OPTIMIZING PARENTERAL ITRACONAZOLE LIPOSOMES: A STATISTICAL EXPERIMENTAL DESIGN APPROACH

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Abstract
The current study aimed to systematically develop stable parenteral itraconazole liposomes using a statistical design of experiment approach. A 3^2 full factorial design was constructed to prepare nine liposomal batches by varying concentrations of hydrophobic lipid (hydrogenated soy phosphatidylcholine (HSPC) or distearoylphosphatidylcholine (DSPC) and cholesterol. Liposomes were formulated employing spray drying and lyophilization techniques. Itraconazole content was quantified using a validated UV spectrophotometric method. Fourier transform infrared spectroscopy (FTIR) confirmed no interactions between the drug and excipients. The formulated liposomes were evaluated for critical attributes including pH, drug loading efficiency, vesicle size, zeta potential and in vitro drug release. Entrapment efficiencies ranged from 33-95% for HSPC liposomes and 26-90% for DSPC liposomes. Optimal batches LH5 and LD5 exhibited particle size less than 150nm, zeta potential greater than -30mV and sustained release for 24 hours. Statistical analysis suggested the liposomal attributes were influenced by lipid composition and concentrations. The developed liposomes demonstrated desired characteristics for parenteral itraconazole delivery.
Keywords: Itraconazole, liposomes, factorial design, parenteral formulation, drug delivery.

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Introduction
Itraconazole is a broad-spectrum triazole antifungal agent used to treat fungal infections [1]. However, its hydrophobicity poses challenges in developing effective parenteral formulations for patients who cannot receive oral medication [2]. Liposomes are nanoparticulate vesicles composed of phospholipid bilayers that can encapsulate hydrophilic drugs in the aqueous core as well as hydrophobic drugs in the bilayer. Liposomal formulations have shown promise for parenteral delivery of hydrophobic drugs due to their biocompatibility and ability to alter drug pharmacokinetics [3,4]. However, developing reproducible liposomal formulations on an industrial scale poses technical challenges5.This study aimed to develop parenteral itraconazole liposomal formulations using statistical experimental design and characterization. A 32 full factorial design was used to systematically vary concentrations of HSPC/DSPC and cholesterol. Liposomes were prepared by spray drying and characterized for critical quality attributes relevant for parenteral delivery such as particle size, zeta potential, drug loading and release. The developed formulations have potential to improve therapeutic efficacy of itraconazole for parenteral administration.

Materials and Methods
Materials
Itraconazole was kindly gifted by Unichem Laboratories (Goa, India). Hydrogenated soy phosphatidylcholine (HSPC) and distearoylphosphatidylcholine (DSPC) were obtained from Lipoid GmbH Sigma Aldrich, Bangalore. Cholesterol, α-tocopherol and stearic acid were purchased from Merck Chemicals, Hyderabad. All the chemicals used were of analytical grade.

Analytical method for estimation of Itraconazole
Various analytical techniques exist for itraconazole estimation, such as UV spectrophotometric6, thin-layer chromatography [7], and HPLC-UV [8] methods. For routine analysis, a practical, swift, and cost-effective method is favored. In this context, the UV

spectrophotometric technique, as previously outlined by Parikh et al [9]., was adopted for this study. A 100 mL volumetric flask contained 50 mg itraconazole dissolved in 20 mL methanol, adjusted with pH 7.4 phosphate buffer (stock solution I). From this, 1 mL was diluted with pH 7.4 phosphate buffer to produce a 5 µg/mL concentration (stock solution II). The UV-visible spectrometer (Perkin Elmer Model Lambda 950) analyzed Stock solution II at 264 nm, where the maximum absorbance occurred. Standard solutions were prepared by diluting 0.2, 0.4, 0.6, 0.8, and 1.0 µg/mL solutions of stock solution II. The UV spectrophotometer measured absorbance at 264 nm using pH 7.4 phosphate buffer as the blank. Replicated thrice for each trial, the absorbance values formed the basis of the calibration curve. A plot of absorbance versus itraconazole concentration yielded the regression equation for itraconazole estimation in pH 7.4 phosphate buffer [9].

Drug excipient compatibility studies using FTIR

The drug and excipients were individually weighed according to predetermined ratios based on the intended formulation. The drug-excipient mixtures were prepared by physically blending the drug with each excipient using a mortar and pestle. The mixtures were thoroughly homogenized to ensure uniform distribution. Control samples consisting of the drug alone and individual excipients were also analyzed using FTIR to establish their spectral and thermal profiles [10]. This served as a reference for comparison with the drug-excipient mixtures and helped identify any significant deviations or interactions. Similarly, pellets were prepared by blending lyophilized itraconazole liposomes powder (approximately 2 mg) with KBr (approximately 300 mg). The FTIR spectra thermograms obtained from the drug-excipient mixtures and control samples were carefully analyzed. Any changes or shifts in characteristic peaks, appearance of new peaks, or melting point depression were noted and compared [11]. A Fourier Transform Infrared (FTIR) spectrometer (Bruker Alpha II, Hallmark mechatronics, Kerala) was used for the analysis of drug-excipient compatibility. The drug-excipient mixtures were subjected to FTIR analysis to investigate any potential chemical interactions or incompatibilities. A small amount of each drug-excipient mixture was placed on the sample holder of the FTIR spectrometer [12]. The FTIR spectra were recorded over a specific spectral range and analyzed for any changes or shifts in characteristic peaks that might indicate interaction or incompatibility between the drug and excipients.

Experimental design

A 32 full factorial design was constructed with 2 independent variables (amount of HSPC/DSPC (X1) and amount of cholesterol (X2)) each at 3 levels (-1, 0, +1). 9 formulations were designed based on all possible combinations of variable levels. Dependent variables measured were particle size (Y1), % entrapment efficiency (Y2) and in vitro drug release at 24 h (Y3) were used as dependent variables [13]. Design Expert® DX8.0.7.1 trial version software was used for the generation and evaluation of statistical experimental design. Table 1 shows the composition of itraconazole liposomes.All the liposomal batches are incorporated with itraconazole at a constant concentration of 2 mg/mL and each batch size is 500 mL

Table 1: Composition of itraconazole liposomes

Liposomes-H			Liposomes-D		
Batch code	**Amount of HSPC (X1)**	**Amount of cholesterol (X2)**	**Batch code**	**Amount of DSPC (X1)**	**Amount of cholesterol (X2)**
LH1 (-1, -1)	30	15	LD1 (-1, -1)	30	15
LH2 (-1, 0)	30	30	LD2 (-1, 0)	30	30
LH3 (-1, 1)	30	45	LD3 (-1, 1)	30	45
LH4 (0, -1)	60	15	LD4 (0, -1)	60	15
LH5 (0, 0)	60	30	LD5 (0, 0)	60	30
LH6 (0, 1)	60	45	LD6 (0, 1)	60	45
LH7 (1, -1)	90	15	LD7 (1,-1)	90	15
LH8 (1, 0)	90	30	LD8 (1, 0)	90	30
LH9 (1, 1)	90	45	LD9 (1, 1)	90	45

Preparation of liposomes by spray drying

a) Preparation of Itraconazole and Lipid Solution

A solution of hydroxylpropyl phosphatidylcholine (HSPC) or distearoylphosphatidylcholine (DSPC), stearic acid, cholesterol, and itraconazole is dissolved in a 1:1 v/v mixture of methanol and chloroform within a steel jacketed vessel at 25°C. The solution is heated to 50°C with continuous stirring to achieve complete dissolution of the itraconazole-HSPC/DSPC-lipid complex. The pH of the complex is adjusted to 4.0 through stirring at 50°C. To enhance liposome retention in target tissues, α-tocopherol (0.05 mg/mL) is incorporated into the organic solution at

pH 4.0 and subsequently filtered using a 0.5 µm stainless steel sintered filter. The resulting clear organic solution is then subjected to the spray drying process to yield a spray-dried itraconazole liposome complex powder. All experiments are conducted on a 500 mL scale, with bulk dispersion prepared in a 1 L stainless steel vessel14. The flow diagram depicting the organic solution preparation for spray drying is presented in Figure 1.

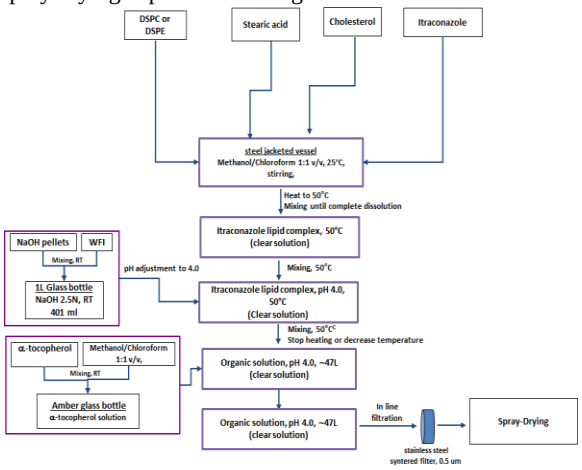

Figure 1: Flow diagram for spray drying of itraconazole liposomes

b) Preparation of Spray-Dried Itraconazole-Lipid Complex Powder

Optimization of the spray drying process involves considerations of feed rate, inlet temperature, nitrogen gas flow, and outlet temperature. A benchtop spray dryer (Mini Buchi) is employed for lab-scale spray drying experiments. Initial trials encompass varying inlet temperature, feed rate, and nitrogen flow rate to optimize the process. Based on these preliminary experiments, critical process parameters (CPP), including feed flow rate, nitrogen flow, and inlet temperature, are optimized to ensure reproducible high yield. The optimized process parameters for spray drying included a pump setting of 3 mL/min to control the feed rate of the organic solution into the spray dryer15. The nitrogen gas flow was maintained at 450 L/h to efficiently atomize the solution into fine droplets and facilitate solvent evaporation. An inlet temperature of 58°C and outlet temperature of 43°C within the drying chamber were conducive for the evaporation process. The run time for each batch was kept at 80 minutes for completeness of the drying cycle. Under these critical conditions, the resulting spray drying rate averaged 2.6 g/min with a high recovered yield of 80-85% of the processed dispersion [16].

c) Hydration of Spray-Dried Itraconazole-Lipid Complex Powder for Liposome Formation

The spray-dried itraconazole-lipid complex powder is dissolved in pH 6.0 succinate buffer and heated to 60°C. Stirring for 60-90 minutes facilitates hydration, utilizing a top-mounted, flange blade stirrer operating at 300-350 RPM. pH adjustment to 5.5 is achieved using 1M NaOH 17].

d) Liposome Sizing

During the hydration stage, multi-lamellar vesicles (MLV) are generated. To achieve uniform unilamellar vesicles, a homogenization technique is employed. A benchtop Nerosavi Panda PLUS 2000 homogenizer is utilized, with initial lab experiments conducted to assess the impact of various process parameters on the vesicle size distribution of the bulk suspension. Recirculation-based homogenization is chosen, wherein the bulk suspension is reprocessed through the homogenizer. Vesicle size analysis is performed using a zeta sizer (Zeta sizer Model ZS-ZEN 3600), with homogenization ceasing upon reaching the desired vesicle size. Optimization of the homogenization process was critical to achieve uniform unilamellar vesicles of the desired size. Initial trials evaluated varying homogenization pressures and temperatures. The optimized parameters included homogenizing the bulk liposomal suspension at a high pressure of 1000 bar to effectively downsize the multi-lamellar vesicles produced during hydration. A circulating water bath controlled the temperature of the inlet and outlet of the homogenizer at 60°C to maintain heat generated by the mechanical shearing forces18. Within the compounding vessel where the liposomes circulated for repeated passes through the homogenizer, gentle stirring at 300-350 rpm combined with a regulated temperature of 62°C ensured uniform processing without disruption of the vesicles. These optimized conditions reliably generated liposomes of the target particle size range during scale-up [19].

Characterization of liposomes

The comprehensive characterization of the prepared liposomes encompasses a range of parameters to evaluate their quality and performance, including pH, percent drug content, percent entrapment efficiency, vesicle size, polydispersity index (PDI), zeta potential (ζ), and in vitro drug release kinetics.

a) Bulk Suspension pH Measurement

To determine the pH of the bulk suspension, 10 mL of itraconazole suspension is transferred to a test tube at a temperature of 25°C20. The pH is measured using a calibrated pH meter (Elico pH meter LI 127).

b) Percent Drug Content Determination

A volume of 2 mL from the itraconazole liposomal dispersion is appropriately diluted with pH 7.4 phosphate buffer to achieve a final volume of 100 mL. Spectrophotometric analysis is conducted at a wavelength of 264 nm to quantify itraconazole concentration. This

procedure is repeated three times, and the average values are reported [21].

c) Percent Entrapment Efficiency (% EE) Calculation

The entrapment efficiency (% EE) of liposomes is assessed through the utilization of an ultra-centrifugation technique16. A prepared volume of 4 mL liposome dispersion is subjected to centrifugation at 7000 rpm for 2 hours at a controlled temperature of 4°C using a Remi cooling centrifuge [22]. The clear supernatant, containing the entrapped drug, is withdrawn and analyzed spectrophotometrically at 264 nm against pH 7.4 phosphate buffer. % EE is calculated using Equation 1,

$$\%EE = \frac{C_d - C}{C_d} \times 100 \text{ (Eq. 1)}$$

where Cd represents the concentration of total drug and C represents the concentration of entrapped drug.

d) Vesicle Size, PDI, and Zeta Potential(ζ) Measurement

For vesicle size, PDI, and zeta potential analysis, 0.5 mL of a 1:100 diluted liposomal dispersion is placed in a cuvette. The cuvette is then positioned within the sample holder of the Zeta sizer Model ZS-ZEN 3600 instrument. Photon correlation spectroscopy is employed to determine the hydrodynamic diameter of the vesicles based on Brownian motion. Vesicle size measurements are taken at a 90° light scattering angle and at a temperature of 25°C. Zeta potential measurements are conducted using the Helmholtz–Smoluchowski equation, which relies on the electrophoretic mobility of vesicles [23].

e) *In Vitro* Drug Release Studies

In vitro drug release studies are conducted using a diffusion cell setup. A cellulose dialyzing membrane (dialysis membrane 60 from HI Media, Mumbai, India) with a molecular cutoff of 12000 Da is soaked in pH 7.4 phosphate buffer overnight. A volume of 2 mL of vesicular dispersion, equivalent to 4 mg of itraconazole, is placed within the dialysis membrane, which is then suspended in a beaker containing 500 mL of pH 7.4 phosphate buffer. This setup is maintained at 37±0.5°C on a temperature-controlled magnetic stirrer, stirring at 400 rpm [17]. Samples are withdrawn at predetermined intervals, and the released itraconazole is quantified spectrophotometrically at 264 nm after suitable dilution with pH 7.4 phosphate buffer.

f) *In vitro* Drug Release Kinetics and Mechanism

The release kinetics and mechanism of itraconazole from liposomes are investigated through model-dependent methods. The in vitro release data is fitted to zero-order (cumulative % released vs. time) and first-order (log % drug remaining vs. time) models to assess release kinetics. Additionally, the release mechanism is analyzed using Higuchi's model (cumulative % drug released vs. square root of time) and Korsmeyer-Peppas model (log % drug released vs. log time)24–26. The model with the highest correlation coefficient (r) is selected as the most appropriate model for describing the dissolution data.

Statistical Analysis of the Data and Optimization

The statistical analysis and optimization of the experimental data were conducted using Design Expert® DX 8.0.7.1 trial version software. Response surface modeling, guided by the principles of multiple linear regression analysis, was employed to assess the quality of fit for the current study27. Polynomial models that incorporated linear, interaction, and quadratic terms were generated for all response variables. The second-order polynomial equation, which provides insight into the effect of independent factors on the response, was expressed as follows:

Linear Model: $Y=\beta0+\beta1X1+\beta2X2$(Eq.2)

2FI (Interaction)

Model: $Y=\beta0+\beta1X1+\beta2X2+\beta12X1X2$(Eq. 3)

Quadratic

Model:$Y=\beta0+\beta1X1+\beta2X2+\beta12X1X2+\beta11X12+\beta22X22$ (Eq. 4)

Here, Y represents the dependent variable, and β represents the estimated coefficient for the corresponding factor X.

The main effects (X1 and X2) reflect the average outcomes of changing one factor at a time, while the interaction terms (X1X2) illustrate the simultaneous influence of two factors. The inclusion of quadratic terms (X12 and X22) enables the exploration of nonlinearity. Positive or negative mathematical signs of coefficients indicate synergistic or antagonistic effects, respectively. The selection of the best fitting mathematical model was based on statistical parameters including the coefficient of variation (CV), coefficient of determination (R2), adjusted coefficient of determination (Adjusted R2), and the predicted residual sum of squares (PRESS) provided by Design Expert software. A low PRESS value signifies a favorable model fit, with significance considered at $p<0.0528$.

Results and Discussion

Analytical method for estimation of Itraconazole

The UV spectroscopic method demonstrated adherence to Beer's law within the concentration span of 0.2-1 µg/mL. The calculated regression equation for the linear relationship was determined as $y = 0.5574x + 0.0146$, with 'y' representing the absorbance and 'x' denoting the itraconazole concentration in µg/mL. A substantial correlation was indicated by the high correlation coefficient (r) value of 0.9973, signifying a strong positive relationship between itraconazole concentration and corresponding absorbance readings 9. The results are shown in figure 2

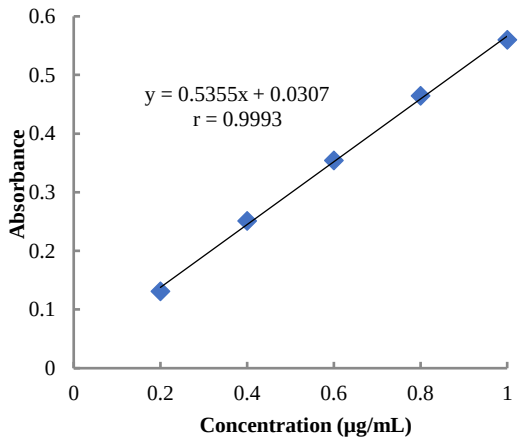

Figure 2. Calibration curve for the estimation of itraconazole

Drug-Excipient Compatibility Studies

Drug-excipient compatibility investigations encompassed Fourier Transform Infrared Spectroscopy (FTIR). FTIR analysis probed chemical interactions between itraconazole and excipients. Signature bands of itraconazole, excipients, and liposomal formulations are detailed in Figure 5 and Table 6. Itraconazole exhibited distinctive bands at specific wavenumbers, including C-O stretching at 1697 cm-1, C-N stretching at 1551 cm-1, aromatic C-C stretching at 1463 cm-1, C-0 and C-N stretching at 1227 cm-1, aromatic C-Cl stretching at 1042 cm-1, and C-H bending at 824, 793, and 722 cm-1. These bands aligned with reported values16. The optimized lyophilized itraconazole parenteral liposomes also presented the characteristic itraconazole bands. HSPC featured CH2 stretching at 2916 cm-1, C=0 ester stretching at 1736.25 cm-1, and CH2 scissor at 1467 and 1253 cm-1, in accordance with literature17. Similar characteristic bands were observed for DSPC18. Cholesterol19 displayed bands at 1022.58 cm-1 for C-O alcoholic stretching, O-H stretching vibration at 3398.38 cm-1, aliphatic C-H stretching from 2933.42 to 2849.37 cm-1, and C=C stretching at 1467.47 and 1375.42 cm-1. Stearic acid20 exhibited bands at 2955 cm-1 for C-H stretching and C=0 bending at 1703 cm-1.

Principal bands for the drug and excipients aligned well with theoretical reported bands of functional groups in the optimized formulations. The presence of DSPC, HSPC, cholesterol, and stearic acid didn't cause significant shifts in the primary bands of itraconazole. Moreover, the presence of one ingredient didn't induce shifts in the bands of other ingredients within the prepared liposomes. This confirmed the absence of chemical interaction between the drug and the used excipients. FTIR spectral analysis, therefore, confirmed the compatibility of the drug and excipients utilized in the study2. Results are shown in Figure 3

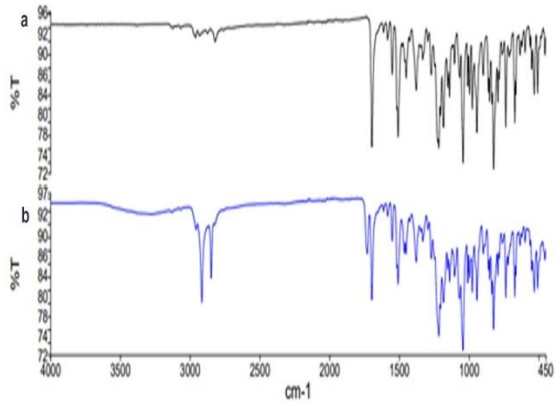

Figure 3. FTIR spectra of a. itraconazole pure drug and b.drug-excipient mixture

***In vitro* Characterization of Itraconazole Liposomes**

The *in vitro* characterization of itraconazole liposomes offers insight into the heterogeneity of these vesicles, encompassing variations in charges, sizes, and encapsulation capacity, even under uniform critical process parameters (CPPs). In-depth characterization is a pivotal aspect of drug formulation development, aiding in the generation of essential physico-chemical data concerning the liposomal system.

a) pH of Formulated Liposomes

The pH of the formulated liposomal dispersion is strategically adjusted to attain a pH of 7.4, facilitated by 0.1N HCl or 0.1N NaOH. This adjustment aligns with the requisite pH range of 7.35 to 7.45 for injectable intravenous formulations, aimed at minimizing irritation at the injection site and in surrounding tissues29. Upon analysis, no significant differences are discerned in the pH values across various formulations. Detailed results are presented in Table2.

b) Percent Drug Content

The quantification of percent drug content across all liposome formulations is detailed in Table 2. The percent drug content ranged from 96% to 100% for HSPC liposomes and from 97% to 101% for DSPC liposomes. This range signifies the uniform distribution of itraconazole within the liposomal matrix [30].

c) Entrapment Efficiency of Formulated Liposomes

The entrapment efficiency (%EE) of the vesicles is determined using an ultra-centrifugation method. The %EE values for HSPC vesicular dispersion formulations range from 33% to 95%, while those for DSPC vesicular dispersion formulations range from 26% to 90%. Remarkably, the formulations LH5 and LD5 exhibit the highest %EE of 95% and 90% respectively. This outcome underscores the significant impact of critical formulation attributes, such as lipid/surfactant composition and vesicle size, on entrapped drug content. The influence of cholesterol content on %EE is evident, with higher

cholesterol content leading to reduced %EE due to increased vesicle rigidity31. Detailed values are presented in Table 2. The complexities of vesicular drug encapsulation involve multifaceted interactions, including the partition coefficient of the drug between aqueous compartments and lipid bilayers, and the total solubility of the drug in each phase. Factors such as lipid/surfactant surface charge, vesicle size, and aqueous volume further contribute to entrapment efficiency. The careful selection of lipid constituents and their attributes significantly influence the encapsulation of drugs, whether polar or nonpolar, within vesicles [32].

Table 2: pH, % drug content and % EE values of itraconazole liposomes (mean±s.d., n=3)

Formulation code	pH	% Drug content	% EE
LH1	7.4±0.1	96±0.5	37±1.0
LH2	7.4±0.3	100±0.9	49±1.1
LH3	7.4±0.1	98±0.1	33±2.0
LH4	7.4±0.2	97±0.3	80±1.2
LH5	7.4±0.6	99±0.5	95±1.4
LH6	7.4±0.9	96±0.6	72±1.3
LH7	7.4±0.2	100±0.7	54±1.0
LH8	7.4±0.1	98±0.8	67±1.1
LH9	7.4±0.4	97±1.0	45±0.9
LD1	7.4±0.6	99±0.5	31±1.1
LD2	7.4±0.1	97±0.9	39±1.2
LD3	7.4±0.4	101±0.1	26±1.5
LD4	7.4±0.0	98±0.8	67±1.4
LD5	7.4±0.2	99±0.5	90±1.2
LD6	7.4±0.5	99±0.6	61±1.2
LD7	7.4±0.2	97±0.7	50±1.5
LD8	7.4±0.1	98±0.8	56±1.6
LD9	7.4±0.8	97±1.0	39±0.9

d) Vesicle Size Distribution and PDI of Itraconazole Liposomes

The investigation of vesicle size distribution and polydispersity index (PDI) determines critical factors influencing the release kinetics of the drug-loaded liposomes. The total interfacial surface area assumes a paramount role in dictating the drug release rate, rendering the assessment of particle size distribution instrumental in understanding the average vesicle size of itraconazole liposomes. The direct influence of vesicle size on itraconazole release and, consequently, its bioavailability upon injection, is particularly pertinent for the development of intravenous formulations. In this context, an average vesicle size of less than 150 nm (100-150 nm) was targeted to enable the successful sterilization through 0.22-micron filters.

The average size and size distribution of liposomes hold sway over their physical attributes, biological fate, and entrapment efficiency. The inherent stability of liposomes is intricately tied to the mean size, with formulations demonstrating lower size distributions exhibiting enhanced stability compared to those with larger vesicle size distributions3,33. In the case of liposomes prepared with HSPC, the mean average size spanned from 39 nm to 179 nm, with a corresponding polydispersity index (PDI) ranging from 0.01 to 0.52. In contrast, liposomes formulated with DSPC exhibited mean average sizes ranging from 29.1 nm to 180 nm, accompanied by PDIs within the range of 0.01 to 0.42. The vesicle size distribution data for all formulation batches are meticulously documented in Table 3.

The pronounced influence of cholesterol content on liposomal vesicle size is evidenced by the vesicle size distributions. Reduced cholesterol concentrations were found to promote the uptake of water within the aqueous compartment of vesicles, engendering increased vesicle size, while higher cholesterol levels induced greater lipophilicity, thereby restricting water penetration and resulting in smaller vesicle sizes [34].

Table 3: Vesicle size distribution, PDI and zeta potential of itraconazole liposomes (mean±s.d., n=3)

Formulation code	Vesicle size (nm)			Z average	PDI	Zeta potential (mV)
	D10	D50	D90			
LH1	55.5	98	269	160	0.32	-23±1.1
LH2	50.1	70.0	255	129	0.16	-31±0.1
LH3	50	100	260	145	0.25	-21±0.1
LH4	51	78	121	72	0.012	-34±0.4
LH5	7.33	32.4	79.4	39	0.01	-36±1.0
LH6	55	93	170	92	0.19	-23±0.2
LH7	50.3	115	330	179	0.17	-29±0.3
LH8	49.0	110	320	161	0.18	-31±0.4
LH9	52.9	112	338	175	0.16	-22±0.2
LD1	50.3	87.4	168.1	131	0.42	-21±0.8
LD2	49.9	76.0	137	102	0.12	-31±1.2
LD3	41.	80.	131.	112	0.04	-

	0	0	3			24±0.4
LD4	49.0	71.2	111	65.0	0.12	-36±1.0
LD5	6.20	25.2	57.1	29.1	0.01	-36±1.1
LD6	37.2	82.0	134.0	79.0	0.13	-31±1.2
LD7	52.0	88.2	240	138	0.22	-24±0.8
LD8	47.9	85.5	140.0	114	0.16	-31±0.4
LD9	62.5	90.0	210.0	180	0.19	-26±0.7

D10: The point on the size distribution curve below which 10% of the particles fall

D50: The corresponding vesicle size when the cumulative percentage reaches 50%

D90: 90% of the total particles are smaller than this size

PDI: The standard deviation of the particle diameter distribution divided by the mean particle diameter.

e) Zeta Potential

The surface charge of liposomes holds substantial sway over drug product clearance, tissue distribution, and cellular uptake. Zeta potential, a direct outcome of electrophoretic mobility measurements, serves as a key indicator of the stability of particles in suspension. The range of zeta potential values for itraconazole liposomal dispersions prepared with HSPC and DSPC spanned from -21 mV to -36 mV, highlighting the negatively charged nature of the liposome surfaces. The notable stearic acid contribution to liposome surface charge is evident in batches LH5 and LD5, where higher zeta potential values of -36 mV were achieved. These findings affirm that the vesicles possess sufficient charge to avert aggregation due to the electric repulsion effect35. The detailed zeta potential values for all liposomal formulations are cataloged in Table3, and the zeta potential distributions for LH5 and LD5 are presented in Figure4a and 4b respectively.

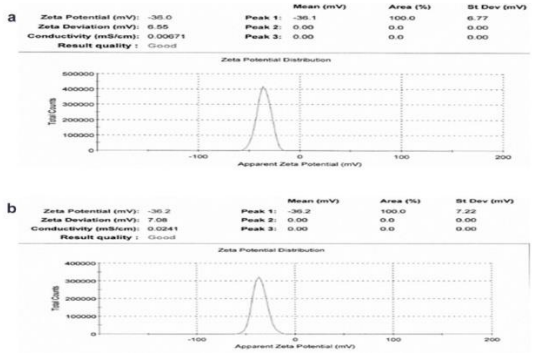

Figure 4: Zeta Potential Histogram of a. LH5 Itraconazole Liposomes and b. LD5 Itraconazole Liposomes

f) *In vitro* Release Studies by Dialysis

In-depth investigation of drug release from liposomes constitutes a pivotal parameter in drug product development and manufacturing, serving as a pivotal tool for quality control to ensure batch-to-batch consistency in drug release profiles from vesicular systems. Through an intricate examination employing a semi-permeable cellulose membrane, the in vitro drug release profiles of all liposomal formulations were scrutinized. The data unveiled a pronounced dependency of drug release on the relative composition of lipid/surfactant and cholesterol within the formulation. The in vitro drug release profiles for liposome formulations utilizing HSPC and DSPC are elucidated in Table 8

An intricate interplay of factors emerged, where the concentration of lipid up to a certain threshold proportion influenced the percent drug release, exhibiting an escalating trend, which subsequently attenuated beyond that point. Conversely, higher cholesterol levels exhibited a retarding effect on drug release. This can be attributed to the stiffening effect imparted by elevated cholesterol concentrations on the lipid bilayers, leading to a deceleration in the drug's release36. Notably, formulations LH1 and LD1, characterized by low cholesterol and lipid concentrations, experienced an altered release profile due to the formation of a stagnant lipid surface layer. In contrast, LH5 and LD5 formulations, with optimal cholesterol and lipid concentrations, exhibited substantial drug release, reaching 99.0% and 91.0% respectively at the 24-hour mark.

Table 4: Cumulative percent of itraconazole liposomes (mean±s.d., n=3)

HSPC-itraconazole liposomes									
Time (hours)	**LH1**	**LH2**	**LH3**	**LH4**	**LH5**	**LH6**	**LH7**	**LH8**	**LH9**
1	16.46±1.0	20.82±1.0	12.68±1.1	19.71±1.0	18.47±1.1	16.86±0.8	18.67±1.0	21.36±0.2	18.40±1.2
2	18.84±0.6	25.53±1.1	22.09±1.0	28.83±1.1	25.10±1.0	23.15±0.2	26.43±1.1	30.48±0.1	23.21±1.0
4	23.05±0	34.60±1	25.53±1	42.93±1	43.41±1	30.40±1	34.56±1	34.76±0	25.12±0

	.2	.3	.5	.2	.0	.1	.0	.3	.4
6	27.33 ±0.4	40.10 ±1.5	33.20 ±1.3	57.58 ±1.0	50.39 ±0.8	41.23 ±1.0	40.61 ±1.4	41.21 ±1.1	33.20 ±0.7
8	31.75 ±1.0	45.65 ±1.1	36.22 ±1.1	64.74 ±1.2	60.17 ±1.1	50.51 ±1.2	48.75 ±1.1	50.13 ±1.0	36.15 ±1.0
10	33.16 ±0.2	52.31 ±1.1	41.44 ±1.1	71.86 ±1.2	69.14 ±1.4	57.50 ±1.1	53.51 ±0.4	58.49 ±1.2	42.34 ±1.0
12	34.29 ±1.0	54.5± 1.0	42.41 ±1.1	84.10 ±1.3	80.36 ±1.0	64.74 ±0.4	59.42 ±0.5	61.57 ±1.1	49.65 ±1.2
24	45.06 ±0.4	62.07 ±1.3	53.09 ±0.7	86.08 ±1.0	99.07 ±1.1	72.04 ±1.0	68.04 ±0.8	74.08 ±1.4	58.02 ±1.0
DSPC-itraconazole liposomes									
Time (hours)	**LD1**	**LD2**	**LD3**	**LD4**	**LD5**	**LD6**	**LD7**	**LD8**	**LD9**
1	15.22 ±0.5	18.88 ±1.2	15.20 ±1.0	20.98 ±1.1	19.39 ±0.2	16.64 ±0.3	17.45 ±0.4	19.12 ±0.3	18.46 ±1.0
2	17.56 ±1.0	22.65 ±1.2	20.32 ±1.2	29.17 ±1.2	29.89 ±1.2	29.12 ±1.1	27.83 ±0.5	23.12 ±0.8	22.46 ±1.2
4	21.38 ±1.1	29.32 ±1.2	25.10 ±1.3	38.95 ±1.0	40.73 ±1.0	36.22 ±1.0	34.72 ±1.1	39.23 ±1.0	32.26 ±1.2
6	25.39 ±1.2	33.25 ±1.0	37.03 ±1.0	49.89 ±1.4	49.68 ±1.4	48.40 ±1.0	44.41 ±1.0	45.33 ±1.1	35.11 ±1.1
8	29.71 ±0.5	46.73 ±1.2	39.94 ±1.2	59.03 ±1.0	54.55 ±1.2	57.48 ±1.0	49.73 ±1.0	58.51 ±1.0	39.35 ±1.2
10	34.75 ±1	52.45 ±1	44.69 ±1	70.14 ±1	63.45 ±1	66.10 ±1	58.32 ±1	68.43 ±1	48.66 ±1
	.0	.0	.0	.3	.1	.1	.2	.0	.0
12	39.21 ±0.3	58.45 ±1.1	56.75 ±1.0	78.66 ±1.4	70.39 ±1.2	69.30 ±1.2	62.13 ±1.3	70.55 ±1.0	52.46 ±1.0
24	41.07 ±0.4	59.03 ±1.0	46.07 ±1.2	80.02 ±1.0	91.08 ±1.0	69.08 ±0.2	61.03 ±0.8	74.00 ±1.1	50.08 ±1.1

g) *In vitro* Release Kinetics

The elucidation of drug release kinetics assumes paramount significance in understanding and optimizing the release behavior of pharmaceutical formulations. Model-dependent methodologies delve into mathematical equations that offer a systematic framework to describe the release profiles, thereby facilitating the quantitative interpretation of pertinent values. The release kinetics are profoundly influenced by a myriad of factors, encompassing the nature of the incorporated drug, dosage, excipient composition, preparation technique, and the intricate interplay of environmental conditions. A comprehensive exploration of these factors is indispensable to unravel the intricate control mechanisms governing drug release from the dosage forms.Among the suite of kinetic models employed, the zero-order model reflects systems where the release rate remains independent of the drug's concentration. Conversely, the first-order model depicts the dependence of drug release on the concentration of the dissolving drug. The Higuchi model is indicative of diffusion-driven release mechanisms, while the Korsmeyer-Peppas model extends further insights into the release mechanisms.

In accordance with the Korsmeyer-Peppas equation, the release exponent 'n' emerges as a critical descriptor characterizing diverse release mechanisms. In geometrically spherical vesicular systems, the 'n' value provides a definitive guide. Values of 'n' ranging from ≤0.43 suggest Fickian diffusion, while values >0.43 yet <0.85 indicate non-Fickian (anomalous) diffusion. When 'n' equals 0.85, it indicates non-Fickian Case II transport, while values exceeding 0.85 signify non-Fickian Super Case II transport [24–26]. In our investigation, it is noteworthy that all liposomal formulations conformed to first-order release kinetics, except for LH4 liposomes, which followed a zero-order release as evidenced by its correlation coefficient ('r') value.

Table 5: Release Rate Constants and Correlation Coefficient (r) Values of Itraconazole Liposomes

Formulation code	Zero order		First order		Higuchi	Korsmeyer–Peppas	
	k0 (% hr-1)	r	r	r	r	r	n
LH1	1.531	0.784	0.0507	0.9718	0.9817	0.9830	0.520
LH2	2.226	0.8518	0.0687	0.9916	0.9742	0.9842	0.580
LH3	1.945	0.826	0.0629	0.9893	0.9851	0.9926	0.558
LH4	3.430	0.8519	0.0606	0.9886	0.9662	0.9923	0.642
LH5	3.928	0.9266	0.0713	0.9771	0.9932	0.9968	0.646
LH6	2.857	0.8891	0.0810	0.9763	0.9803	0.9946	0.607
LH7	2.530	0.8771	0.0785	0.9852	0.9833	0.9896	0.593
LH8	2.716	0.8898	0.0687	0.9852	0.9854	0.9861	0.598
LH9	2.115	0.8993	0.0565	0.9673	0.9845	0.9860	0.558
LD1	1.492	0.8488	0.0663	0.9719	0.9663	0.9857	0.521
LD2	2.282	0.8436	0.1102	0.9342	0.9574	0.9872	0.579
LD3	1.829	0.7620	0.0818	0.9211	0.9098	0.9858	0.563
LD4	3.154	0.8529	0.0693	0.9815	0.9652	0.9903	0.623
LD5	3.440	0.9242	0.0572	0.9917	0.9652	0.9935	0.627
LD6	2.713	0.8130	0.0951	0.9875	0.9475	0.9897	0.617
LD7	2.311	0.8031	0.0998	0.9777	0.9633	0.9869	0.595
LD8	2.949	0.8418	0.1172	0.9708	0.9459	0.9915	0.621
LD9	1.834	0.7920	0.0732	0.9760	0.9391	0.9824	0.562

Furthermore, the drug release mechanisms were systematically explored by fitting the release data to the Higuchi and Korsmeyer-Peppas equations. The results, encapsulated in Table 5, unequivocally reveal that all formulated liposomes, whether based on HSPC or DSPC, followed a diffusion-based mechanism. The subsequent analysis, based on 'n' values ranging from 0.52 to 0.646, indicates a further classification of non-Fickian (anomalous) diffusion.

h) Results of Statistical Analysis and Optimization

The investigation was initiated by identifying key factors affecting critical quality attributes of liposome formulations based on a literature review. The amount of lipids (HSPC/DSPC) and cholesterol emerged as significant factors influencing vesicle size, percent entrapment efficiency (% EE), and in vitro drug release profiles. Consequently, these factors were designated as independent variables (X1 and X2), while vesicle size, % EE, and 100% in vitro drug release at 24 hours were selected as dependent variables. A 32 full factorial design was used, resulting in nine experimental runs for each liposome formulation.

The observed responses from the experimental runs were subjected to regression analysis using linear, interaction, and quadratic models. The suitability of each model was evaluated based on statistical parameters, and a quadratic model was chosen for both HSPC and DSPC liposome formulations. The fitted polynomial equations and their corresponding coefficients were derived and provided insights into the relationships between independent variables and dependent responses [37].

The significance of X1 and X2 ($p < 0.05$) on all responses, as determined by analysis of variance (ANOVA), shows their substantial influence. The relationship between variables was visualized using contour and response surface plots, revealing the effects of varying two factors simultaneously on response variables. Moreover, numerical and graphical optimization techniques were employed to generate an optimized formulation with desired responses.

i) Quadratic Equations and Model Evaluation

The factorial design studies for itraconazole-loaded vesicles revealed that the impact of X1 and X2 (pertaining to HSPC/DSPC-itraconazole liposomes) on vesicle size, % EE, and 100% drug release at 24 hours was best fitted by quadratic models. These models were preferred over linear and interactive models due to their smaller p-values, signifying better fitting. The quadratic model exhibited strong correlation as indicated by R2 values. For HSPC-itraconazole liposomes, the R2 values were 0.9145, 0.8674, and 0.7858 for vesicle size, % EE, and 100% drug release at 24 hours, respectively. Similarly, for DSPC-itraconazole liposomes, the R2 values were 0.8273, 0.8161, and 0.8219, demonstrating robust correlation.ANOVA results indicated model significance ($p < 0.05$) for all three responses in both HSPC and DSPC liposomes. Predicted R2 values were well in line with adjusted R2 values, strengthening the validity of the model [38].

j) Optimization

A multi-criteria decision approach was employed for optimization, utilizing both numerical and graphical techniques. The desirability function and overlay plot

methods were applied to optimize three distinct responses: minimum vesicle size, maximum % EE, and 100% drug release at 24 hours. The formulation optimization was achieved by imposing constraints on dependent variables. Through feasibility and grid searches, a formulation with near-perfect desirability (approximately 1.0) was identified, highlighting its suitability39,40.The Design Expert software facilitated the determination of recommended lipids (HSPC/DSPC) and cholesterol for the optimized formulation. The desirability and overlay plots for both HSPC and DSPC liposomes are depicted in Figure 5 showcasing the optimal formulations.

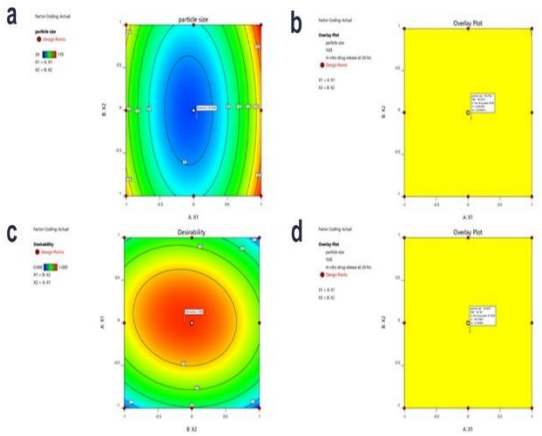

Figure 5. a.) Contour plot b) Overlay plot of HSPC-itraconazole liposomes c) Contour plot and d) Overlay plot of DSPC-itraconazole liposomes

k) Cross-Validation of Model

Predicted formulations closely aligned with LH5 and LD5 for HSPC and DSPC liposomes.The optimized formulations of HSPC and DSPC liposomes were statistically validated by comparing their observed and predicted responses of critical quality attributes. For HSPC liposomes, the observed vesicle size of 39.0 nm closely matched the predicted size of 50.06 nm, showing a percent relative error (%RE) of only 2.2%. An observed entrapment efficiency of 95% was nearly identical to the predicted 95.02% with %RE of 1.07%. Likewise, the observed and predicted times to achieve 100% drug release (T100) were 99% and 99.84% respectively, with low %RE of 0.85%. In case of DSPC liposomes, the vesicle size, entrapment efficiency and T100 values followed a similar trend, with %RE ranging from 0.74% to 1.6%, validating the design space of the formulated liposomal batches41. Although the formulations were freshly prepared and evaluated for three variables, the percent relative error (% RE) between predicted and experimental values was computed. The optimized formulations demonstrated exceptional quality, with % RE values for all response parameters remaining under 5%. The experimental results closely matched the predicted values, underscoring the reliability and validity of the model.

Discussion

The present study aimed to optimize itraconazole-loaded vesicles using a factorial design approach, investigating the influence of key factors on vesicle size, percent entrapment efficiency (% EE), and 100% in vitro drug release at 24 hours. Our findings revealed the significant impact of lipid type (HSPC/DSPC) and cholesterol content (X1 and X2, respectively) on the response variables. The quadratic models demonstrated superior fitting for all responses, elucidating the intricate interplay between these factors.

The selection of lipid type and cholesterol content was grounded in literature, where these components were shown to profoundly affect critical quality attributes of liposome formulations, such as vesicle size, stability, in vitro/in vivo behavior, and % EE16,21.Our preliminary studies further validated the role of these factors in influencing vesicle size, % EE, and drug release profiles.

The quadratic models, which were derived from the factorial design studies, exhibited strong correlation with R2 values ranging from 0.7858 to 0.9145 for HSPC-itraconazole liposomes and 0.8161 to 0.8273 for DSPC-itraconazole liposomes. The ANOVA results confirmed the significance of the models, indicating that the models adequately represented the relationship between the independent factors and the dependent responses [40].

The optimization phase was undertaken using both numerical and graphical techniques, providing a comprehensive approach to achieving desired responses. The desirability function and overlay plot methods enabled the identification of an optimal formulation that concurrently minimized vesicle size, maximized % EE, and ensured 100% drug release at 24 hours. The recommended lipid types and cholesterol quantities for the optimized formulations were established through Design Expert software, resulting in formulations with near-perfect desirability scores [27].

Cross-validation of the model with experimentally prepared optimized formulations confirmed the accuracy and predictability of the established models. The observed responses closely aligned with the predicted values, with percent relative error (% RE) values remaining within 5% for all parameters. This robust agreement between predicted and experimental outcomes validated the reliability of the quadratic models and the optimization process28.The preparation and stabilization of liposomal formulations continue to pose significant challenges despite substantial advancements and thorough investigations. Notably, the industrial-scale production of liposomes remains particularly intricate, given the complexity and multi-step nature of the production process [16].

This study demonstrated the successful optimization of itraconazole-loaded vesicles using a factorial design approach. The quadratic models effectively captured the intricate relationship between lipid type, cholesterol content, and critical quality attributes. The optimization process, guided by the desirability function and overlay plot methods, yielded formulations with outstanding desirability scores, which were subsequently validated through cross-validation experiments. The findings of this study provide a robust framework for the rational design and optimization of liposome formulations, offering potential benefits for drug delivery applications.

Conclusion

The results of this study showcases the importance of considering lipid type and cholesterol content in the design of liposomal drug delivery systems. The factorial design approach coupled with quadratic models facilitated the systematic exploration of the effects of these factors on vesicle size, % EE, and drug release kinetics. The successful optimization process further confirmed the utility and accuracy of the established models, leading to the development of optimized formulations that met desired criteria. This study contributes valuable insights into the rational design and optimization of liposomal formulations, enhancing our understanding of the interplay between formulation parameters and critical quality attributes.

Conflict of Interest

The authors declare no conflicts of interest regarding this work**.**

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