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Development And Characterization Of Liposomes Containing Relugolix

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ABSTRACT

Research study investigates the formulation and characterization of Relugolix-loaded liposomes using a systematic approach involving various analytical methods. Relugolix, an BCS IV anticancer, was assessed for its physical properties, melting point, and solubility across multiple solvents, revealing optimal solubility in ethanol. UV spectroscopy established the absorption maxima at 247 nm, facilitating the development of a calibration curve in phosphate buffer pH 6.8. Fourier Transform Infrared Spectroscopy (FTIR) and Differential Scanning Calorimetry (DSC) provided insights into the drug's chemical interactions and thermal behavior, respectively. Liposomes were prepared using the solvent evaporation method, and their formulation was optimized through a Box-Behnken design focusing on soya lecithin, cholesterol, and rotational speed. The optimized formulation (F8) exhibited a drug content of 97.56% and an entrapment efficiency of 95.78%, alongside promising in vitro drug diffusion profiles reaching 96.87% over 12 hours. Stability studies demonstrated that these parameters remained consistent over one month. Overall, this research highlights the potential of Relugolix-loaded liposomes for effective drug delivery, paving the way for future in vivo efficacy studies.

KEYWORDS: Relugolix, Liposomes, Solvent evaporation method, Box-Behnken design, Stability studies

INTRODUCTION

Relugolix is a novel, orally bioavailable GnRH receptor antagonist primarily used in the treatment of prostate cancer. As a BCS Class IV drug, it faces significant solubility and permeability challenges, which can limit its therapeutic effectiveness. Liposomes, as nanocarrier systems, offer a promising solution by enhancing drug stability, solubility, and controlled release, potentially improving Relugolix's bioavailability while reducing side effects.
Liposomes are nanocarrier systems composed of phospholipid bilayers that encapsulate drugs, enhancing their stability, solubility, and controlled release. They can improve the pharmacokinetics and bioavailability of poorly soluble drugs while reducing systemic toxicity. By utilizing liposomes for Relugolix delivery, this research aims to enhance its therapeutic effectiveness and minimize potential side effects.
This study focuses on the formulation and evaluation of Relugolix-loaded liposomes.

MATERIALS AND METHODS

MATERIALS
Relugolix by MSN laboratories pvt. Ltd., Sangareddy, Telangana. Soya Lecithin, Cholesterol, Chloroform, Acetone, Methanol, Ethanol and other chemicals provided by Cosmo Chem. Pvt. Ltd.

METHODS
Appearance:
Relugolix was observed visually by using watch glass for its physical appearance such as texture, color and odor.

Melting Point
The melting point of Relugolix was determined using the capillary tube method. Thieles tube containing liquid paraffin solution and then a small amount of pure drug was filled in the capillary tube which is sealed at one end using a flame. Filled capillary is tied with thread to the thermometer suspended in Thieles tube and heated till drug powder melts. The temperature at which the pure drug powder started melting was noted.

Solubility study
The solubility of Relugolix was performed in Methanol, ethanol, distilled water, phosphate buffer pH 7.4, Phosphate buffer pH 6.8, and Acidic buffer pH 1.2 were taken in different100 ml conical flasks & 50 mg of Relugolix were added

in it. The conical flask was stirred for 24 hrs. On mechanical shaker at 150 RPM. After 24 hrs. The flask was removed solutions were filtered and absorbance was measured at 247 nm.

UV Spectroscopy:

Detection of Absorption Maxima (λ max)

The sample of the standard solution was scanned between 200-400 nm regions on a UV spectrophotometer (SHIMADZU). Stock solutions of the Relugolix sample were prepared by dissolving 25 mg of the drug in 25 ml of, Distilled water respectively. The absorption maximum for distilled water was found to be 247 nm.

Standard calibration curve of Relugolix in Phosphate buffer pH 6.8

Preparation of stock solution in Phosphate buffer pH 6.8:

Standard stock solution was prepared by taking 25 mg in 25 ml of Phosphate buffer pH 6.8 (1000µg/ml). The stock solution scanned in the range 400-200 nm by UV spectrophotometer the solution showed maximum absorbance at 247 nm.

Preparation of dilutions for the standard curve:

From 1000µg/ml, diluted 10 ml to 100 ml (100 µg/ml), from this solution 2-12 µg/ml dilutions prepared. Absorbance was taken at 247 nm using water as a blank. The absorbance v/s concentration graph is plotted.

Fourier transform infra-red spectroscopy (FTIR)

The FTIR spectra of Relugolix were recorded and interpreted for the possible chemical interactions. The transparent pellets of these samples were prepared by mixing each of these components with potassium bromide, and FTIR spectra were recorded in the region of 4000–400 cm-1 (PerkinElmer FTIR).

FORMULATION

Preparation of Liposomes

Liposomes were formed by using the solvent evaporation method. A mixture of lipids (lecithin -cholesterol) dissolved in organic solvent with 120 mg of Relugolix and evaporated at 45°C. The resulting thin film; mixed with phosphate buffer pH 7.4 and stirred using glass beads to form small vesicles.

Optimization Using Box-Behnken Design

Response Surface Methodology (RSM) via Box-Behnken design was employed, focusing on three variables: soya lecithin, cholesterol, and rotational speed. Thirteen experimental runs were analysed for drug content, entrapment efficiency, and drug diffusion.

Table 1: Box-Behnken Design of Liposomal Formulation

Formulation code	Relugolix (mg)	Soya lecithin (mg)	Cholesterol	Rotational speed (rpm)	Chloroform: Methanol	Phosphate buffer pH 7.4
F1	120	25	30	150	1:1	10
F2	120	112.5	30	100	1:1	10
F3	120	200	175	150	1:1	10
F4	120	25	175	150	1:1	10
F5	120	112.5	102.5	150	1:1	10
F6	120	112.5	30	200	1:1	10
F7	120	112.5	175	200	1:1	10
F8	120	200	102.5	200	1:1	10
F9	120	25	102.5	100	1:1	10
F10	120	112.5	175	100	1:1	10
F11	120	25	102.5	200	1:1	10
F12	120	200	102.5	100	1:1	10
F13	120	200	30	150	1:1	10

EVALUATION:

Entrapment Efficiency (%EE)

The entrapment efficiency of Relugolix loaded liposomal formulation was determined by centrifugation method, in which 2ml in 10 ml of the prepared liposomal suspension was placed in the Eppendorf for centrifugation (REMI) at 1400 rpm for 30min after centrifugation, the supernatant liquid was separated, containing the un-entrapped drug, diluted with phosphate buffer pH 6.8 and analyzed using UV spectrophotometer at 247 nm. By using that absorbance entrapment efficiency (%EE) are calculated by using the following formula.

$$Entrapment\ efficiency(EE\ \%) = \frac{Total\ amount\ of\ drug\ - Amount\ of\ the\ un-entrapped\ drug}{Total\ amount\ of\ drug} \times 100$$

In vitro drug diffusion study

Cellophane membrane diffusion technique was used to study in-vitro diffusion of drug from the prepared nanolipisomal formulations. The receptor medium used was freshly prepared phosphate buffer pH 7.4. Cellophane membrane soaked overnight in the receptor medium was on the Franz's Diffusion cell assembly 0.5 g of lyophilized formulation was placed in the donor compartment and the assembly was kept on the diffusion study apparatus at 37°C ± 2°C and stirred at 700 RPM. Aliquots of 0.5 ml were withdrawn at pre-determined time intervals (0.5, 1, 2, 4, 8, and 12 hrs.) and immediately replaced by same volume of the fresh medium. The aliquots were suitably diluted with the dissolution medium and analyzed by UV Spectrophotometer at 247nm (λ max).

Particle size analysis

The 2 ml of optimized liposome formulation was taken and mixed with distilled water and sonication was kept for 30 min. The analysis was performed at a temperature of 25°C same procedure repeated at zeta potential. The prepared formulations were characterized for zeta-potential in order to know the stability of the formulations.

TEM analysis

TEM is done to study ultrastructure of formulation. The TEM analysis of the optimized batch F8 was established by using Transmission Electron Microscopy (TEM). TEM images of samples were taken by using Soft Imaging Viewer Software.

Stability study

The Relugolix loaded liposomal formulation were stored at room temperature (25°C) and refrigerator temperature (2–8°C) for 1 month and Drug content, Entrapment efficiency (EE%) and Drug Diffusion (%) were determined.

RESULTS AND DISCUSSION

Preformulation study

Appearance

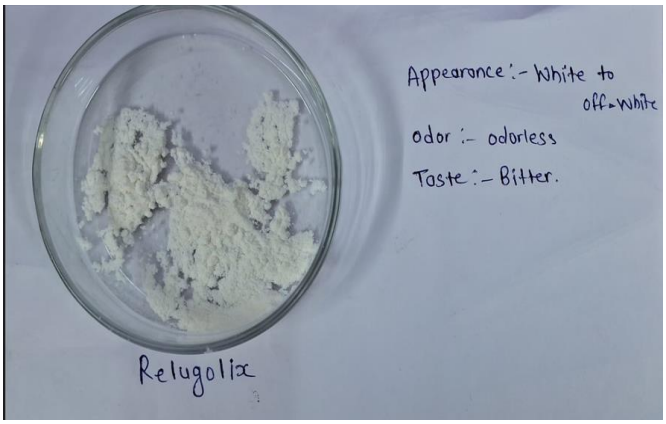

Figure 1: Appearance

Melting point

The observed melting point of Relugolix is 228°C, which matches the reported melting point value of 228°C. This consistency suggests that the purity and identity of the Relugolix sample are in line with the expected standards. The observed melting point aligns with the reported value, indicating that the sample is likely of high purity and has not undergone significant degradation or contamination.

Table 2: Observation of melting point

Drug name	Observed value	Reported value
Relugolix	228 °C	228 °C

Solubility study of Relugolix

The solubility study of Relugolix across various media reveals that ethanol provides the highest solubility for the drug, with a value of 50 µg/mL. This suggests that ethanol is the most effective solvent for dissolving Relugolix among the tested options. In aqueous media, Relugolix exhibits moderate solubility, with phosphate buffer at pH 7.4 and pH 6.8 showing solubilities of 35 µg/mL and 30 µg/mL, respectively. This indicates that Relugolix remains reasonably soluble in conditions that mimic physiological pH. Conversely, the solubility of Relugolix in acidic buffer is the lowest at 20 µg/mL, indicating reduced solubility in more acidic environments. These findings suggest that for formulations requiring high solubility, ethanol is the preferred solvent, while phosphate buffers are suitable for aqueous formulations that need to simulate physiological conditions.

Table 3: Solubility in different Medium

Medium	Solubility(µg/ml)
Distilled water	25
Methanol	40
Ethanol	50
Phosphate buffer ph 6.8	30
Phosphate buffer ph 7.4	35
Acidic buffer	20

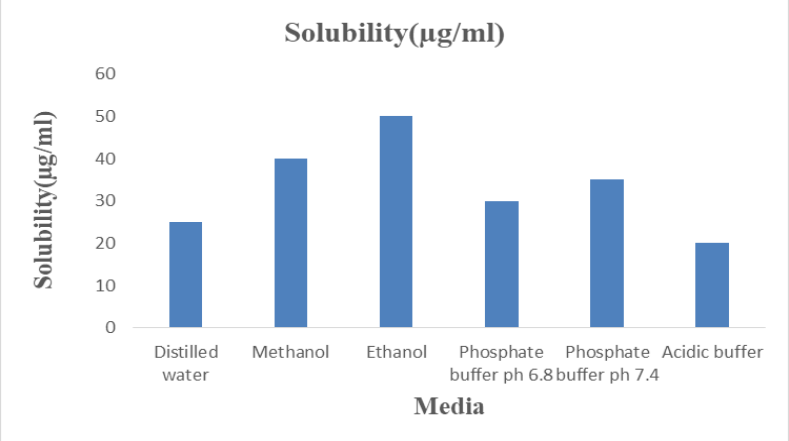

Figure 2: Solubility study in different Medium

Spectrophotometric characterization of Relugolix in UV Spectroscopy

Detection of Absorption Maxima (λ max)

The observed λ max (maximum wavelength of absorbance) for Relugolix is 247 nm, which matches the reported λ max of 247 nm. This indicates that the λ max for Relugolix in this study is consistent with previously reported values. This agreement validates the wavelength used for absorbance measurements and confirms that the spectrophotometric analysis is being performed correctly for this drug. The consistency in λ max ensures reliable and accurate quantification of Relugolix in various solvent systems.

Table 4: Observation of λ max

Drug name	Observed value (nm)	Reported value (nm)
Relugolix	247	247

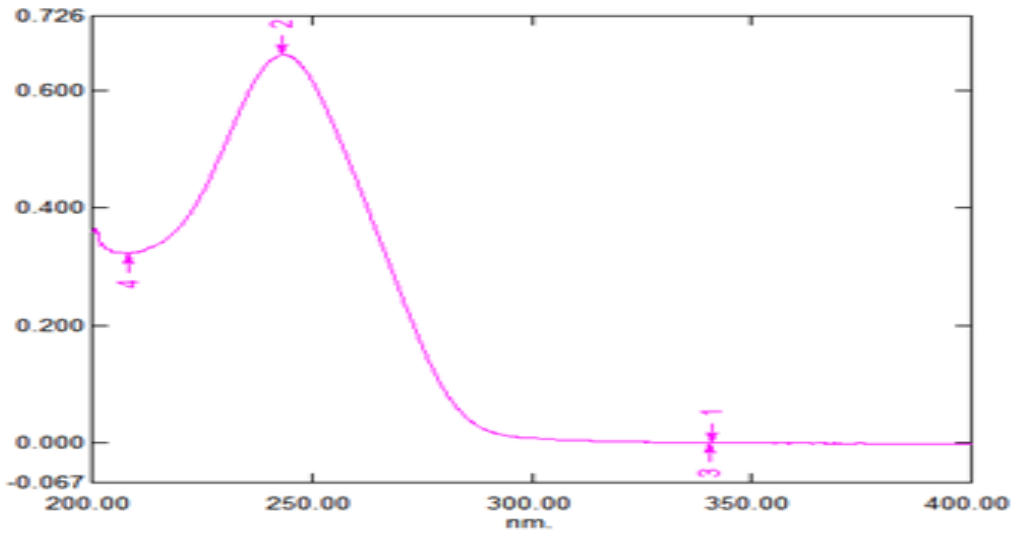

Figure 3: UV spectra of Relugolix in ethanol

Calibration curve in Phosphate buffer pH 6.8

Table 5: Calibration curve in Phosphate buffer pH 6.8

Concentration (µg/ml)	Absorbance
0	0
2	0.125
4	0.324
6	0.425
8	0.520
10	0.637
12	0.745

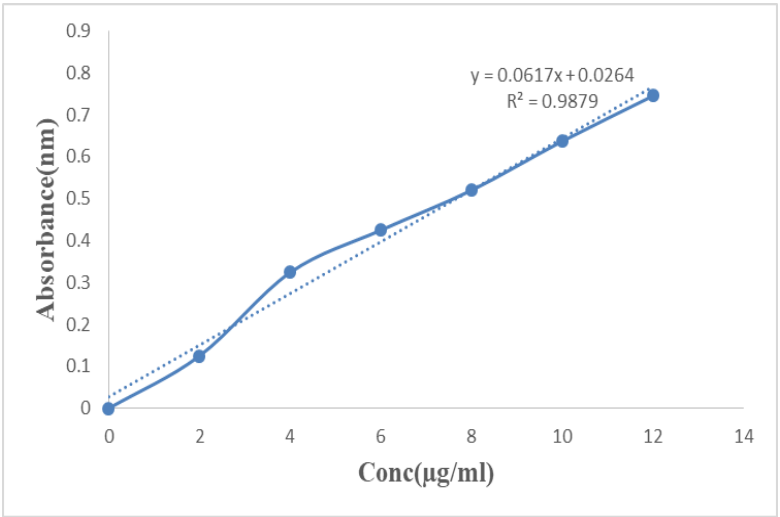

Figure 4: Calibration curve in Phosphate buffer pH 6.8

Table 6: Linearity equation

Equation	y = 0.0617x + 0.0264
Correlation coefficient	0.9879

Fourier transform infra-red spectroscopy (FTIR)

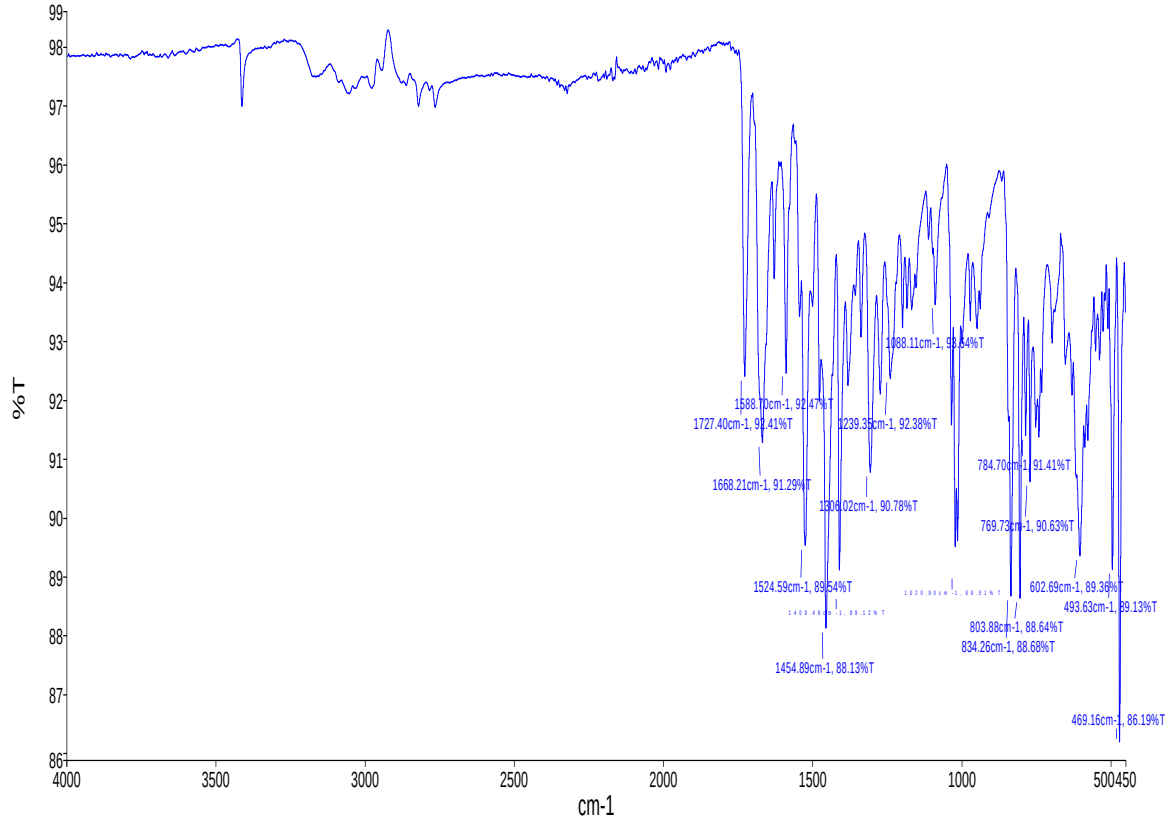

Figure 5: FTIR of Relugolix

Table 7: FTIR interpretations

Peaks obtained (cm-1)	Functional group
1727.4	C=O (Carbonyl, ketones/esters)
1668.21	C=O (Amide)
1588.7	C=C (Aromatic rings)
1524.59	C=C (Aromatic rings)
1454.89	C–H (Alkane, bending vibrations)
1409.48	C–H (Methyl bending)
1306.02	C–N (Amine, C–N stretching)
1239.35	C–O (Ester, ether)
1088.11	C–O (Alcohol, ether)
1020.9	C–O (Alcohol, C–O stretching)
834.26	C–H (Out-of-plane bending, aromatic)
803.88	C–H (Out-of-plane bending, aromatic)
784.7	C–H (Out-of-plane bending, aromatic)
769.73	C–H (Out-of-plane bending, aromatic)
602.69	C–Cl (Chlorinated compounds)
493.63	Out-of-plane bending (various)
469.16	C–C (Bending vibrations)

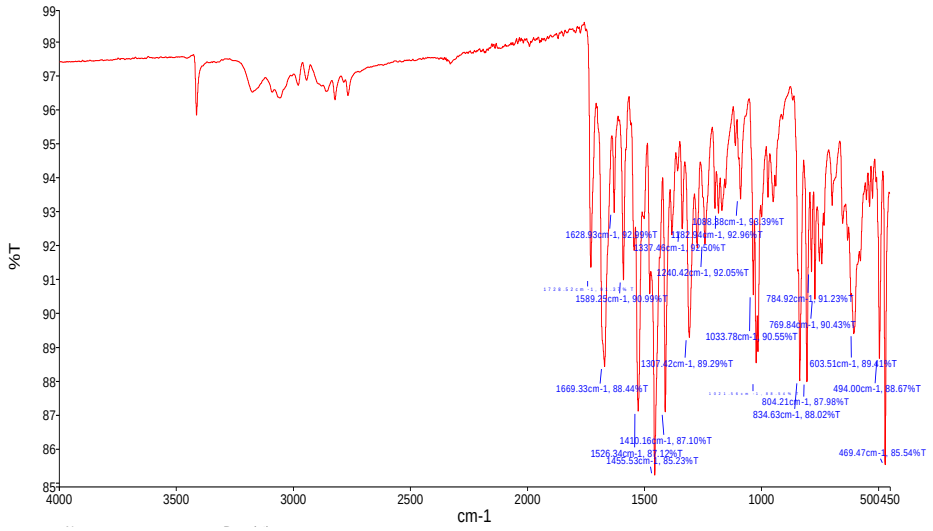

Figure 6: FTIR of Drug and excipients

FTIR analysis successfully identified key functional groups present in the drug, providing valuable insights into its molecular structure. Also, the FTIR of Drug and excipients prove to be compatible to be each other.

Evaluation of Relugolix loaded liposomes

Entrapment Efficiency (%EE)

The entrapment efficiency (%EE) data for Relugolix-loaded liposomes (F1-F13), formulation F8 exhibits the highest entrapment efficiency at 95.78%, indicating that it is the most effective in encapsulating the drug within the liposome structure. This makes F8 a leading candidate for the optimized formulation, assuming other properties such as stability and diffusion profile are also satisfactory. Formulations F12 (92.66%), F13 (93.56%), F7 (90.54%), and F6 (90.12%) also show high entrapment efficiency, suggesting these formulations are efficient in retaining the drug.

On the other hand, formulations like F1 (60.56%), F4 (60.23%), and F11 (66.45%) have the lowest entrapment efficiencies, indicating less effective drug encapsulation, which may require modifications to improve their performance. The range of entrapment efficiency across all formulations is from 60.23% to 95.78%, highlighting a significant variation in the ability of different liposome formulations to effectively trap the drug. Overall, F8 stands out as the most promising formulation for further development due to its highest entrapment efficiency, while those with lower efficiency may need further optimization to enhance their drug retention capacity.

Table 8: Entrapment Efficiency of Relugolix loaded liposomes (F1-F13)

Formulation code	Entrapment Efficiency (%EE)
F1	60.56
F2	86.56
F3	89.56
F4	60.23
F5	89.54
F6	90.12
F7	90.54
F8	95.78
F9	78.87
F10	79.45
F11	66.45
F12	92.66
F13	93.56

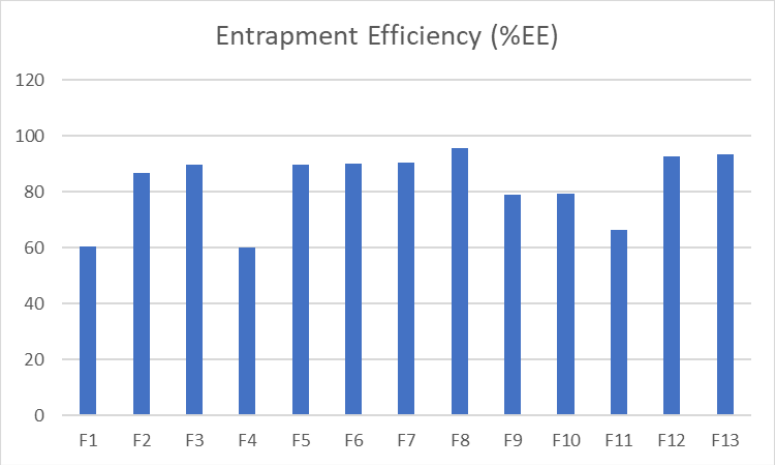

Figure 7: Entrapment Efficiency of Relugolix loaded liposomes (F1-F13)

In vitro diffusion Study

The drug diffusion data of Relugolix-loaded liposomes (F1-F13) over time, formulation F8 shows the highest cumulative drug diffusion, reaching 96.87% at 12 hours. This indicates that F8 provides the most efficient and sustained drug diffusion among all the formulations, making it a leading candidate for optimized drug delivery. Other formulations, such as F7 (93.56%), F12 (92.56%), F3 (92.45%), and F13 (92.45%), also demonstrate high drug diffusion percentages by the end of 12 hours, suggesting they are effective alternatives for sustained diffusion.

In contrast, formulations such as F1 (67.56%), F4 (65.23%), and F11 (63.78%) show comparatively lower cumulative drug diffusion at 12 hours, indicating slower drug diffusion profiles. These formulations may require further modification or optimization to enhance their diffusion rates if a faster or more complete drug diffusion is desired.

Overall, F8 emerges as the most promising formulation for achieving efficient and sustained drug diffusion, while formulations with lower diffusion percentages may need adjustments to meet the required therapeutic goals.

Table 9: Drug diffusion (%) of Relugolix loaded liposomes (F1-F13)

Time(hrs)	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13
0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.5	15.12	14.56	16.23	13.45	15.67	17.12	18.23	20.45	14.12	13.67	12.78	19.34	18.78
1	22.45	23.34	24.56	20.12	25.34	27.45	28.34	30.56	21.45	22.12	20.45	29.67	28.89
2	30.23	32.12	35.45	28.56	36.23	38.12	40.23	42.78	31.56	33.23	29.34	41.45	39.56
4	45.56	50.23	53.78	47.12	55.45	57.89	60.12	62.34	49.34	51.56	46.78	60.78	58.12
8	60.34	68.12	70.12	55.23	72.45	75.23	77.89	80.56	76.45	69.34	53.45	78.23	76.12
12	67.56	80.12	92.45	65.23	89.45	90.45	93.56	96.87	79.87	78.49	63.78	92.56	92.45

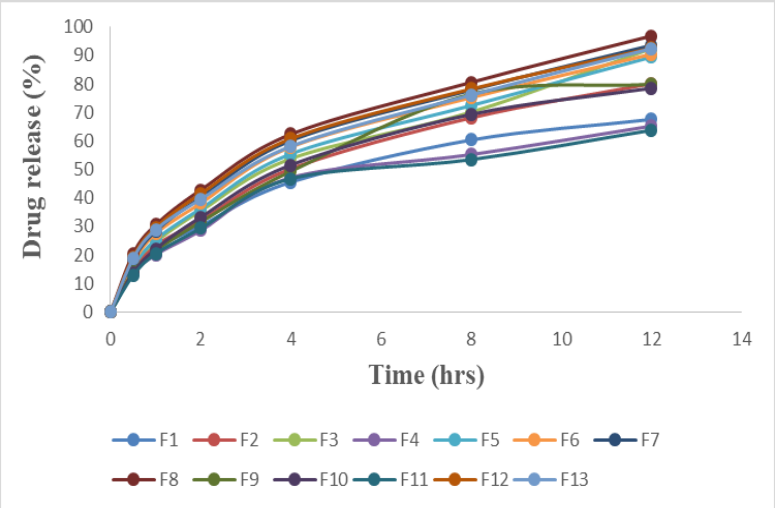

Figure 8: Drug diffusion (%) of Relugolix loaded liposomes (F1-F13)

Table 10: ANOVA for Linear model of Response 1: Entrapment efficiency

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1408.72	3	469.57	8.64	0.0051	significant
A- soya lecithin	1389.96	1	1389.96	25.58	0.0007	
B-Cholesterol	15.18	1	15.18	0.2794	0.6099	
C-rotational speed	3.58	1	3.58	0.0658	0.8033	
Residual	489.03	9	54.34			
Cor Total	1897.75	12				

P-values less than 0.0500 indicate model terms are significant. In this case A is a significant model term. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

Table 11: Fit Statistics of Response 1: Entrapment efficiency

Std. Dev.	7.37		R^2	0.7423
Mean	82.61		Adjusted R^2	0.6564
C.V. %	8.92		Predicted R^2	0.4574
			Adeq Precision	7.1211

The Predicted R^2 of 0.4574 is in reasonable agreement with the Adjusted R^2 of 0.6564; i.e. the difference is less than 0.2.

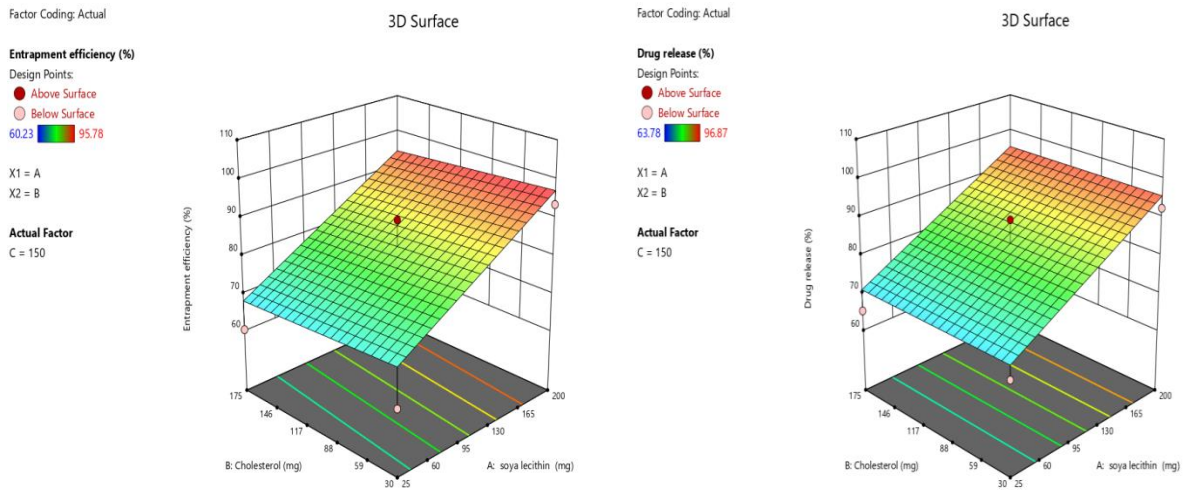

Figure 9:3D Surface response plot for response 1&2

Table 12: ANOVA for Linear model for Response 2: Drug diffusion

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1221.08	3	407.03	8.92	0.0046	significant
A- soya lecithin	1197.81	1	1197.81	26.24	0.0006	
B-Cholesterol	0.0903	1	0.0903	0.0020	0.9655	
C-rotational speed	23.19	1	23.19	0.5080	0.4941	
Residual	410.78	9	45.64			
Cor Total	1631.87	12				

P-values less than 0.0500 indicate model terms are significant. In this case A is a significant model term. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

Table 13: Fit Statistics for Response 2: Drug diffusion

Std. Dev.	6.76		R^2	0.7483
Mean	83.30		Adjusted R^2	0.6644
C.V. %	8.11		Predicted R^2	0.4684
			Adeq Precision	7.4389

The Predicted R^2 of 0.4684 is in reasonable agreement with the Adjusted R^2 of 0.6644; i.e. the difference is less than 0.2.

Particle size analysis

The F8 batch is spot-on. The particle size of 187.1 nm falls well within the ideal range for Nano liposomes, which is crucial for effective drug delivery. The PDI of 0.235 indicates that the liposomes have a narrow size distribution, which is excellent for ensuring uniformity and consistency in drug delivery. Overall, the F8 formulation shows promising characteristics for stability, cellular uptake, and prolonged circulation, making it a strong candidate for drug delivery applications.

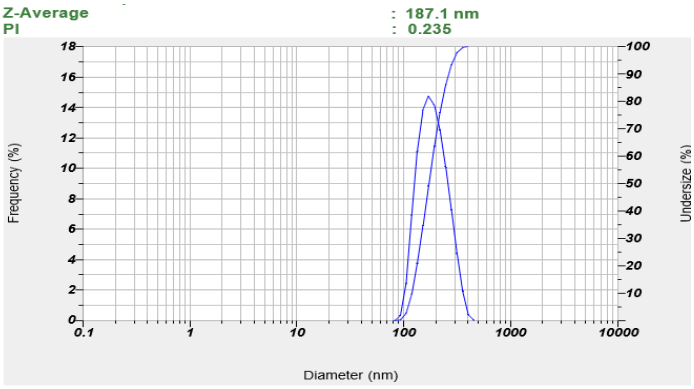

Figure 10: Particle size of optimized F8 batch

TEM

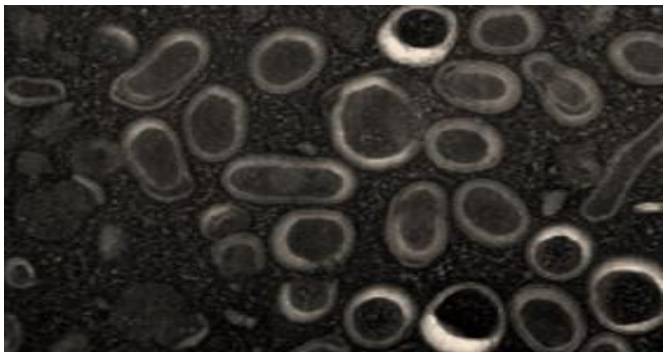

Figure 11: TEM image of optimized liposome

Stability study

The stability study of the optimized batch F8 for Relugolix-loaded liposomes shows that all key parameters like drug content, entrapment efficiency, and drug diffusion remain consistent over the initial and 1-month time points. The drug content remains at 97.56%, with minimal variation (±0.25 initially and ±0.36 after 1 month), indicating excellent stability in maintaining the drug's integrity over time. The entrapment efficiency also remains unchanged at 95.78%, with only a slight increase in variability (±0.15 initially and ±0.54 after 1 month), suggesting that the formulation is stable and retains the drug effectively within the liposomes.

Similarly, the drug diffusion percentage remains consistent at 96.87%, with a small increase in variability (±0.36 initially and ±0.87 after 1 month), indicating that the diffusion profile of the drug is not significantly affected over time.

Overall, these results suggest that the optimized batch F8 exhibits excellent stability under the tested conditions, maintaining its drug content, entrapment efficiency, and drug diffusion profiles over at least one month. This stability indicates the formulation's suitability for further development and potential commercial use.

Table 14: Stability study of optimized batch F8

Stability parameter	Initial	Final
Drug Content (%)	97.56±0.25	97.56±0.36
Entrapment efficiency (%)	95.78±0.15	95.78±0.54
Drug diffusion (%)	96.87±0.36	96.87±0.87

CONCLUSION

In conclusion, F8's exceptional entrapment efficiency and release profile make it the most promising candidate for further development. This optimization process underscores the crucial role of formulation parameters in improving liposomal drug delivery. The Relugolix-loaded liposomes produced via the thin film hydration method show significant potential for effective drug delivery. Future research will be necessary to evaluate their in vivo efficacy and safety profiles.

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