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Advanced Bilosomal Nanocarriers for Tenofovir Delivery: Formulation and Evaluation for Enhanced Antiviral Therapy

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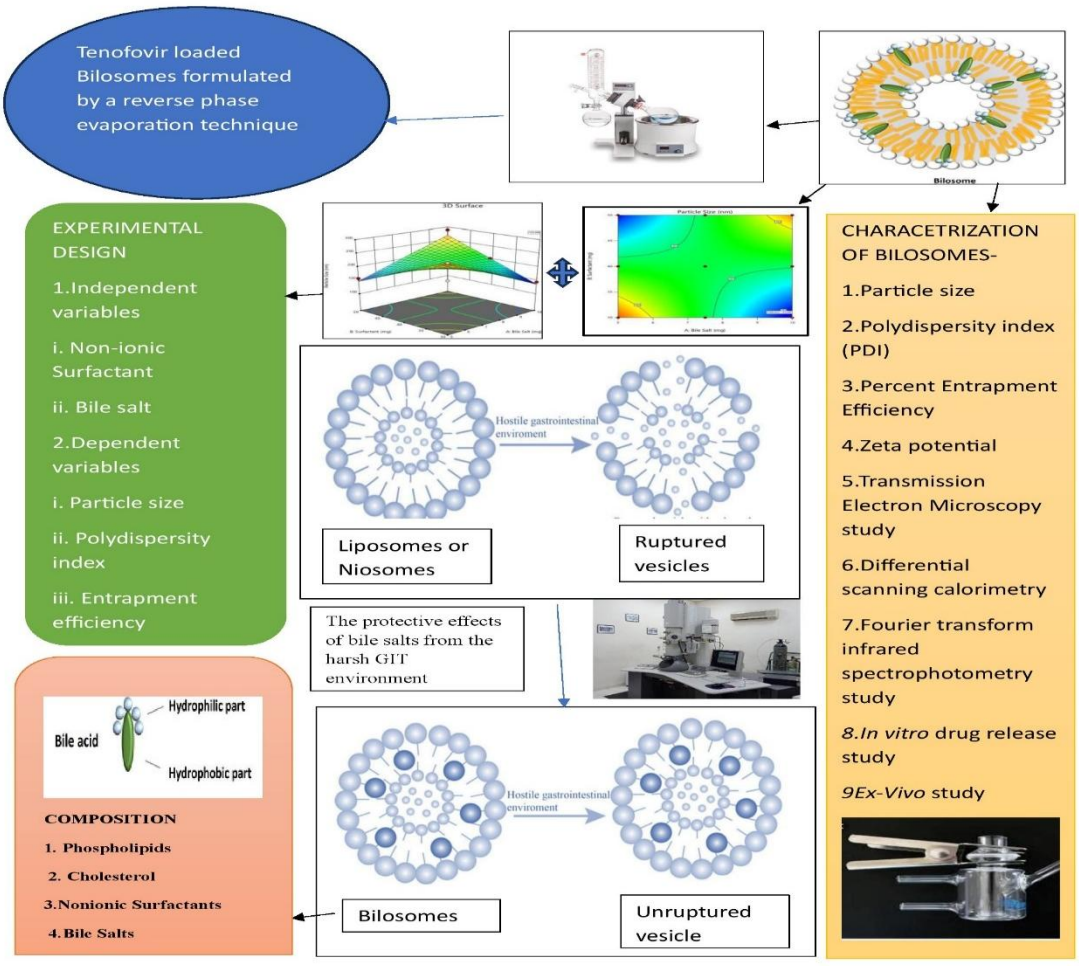
Graphical Abstract:



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KEYWORDS

Bilosomes, Anti-viral, Tenofovir, Reverse phase evaporation.

ABSTRACT:

Tenofovir is a BCS class III antiviral medication with poor permeability. Tenofovir's oral bioavailability is also modest, at 25%. Since the Tenofovir with low oral bioavailability they were formulated as Bilosomes to improve oral bioavailability because bile salt present in the Bilosomes prevent the gastric degradation of drug. Tenofovir loaded bilosomes were formulated by a reverse phase evaporation technique. Tenofovir loaded Bilosomes was prepared and optimized using Design of Experiment software. By using ANOVA model F7 batch was decided as optimized batch. The optimized batch (F7)'s particle size, polydispersity index, and entrapment efficiency percentage were 143.5 nm, 0.735, and 86.04, respectively. TEM (Transmission Electron Microscopy) image of optimized Tenofovir loaded Bilosomes formulation showed spherical shape. By using In-Vitro study the release of drug from Tenofovir loaded Bilosome and normal Tenofovir drug solution was calculated. This showed 84.42% drug release from Tenofovir loaded Bilosomes. The Ex-vivo study showed effective diffusion of Tenofovir across the intestinal tissue, making it a promising candidate for oral absorption. Since the current study showed that drug release studies showed that Tenofovir-loaded bilosomes exhibited a higher release compared to the Tenofovir solution, the research's goals were successfully met. Therefore, bilosomes are a promising drug delivery vehicle to address issues with Tenofovir's low oral bioavailability, poor absorption, and multiple-dose regimen.

INTRODUCTION

Bile salts are integrated into the membrane of bilosomes, one of the most inventive vesicular nanocarriers. Compared to alternative nano-vesicular carrier systems, these are more elastic, flexible, and ultra-deformable. [1] The hydrated non-ionic surfactant monomers self-assemble to create the bilayered structures known as Bilosomes, which are flexible and ultradeformable niosomes. Because of their amphiphilic nature, they can ensnare pharmaceuticals that are hydrophilic inside and lipophilic outside, which are contained in bilayered membranes and hydrophilic compartments, respectively. [2] Although Bilosomes and liposomes have structural similarities, Bilosomes are more stable than liposomes due to the non-ionic surfactants that are essential to their manufacture. As they get past the physical instability issues that liposomes have because of their lipid composition, like oxidation susceptibility and the challenge of achieving high purity levels that impact the size, stability, and shape of vesicles [3]

A non-ionic surfactant, bile salt, lipophilic substances like cholesterol and phospholipids, and other additions like hyaluronic acid and charge inducers make up Bilosomes. [4] They are drug delivery systems that use nonionic surfactants and contain bile salts in their bilayer membrane, either with or without cholesterol. [5]

The pharmaceutical industry primarily uses bile salts as intestinal penetration enhancers to increase the oral bioavailability of medications with limited intestinal permeability and water solubility. [6] Bilosomes are also biocompatible due to their naturally occurring lipids, such as cholesterol. All things considered, the addition of cholesterol affects the properties of bilosomes, including membrane permeability, stiffness, encapsulation effectiveness, rehydration of freeze-dried bilosomes, and decreased toxicity. They work just as well for administering hydrophilic and hydrophobic medications, therapeutic proteins and peptides, and vaccinations orally.[7]

STRUCTURE OF BILOSOMES

Bile salts and hydrophobic medications are found in the outer layer of the bilosomes, while hydrophilic medications and antigens are found in the interior layer. The bile salts have a unique closed form because they are encased in lipid layers inside the bilosomal structure. In a bilosome vesicle, the hydrophobic end of the bile acid molecule is located inside the hydrophobic region of the lipid bilayer, whilst the hydrophilic portion of the molecule is orientated towards the hydrophilic section of the lipid bilayer. [8,9,10] Bilosomes, which are synthetic nanoscale vesicular carriers, present a promising approach to improve the effectiveness of oral drug delivery for a wide range of

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pharmaceutical substances. These vesicular nanostructures were examined for their capacity to encapsulate hydrophilic and hydrophobic compounds within their aqueous core and phospholipid bilayer, accordingly. Bile salts, non-ionic surfactants, and lipids serve as key vesicular elements in the production of bilosomes. [11]

COMPOSITION OF BILOSOMES

COMPOSITION OF BILOSOMES

- Phospholipids
- Cholesterol
- Nonionic Surfactants
- Bile Salts

Figure 1. Composition of Bilosomes

Phospholipids are highly biocompatible with cell membranes, making them a natural fit for various biological applications. Due to their amphiphilic nature (having both hydrophilic and hydrophobic parts), they have a unique ability to self-assemble. This property allows them to facilitate wetting and emulsification. When exposed to water, phospholipids can form stable, concentric bilayer structures. Their strong emulsifying capabilities also make them effective at stabilizing emulsions, ensuring consistency, and preventing separation. [12,13]

1) Cholesterol:

The cellular membrane contains cholesterol, a molecule having amphiphilic properties, with hydroxyl groups at the aqueous surface and aliphatic chains parallel to the acyl chains at the heart of the bilayer. This makes bilosomes stiff [14].

2) Nonionic Surfactants:

Since nonionic surfactants are more stable and compatible than anionic, cationic, or amphoteric ones, they are frequently utilized in the formulation of bilosomes. [15,16] They help maintain the closest physiological pH in solution and are less irritant to cellular surfaces and hemolytic. They serve as permeability enhancers, emulsifiers, wetting agents, and solubilizers. They are also strong inhibitors of P-glycoprotein. Better drug absorption and tissue targeting are made possible by them. Nonionic surfactants have strong interfacial activity and are composed of both polar and non-polar regions. The overall entrapment efficiency of the medication is influenced by the length of the nonionic surfactant chain and the size of the hydrophilic head groups. The entrapment efficiency of nonionic surfactants with stearyl (C18) chains is higher than that of those with lauryl (C12) chains. [17, 18]

3) Bile Salts:

The gut lumen contains endogenous biosurfactants called bile salts, which are essential for digestion and lipid absorption. The increase of bile secretion improves the absorption of biologically active substances. This allowed for the effective use of various mixed micelle systems to improve the solubility of extremely lipophilic medications. By causing repulsion between bile salts inside the bilosomes and between external bile salts in the intestinal lumen, bile salts also make bilosomes more stable in simulated fluids [19–21].

MATERIALS AND METHODS

MATERIALS:

Tenofovir was purchased from Dhamtec Pharma and Consultants, Navi Mumbai. Phosphatidylcholine was purchased from Research Lab Fine Chem Industries. Sodium Taurocholate, Cholesterol, Span 80 and Tween 20 was purchased from Loba Chemie, Mumbai. Choloform and Diethyl ether was purchased from Molychem, Mumbai.

METHODS:

Tenofovir -loaded bilosomes were formulated by a reverse phase evaporation technique. Weighed required quantity of Phosphatidylcholine Cholesterol and surfactant. Added into beaker containing Choloform and diethyl ether. Dissolved that using magnetic stirrer. Aqueous solution of Drug and Bile salt was prepared. That aqueous phase added dropwise into lipid phase on magnetic stirrer (1500 rpm). Sonicated for till formation of milky w/o emulsion. Kept aside for 30 min. After that rotary evaporator used for 30 min. Hydrated that formulation by using sufficient quantity of water and rotated on hot plate magnetic stirrer for 10 min.

EXPERIMENTAL DESIGN

Design-Expert software was employed for the optimization of different independent variables in the

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development of bilosomes. A 3^2 Factorial Design was used that produced 9 experimental runs. The developed preparations were optimized using 2 factors and 3 levels & experimental trials performed at all nine combinations. The volume of (X1-Nonionic Surfactant) and (X2- Bile salt) was selected as independent parameters. The Particle size & entrapment efficiency and polydispersity index were selected as the dependent parameters. The coding of the actual value of variables shown in the Table 1.

Table 1. Factorial Design for Bilosomes

Factor	**Levels**		
Independent variables	**Low (-1)**	**Medium (0)**	**High (+1)**
A (Nonionic Surfactant)	5	7.5	10
B (Bile salt)	30	40	50
Dependent variables		Goal	
Y1(Particle size)	Minimum		
Y2(Polydispersity index)	In range		
Y3(Entrapment efficiency)	Maximum		

Table 2. Formulation table for Tenofovir Loaded Bilosomes preparation

RUN	**Tenofovir (API) in mg**	**Bile salt in mg**	**Nonionic surfactant in mg**	**Cholesterol in mg**	**Phospholipid in mg**
F1	25	5	30	10	40
F2	25	5	40	10	40
F3	25	5	50	10	40
F4	25	7.5	30	10	40
F5	25	7.5	40	10	40
F6	25	7.5	50	10	40
F7	25	10	30	10	40
F8	25	10	40	10	40
F9	25	10	50	10	40

OPTIMIZATION OF BILOSOMES

The goal was to have the smallest possible vesicle size, a PDI within the range, and the highest possible drug entrapment while choosing the best formulation for additional research.

CHARACTERIZATION OF TENOFOVIR-LOADED BILOSOMES

Particle size and polydispersity index (PDI):[22]

To evaluate the average particle size and PDI of Tenofovir loaded Bilosomes the HORIBA SZ 100 zeta sizer was utilized in the dynamic light scattering approach. The formulation was further diluted with distilled water to remove multiscattering events, and the measurements were performed. The PDI was employed as a standard for uniformity purpose.

Percent Entrapment Efficiency: [23,24]

The ultracentrifugation method was employed to determine the percentage of drug entrapment in the Tenofovir loaded Bilosome formulation. Centrifuge 1 ml of the Tenofovir loaded Bilosome formulation for 30 minutes (at 15000 rpm). The supernatant was further diluted with Methanol. Tenofovir content was determined using UV spectrophotometry with a wavelength of 260 nm. The percentage drug entrapment was estimated using the equation:

$$\% \, EE = \frac{\text{Total amount of drug} - \text{Unentrapped drug}}{\text{Total amount of drug}} * 100$$

Zeta potential: [25]

Using (HORIBA SZ 100), the zeta potential of Tenofovir loaded Bilosome was determined at 25°C. One milliliter of the ten-fold diluted formulation was employed in a disposable, foldable capillary cell for the measurement. The sample was analyzed three times, with each reading being made for accuracy. The mean voltage is the result that is displayed.

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Transmission Electron Microscopy study: [23]

The shape of Tenofovir loaded Bilosomes vesicles was investigated using a TEM. To prepare the sample for TEM investigation, a small drop of an optimum batch dispersal was applied to a 400-mesh carbon film covered grid (copper) after being diluted fifty times with double distilled H2O. The carbon film (-ve) was dyed with 1% phosphotungstic acid for ten seconds before drying on the grid. Prior to the TEM inspection, the sample was air dried.

Differential scanning calorimetry (DSC) Analysis-

DSC study was carried to analyse the thermal behaviour of Tenofovir loaded Bilosomes.

Fourier transform infrared spectrophotometry (FTIR) study-

FTIR spectra of optimized batch of Tenofovir loaded Bilosomes was studied using Agilent Cary 630 Alpha and Bruker, Japan.

***In vitro* drug release study:** [22]

The dialysis bag approach was used to carry out the medication release experiment. Before the trial, the dialysis bag was pre-treated by soaking it in the dissolving medium for 24 hours, which consisted of 100 milliliters of simulated intestinal fluid (Phosphate buffer, pH 6.8). The pre-treated dialysis sac was filled with an optimized Tenofovir- loaded bilosome and submerged in 100 mL of newly made SIF (pH=6.8) at 37±1°C in a dissolution flask. At specified intervals (1, 2, 4, 6, 12, and 24 hours), 2 mL samples were removed, and the digital magnetic stirrer's rpm was set to 50. Tenofovir-loaded bilosomes and Tenofovir solution were the subjects of this comparative investigation.

***Ex-Vivo* study:** [23]

The ex vivo intestinal permeation investigation was conducted using a modified Franz diffusion cell. Between the donor and receptor compartments were freshly removed rat intestinal segments, usually the ileum or jejunum. PBS (pH 7.4) was used to fill the receptor chamber, and a circulating water bath was used to keep the temperature at 37 ± 0.5 °C. A magnetic bead was used to maintain constant stirring. The test formulation was in the donor compartment. At certain intervals (e.g., 0, 0.5, 1, 3, 6, 8, and 12 minutes), samples were taken out and subjected to appropriate analytical methods. UV-visible spectrophotometry was used to filter and evaluate the samples that were gathered. To evaluate tissue integrity, the intestinal tissue was inspected under a microscope for any morphological alterations at the conclusion of the experiment.

RESULTS & DISCUSSION

Experimental design optimization:

Factorial designs such as ANOVA model are used to simultaneously study the influence of variables on the response. Here, the effect of A (Nonionic Surfactant) and B (Bile salt) were studied through 9 experimental runs obtained for responses: particle size, PDI and % EE (Table3).

Table 3. Optimization of Tenofovir loaded Bilosomes

Formulation code	Independent Variables		Dependent Variables		
	Factor 1 A: Bile salt (mg)	Factor 2 B: Nonionic surfactant (mg)	Response 1 Particle size (Nm)	Response 2 Polydispersity index	Response 3 Entrapment Efficiency (%)
F1	5	30	276.4	0.516	80.52
F2	5	40	230	0.498	82.32
F3	5	50	157.6	0.831	85.92
F4	7.5	30	219	0.513	82.6

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F5	7.5	40	148.6	0.357	81.96
F6	7.5	50	195	0.485	83.84
F7	10	30	143.5	0.735	86.04
F8	10	40	202.3	0.588	81.84
F9	10	50	288.6	0.586	80.44

Effect of dependent variable on particle size

The **Model F-value** of 6.31 implies the model is significant. The **Model P-value** is 0.0375 which is less than 0.05. This shows that model is significant. The quadratic equation given below explains how independent variables affect particle size.

Final Equation = +206.78-4.93A+0.3833B+65.97AB

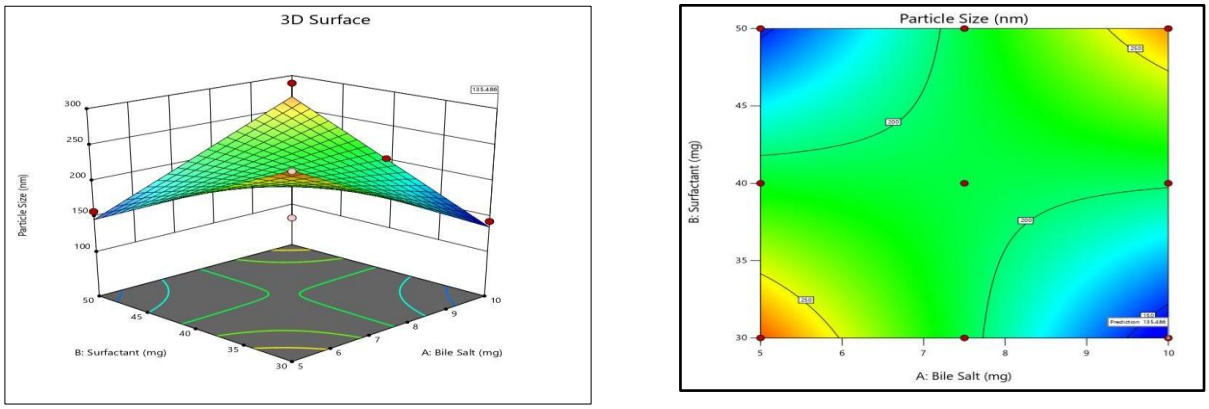

Figure 2. A) 3D Graph of particle size B) Contour plot of particle size

Effect of in dependent variable on Polydispersity Index

Model F-value of 11.71 implies the model is significant. The **Model P-value** is 0.0349 which is less than 0.05. This shows that model is significant. The quadratic equation given below explains how independent variables affect Polydispersity Index.

Final Equation = $+0.3650+0.0107A+0.0230B-0.1160AB+0.1740A^2+0.1300B^2$

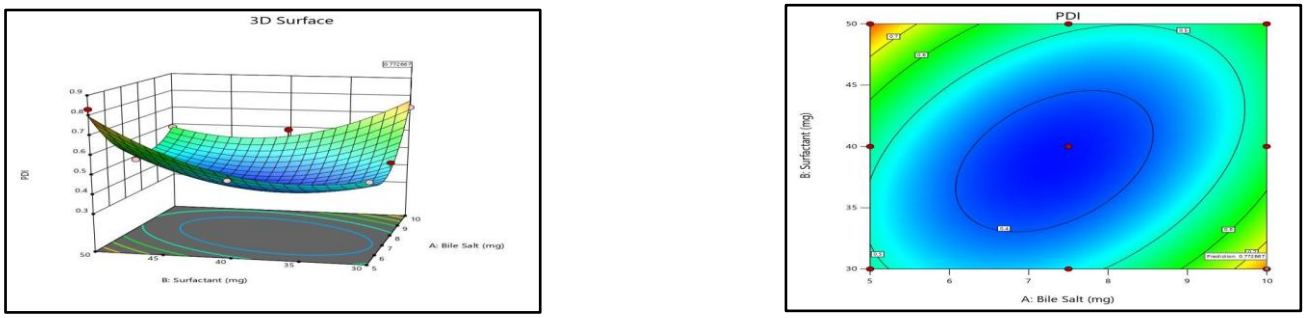

Figure 3. A) 3D Graph of Polydispersity Index. B) Contour plot of Polydispersity Index

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Effect of in dependent variable on %Entrapment Efficiency

The Model F-value of 8.13 implies the model is significant. The Model P-value is 0.0228 which is less than 0.05.

Final Equation = +83.32-0.2400A+0.3400B-3.00AB

This shows that model is significant. The quadratic equation given below explains how independent variables affect % Entrapment Efficiency.

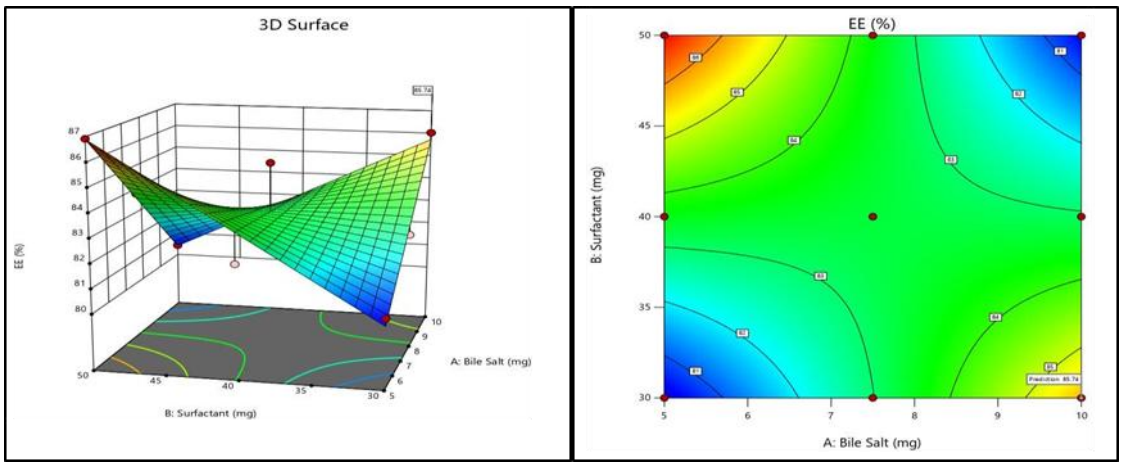


Figure 4. A) 3D Graph of %Entrapment Efficiency. B) Contour plot of %Entrapment Efficiency

CHARACTERIZATION OF OPTIMIZED BATCH

Particle Size, Polydispersity Index (PDI) And % Entrapment Efficiency:

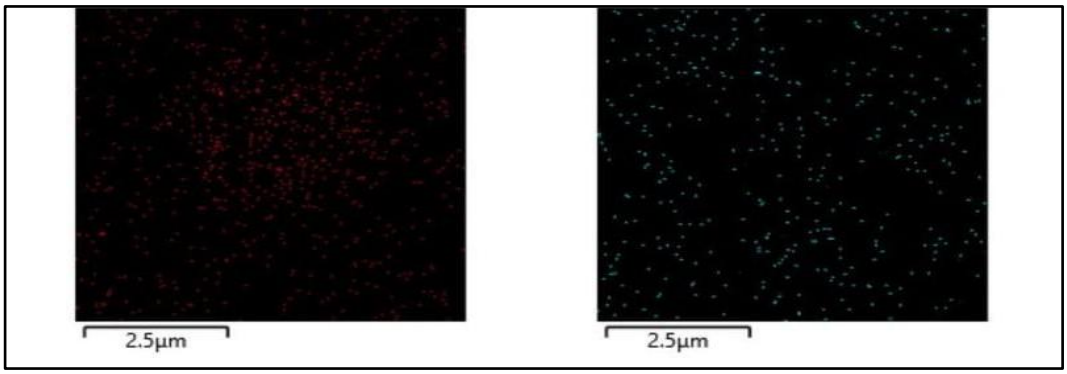
Particle size, PDI, and %EE of the optimized Tenofovir -loaded bilosome batch (F7) was found to be in range 143.5nm to 288.6 nm, 0.357 to 0.831, and 80.44 to 86.04% respectively (Table 3). Small vesicle size and high %EE is one of the most important criteria of bilosomes for improved bioavailability. PDI indicates the size homogeneity of bilosome vesicles in the formulation.

Zeta Potential:

A zeta potential of optimized Tenofovir -loaded bilosomes batch (F7) was -50.7 mV. This indicates that the sample has high stability. In colloidal systems, values greater than ±30 mV typically suggest good electrostatic repulsion between particles, reducing the risk of aggregation. The negative sign indicates that the particles are negatively charged.

The Tenofovir loaded Bilosomes was highly stable, with uniformly negatively charged particles. This stability is favorable in applications like drug delivery, suspensions, and emulsions where particle aggregation must be avoided.

Transmission Electron Microscopy study:



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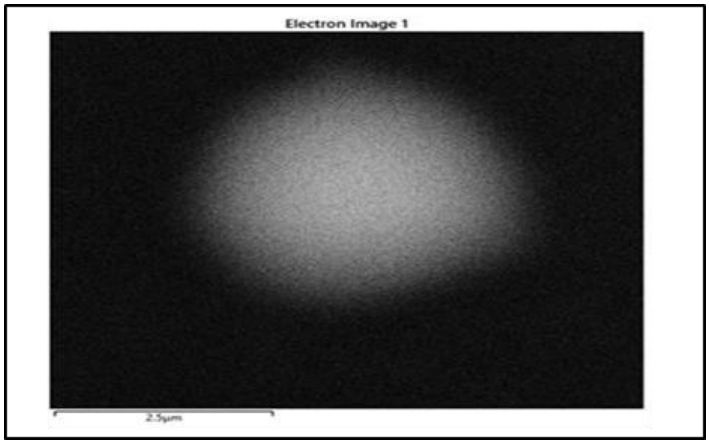

Figure 3. TEM of optimized Tenofovir loaded bilosome

The shape of Bilosomes vesicles was investigated using a TEM. Image of optimized Tenofovir formulation shows spherical shape and Aggregation was not observed in spherical form.

Differential Scanning Calorimetry (DSC) Analysis:

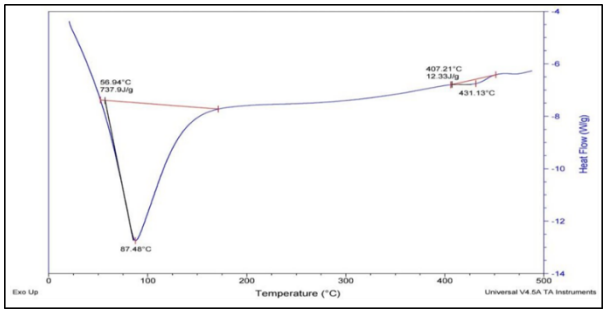

Figure 4. DSC of optimized Tenofovir loaded bilosome

The formulation melts around 87.48 °C, showing a strong endothermic transition. Thermal stability is good up to 400 °C, after which degradation begins.

Fourier Transform Infrared Spectrophotometry study (FTIR) study:

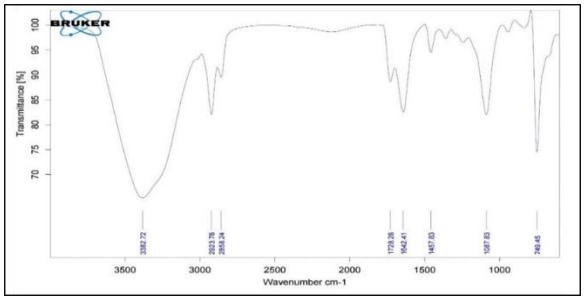

Figure 5. FTIR of optimized Tenofovir -loaded bilosome batch

Peak at 3382.72 cm^{-1} shows O-H or N-H Stretching that typically indicating the presence of hydroxyl or amine groups. C-H Stretching at 2923.78 cm^{-1} and 1457.83 cm^{-1} correspond to C-H bonds in alkanes. C=O Stretching peak shows at 1728.28 cm^{-1}, characteristic of the carbonyl group. Peak at 749.45 cm^{-1} shows presence of aromatic hydrocarbons.

***In vitro* drug release study of optimized batch Bilosomes:**

The release of drug from Tenofovir loaded Bilosome and normal Tenofovir drug solution was calculated. Tenofovir- loaded Bilosomes showed superior and sustained drug release compared to the plain Tenofovir solution. At first hour, Tenofovir-loaded Bilosomes released 15.72% of drug while the solution released only 4.52%. Tenofovir- loaded Bilosomes showed a gradual and extended-release pattern, reaching 84.42% at 24 hours. The Tenofovir-loaded Bilosomes consistently performed better than the plain solution, especially after the 2nd hour, showing better encapsulation and prolonged drug availability. This Indicates Tenofovir-loaded Bilosomes can potentially reduce dosing frequency and enhance therapeutic efficacy.

Table 4. In vitro drug release study of optimized Tenofovir loaded bilosome

Time (Hr.)	% DR of Tenofovir Solution	% DR of Tenofovir loaded Bilosomes
0	0	0

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1	4.52	15.72
2	13.53	23.8
3	15.84	33.91
4	21.2	45.02
5	25	55.12
6	27.27	62.2
12	40	69.27
24	54.44	84.42

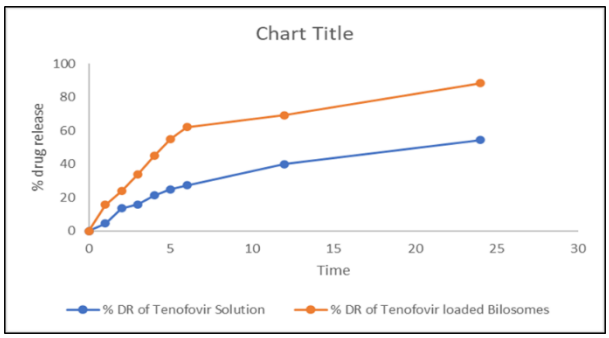

Figure 6. In vitro drug release study of optimized Tenofovir -loaded bilosome batch

***Ex-Vivo* study:**

Table 5. Ex-Vivo study of optimized Tenofovir -loaded bilosome

Sr. No.	Time (min)	Absorbance
1	0	0.455
2	0.5	1.106
3	1	1.972
4	3	4.490
5	6	4.560
6	8	3.824
7	12	9.999

The absorbance first increased from 0.455 at 0 minutes to 1.972 at 1 minute, which indicates that drug penetration started quickly. Active permeation during the early phase is indicated by a steep rise that lasts for three minutes (4.490). The absorbance increased slightly from 4.490 to 4.560 between 3 and 6 minutes, then decreased slightly at 8 minutes (3.824). A noticeable increase in absorbance to 9.999 at 12 minutes, however, suggests a second stage of improved permeation. Tenofovir is a viable option for oral absorption based on this pattern, which indicates efficient diffusion of the drug across intestinal tissue.

DISCUSSION:

The development of Tenofovir-loaded bilosomes presents a promising strategy to address the drug's poor oral bioavailability and limited permeability, attributed to its BCS Class III classification. This study aimed to optimize and characterize bilosomal formulations using a 3^2 factorial design, focusing on two independent variables—bile salt (A) and non-ionic surfactant (B) and evaluating their effects on particle size, polydispersity index (PDI), and entrapment efficiency (%EE).

ANOVA results confirmed the statistical significance of all models ($p < 0.05$). Particle size varied from 143.5 nm to 288.6 nm, which is suitable for mucosal absorption. PDI values ranged between 0.357 and 0.831, indicating moderate to good size uniformity. Entrapment efficiency was high across formulations, with %EE ranging from 80.44% to 86.04%. The optimized formulation (F7) exhibited the highest %EE, attributed to the bilayer-forming capacity of phosphatidylcholine and the stabilizing effects of bile salts and surfactants.

F7 showed desirable characteristics for oral delivery. Its zeta potential was -50.7 mV, indicating good colloidal stability. TEM images confirmed spherical, non-aggregated vesicles, aligning with PDI results. DSC analysis showed thermal stability up to 400°C, and FTIR studies revealed no chemical interaction between drug and excipients. In vitro drug release studies demonstrated sustained release up to 24 hours (84.42%) compared to 54.44% from the plain drug solution. This controlled release is likely due to the protective lipid bilayer that minimizes enzymatic degradation and slows drug diffusion.

Ex vivo intestinal permeation studies revealed a biphasic absorption pattern—an initial rapid phase followed by prolonged absorption—suggesting enhanced membrane permeation and sustained mucosal interaction. Bile salts played a key role by enhancing

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vesicle stability, permeability, and resistance to degradation. Their amphiphilic nature improves lipid bilayer fluidity and promotes absorption through the intestinal epithelium.

In summary, Tenofovir-loaded bilosomes demonstrated enhanced entrapment, stability, and controlled release, making them a viable oral delivery system to improve the bioavailability of Tenofovir and support more effective antiretroviral therapy.

CONCLUSION:

The present study demonstrated the successful development and optimization of a bilosome-based nanocarrier system for the oral delivery of Tenofovir, a BCS Class III antiviral drug with poor permeability and limited bioavailability. Using a factorial design approach, the effects of bile salts and non-ionic surfactants on formulation parameters such as particle size, PDI, and entrapment efficiency were systematically evaluated. The optimized formulation (F7) exhibited favourable characteristics including a small vesicle size (143.5 nm), high entrapment efficiency (86.04%), and excellent colloidal stability (zeta potential of -50.7 mV). Physicochemical characterization through TEM, DSC, and FTIR confirmed the structural integrity, thermal stability, and drug-excipient compatibility of the bilosome formulation. The vesicles displayed a uniform spherical shape and showed no signs of aggregation or chemical incompatibility, which is critical for maintaining stability during storage and transit through the gastrointestinal tract. The bilosome formulation demonstrated a sustained in vitro drug release of 84.42% over 24 hours, significantly outperforming the plain Tenofovir solution. Ex vivo permeation studies further confirmed enhanced intestinal absorption, likely due to the combined effects of nanoscale size, vesicle flexibility, and the membrane-permeabilizing properties of bile salts.

Overall, the inclusion of bile salts played a crucial role in improving drug stability, permeation, and release, making bilosomes a promising oral delivery vehicle for Tenofovir. The sustained release profile and enhanced permeation suggest potential for reducing dosing frequency and improving patient compliance in antiretroviral therapy. Additionally, the use of GRAS-status excipients supports the formulation's safety and scalability. In conclusion, Tenofovir-loaded bilosomes offer a viable and effective strategy to overcome the challenges associated with its oral administration.

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