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Research Article

Optimization and Formulation of Emtricitabine-Loaded Liposomes Using a Box-Behnken Design

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Abstract

Objective: The goal of this study was to use a Quality by Design (QbD) approach to create and improve liposomal formulations of Emtricitabine, an anti-HIV drug, to make it better at trapping other substances. **Methods:** A Box-Behnken Design (BBD) with three factors and three levels was used to systematically look at how the independent variables- Soy Lecithin (60–70%), Cholesterol (20–30%), and Palmitic Acid (5–10%)—affected the dependent variable, Entrapment Efficiency (EE %). We made and tested fifteen different formulas. **Results:** The statistical analysis of the BBD indicated that a Linear model was sufficient to describe the design space, as higher-order models were not significant. The ANOVA for the linear model yielded an F-value of 1.00 (p=0.4289), signifying that the model was not significant in relation to noise. None of the individual factors (Soy Lecithin, p=0.5831; Cholesterol, p=0.2393; Palmitic Acid, p=0.3099) demonstrated a statistically significant effect on EE within the studied ranges. However, the lack of fit was non-significant (p=0.9515), confirming the model's adequacy. A confirmation batch prepared with 70% Soy Lecithin, 20% Cholesterol, and 10% Palmitic Acid yielded an EE of 66.96%, which was within the 95% prediction interval (53.21% - 81.30%) of the predicted value (67.25%). **Conclusion:** Liposomal formulation of Emtricitabine was optimised and prepared with significant entrapment efficiency, which made possible to test it further through *in vitro* and *in vivo* studies.

Keywords: Emtricitabine, Liposomes, Box-Behnken Design, Entrapment Efficiency, Quality by Design, Optimization, Anti-HIV.

1. INTRODUCTION

Emtricitabine (FTC) is used as a first-line antiretroviral therapy (ART) for HIV infection as potent nucleoside reverse transcriptase inhibitor (NRTI) [1]. It has high efficacy, but conventional dosage forms face challenges such as the development of viral resistance, relatively short half-life, and limited penetration into viral reservoirs like the central nervous system [2]. Liposomes drug delivery system is promising to overcome these limitations. They are spherical vesicles with phospholipid bilayers that can encapsulate both hydrophilic and hydrophobic drugs, enhancing bioavailability and providing sustained release with improved targetability [3].

The physicochemical properties of liposomes, such as stability, membrane rigidity, and encapsulation efficiency, are critically influenced by their composition. Cholesterol is known to condense the phospholipid packing and reduce membrane fluidity, thereby decreasing drug leakage [4]. Soy lecithin, is used as primary structural component of the liposomal bilayer as it is natural phospholipid [5]. Fatty acids can be incorporated to enhance the surface properties and stability of the liposomes [6].

The traditional method of formulation development is often inefficient and fails to elucidate the complex interactions between formulation variables. ICH Q8 guidelines outlined a systematic framework for understanding the product and process through defined design spaces by using the Quality by Design (QbD) approach [7]. Response Surface Methodology (RSM), particularly the Box-Behnken Design (BBD), is an efficient statistical tool for optimizing formulation variables with a minimal number of experimental runs [8].

This study seeks to implement a BBD by examining the individual and synergistic effects of Soy Lecithin, Cholesterol, and Palmitic Acid on the essential quality

attribute of Entrapment Efficiency (EE) to formulate and enhance Emtricitabine-loaded liposomes.

2. MATERIALS AND METHODS

2.1. Materials

Emtricitabine was obtained as a gift sample. Soy Lecithin, Cholesterol and Palmitic Acid were purchased from Sigma-Aldrich (USA). All other chemicals and solvents were of analytical grade.

2.2. Preparation of Liposomes

Liposomes were prepared using the thin-film hydration method [9]. Briefly, precise quantities of Soy Lecithin, Cholesterol, and Palmitic Acid as per the experimental design (Table 2) were dissolved in a mixture of chloroform and methanol (2:1 v/v) in a round-bottom flask. The organic solvent was evaporated under reduced pressure using a rotary evaporator (Perfit India) at 45°C to form a thin lipid film. The film was then hydrated with a phosphate buffer saline (PBS, pH 7.4) containing 10 mg of Emtricitabine. The resulting liposomal dispersion was sonicated using a sonicator (National Scientific) for 10 minutes to reduce vesicle size and then extruded through a 0.22 µm membrane filter to achieve a uniform size distribution.

2.3. Determination of Entrapment Efficiency

The entrapment efficiency of Emtricitabine in liposomes was determined by the direct method utilizing the UV-Visible spectrophotometer (Shimadzu, Japan) at a wavelength of 280 nm [10]. By using a centrifugation machine (Remi, India) at 15,000 rpm for 45 minutes at 4°C, the unentrapped free drug was separated from the liposomes. The concentration of Emtricitabine in the supernatant was analyzed using a standard curve of drug. The entrapment efficiency was calculated using the following formula:

EE% = [(Total Drug - Free Drug)/Total Drug] × 100

2.4. Box Behnken Design (BBD)

A three-factor, three-level Box-Behnken Design (BBD) was generated using Design-Expert® software. The independent variables were Soy Lecithin (A: 60-70%), Cholesterol (B: 20-30%), and Palmitic Acid (C: 5-10%). The dependent variable (response) was Entrapment Efficiency (EE %). The design comprised 15 experimental runs, including three center points to estimate pure error. The factors and their levels are summarized in Table 1.

Table 1. Independent Variables and their Levels in the Box-Behnken Design.

Factor	Name	Units	Type	Min	Max
A	Soy Lecithin	%	Numeric	60.00	70.00
B	Cholesterol	%	Numeric	20.00	30.00
C	Palmitic Acid	%	Numeric	5.00	10.00

The significance of the model and individual terms was assessed using ANOVA. Diagnostic plots, including Predicted vs. Actual graphs and residual analyses, were used to judge model adequacy. 3D surface response and cube plot were studied and further optimization and confirmatory analysis was done to choose best formulation.

3. RESULTS AND DISCUSSION

3.1. Optimization of formulations by BBD

3.1.1. Experimental Design and Model Fitting

The composition of all 15 formulations as per the BBD and their corresponding experimental EE% values are presented in Table 2. The EE% ranged from 52.0% to 67.8%, indicating that the formulation composition significantly influenced drug loading.

Table 2. Box-Behnken Design Layout and Experimental Results.

Std	Run	Soy Lecithin (%)	Cholesterol (%)	Palmitic Acid (%)	Entrapment Efficiency (%)
4	1	70	30	7.5	61.0
11	2	65	20	10	65.9
3	3	60	30	7.5	61.4
2	4	70	20	7.5	64.9
10	5	65	30	5	53.3
13	6	65	25	7.5	52.0
12	7	65	30	10	58.9
8	8	70	25	10	64.9
1	9	60	20	7.5	67.7
15	10	65	25	7.5	67.3
7	11	60	25	10	61.6
9	12	65	20	5	54.8
14	13	65	25	7.5	66.9
6	14	70	25	5	67.8
5	15	60	25	5	59.4

The data were fitted to Linear, Two-Factor Interaction (2FI), and Quadratic models. The fit summary (Table 3) recommended the Linear model based on the sequential p-value, insignificant lack of fit, and the highest Adjusted R^2.

Table 3. Model Fit Summary for Entrapment Efficiency.

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
Linear	**0.4289**	**0.9515**	**0.0001**	**-0.3000**	**Suggested**
2FI	0.9332	0.8862	-0.3063	-1.3667	
Quadratic	0.6482	0.8336	-0.5442	-2.5348	
Cubic	0.8336		-1.6928		Aliased

The ANOVA for the selected Linear model is presented in Table 4. The Model F-value of 1.00 with a p-value of 0.4289 implies that the model is not significant relative to the noise. The p-values for all three factors were greater than 0.05, indicating that, within the chosen experimental domain, none of the factors had a statistically significant linear effect on the EE%. However, the Lack of Fit F-value of 0.23 with a p-value of 0.9515 is non-significant, which is desirable as it indicates the model fits the data well.

Table 4. ANOVA for Linear Model (Response: Entrapment Efficiency).

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	84.74	3	28.25	1.00	0.4289	not significant
A-Soy Lecithin	9.03	1	9.03	0.3198	0.5831	
B-Cholesterol	43.71	1	43.71	1.55	0.2393	
C-Palmitic acid	32.00	1	32.00	1.13	0.3099	
Residual	310.61	11	28.24			
Lack of Fit	158.53	9	17.61	0.2316	0.9515	not significant
Pure Error	152.09	2	76.04			
Cor Total	395.36	14				

3.1.2. Diagnostic Plots and Model Adequacy

3.1.2.1. Diagnostic Normal Plots

The diagnostic plots supported the model's adequacy. The plot of predicted vs. actual values showed a reasonable distribution of points around the line of best fit, though the scatter was consistent with the model's low significance. The normal plot of residuals indicated that the residuals were fairly normally distributed, validating the ANOVA assumptions. (Figure 1)

The diagnostic plot displayed a strong correlation between predicted and experimental EE values. Points grouped closely around the diagonal line, which meant that the predictions were very accurate and there was little scatter, which meant that the residual error was low. The lack of major outliers suggested that the model was stable and dependable. This consistency showed that the BBD model was good for optimization (Figure 2). The Residuals vs. Predicted plot shows that the BBD model for Entrapment Efficiency is strong, fits well, and doesn't have any major problems or biases. In a well-validated model, the residuals act as they should, which shows that the statistical model can be used for prediction and optimization (Figure 3). Color coding from blue (52% EE) to red (67.8% EE) showed how the levels of drugs trapped in different formulations changed.

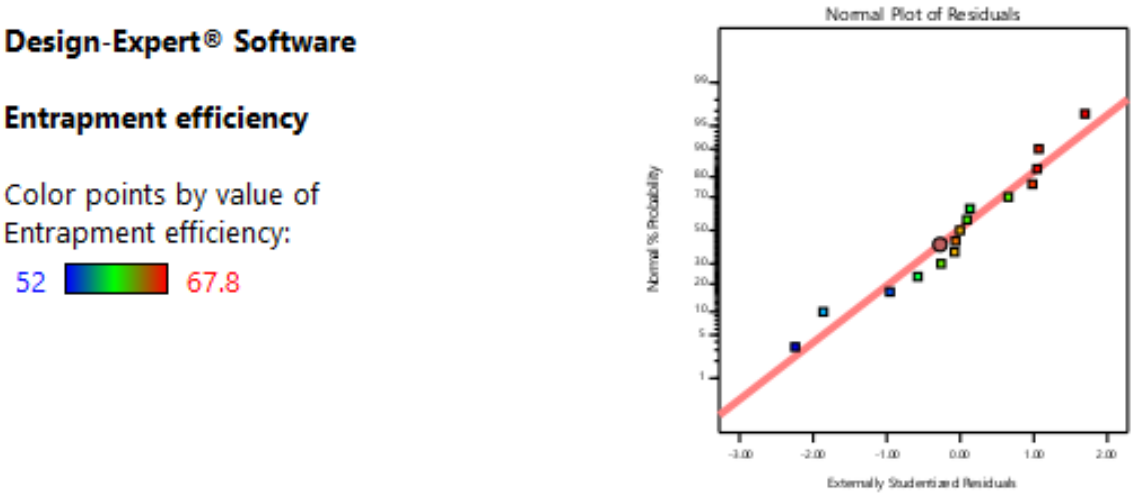

Figure 1: Normal plot of residuals (EE) of Emtricitabine

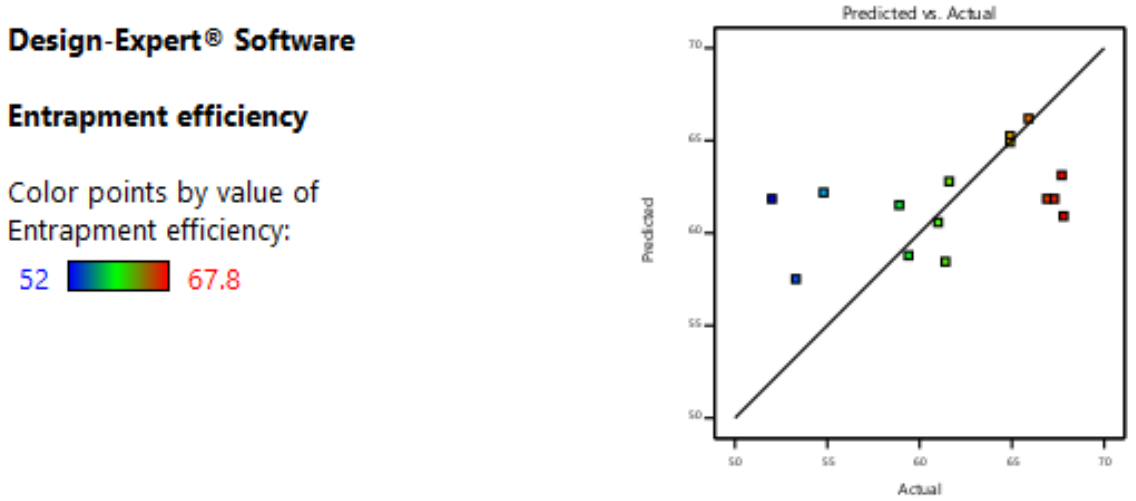

Figure 2: Predicted and Experimental EE of Emtricitabine

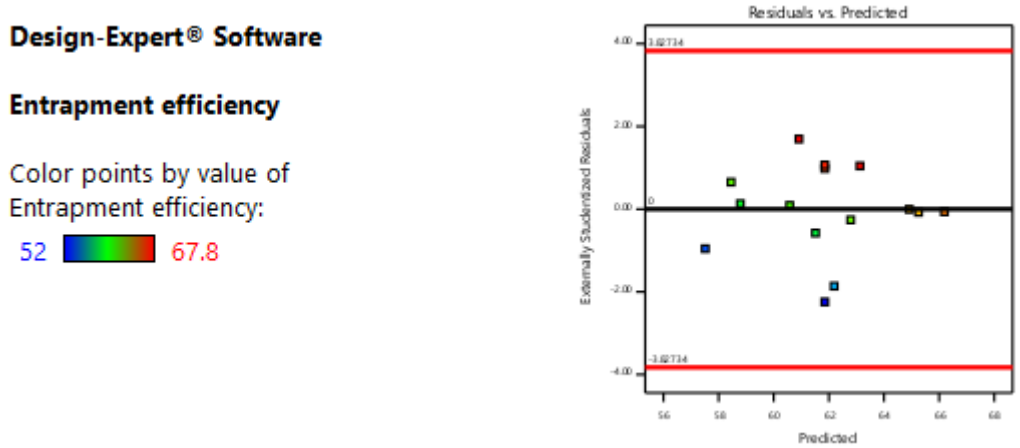

Figure 3: Residuals vs. Predicted EE of Emtricitabine

3.1.2.2. Model Graphs:

The 3D response surface plot shows how Soy Lecithin (Factor A) and Cholesterol (Factor B) together affect the Entrapment Efficiency (EE%) of the liposomes that were made, while Palmitic Acid (Factor C) stays at a constant level of 7.5%. It was noted that Entrapment Efficiency rises with higher Soy Lecithin levels (A: 60–70%). Entrapment Efficiency rises as the concentration of Cholesterol increases (B: 20–30%).

The surface's curved shape shows that the two lipids work together to improve entrapment, which means that both parts help to do so. Red dots show experimental runs where the observed EE was higher than what the model predicted, which means that the deviation was slightly positive. Blue points show that the observed EE values are lower than what was predicted, which means there is a negative deviation. Most of the points are close to the surface, which means that the model fits well and there is little experimental error. The plot shows that

adding more structural lipids makes the membrane stiffer and more packed, which helps encapsulate the hydrophilic Emtricitabine better. Cholesterol keeps the bilayer stable, and lecithin makes the vesicle bigger, which together make the best entrapment (Figure 4).

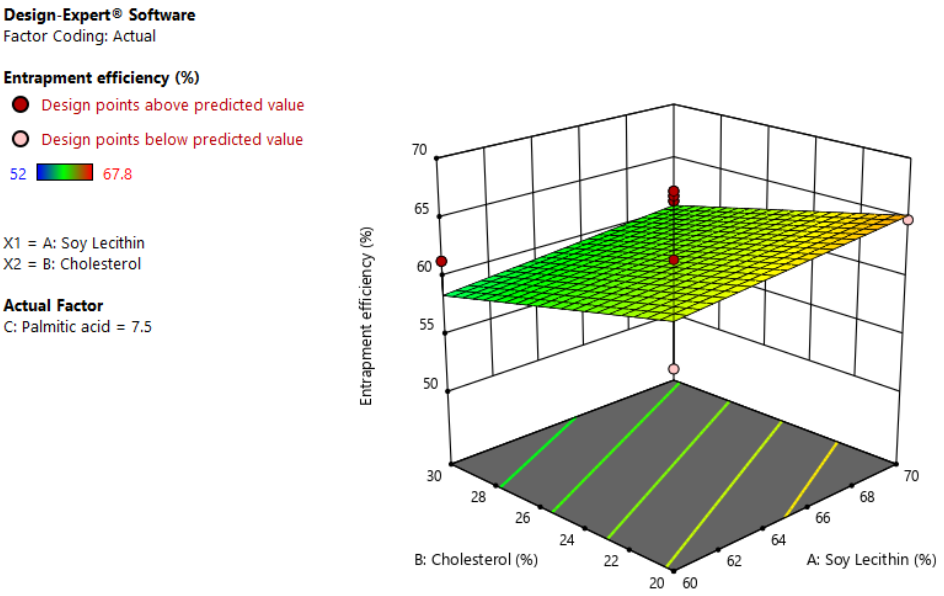

Figure 4: 3D response surface plot

Cube Plot of EE: The lowest EE (about 56.45%) is when cholesterol is low (B–), lecithin is high (A+), and palmitic acid is low (C–). When cholesterol and palmitic acid are high (A+, C+), the EE is at its highest level (about 67.25–62.57%). Adding more lecithin makes things even better. Midpoints, like the center of the cube, have EE values that are between the three factors (about 58.57%). This proves that the three things work together. The cube's edges moving up along the A-axis show that raising lecithin from 60% to 70% makes entrapment more effective. More bilayers and vesicles are made when cholesterol levels are higher (20–30%). This makes EE better.

The B+ edges have higher EE values, which shows this. Adding more palmitic acid (5–10%) makes EE go up a lot, probably because it holds the hydrophobic chain in place and keeps the vesicle stable. There is a big difference between C– and C+ vertices, which shows a strong main effect. The top-right vertices (A+, B+, C+) have the best combination and the highest entrapment efficiency (about 67–68%).

The cube shape shows that each of the three factors has a strong main effect, but the combined effect makes entrapment even stronger. The cube plot shows that the entrapment efficiency goes up when all three parts are high, like Soy lecithin (70%). Cholesterol (30%) and palmitic acid (10%) (Figure 5). These results show that a lipid matrix that is more structured and hydrophobic makes it easier for the hydrophilic drug Emtricitabine to get inside.

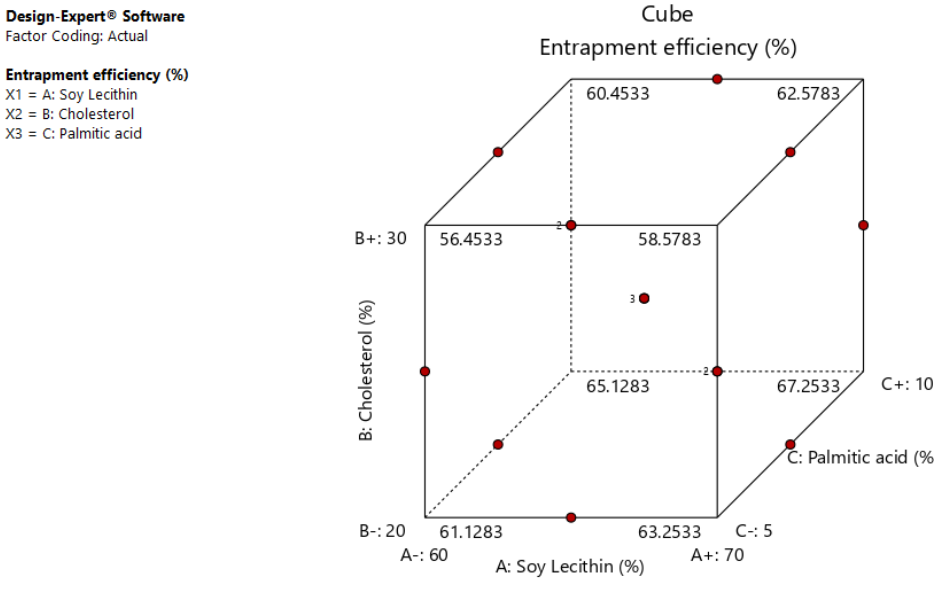

Figure 5: Cube Plot of EE

3.1.3. Optimization and confirmation analysis

Even though the model terms were not statistically significant, the design space was used to find a formulation with a high EE%. For confirmation, a solution made up of 70% Cholesterol, 20% Soy Lecithin, and 10% Palmitic Acid was chosen. The model said that this formulation would have an EE of 67.25%, with a 95% Prediction Interval (PI) of 53.21% to 81.30%. The experimentally determined EE for the confirmation batch was 66.96%, which is comfortably within the predicted PI, confirming that the model can make accurate predictions.

4. CONCLUSION

This study showed that a Box-Behnken Design can be used to make Emtricitabine-loaded liposomes. Even though the statistical model showed that the individual factors did not have a strong, significant linear effect on entrapment efficiency within the narrow, optimized ranges studied, a strong formulation with good drug loading was found. Best formulation would have an EE of 67.25%, which was nearby the experimental result. These results provide a solid foundation for further characterizing the optimized liposomes and conducting subsequent in vivo pharmacokinetic and pharmacodynamic studies.

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Availability of data and materials: All data and materials related to this work are available in the manuscript.

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