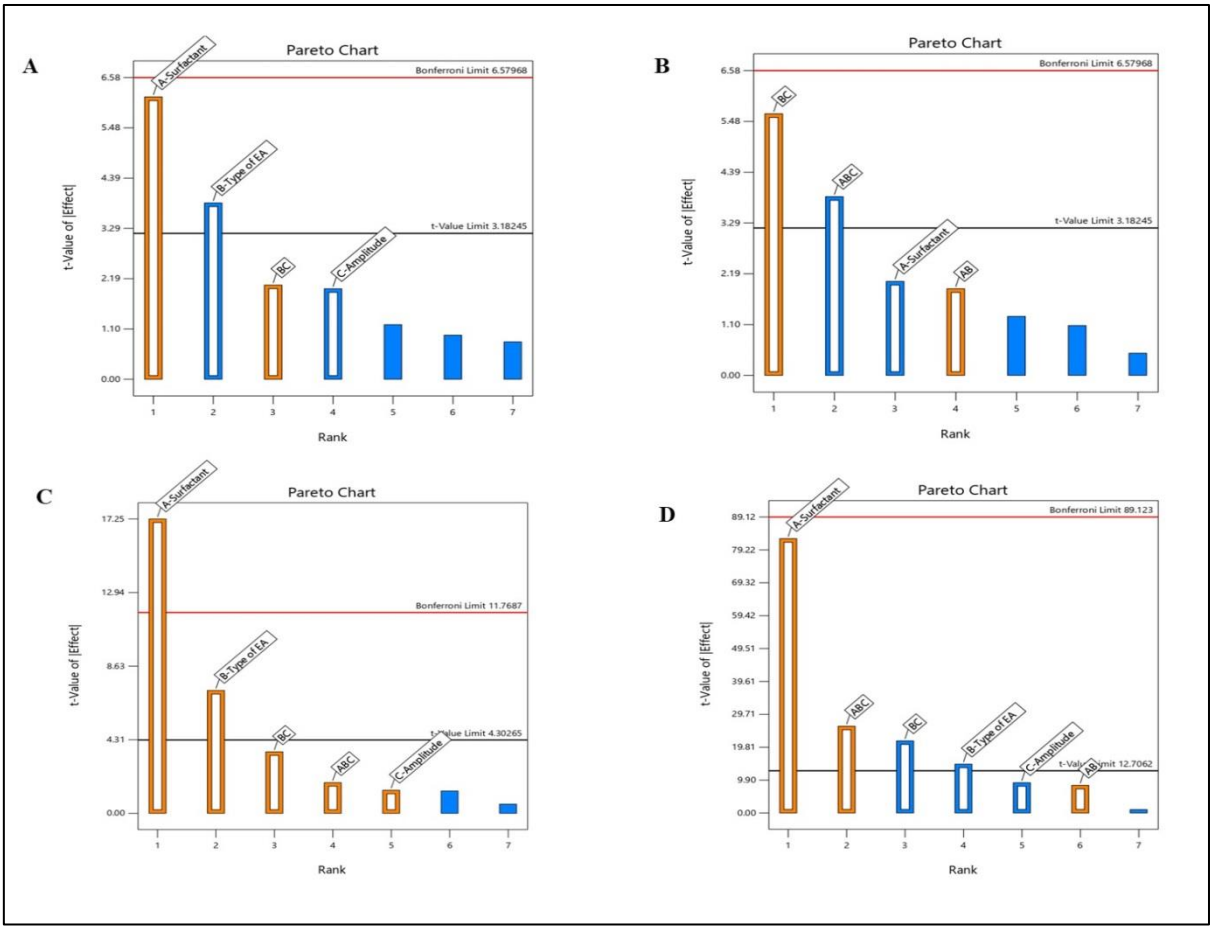

Fig. 2: Pareto chart illustrating the impact of independent factors on responses: A. Particle size, B. Polydispersity index, C. Zeta potential, D. Entrapment efficiency

The resulting are polynomial equations for PS, PDI, EE%, and ZP:

$$PS = +182.17 + 45.78A - 28.58B - 14.67C + 15.27BC - \text{(Eq. 2)}$$

$$PDI = +0.5435 - 0.0128A + 0.0118AB + 0.0355BC - 0.0243ABC - \text{(Eq. 3)}$$

$$ZP = -45.74 + 7.72A + 3.22B + 0.6037C + 1.61BC + 0.8038ABC - \text{(Eq. 4)}$$

$$EE\% = +57.32 + 21.06 - 3.75B - 2.32C + 2.12AB - 5.53BC + 6.66AB - \text{(Eq. 5)}$$

The negative and positive terms in the polynomial equation indicate independent variables' antagonistic and synergistic effects on responses [30]. Where A-Surfactant, B-Type of edge activator, and C-Amplitude and AB, BC, and ABC are the combined effects.

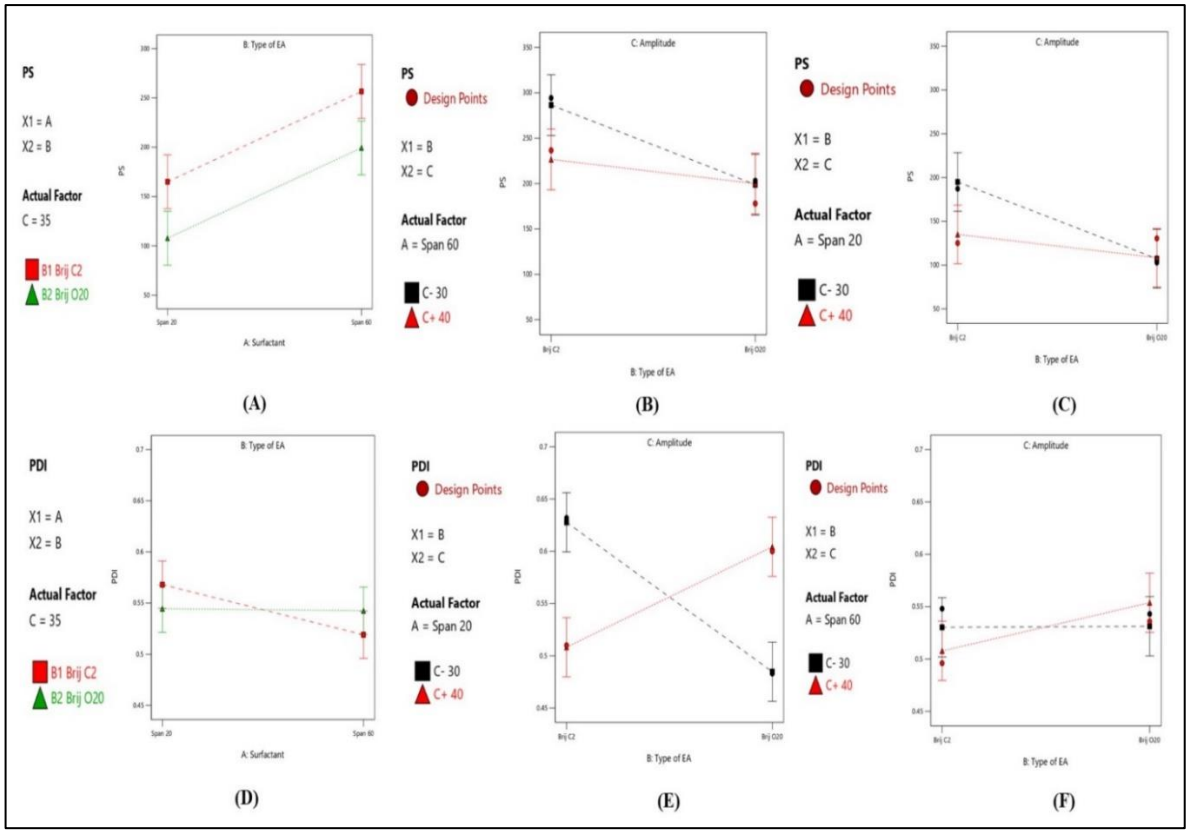

Fig. 3: Response interaction plots illustrate the intertwined impact of AB factors (surfactant type and edge activator type) and BC factors (edge activator type and amplitude) on particle size and polydispersity index, respectively, PS: Particle size, PDI: Polydispersity Index, EA: Edge activator

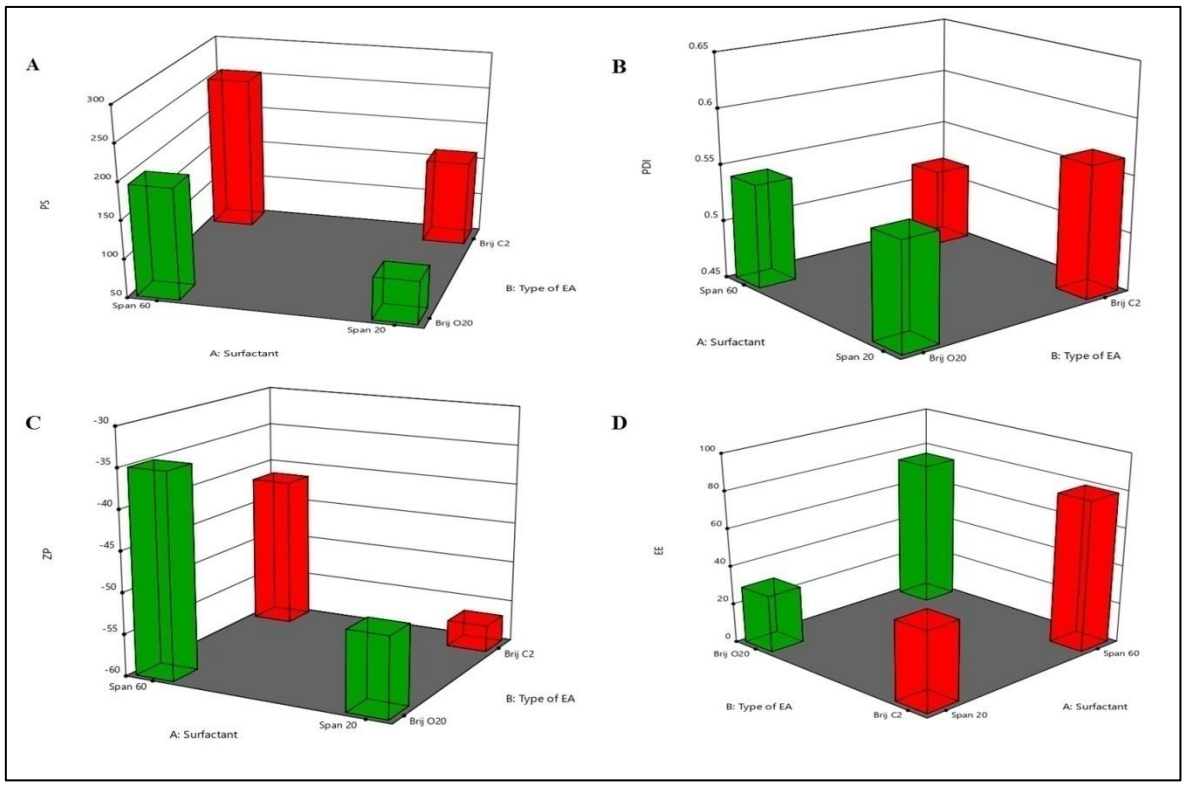

Fig. 4: 3D plots illustrating the impact of independent variables on dependent responses of ketoconazole-loaded pegylated bilosomes. A. Effect of surfactant and pegylated edge activator on particle size. B. Effect of surfactant and pegylated edge activator on polydispersity index C. Effect of surfactant and pegylated activator on zeta potential. D. Effect of surfactant and pegylated edge activator on entrapment efficiency, PS: Particle size, PDI: Polydispersity Index, ZP: Zeta potential, EE: Entrapment efficiency, EA: Edge activator

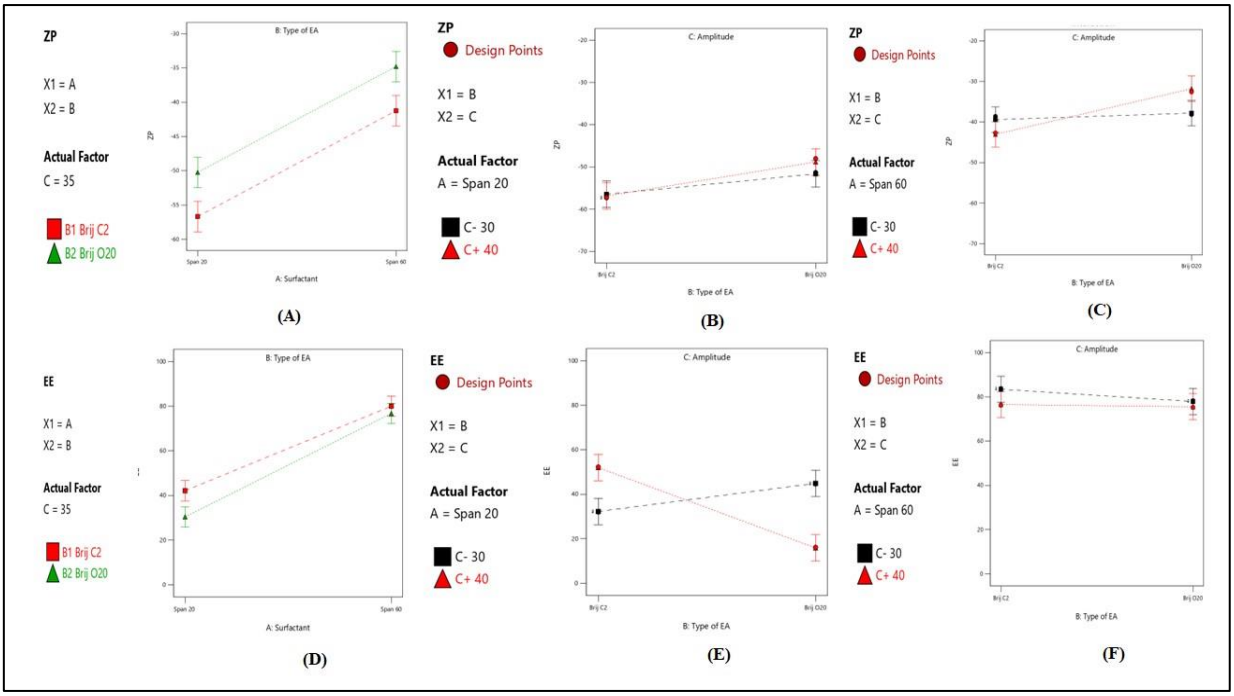

Fig. 5: Response interaction plots illustrating the combined impact of AB factors (surfactant type and edge activator type) and BC factors (edge activator type and amplitude) on zeta potential and entrapment efficiency, respectively, ZP: Zeta potential, EE: Entrapment Efficiency, EA: Edge activator

Validation of model and confirmation of software-derived optimized solution

The software DOE utilized the obtained response to predict the significant factors (formulation and process variables) with an optimal solution and desirability, which was subsequently prepared and subjected to further characterization study. The obtained predicted and actual values of the developed preparation from the software were substituted into the following equation 6.

$$\% \text{ Residuals} = \frac{\text{Predicted} - \text{Actual}}{\text{Predicted}} (\times 100) \ldots\ldots \text{Eq. 6}$$

The findings and the residual % error of PDI, PS, %EE, and ZP of the optimized solution are presented in table 4.

Table 4: Validation of the developed ketoconazole-loaded pegylated bilosome formulation

Formulation	Independent variables			Dependent responses				Desirability
	Type of surfactant	Type of edge activator	Amplitude (%)	PS (nm)	PDI	ZP (mV)	EE%	
Software suggested composition	Span 60	Brij C2	30	286.470	0.530	-39.425	83.445	0.965
Practically performed composition	Span 60	Brij C2	30	299.1	0.481	-36.6	79.25	
Residual error (%)				-4.40	9.24	7.16	5.02	

PS: Particle size, PDI: Polydispersity index, ZP: Zeta potential, EE%: Entrapment efficiency

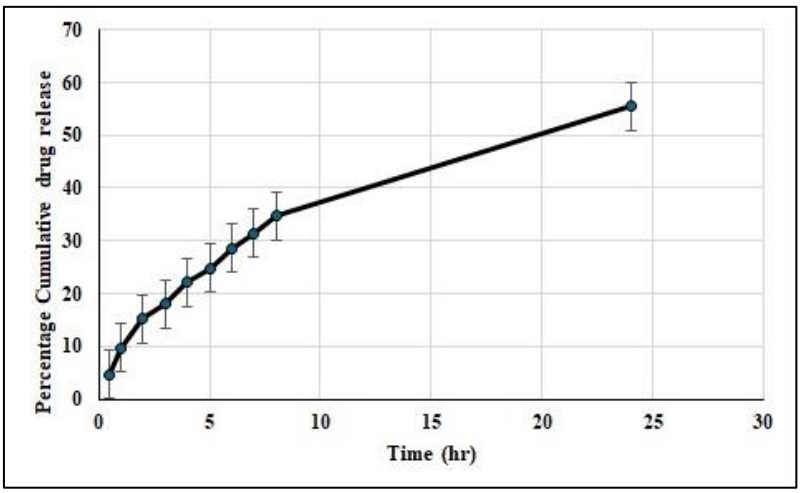

Fig. 6: *In vitro* drug release study of the screened and validated formulation, the data is presented as mean±SD (n = 3), where n represents the total number of observations

In vitro drug release

The *in vitro* drug release profile from the optimized and validated formulation demonstrated a slow and sustained release over a 24 h period (fig. 6). Specifically, the formulation exhibited a release of 34.79±6.47% at 8 h and 55.53±6.75% at 24 h from ketoconazole loaded pegylated bilosomes. To analyze the release kinetics, the *in vitro* release data were fitted to various mathematical models, and the regression coefficients (R^2) were calculated, as shown in table 5. The model with the highest R^2 value was selected to elucidate the drug release mechanism from the pegylated bilosome system.

Table 5: Kinetic modeling data of *in vitro* drug release study

Type of kinetic model	R^2
Korsmeyer Peppas model	0.967
Higuchi Model	0.9939
Zero-order Model	0.8952
First-Order Model	0.9557

DISCUSSION

Risk assessment by ishikawa diagram

It is commonly performed in the early stages of pharmaceutical product development. A fishbone diagram is a tool for identifying and analyzing risks, offering a structured approach to examine the causes generating or influencing specific effects, also termed a cause-and-effect diagram [12, 24]. Based on the Ishikawa diagram, the selected CPP and CMAs were screened using the factorial design.

Screening study of ketoconazole-loaded pegylated bilosomes

The factorial designs are commonly employed to identify the factors that could impact the attributes of a novel drug delivery system. They are beneficial as they can simultaneously analyze the multiple variable effects on the characteristics of the drug delivery methods [31, 32]. The study used a three-factor interaction model to analyze the dependent variables, demonstrating the highest R^2 prediction value. The adequate precision value of the model, which determines the ratio of signal-to-noise, confirms its adequacy in navigating the design space, with a preferred ratio (>4) for all the dependent variables [31, 33]. A reasonable agreement between the adjusted and predicted R^2 values, about 0.20, was necessary to confirm a good fit [34]. In all the responses, the R^2 adjusted values agreed well with the R^2 predicted values except for PS and PDI, possibly due to a large block effect [35, 36].

Influence of surfactants and pegylated edge activator on particle size

Equation 2 and the Pareto chart (fig. 2A) showed the synergistic effects of A and BC terms on PS, whereas ABC and a expressed antagonistic effects on PS. The confounding of two-factor interactions was observed in an interaction plot. The absence of interactions between the surfactant and edge activator types was observed, as indicated by two parallel lines (fig. 3A). By employing a suitable surfactant, a minor interaction effect was observed amongst the material attributes [type of edge activator (Brij C2 and Brij O20)] and process (30 and 40%) on PS (fig. 3B and 3C). It showed that combining the surfactant (span 20 or 60) with the Brij O20 edge activator and formulating at a lesser amplitude of 30% results in a particle with a lesser vesicle size.

The 3D plots showed that Span 60-based pegylated bilosomes result in larger particle sizes than Span 20. The size of the vesicles could influence C–H bonds present in the alkyl chain, potentially due to the longer chain length of Span 60 (C16) compared to the C12 chain of Span 20 [37]. A higher HLB (Hydrophilic-Lipophilic Balance) value of 15.3 containing an edge activator (Brij 020) increased the surface free energy with decreased vesicle PS [9, 38] (fig. 4A). In contrast, an edge activator with a lower HLB of 5.3 (Brij C2) increased the PS. An increase in the PEG (Polyethylene Glycol) content from 2 to 20 units may slow the rate of vesicle precipitation, thereby preventing vesicle aggregation [9, 39]. In the case of the ANOVA factorial model for PS, the model's F-value of 15.19 and A and B terms implies the model is significant (table 3). There is a mere 2.48% probability that F-value could be attributed to random noise. The R^2 predicted value of 0.6655 differed significantly from the adjusted R^2 value of 0.8902, which could indicate a more considerable block effect.

Influence of surfactants and pegylated edge activator on polydispersity index

Regarding PDI, a value of 1 denotes a polydisperse particle, whereas a value nearer to 0 signifies monodispersity. The ketoconazole-loaded pegylated bilosome formulation showed a PDI range from 0.483 to 0.632, depicting a narrow to polydisperse distribution of particles.

As shown in Equation 3 and the Pareto chart (fig. 2B), the terms A and ABC negatively impacted the PDI. In contrast, BC and AB had a positive effect, exceeding the Bonferroni limit. The sonication amplitude positively impacted PDI, leading to an elevation in PDI values, which could be due to the particles' irregular shape [40].

The interaction plot was used to examine the impact of the pegylated edge activator and amplitude on the formulation's PDI by employing two distinct surfactants, which depicted strong interaction (fig. 3D). A robust interaction was identified between the edge activator type and amplitude when utilizing span 20 as the surfactant (fig. 3E). Conversely, span 60 also exhibited an interaction effect, which was comparatively less pronounced than that observed with span 20 (fig. 3F). The 3D graphs (fig. 4B) demonstrated that amplitude, pegylated edge activator, and surfactant type all had an equal individual impact on the PDI of the formulation. The model has an F-value (13.59), indicating that the model is not only significant but also well-fitted, as the p-value<0.05. The large F-value is attributed to random noise with only a 2.90% probability. BC and ABC were observed as significant model terms (table 3). The predicted R^2 value of 0.6282 deviated significantly from the adjusted R^2 of 0.8780, indicating a larger block effect than the PS ANOVA model.

Influence of surfactants and pegylated edge activator on zeta potential

Equation 4 and the Pareto chart (fig. 2C) showed that the A, B, C, BC, and ABC terms positively influenced the ZP of the formulation, in which the term BC (Type of edge activator and amplitude) had a much higher impact. Two parallel lines denoted the absence of interactions between the surfactant and edge activator types (fig. 5A). A stronger interaction was identified between the edge activator type and amplitude when using span 60 as the surfactant than in the span 20-based formulation (fig. 5B and 5C).

The 3D graphs illustrated decreased ZP values for the Brij O20-containing formulation, whereas Brij C2-based formulations observed higher ZP (fig. 4C). The Transition Temperature (Tc) of Brij C2 is 36-38 °C and Brij O20 of 25-30 °C, respectively. A higher Tc-containing edge activator causes more ordered and stable vesicles with higher ZP values. Brij O20 contains 20 repeated PEG units. An increased hydrophilic PEG steric shield on the surface of the vesicles clears the carboxyl groups' surface charge, lowering ZP [9]. In the study by Ammar *et al.*, 2018, it was reported that formulations based on span 60 exhibit higher EE% and tend to acquire more charge due to the ionization of Span 60 into a negatively charged molecule under alkaline or neutral pH conditions [41]. A and B terms in the selected ANOVA factorial model and the F-value (73.47) imply a significant model (table 3). There is only a mere 1.35% chance that the larger F-value arises from noise. The R^2 predicted value of 0.9134 was in reasonable concordance with the R^2 adjusted value of 0.9810 with a difference of<0.2.

Influence of surfactants and pegylated edge activator on entrapment efficiency

Equation 5 and the Pareto chart of EE% (fig. 2D) illustrated the detrimental influence of terms B, C, and BC, whereas the A, AB, and ABC terms exhibited a positive effect. No interaction was observed between the edge activator and surfactant (fig. 5D). However, a strong interaction between the edge activator type and amplitude was found while employing span 20 as the surfactant (fig. 5E and 5F).

As illustrated in fig. 4D, the long-saturated alkyl chain length of Span 60 (C18) showed higher EE% than the Span 20 (C12) based formulations. Higher drug entrapment increases the bilayer distance by including the drug within the vesicles in the hydrophobic zones, thus increasing PS [20]. The HLB value of the surfactant and pegylated edge activator also affects the EE% values. Span 20, Span 60, Brij C2, and Brij O20 have an HLB of 8.7, 4.7, 5.3, and 15.3, respectively. The lower HLB value results in higher EE%. In Brij O20, the presence of a carbon chain with an unsaturated double bond (C=C) and the loose packing of the molecules may cause the vesicle membrane to twist and become leakier, resulting in a decreased EE% [9]. The surfactant and pegylated edge activator's Tc also affected EE%. The Tc of Span 60 (53 °C), Span 20 (16 °C), Brij C2 (36-38 °C), and Brij O20 (25-30 °C), respectively. Increasing Tc increases the capacity to establish a structured and organized bilayer, ultimately contributing to higher EE% values [42]. The selected ANOVA factorial model for EE% showed that the A, B, BC, and ABC terms were significant. The higher F-value of 1389.85 also implies that the model demonstrates statistical significance ($P<0.05$) and that there is a mere 2.05% chance that this larger F-value owning to noise (table 3). A difference of less than 0.2 was observed between the predicted R^2 of 0.9923 and the adjusted R^2 value of 0.9992.

The high-frequency vibrations (amplitude) during sonication result in the formation of a cavity with a decrease in vesicle PS. The continuous formation and implodation of intense microscopic vacuum bubbles lead to high collapse and, thus, shear, eventually converting large multilamellar vesicles to small unilamellar vesicles [43]. High-power probe sonication also causes a little temperature elevation due to the high energy acquisition, resulting in drug leakage and low EE% [44].

Validation of model and confirmation of software-derived optimized solution

The screening study revealed that utilizing a surfactant and a pegylated edge activator, which has a lower HLB value, along with a reduced amount of PEG molecules in the pegylated edge activator structure and application of lower power (amplitude) during sonication, would result in the optimum formulation of ketoconazole-loaded pegylated bilosomes. It showed that a combination of Span 60 and Brij C2 at an amplitude of 30% resulted in optimum PDI, PS, EE%, and ZP. The residual % error of PDI, PS, EE%, and ZP was within ±15% and in close agreement with the values predicted by the software (table 4).

In vitro drug release study

The evaluation of the release kinetic model depicted first-order kinetics and the Higuchi model, and the results were similar to the study by Subair *et al.* [45]. The observed slow drug diffusion and penetration of the drug from the dissolution medium align with the Higuchi model, suggesting diffusion-controlled drug release, which may be likely due to an increased path length for the diffusion of the drug associated with a slower erosion rate of the formulation. Additionally, the linearity of the log cumulative percentage of drug released from the pegylated bilosomes with respect to time confirms the Korsmeyer–Peppas model (table 5). With an "n" value of more than 0.45, the system displayed anomalous or non-Fickian diffusion of the drug [28]. Non-Fickian implies that the release of the drug is governed by both erosion/dissolution of the lipid matrix and diffusion of the drug rather than solely by concentration gradients of the drug. The release is ruled by both diffusion of the drug and dissolution/erosion of the lipid matrix. These findings suggest a shift from a purely diffusion-controlled mechanism to anomalous transport, where both erosion and diffusion play significant roles. Therefore, the results clearly suggest first-order characteristics with a diffusion-dominant mechanism of drug release from ketoconazole-loaded pegylated bilosomes [46–48].

CONCLUSION

This research aimed to systematically identify suitable independent variables for developing pegylated bilosomes loaded with an antifungal drug using a quality-by-design approach. The formulation process utilized the thin film hydration method. A 2^3factorial design was implemented to systematically assess various independent variable and their impact on key dependent responses, including PS, PDI, %EE, and ZP. A blend of Brij C2 and Span 60 at an amplitude of 30% was chosen as the optimum variable to formulate and develop antifungal drug-loaded pegylated bilosomes through the statistical data analysis (Pareto, 3D and interaction plots) generated by the DOE software. The *in vitro* drug release demonstrated diffusion-controlled release of developed and validated formulation. The study concluded by exhibiting the significance of DOE and QBD in optimizing the appropriate independent variables. This research showcases the effectiveness of QBD principles in pharmaceutical formulation development, emphasizing the importance of a systematic and scientific approach to achieving desirable product attributes.

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AUTHORS CONTRIBUTIONS

Devika Nayak: Writing original draft, Data curation, Conceptualization and Methodology. Mahalaxmi Rathnanand: Investigation, Visualization. Vamshi Krishna Tippavajhala: Supervision, Investigation, Visualization.

CONFLICT OF INTERESTS

Declared none

REFERENCES

1. Bongomin F, Gago S, Oladele RO, Denning DW. Global and multinational prevalence of fungal diseases estimate precision. J Fungi (Basel). 2017;3(4):1-29. doi: 10.3390/jof3040057, PMID 29371573.
2. Xiao Z, Wang Q, Zhu F, An Y. Epidemiology species distribution antifungal susceptibility and mortality risk factors of candidemia among critically ill patients: a retrospective study from 2011 to 2017 in a Teaching Hospital in China. Antimicrob Resist Infect Control. 2019;8(1):89. doi: 10.1186/s13756-019-0534-2, PMID 31161036.
3. Lakshmanan A, Ganeshkumar P, Mohan SR, Hemamalini M, Madhavan R. Epidemiological and clinical pattern of dermatomycoses in rural India. Indian J Med Microbiol. 2015;33 Suppl:134-6. doi: 10.4103/0255-0857.150922, PMID 25657132.
4. Aaron D. Candidiasis (mucocutaneous) dermatological disorders. MSD manual professional edition; 2022. Available from: https://www.msdmanuals.com/en-in/professional/dermatologic-disorders/fungal-skin-infections/candidiasis-mucocutaneous.
5. Ketoconazole. Drugbank. Available from: https://go.com/drugs/DB01026.
6. FDA drug safety communication: FDA limits usage of nizoral (ketoconazole) oral tablets due to potentially fatal liver injury and risk of drug interactions and adrenal gland problems. Available from: https://www.fda.gov/drugs/drug-safetyand-availability/fda-drug-safety-communication-fda-limits-usage-nizoral-ketoconazole-oral-tablets-due-potentially.
7. Guo F, Wang J, MA M, Tan F, LI N. Skin targeted lipid vesicles as novel nano carrier of ketoconazole: characterization *in vitro* and *in vivo* evaluation. J Mater Sci Mater Med. 2015 Apr 1;26(4):175. doi: 10.1007/s10856-015-5487-2, PMID 25825320.
8. Saifi Z, Rizwanullah M, Mir SR, Amin S. Bilosomes nanocarriers for improved oral bioavailability of acyclovir: a complete characterization through *in vitro* ex-vivo and *in vivo* assessment. J Drug Deliv Sci Technol. 2020 Jun;57:101634. doi: 10.1016/j.jddst.2020.101634.
9. Albash R, El Nabarawi MA, Refai H, Abdelbary AA. Tailoring of PEGylated bilosomes for promoting the transdermal delivery of olmesartan medoxomil: *in vitro* characterization ex vivo permeation and *in vivo* assessment. Int J Nanomedicine. 2019 Aug 15;14:6555-74. doi: 10.2147/IJN.S213613, PMID 31616143.

10. Alshaer W, Nsairat H, Lafi Z, Hourani OM, Al Kadash A, Esawi E. Quality by design approach in liposomal formulations: robust product development. Molecules. 2022 Dec 20;28(1):10. doi: 10.3390/molecules28010010, PMID 36615205.
11. International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use. Pharmaceutical. Development. Vol. Q8(R2); 2009.
12. International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use. Quality. Risk Manag. Vol. Q9; 2005.
13. Ibrahim MS, Elmahdy Elsayyad NM, Salama A, Noshi SH. Quality by design (QBD) as a tool for the optimization of indomethacin freeze-dried sublingual tablets: *in vitro* and *in vivo* evaluation. Int J App Pharm. 2021;13(5):160-71. doi: 10.22159/ijap.2021v13i5.42216.
14. Woods DC, Lewis SM. Handbook of uncertainty quantification. Handbook of uncertainty quantification; 2015.
15. Singh A, Chauhan CS. Factorial designing an essential tool in pharmaceutical optimization. YMER. 2022;21(7):519-25. doi: 10.37896/YMER21.07/41.
16. Dhoot AS, Fernandes GJ, Naha A, Rathnanand M, Kumar L. Design of experiments in pharmaceutical development. Pharm Chem J. 2019;53(8):730-5. doi: 10.1007/s11094-019-02070-4.
17. Chinta R, Rohini P. Formulation development of empagliflozin and metformin hydrochloride extended-release tablets: optimization of formulation using statistical experimental design. Res J Pharm Technol. 2021;14(3):1201-8. doi: 10.5958/0974-360X.2021.00214.6.
18. Aziz DE, Abdelbary AA, Elassasy AI. Investigating superiority of novel bilosomes over niosomes in the transdermal delivery of diacerein: *in vitro* characterization ex vivo permeation and *in vivo* skin deposition study. J Liposome Res. 2019 Jan 2;29(1):73-85. doi: 10.1080/08982104.2018.1430831, PMID 29355060.
19. El Nabarawi MA, Shamma RN, Farouk F, Nasralla SM. Bilosomes as a novel carrier for the cutaneous delivery for dapsone as a potential treatment of acne: preparation characterization and *in vivo* skin deposition assay. J Liposome Res. 2020 Jan 2;30(1):1-11. doi: 10.1080/08982104.2019.1577256, PMID 31010357.
20. Al Mahallawi AM, Abdelbary AA, Aburahma MH. Investigating the potential of employing bilosomes as a novel vesicular carrier for transdermal delivery of tenoxicam. Int J Pharm. 2015 May 15;485(1-2):329-40. doi: 10.1016/j.ijpharm.2015.03.033, PMID 25796122.
21. Dobo DG, Nemeth Z, Sipos B, Cseh M, Pallagi E, Berkesi D. Pharmaceutical development and design of thermosensitive liposomes based on the QBD approach. Molecules. 2022;27(5):1536. doi: 10.3390/molecules27051536, PMID 35268637.
22. Kis N, Kovacs A, Budai Szucs M, Gacsi A, Csanyi E, Csoka I. Investigation of silicone-containing semisolid in situ film forming systems using QBD tools. Pharmaceutics. 2019;11(12):660. doi: 10.3390/pharmaceutics11120660, PMID 31817871.
23. Shreya AB, Managuli RS, Menon J, Kondapalli L, Hegde AR, Avadhani K. Nano-transpersonal formulations for transdermal delivery of asenapine maleate: *in vitro* and *in vivo* performance evaluations. J Liposome Res. 2016;26(3):221-32. doi: 10.3109/08982104.2015.1098659, PMID 26621370.
24. Waghule T, Dabholkar N, Gorantla S, Rapalli VK, Saha RN, Singhvi G. Quality by design (QBD) in the formulation and optimization of liquid crystalline nanoparticles (LCNPs): a risk based industrial approach. Biomed Pharmacother. 2021 Sep;141:111940. doi: 10.1016/j.biopha.2021.111940, PMID 34328089.
25. Miriam Marques S, Shirodkar RK, Kumar L. Analytical quality by design paradigm in development of a RP-HPLC method for the estimation of cilnidipine in nanoformulations: forced degradation studies and mathematical modelling of *in vitro* release studies. Microchem J. 2023 Oct;193:109124. doi: 10.1016/j.microc.2023.109124.
26. Teaima MH, Alsofany JM, El Nabarawi MA. Clove oil endorsed transdermal flux of dronedarone hydrochloride loaded bilosomal nanogel: factorial design *in vitro* evaluation and ex vivo permeation. AAPS Pharm Sci Tech. 2022;23(6):182. doi: 10.1208/s12249-022-02337-2, PMID 35773361.
27. El Shenawy AA, Abdelhafez WA, Ismail A, Kassem AA. Formulation and characterization of nanosized ethosomal formulations of antigout model drug (febuxostat) prepared by cold method: *in vitro*/ex vivo and *in vivo* assessment. AAPS Pharm Sci Tech. 2019;21(1):31. doi: 10.1208/s12249-019-1556-z, PMID 31858305.
28. Shinde UA, Shete JN, Nair HA, Singh KH. Design and characterization of chitosan alginate microspheres for ocular delivery of azelastine. Pharm Dev Technol. 2014;19(7):813-23. doi: 10.3109/10837450.2013.836217, PMID 24032373.
29. Zafar A, Alruwaili NK, Imam SS, Hadal Alotaibi N, Alharbi KS, Afzal M. Bioactive apigenin loaded oral nano bilosomes: formulation optimization to preclinical assessment. Saudi Pharm J. 2021;29(3):269-79. doi: 10.1016/j.jsps.2021.02.003, PMID 33981176.
30. Ani JU, Okoro UC, Aneke LE, Onukwuli OD, Obi IO, Akpomie KG. Application of response surface methodology for optimization of dissolved solids adsorption by activated coal. Appl Water Sci. 2019;9(3):1-11. doi: 10.1007/s13201-019-0943-7.
31. Abdelbary AA, Abd Elsalam WH, Al Mahallawi AM. Fabrication of novel ultra deformable bilosomes for enhanced ocular delivery of terconazole: *in vitro* characterization ex vivo permeation and *in vivo* safety assessment. Int J Pharm. 2016;513(1-2):688-96. doi: 10.1016/j.ijpharm.2016.10.006, PMID 27717916.
32. Araujo J, Gonzalez Mira E, Egea MA, Garcia ML, Souto EB. Optimization and physicochemical characterization of a triamcinolone acetonide loaded NLC for ocular antiangiogenic applications. Int J Pharm. 2010;393(1-2):167-75. doi: 10.1016/j.ijpharm.2010.03.034, PMID 20362042.
33. DE Lima LS, Araujo MD, Quinaia SP, Migliorine DW, Garcia JR. Adsorption modeling of Cr Cd and Cu on activated carbon of different origins by using fractional factorial design. Chem Eng J. 2011;166(3):881-9. doi: 10.1016/j.cej.2010.11.062.
34. Annadurai G, Ling LY, Lee JF. Statistical optimization of medium components and growth conditions by response surface methodology to enhance phenol degradation by pseudomonas putida. J Hazard Mater. 2008;151(1):171-8. doi: 10.1016/j.jhazmat.2007.05.061, PMID 17618738.
35. Mosallam S, Sheta NM, Elshafeey AH, Abdelbary AA. Fabrication of highly deformable bilosomes for enhancing the topical delivery of terconazole: *in vitro* characterization microbiological evaluation and *in vivo* skin deposition study. AAPS Pharm Sci Tech. 2021;22(2):74. doi: 10.1208/s12249-021-01924-z, PMID 33586022.
36. Albash R, Yousry C, Al Mahallawi AM, Alaa Eldin AA. Utilization of PEGylated cerosomes for effective topical delivery of fenticonazole nitrate: *in vitro* characterization statistical optimization and *in vivo* assessment. Drug Deliv. 2021;28(1):1-9. doi: 10.1080/10717544.2020.1859000, PMID 33322971.
37. Asgharkhani E, Fathi Azarbayjani A, Irani S, Chiani M, Saffari Z, Norouzian D. Artemisinin loaded niosome and pegylated niosome: physic chemical characterization and effects on MCF-7 cell proliferation. J Pharm Investig. 2018;48(3):251-6. doi: 10.1007/s40005-017-0331-y.
38. Tagami T, Ernsting MJ, LI SD. Optimization of a novel and improved thermosensitive liposome formulated with DPPC and a Brij surfactant using a robust *in vitro* system. J Control Release. 2011;154(3):290-7. doi: 10.1016/j.jconrel.2011.05.020, PMID 21640149.
39. Caliceti P, Salmaso S, Elvassore N, Bertucco A. Effective protein release from PEG/PLA nano particles produced by compressed gas anti-solvent precipitation techniques. J Control Release. 2004;94(1):195-205. doi: 10.1016/j.jconrel.2003.10.015, PMID 14684283.
40. Fernandes AV, Pydi CR, Verma R, Jose J, Kumar L. Design preparation and *in vitro* characterizations of fluconazole loaded nanostructured lipid carriers. Braz J Pharm Sci. 2020;56:1-14. doi: 10.1590/s2175-97902019000318069.
41. Ammar HO, Mohamed MI, Tadros MI, Fouly AA. Transdermal delivery of ondansetron hydrochloride via bilosomal systems: *in vitro* ex vivo and *in vivo* characterization studies. AAPS Pharm

Sci Tech. 2018;19(5):2276-87. doi: 10.1208/s12249-018-1019-y, PMID 29845503.

42. Khalil RM, Abdelbary A, Kocova El Arini S, Basha M, El Hashemy HA. Evaluation of bilosomes as nanocarriers for transdermal delivery of tizanidine hydrochloride: *in vitro* and ex vivo optimization. J Liposome Res. 2019 Apr 3;29(2):171-82. doi: 10.1080/08982104.2018.1524482, PMID 30221568.
43. Jain S, Indulkar A, Harde H, Agrawal AK. Oral mucosal immunization using glucomannosylated bilosomes. J Biomed Nanotechnol. 2014;10(6):932-47. doi: 10.1166/jbn.2014.1800, PMID 24749389.
44. Zafar A, Alsaidan OA, Imam SS, Yasir M, Alharbi KS, Khalid M. Formulation and evaluation of moxifloxacin loaded bilosomes in situ gel: optimization to antibacterial evaluation. Gels. 2022;8(7):418. doi: 10.3390/gels8070418, PMID 35877503.
45. Subair TK, Mohanan J. Development of nano-based film forming gel for prolonged dermal delivery of luliconazole. Int J Pharm Pharm Sci. 2022;14(2):31-41. doi: 10.22159/ijpps.2022v14i2.43253.
46. Ramasamy T, Khandasami US, Ruttala H, Shanmugam S. Development of solid lipid nanoparticles enriched hydrogels for topical delivery of anti-fungal agent. Macromol Res. 2012;20(7):682-92. doi: 10.1007/s13233-012-0107-1.
47. Naveentaj S, Muzib YI, Radha R. Design and optimization of fluconazole loaded pharmacosome gel for enhancing transdermal permeation and treating fungal infections through box behnken design. Int J App Pharm. 2023;15(1):131-40. doi: 10.22159/ijap.2023v15i1.46413.
48. MD S, Mehaboob SZ, Doddayya H. Preparation and characterization of fluconazole topical nanosponge hydrogel. Int J Pharm Pharm Sci. 2024;16(4):18-26.