RESEARCH PAPER

# Preformulation Studies of Amphotericin B for Formulation and Development of Lipid Nanoparticles for Macrophage Targeted Delivery

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**ABSTRACT**

Amphotericin B (AmB) is a potent antifungal agent whose clinical use is limited by poor aqueous solubility and high lipophilicity, making lipid nanoparticles a promising delivery platform. The present study aimed to establish a comprehensive preformulation profile of AmB relevant to lipid nanoparticle development and to develop and validate a sensitive HPLC method for its quantitative analysis. Preformulation studies including drug identification, melting point determination, saturation solubility studies in aqueous, organic, and solid lipid matrices, and determination of the partition coefficient were performed. AmB exhibited extremely low aqueous solubility, significantly higher solubility in lipid media, and a high partition coefficient with a log P value of approximately 4.6, confirming its strong affinity for lipid-based carriers. An HPLC method was successfully developed and optimized by appropriate selection of mobile phase composition, flow rate, and detection wavelength, producing a sharp and well-resolved AmB peak with a retention time of 5.9 min. The method showed excellent linearity over the studied concentration range with a correlation coefficient ($R^2$) greater than 0.999. The limit of detection (LOD) and limit of quantitation (LOQ) were found to be 0.04 µg/mL and 0.12 µg/mL, respectively, demonstrating high analytical sensitivity. System suitability parameters were within acceptable limits, confirming method reliability. FT-IR compatibility studies revealed no significant interactions between AmB and selected lipid excipients. Overall, these findings provided strong preformulation and analytical support for the rational development of AmB loaded lipid nanoparticles.

**Keywords:** Amphotericin B; Lipid nanoparticles; Preformulation studies; HPLC method development; Method validation

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## INTRODUCTION

Amphotericin B (AmB) is an essential, broad-spectrum polyene antifungal agent that has remained a cornerstone of therapy for over 50 years due to its high efficacy and low incidence of resistance. It is primarily indicated for life-threatening systemic fungal infections, serving as a first-line treatment for invasive mucormycosis (including COVID-19 associated cases), cryptococcal meningitis, and severe forms of aspergillosis, candidiasis, and blastomycosis. Beyond its antifungal role, AmB is a critical antiprotozoal agent, utilized as the treatment of choice for visceral leishmaniasis (Kala-azar) and primary amoebic meningoencephalitis. Its utility extends to various refractory or disseminated diseases such as histoplasmosis, coccidioidomycosis, and sporotrichosis, making it indispensable for managing severe mycotic infections in immunocompromised and critically ill patients [1]

However, the clinical utility of AmB is severely restricted by its extreme physicochemical properties and high systemic toxicity, particularly nephrotoxicity. As a BCS Class IV drug, AmB is characterized as "practically insoluble" in both water and common organic solvents, a challenge rooted in its amphiphilic structure which contains both a lipophilic polyene chain and a hydrophilic polyol region. These hurdles necessitate the development of advanced delivery systems that can improve solubility while minimizing the adverse effects associated with conventional deoxycholate-based formulations [2]

Currently, several commercial formulations of AmB are available, primarily categorized into the conventional

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deoxycholate form and advanced lipid-based systems. The conventional formulation, Fungizone, is cost-effective and remains widely used in resource-limited settings; however, its clinical utility is severely hampered by a high incidence of infusion-related toxicity and dose-limiting nephrotoxicity [3,4]. To mitigate these effects, lipid-based formulations such as AmB lipid complex (ABLC), AmB colloidal dispersion (ABCD), and liposomal AmB (AmBisome) were developed. While these lipid-based carriers significantly reduced renal toxicity and allowed for higher dosing regimens, they are often characterized by high manufacturing costs, requirements for cold-chain storage, and potential physical instability during long-term storage [5]. These limitations highlight the urgent need for alternative delivery systems like lipid nanoparticles (LNPs), which aim to combine the safety of lipid-based delivery with the structural stability and cost-efficiency of solid-state carriers [6].

LNPs have emerged as a promising alternative to traditional lipid-based carriers like liposomes. LNPs consist of a solid lipid core stabilized by surfactants in an aqueous dispersion. They offer unique advantages, including high physical stability, protection of the entrapped drug from chemical degradation, and the ability to provide a sustained release profile. By using a solid lipid matrix instead of a liquid oil, the mobility of the drug is restricted, which prevents drug leakage and allows for better control over the release kinetics of the highly lipophilic AmB [7,8].

The preformulation phase serves as the critical scientific foundation for the development of AmB LNPs, providing the essential physicochemical data required to ensure a stable and effective delivery system. By systematically evaluating parameters such as the partition coefficient (log P), which confirms the drug's high lipophilic affinity for the solid lipid core, and its pH-dependent solubility, researchers can strategically optimize the drug-to-lipid ratio for maximum entrapment efficiency [9]. Furthermore, utilizing advanced analytical tools like FT-IR and DSC during this stage allows for the early detection of potential drug-excipient interactions and the confirmation of the drug's transition into a beneficial amorphous state, thereby preventing physical instability or drug expulsion during storage [10]. Ultimately, a robust preformulation study supported by validated UV-Vis and HPLC methods minimizes the risk of formulation failure and ensures that the final LNPs product delivers the intended therapeutic response for systemic fungal therapy [11]. In this research, we performed comprehensive preformulation studies of AmB to facilitate development of LNPs based formulation to alleviate challenges associated with its delivery.

## MATERIALS AND METHODS

Amphotericin B was procured from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). Acetone, methanol, and dimethyl sulfoxide (DMSO) were obtained from Qualikems Lifesciences Private Limited (Mumbai, India). Stearic acid and palmitic acid were purchased from SD Fine Chemicals Ltd. (Mumbai, India), while glycerol monostearate was obtained from Loba Chemie Pvt. Ltd. (Mumbai, India). All other chemicals and reagents used in this study were of analytical grade and used as received.

### 2.1 Preformulation Studies of Amphotericin B

#### 2.1.1 Drug Identification and Characterization

The organoleptic evaluation of AmB was conducted to establish a baseline for API authenticity and structural integrity. AmB was physically characterized based on its distinctive color, odor and texture [12].

##### 2.1.1.1 Melting Point Determination

The measurement of the melting point is a critical parameter in the preformulation studies of AmB. The primary objective for determining the melting point is to assess the crystalline lattice energy and its direct impact on aqueous solubility. Due to the complex polyene structure of the drug, the melting point was determined by the capillary melting technique, with particular focus on identifying the onset of decomposition. This thermal characterization provides essential data regarding the purity and the solid-state stability of the API prior to its incorporation into the lipid nanoparticle matrix [13].

##### 2.1.1.2 Saturation Solubility Studies

###### 2.1.1.2.1 Aqueous and Organic Phase Solubility

The saturation solubility of AmB was determined using the shake-flask method. An excess amount of the drug was added to 10 mL of various media, including distilled water, phosphate buffers (pH 1.2 and 7.4), and organic solvents (DMSO, Methanol and Ethanol). The mixtures were placed in amber-colored glass vials to prevent photolytic degradation of the heptaene chromophore. The samples were subjected to continuous agitation in a temperature-controlled shaking water bath at 37±0.5°C for 48 hours to ensure equilibrium. After reaching equilibrium, the suspensions were centrifuged at 10,000 rpm for 15 minutes. The supernatant was filtered through a 0.22 µm membrane filter, appropriately diluted, and analyzed using a UV-Visible spectrophotometer at $\lambda_{max}$ of 405 nm to quantify the dissolved drug concentration. All experiments were performed in triplicate [14].

###### 2.1.1.2.2 Solubility in Solid Lipids (Melt Method)

Lipid solubility of AmB was evaluated to determine its suitability for entrapment within a solid lipid matrix hence solubility in various lipids (e.g., stearic acid, palmitic acid, and glycerol monostearate) was determined using the modified transition method. The

solid lipids were heated to 10°C above their respective melting points in a controlled oil bath. AmB was added in small, increments (2 mg) to the molten lipid under constant magnetic stirring. The point at which the drug no longer dissolved observed as the appearance of persistent crystalline particles or turbidity was recorded as the saturation point in the lipid melt. This process was performed in a dark environment to maintain API integrity [14,15].

#### 2.1.1.3 Determination of Partition Coefficient (Log P)

The partition coefficient of AmB was determined using the n-octanol/water shake-flask method. Pre-saturated phases were prepared by agitating n-octanol and phosphate-buffered saline (PBS, pH 7.4) together for 24 hours. A known concentration of the drug was dissolved in the n-octanol phase and mixed with an equal volume of PBS (pH 7.4). The mixture was equilibrated in a shaking water bath at 37 ± 0.5 °C for 24 hours in the dark. After phase separation by centrifugation at 5000 rpm for 10 minutes, the concentration of AmB in both phases was quantified using UV–visible spectrophotometry at $\lambda_{max}$ = 405 nm [16].

### 2.2 Development and Validation of UV–Visible Spectrophotometric Method for AmB

#### 2.2.1 Selection of Detection Wavelength ($\lambda_{max}$)

To determine the wavelength of maximum absorption ($\lambda_{max}$), a 100 µg/mL, stock solution was prepared by initially solubilizing an accurately weighed quantity of AmB in a minimal volume of dimethyl sulfoxide (DMSO), followed by volume adjustment with a mixture of phosphate-buffered saline (PBS, pH 7.4) and methanol (1:1, v/v). The solution was subsequently analyzed using a UV-Visible Spectrophotometer within the range of 300 to 450 nm using 1 cm quartz cuvettes against a blank diluent for baseline correction. The resulting spectrum exhibited the characteristic heptaene triplet with four distinct absorption bands near 345, 365, 385 and 405 nm; the most intense peak at 405 nm was selected as the $\lambda_{max}$ for all subsequent quantitative estimations to ensure maximum sensitivity and minimal interference from lipidic excipients [17].

#### 2.2.2 Preparation of Calibration Curve

##### 2.2.2.1 Preparation of Stock Solution

A primary stock solution 1000 µg/mL was prepared by dissolving 10 mg of accurately weighed AmB in minimum volume of DMSO and then volume was made up to 10 mL with PBS (pH 7.4): methanol (1:1 v/v). From this, a secondary stock solution 100 µg/mL was prepared using PBS (pH 7.4): methanol (1:1 v/v).

##### 2.2.2.2 Preparation of Working Standards

A series of working standard solutions were prepared by appropriately diluting the secondary stock solution to obtain concentrations of 1-10µg/mL. The absorbance of these solutions was measured at the wavelength of maximum absorption ($\lambda_{max}$ 405 nm) against a blank diluent. The procedure was performed in triplicate to ensure reproducibility, and the mean absorbance was plotted against the concentration**.** The quantitative estimation of AmB was performed using a UV-Visible Spectrophotometer at $\lambda_{max}$ = 405 nm [17,18].

#### 2.2.3 Method Sensitivity

##### 2.2.3.1 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The sensitivity of the developed UV-spectrophotometric method in the PBS (pH 7.4): Methanol (1:1 v/v) system was determined by calculating the LOD and LOQ. These values were derived from the standard deviation of the response ($\sigma$) and the slope of the calibration curve (S), in accordance with International Council for Harmonisation ICH Q2 (R1) guidelines, the values were calculated based on the standard deviation of the response $\sigma$ and the slope of the calibration curve (S) using the following equations [19].

$$LOD = \frac{3.3 \times \sigma}{S}$$

$$LOQ = \frac{10 \times \sigma}{S}$$

### 2.3 HPLC Method Development and Validation for Amphotericin B

The development of a robust analytical protocol is a fundamental prerequisite for the characterization of complex drug delivery systems like LNPs.

#### 2.3.1 Optimization of Chromatographic Conditions

##### 2.3.1.1 Selection of Mobile Phase

The stability-indicating HPLC method for AmB was developed through systematic evaluation of different mobile phase compositions to achieve optimal resolution and peak symmetry. Mobile phases containing acetonitrile or methanol in combination with water and various buffer systems were initially screened. Methanol as the organic modifier produced an unstable baseline with significant drift and reduced analytical response, whereas substitution with acetonitrile resulted in a stable baseline and improved AmB peak response. The effects of mobile phase pH (3–7) and buffer strength (5–50 mM) were investigated using phosphate and sodium acetate buffers; extreme pH conditions caused increased peak tailing and poor resolution, while buffer concentration significantly influenced AmB retention. Based on these observations, a mobile phase comprising acetonitrile and sodium acetate buffer (30:70 v/v, pH 4.0) was selected as

optimal, providing well-resolved peaks and acceptable system suitability.

**2.3.1.2 Flow Rate and Detection Wavelength**

Flow rates in the range of 0.8–1.5 mL/min were evaluated during method optimization. Lower flow rates resulted in increased retention times and prolonged analysis, whereas higher flow rates caused excessive column backpressure and reduced chromatographic resolution. A flow rate of 1.2 mL/min was selected as the optimal condition, providing an appropriate balance between resolution and run time. The detection wavelength was optimized by scanning between 303 and 420 nm, and 405 nm was selected due to the high molar absorptivity of the heptaene chromophore of AmB at this wavelength, ensuring maximum sensitivity and reliable detection.

**2.3.2 Preparation of HPLC Calibration Curve**

**2.3.2.1 Preparation of Stock Solution**

A primary stock solution 1000 µg/mL was prepared by dissolving 10 mg of accurately weighed AmB and dissolved in minimum volume of DMSO. Then volume was made up to 10 mL with mobile Phase (acetonitrile and 10 mM sodium acetate buffer (pH 4.0) in the ratio of 30:70 (v/v)). From this, a secondary stock solution 100 µg/mL was prepared using mobile phase

**2.3.2.2 Preparation of Working Standard Solutions**

A series of working standard solutions were prepared by appropriately diluting the secondary stock solution to obtain concentrations in the range of 0.5-10 µg/mL. The calibration curve was generated by plotting the peak area against the corresponding concentration of AmB.

**2.3.3 System Suitability Evaluation**

System suitability testing was conducted to confirm the adequacy of the HPLC system and the reliability of the analytical method. Prior to the analysis of LNPs samples, six replicate injections of a standard AmB solution (10 µg/mL) were performed. Chromatographic performance parameters, including retention time, theoretical plate count (N), tailing factor (T), and peak area reproducibility, were evaluated to ensure compliance with established pharmacopeial acceptance criteria [20,21].

**2.3.4 Method Sensitivity**

**2.3.4.1 Limit of Detection (LOD) and Limit of Quantitation (LOQ)**

The sensitivity of the developed HPLC method was evaluated by determining the limit of detection (LOD) and the limit of quantitation (LOQ). These parameters define the lowest concentration of AmB that can be reliably detected and accurately quantified, respectively, under the optimized chromatographic conditions. In accordance with the ICH Q2 (R1) guidelines, these values were derived from the standard deviation of the response ($\sigma$) and the slope of the HPLC calibration curve (S) utilizing the same statistical equations previously established for the UV method [19,22].

**2.4 FT-IR Studies for Drug–Excipient Compatibility**

The chemical compatibility between AmB and stearic acid was evaluated using Fourier Transform Infrared (FT-IR) spectroscopy. Samples of the AmB, Stearic acid, and their physical mixtures were prepared as KBr pellets. The spectra were recorded in the range of 4000 to 400 $cm^{-1}$ to identify any shifts or disappearances of functional group characteristic peaks that might indicate a chemical interaction

**RESULTS AND DISCUSSION**

The goal of the present work was to investigate preformulation studies of AmB to generate information useful in developing stable and Bioavailable dosage forms.

**3.1 Preformulation Characterization of Amphotericin B**

**3.1.1 Drug Identification and Characterization**

Amphotericin B appeared as a yellow–orange, odorless fine crystalline powder, consistent with its polyene macrolide structure and compliant with pharmacopeial specifications. The stable pigmentation indicated the absence of oxidative degradation during the preformulation stage, as summarized in Table 1 [12,24].

**Table 1: Physical characterization of Amphotericin B**

| Attribute | Experimental Observation | Pharmacopeial/Reference Description | Scientific Interpretation |
|---|---|---|---|

| | | | |
|---|---|---|---|
| Physical form | Fine crystalline powder with a vibrant yellow to orange appearance | AmB occurs as a yellow to orange powder; odorless or almost odorless, consistent with official monographs. | The observed solid state and pigmentation are consistent with the extended conjugated heptaene system of polyene macrolides, confirming the expected API morphology. |
| Odor | No discernible odor detected | Described as odorless or almost odorless in reference standards. | The lack of perceptible odor aligns with official descriptions and indicates absence of volatile degradation products. |
| Color intensity | Intense yellow-orange pigmentation throughout preformulation analysis | Color and appearance as yellow-orange powder reported in official sources. | The intense pigmentation serves as a qualitative indicator that polyene conjugation remains intact, with minimal oxidative degradation of the API. |

**3.1.2 Melting Point**

The thermal analysis of AmB using the capillary method revealed a transition temperature of 172°C to 175°C. Notably, the drug did not exhibit a classical melting transition (solid-to-liquid phase change) but instead underwent simultaneous melting and decomposition. The observed decomposition range of 172°C to 175°C is in high agreement with reported literature values for the crystalline form of AmB. The absence of a sharp melting point is attributed to the high molecular weight and the extensive intermolecular hydrogen bonding within the crystal lattice, which requires significant thermal energy to overcome (Table 2) [25].

**Table 2: Thermal characterization of Amphotericin B**

| Parameter Evaluated | Experimental Observation | Reported Value | Interpretation |
|---|---|---|---|
| Melting point (°C) | 172–175 °C | >170 °C | The observed melting range is consistent with reported values, confirming the crystalline purity of AmB. |
| Physical behavior on heating | Darkening and charring observed | Thermal decomposition reported | The compound undergoes decomposition upon heating, indicating thermal instability above 170 °C. |
| Nature of melting peak | Broad and decomposing peak | Broad and decomposing peak | The broad, decomposing peak is characteristic of polyene macrolide antibiotics such as AmB. |

### 3.1.3 Saturation Solubility Behavior

Amphotericin B exhibited negligible aqueous solubility across the physiological pH range (<1.5 µg/mL), as shown in Figure 1. This poor solubility is attributed to its high lattice energy and amphoteric nature, leading to strong intermolecular zwitterionic interactions that limit hydration. The minimal variation in solubility between pH 1.2 and 7.4 highlights the limitations of conventional oral and intravenous delivery without a carrier system.

In contrast, AmB showed markedly higher solubility in DMSO, likely due to disruption of molecular self-association, identifying DMSO as a suitable solvent for the solvent-emulsification step in LNPs preparation. Among the lipids evaluated, stearic acid demonstrated the highest solubilizing capacity for AmB, followed by glyceryl monostearate and palmitic acid. Among the lipids evaluated, stearic acid showed significantly higher solubility for AmB compared to the aqueous phase, justifying its selection as the lipidic core. In pharmaceutical formulation development, enhanced lipid solubility is a critical prerequisite for achieving higher entrapment efficiency [14,26]

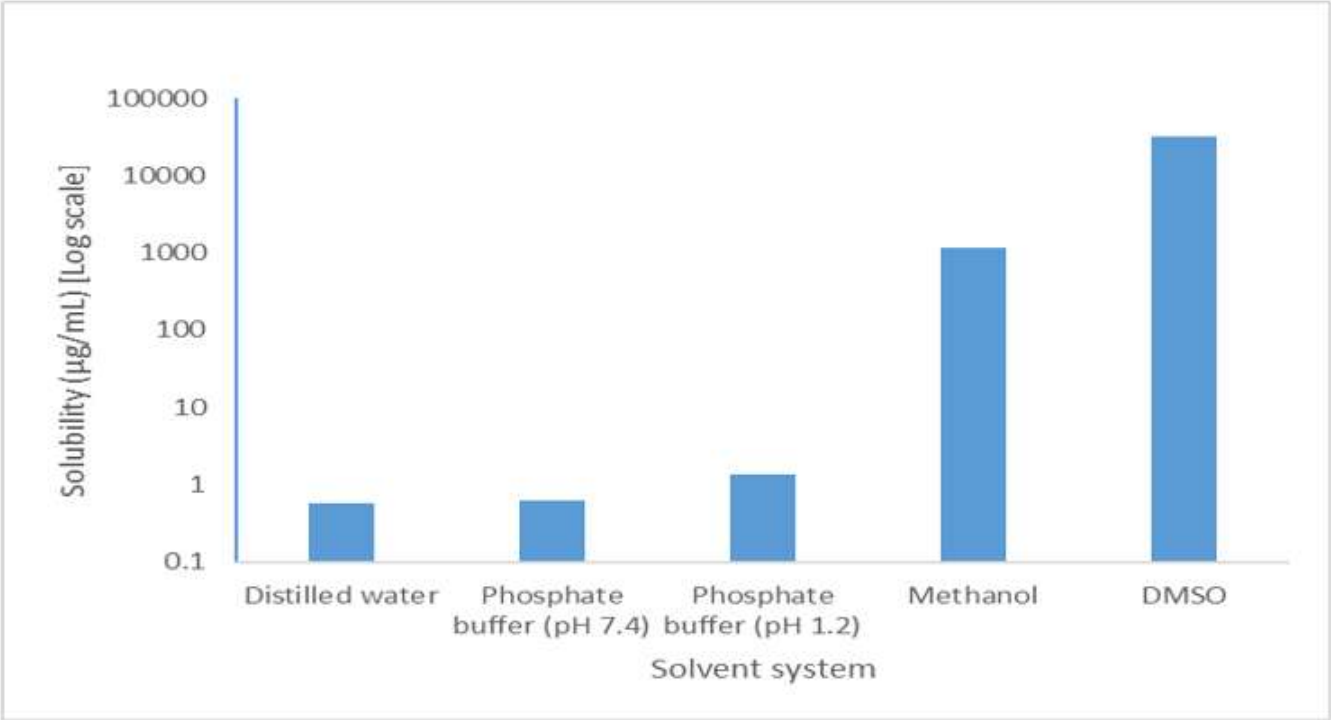


**Figure 1:** Saturation solubility of Amphotericin B in various aqueous and organic solvent media at 25 °C.

### 3.1.3 Partition Coefficient (Log P)

The log P (n-octanol/water) of AmB was determined to be 4.62 ± 0.21, consistent with reported literature values (~4.5), confirming its highly lipophilic and BCS Class IV nature. This high partition coefficient indicates a strong preference of the drug for lipidic environments, which is advantageous for lipid nanoparticle formulation, as AmB is expected to preferentially partition into the molten lipid core during homogenization, resulting in enhanced entrapment efficiency. Moreover, a log P value greater than 4 suggests strong retention of the drug within the lipid matrix, minimizing premature leakage into the aqueous phase and supporting a sustained release profile governed by diffusion through the solid lipid lattice [24,27].

### 3.2 UV–Visible Spectrophotometric Method Validation

The UV–Visible spectrum of AmB exhibits four characteristic absorption bands at approximately 345, 365, 385, and 405 nm, arising from electronic transitions within its conjugated heptaene system. UV–Visible scanning confirmed a typical heptaene spectrum, with maximum absorbance observed at 405 nm, which was selected as the $\lambda_{max}$ for quantitative analysis (Figure 2). This wavelength lies in the visible region, thereby minimizing interference from lipid excipients and surfactants that absorb below 300 nm, making it suitable for LNPs analysis. Furthermore, the 405 nm peak is sensitive to the monomeric form of AmB and serves as a stability-indicating signal for detecting aggregation or degradation, while its high molar absorptivity ensures adequate sensitivity with low LOD and LOQ values [28–30].

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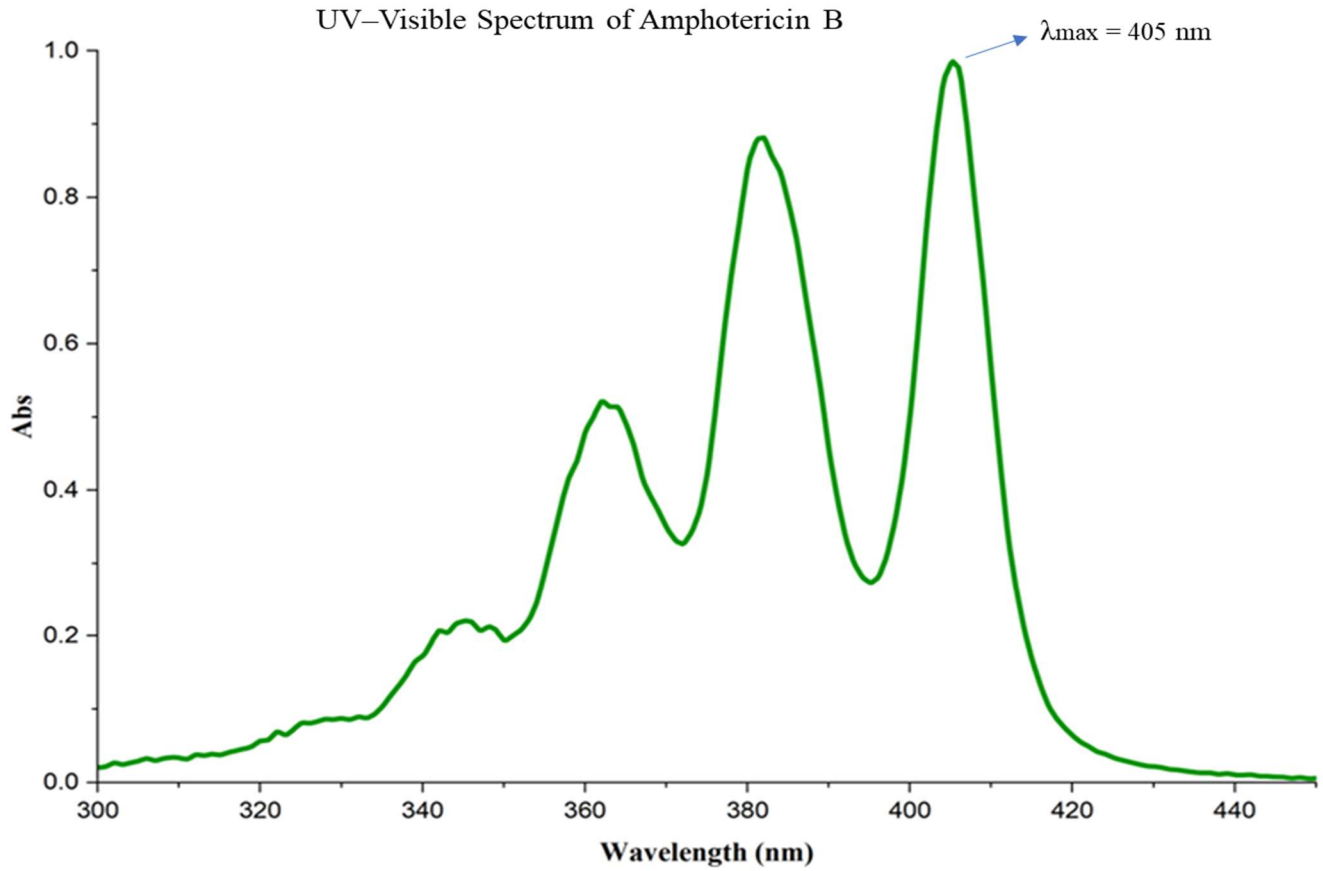


**Figure 2:** UV-Visible absorption spectrum of Amphotericin B

### 3.2.1 Linearity and Calibration Curve

The quantitative estimation of AmB was successfully established using a UV-visible spectrophotometric method, which demonstrated a robust linear relationship between absorbance and concentration across the range of 1–10 µg/mL. The regression analysis yielded a slope (m) of 0.0984 and an intercept (c) of 0.0161, resulting in the regression equation $y = 0.0984x + 0.0161$. The high correlation coefficient ($R^2$ = 0.9984) (Figure 3). confirms that the method strictly adheres to Beer-Lambert's Law, indicating excellent linearity and reliability for the quantification of AmB in this range.

The selection of a solvent system comprising phosphate buffered saline (PBS, pH 7.4) and methanol in a 1:1 (v/v) ratio was instrumental in ensuring the complete solubilization of the lipophilic drug while simultaneously maintaining a stable, physiologically relevant pH. Furthermore, the optimization of the analytical wavelength to 405 nm exploited the characteristic heptaene chromophore of the AmB molecule. This wavelength provided a sharp, intense absorption peak that effectively minimized potential spectral interference from common lipid excipients, such as stearic acid, which are utilized in the LNPs formulations. Consequently, this validated standard curve served as the foundational analytical tool for the subsequent determination of aqueous and organic phase solubility, as well as the partition coefficient [17,31].

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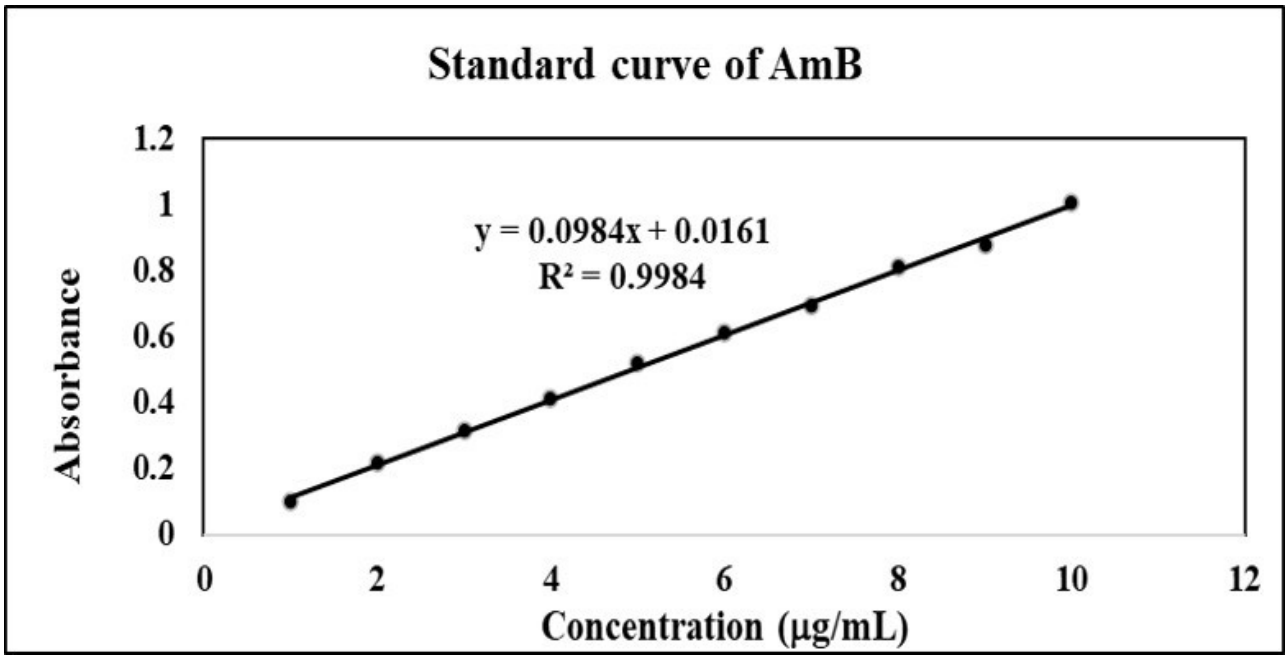


**Figure 3:** Standard calibration curve of AmB obtained by UV–Visible spectrophotometry at 405 nm over the concentration range of 1–10 µg/mL.

**3.2.2 Sensitivity Parameters (LOD and LOQ)**

The sensitivity of the developed UV-Vis spectrophotometric method was substantiated by determining the limit of detection (LOD) and limit of quantitation (LOQ) in accordance with ICH Q2 (R1) guidelines. Based on the regression analysis, the LOD and LOQ were calculated to be 0.019 µg/mL and 0.057 µg/mL, respectively. These low values signify the high sensitivity of the method, confirming its capability to accurately detect and quantify AmB at trace concentrations. This level of sensitivity is essential for monitoring drug partitioning behavior and low-level solubility in lipidic environments during the preformulation phase.

**3.3 HPLC Method Development and Validation**

**3.3.1 Chromatographic Performance and Linearity**

Under the optimized conditions summarized in Table 3, a robust separation of the AmB was achieved with a total run time of less than 8.0 minutes. The average retention time for AmB was found to be 5.9 ± 0.14 minutes (n=10), demonstrating exceptional reproducibility and method precision. The high correlation coefficient ($R^2 = 0.9998$) indicates a strong linear relationship, confirming that the method strictly adheres to the Beer-Lambert law within the concentration range essential for the analysis of LNPs (Figure 4).

Furthermore, the narrow standard deviation in retention time and the high linearity suggest that the developed HPLC protocol is suitable for high-throughput analysis and provides the necessary sensitivity to detect small variations in drug loading and entrapment efficiency.

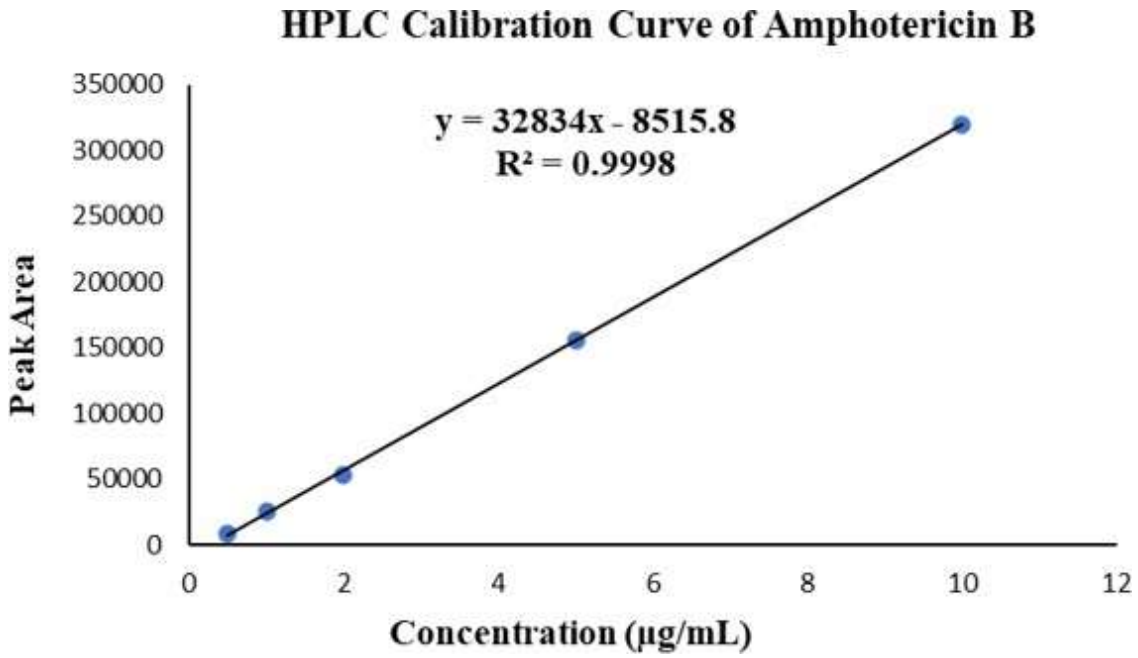


**Figure 4:** HPLC calibration curve of Amphotericin B showing the relationship between concentration (µg/mL) and peak area.

**Table 3:** Optimized Chromatographic Conditions for AmB Quantification

| **Parameter** | **Description / Value** | **Purpose / Remark** |
|---|---|---|
| Chromatographic System | High-Performance Liquid Chromatography (HPLC) system equipped with quaternary pump | Ensures accurate solvent delivery and reproducible injections |
| Stationary Phase | Reversed-phase C18 analytical column (250 mm × 4.6 mm, 5 µm particle size) | Suitable for separation of non-polar to moderately polar compounds |
| Mobile Phase | Acetonitrile: 10 mM<br>Sodium acetate buffer (pH 4.0) | Provides optimal polarity and pH for analyte stability |
| Mobile Phase Mixing Ratio | 30:70 (v/v) | Optimized to achieve adequate retention and peak symmetry |
| Flow Rate | 1.2 mL/min | Maintains optimal resolution and analysis time |
| Elution Mode | Isocratic | Ensures consistent retention time and method simplicity |
| Detection System | UV–Visible detector | Enables sensitive analyte detection |
| Detection Wavelength ($\lambda_{max}$) | 405 nm | Selected based on maximum absorbance of AmB |
| Column Temperature | 37 ± 2 °C | Improves reproducibility and peak shape |
| Injection Volume | 20 µL | Ensures adequate sensitivity and precision |

| | | |
|---|---|---|
| Run Time | 10 min | Allows complete elution of the analyte |

**3.3.2 System Suitability Results**

System suitability testing confirmed the reliability of the developed HPLC method. As summarized in Table 4, all parameters met the predefined acceptance criteria. The high theoretical plate count (N > 5000) and low height equivalent to a theoretical plate (HETP) (0.045 mm) indicate excellent column efficiency under optimized conditions. A tailing factor of 1.12 demonstrates effective peak symmetry, attributed to the use of acetonitrile as the organic modifier and a pH 4.0 buffer, which minimizes silanol interactions and reduces peak broadening of amphoteric AmB. The low %RSD of peak area (0.85%) further confirms the high precision and suitability of the method for routine quantification in complex lipid nanoparticle matrices [32].

**Table 4:** System Suitability Parameters for Amphotericin B Analysis

| **Parameter** | **Observed Value** | **Acceptance Limit** | **Inference** |
|---|---|---|---|
| Retention Time (Rt) | 5.9 min | — | Reproducible retention with stable chromatographic conditions |
| Theoretical Plates (N) | > 5000 | > 2000 | Excellent column efficiency and adequate analyte separation |
| Tailing Factor (T) | 1.12 ± 0.05 | < 2.0 | Symmetrical peak with minimal secondary interactions |
| Peak Area (% RSD) | 0.85% | < 2.0% | High system precision and repeatability |
| Height Equivalent to a Theoretical Plate (HETP) | 0.045 mm | — | Optimized flow rate and minimal band broadening |

**3.3.3 Sensitivity Parameters (LOD and LOQ)**

The resulting LOD of 0.04 µg/mL and LOQ of 0.12 µg/mL underscore the superior sensitivity of the HPLC system compared to routine UV methods. This high degree of resolution and detection is paramount for the precise quantification of AmB in complex matrices, where the drug may be present in trace amounts, such as during long-term stability testing or localized *in vitro* release studies from the solid lipid matrix. Furthermore, the low LOQ ensures that even minor instances of API degradation or low-level partitioning into the aqueous phase can be monitored with high statistical confidence [32].

**3.4 FT-IR Compatibility Assessment**

The FT-IR spectra of pure AmB, stearic acid, and their physical mixture (1:1 w/w) were analyzed to assess chemical compatibility. The spectra were evaluated for the appearance of new peaks, the disappearance of existing functional group signals, or significant shifts in absorption frequencies, any of which would suggest a chemical interaction between the drug and the lipid matrix.

**3.4.1 Interpretation of Characteristic Absorption Peaks**

The FT-IR spectrum of pure AmB exhibited its signature vibrational bands, including a broad peak at 3361.78 $cm^{-1}$ (O-H and N-H stretching), a sharp peak at 1648.70 $cm^{-1}$ (C=O stretching of the carboxylic acid), and the characteristic $NH_2$ in plane band at 1546.98 $cm^{-1}$ (Figure 5). Stearic acid demonstrated its typical long-chain fatty acid profile, with intense C-H stretching bands at 2913.01 $cm^{-1}$ and 2846.83 $cm^{-1}$, and a strong carbonyl (C=O) peak at 1694.58 $cm^{-1}$ (Figure 6).

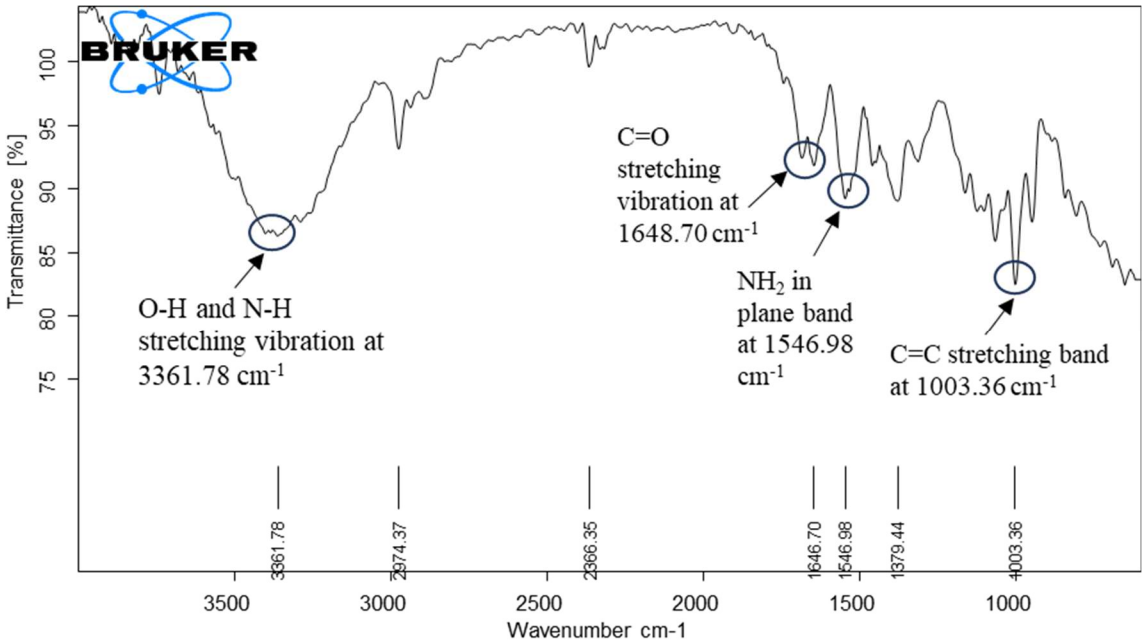


**Figure 5:** FTIR spectrum of Amphotericin B

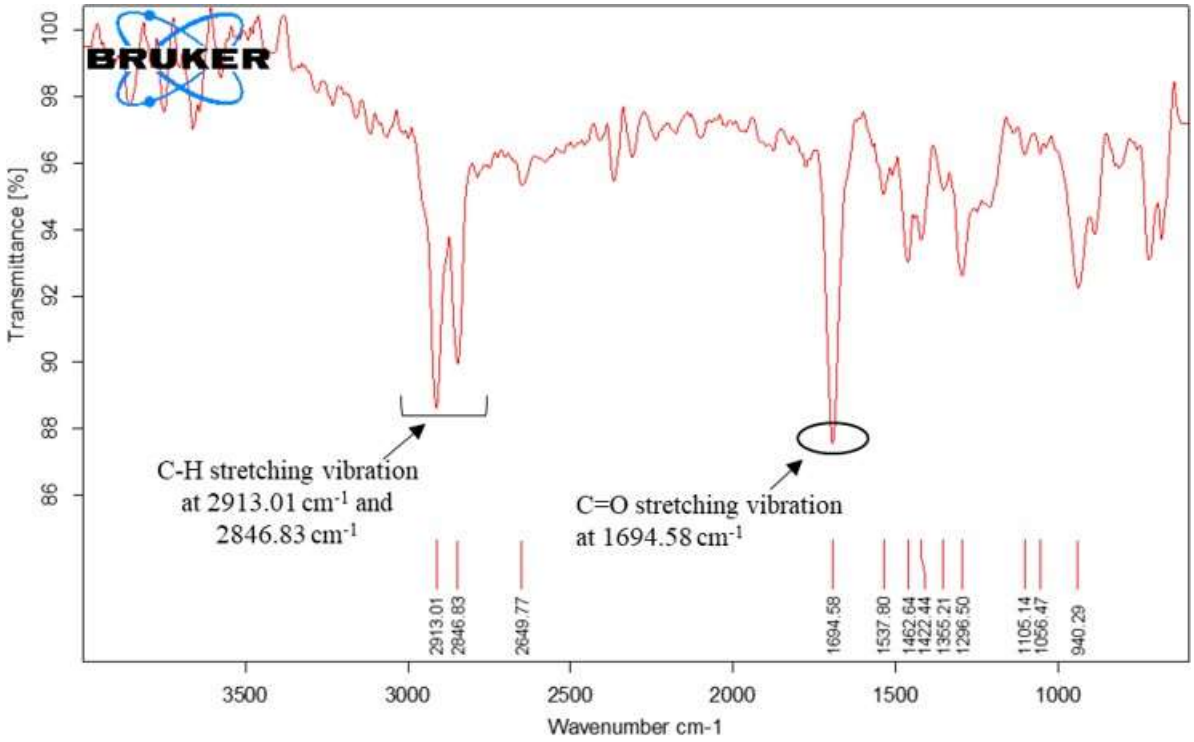


**Figure 6:** FTIR spectrum of Stearic acid

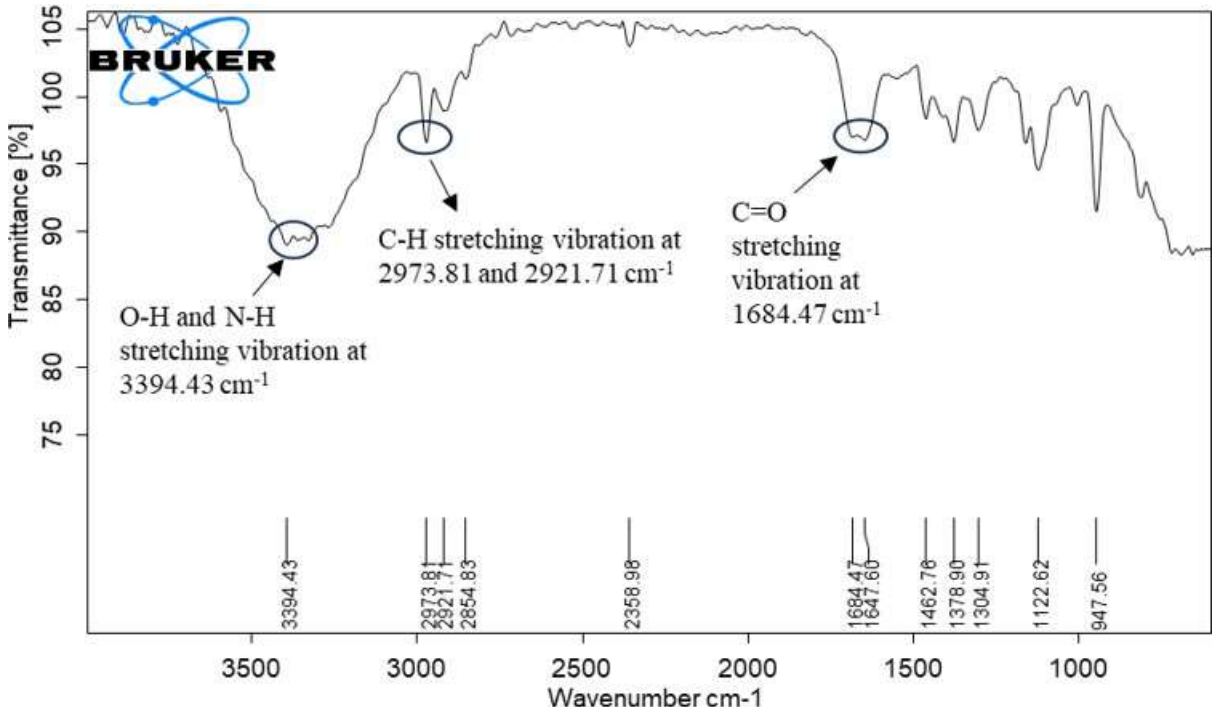


**Figure 7:** FTIR spectrum of Physical Mixture of AmB and Stearic acid

### 3.4.2 Drug–Excipient Compatibility Evaluation

FT-IR analysis of pure AmB, pure stearic acid, and their physical mixture showed no significant shift, disappearance, or formation of new peaks, indicating good compatibility between the drug and excipient. Amphotericin B exhibited a broad O–H/N–H stretching band at 3361.78 $cm^{-1}$, while stearic acid showed characteristic aliphatic C–H stretching vibrations at 2913.01 and 2846.83 $cm^{-1}$. The physical mixture retained these characteristic bands at 3394.43 $cm^{-1}$ for AmB and 2973.81 and 2921.71 $cm^{-1}$ for stearic acid respectively, indicating preservation of functional groups. The carbonyl (C=O) stretching vibration of stearic acid observed at 1694.58 $cm^{-1}$ remained nearly unchanged in the physical mixture at 1684.47 $cm^{-1}$, suggesting the absence of chemical interaction (Figure 7) and further confirming compatibility between AmB and stearic acid [33,34].

## CONCLUSION

The present work provided a systematic preformulation assessment of Amphotericin B and established a robust analytical framework to support its development in lipid nanoparticle–based delivery systems. The physicochemical characteristics of the drug, particularly its strong affinity toward lipid matrices and compatibility with selected excipients, underscore the suitability of lipid nanocarriers for addressing formulation challenges associated with AmB. The validated HPLC method ensures reliable quantification of the drug throughout formulation development and quality evaluation. Importantly, the outcomes of this study lay a solid foundation for the rational design and optimization of AmB-loaded LNPs with the potential to enhance oral or parenteral bioavailability while minimizing drug-related toxicity. Future investigations will focus on formulation optimization, stability assessment, and *in vitro* and *in vivo* performance studies to evaluate the effectiveness of lipid nanoparticles in improving the therapeutic index and safety profile of Amphotericin B.

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