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# Optimizing Amphotericin B Invasomes: Critical Quality Attributes using Plackett Burman Design

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**Abstract:** ***This study investigates the development and optimization of Amphotericin B loaded invasomes for enhanced transdermal drug delivery. Invasomes (IVS) are the novel vesicular carrier of drugs for transdermal delivery and enhanced penetration than conventional liposomes. IVS are flexible vesicles and composed of phospholipids, alcohol, and terpenes. Invasomes were prepared using the mechanical Dispersion method. Plackett - Burman design is used as screening method to identify the critical factors using 12 runs with 7 variables. Hence, critical quality attributes (CQAs) such as phospholipid concentration, terpene concentration, and sonication speed were identified and optimized to maximize entrapment efficiency and control vesicle size. Results showed that the entrapment efficiency (%) and vesicle size (nm) across the 12 batches ranged from 60.63 to 96.82% and 73 to 306 nm, respectively which demonstrated significant improvements in drug entrapment and vesicle size, offering a promising approach for effective transdermal delivery of Amphotericin B.***

**Keywords:** Quality by Design, Invasomes, Amphotericin B, Topical Candidiasis, Mechanical Dispersion, Plackett Burman Design, Critical Quality Attributes, Critical Process Parameters, Critical Material Attributes

## 1. Introduction

Pharmaceutical QbD is a systematic, scientific, risk - based, holistic and proactive approach to pharmaceutical development that begins with predefined objectives and emphases product and processes understanding and process control. [1] Amphotericin B is an effective polyene antifungal considered as a “gold standard” in the management of fungal infections and BCS class – IV drug. Amphotericin B is having oral bioavailability 0.2 to 0.9% and 100% upon IV administration. Oral Half - life is 7 - 8 hours. Amphotericin B Aqueous Solubility is 0.086 mg/ml and Log P value of Amphotericin B is 5.83. [2] Intravenous administration paradoxically leads to side effects that have not been fully mitigated, even with newer formulations. Thus, the need for alternative formulations/route of administration for amphotericin B remains crucial. [3] However, the commercial topical preparation is not significantly properly permeated or absorbed through the skin due to low residence time or less crossing of the stratum corneum layer of the skin. Thus, it has been required to develop a novel drug delivery system (nanotechnology - based) for the improvement of the therapeutic activity of drugs. [4]

Invasomes (IVS) are the novel vesicular carrier of drugs for transdermal delivery and enhanced penetration than conventional liposomes. [5 - 6] IVS is flexible vesicles and composed of phospholipids, alcohol, and terpenes. Alcohol has the property that enhances the flexibility of lipids bi - layer of IVS, producing soft vesicles leading to increasing skin permeability of the drug. [7] Ethanol can fluidize and disturb the stratum corneum lipid leading to an increase in the permeability of the drug. Terpenes also enhance the skin permeation of both lipophilic and hydrophilic drugs by re - arrangement of the stratum corneum layer of skin. Incorporating IVS into the gel system provided the sustained drug release, increased skin contact time and enhanced penetration. [8] There are many reports published on IVS gel, which improved the transdermal delivery of drugs.

**Purpose Statement**

This study aims to optimize the formulation of Amphotericin B loaded invasomes by identifying and controlling critical quality attributes using the Plackett Burman design.

**Significance of research work**

The study significance lies in its potential to enhance the effectiveness of Amphotericin B as a transdermal drug, offering an alternative to traditional administration routes, thereby reducing side effects and improving patient outcomes.

## 2. Materials and Methods

Amphotericin B was received as gift sample from Kivi Lab, Por, India. Phospholipids such as Soyaphosphatidylcholine and Dimyristoylphosphatidylcholine as well as Terpenes such as D - Limonene and Fenchone were procured from Kivi Lab, Por, India. All other reagents were purchased from local suppliers.

**Method of Preparation of Amphotericin B Invasomes:**

Drug and terpene or mixtures of terpenes are dissolved in ethanolic phospholipid solution. The mixture is vortexed for 5 min and then sonicated for 5 min in order to obtain a clear solution. Phosphate buffer saline (PBS) (pH: 7.4) is added to the solution by a syringe under constant vortexing. The vortexing is continued for an additional 5 min. The last step is the extrusion of multilamellar vesicles through polycarbonate membranes of different pore sizes. The invasome dispersions are extruded through each polycarbonate membrane for several times. [9 - 11]

**Characterization of Amphotericin B Invasomes**

**Entrapment efficiency**

Entrapment efficiency of invasomal formulation determined by centrifuging 10 mL invasomal suspension for 10 min. The clear fraction was further used for the determination of free

drug by using UV/visible spectrophotometer at 406 nm.13 The entrapment efficiency was calculated using the following formula:

**Entrapment efficiency = (Ct - Cf) / Ct x100**
Where, Ct: concentration of total drug and; Cf: concentration of unentrapped (free) drug

**Vesicle size**
It is determined by Malvern Instrument at Kivi Labs, Por, Vadodara.

**Statistical analysis:**
All data were subjected to analysis of variance (ANOVA) using Design Expert 13.0.01.

## 3. Results and Discussion

**Calibration curve of Amphotericin B**
A stock solution was prepared by dissolving 100mg of drug in small quantity of methanol & sonicated for few minutes & diluted with 100 ml of phosphate buffer (pH 7.4). Stock solution was serially diluted to get solutions in range of 2 - 10µg/ml & λmax of solution was found out. Absorbance of different diluted solution was measured in UV - visible spectrophotometer scan. Calibration curve was plotted by taking concentration in X axis & absorbance in Y axis & correlation coefficient 'R2' was calculated. [12]

**Identification of Amphotericin B by FTIR Spectroscopy**
Amphotericin B had identified by using FTIR spectroscopy in which it was confirmed from the observed functional groups stated in spectra.

**Formulation of Amphotericin B Invasomes**
Amphotericin B Invasomes was prepared by using Mechanical Dispersion method with QbD Approach. In order to assess risk, the identified QTPPs were %E. E and Vesicle size whereas CQAs were Conc. and Type of Phospholipids, Conc. and Type of terpene, ethanol conc., sonication speed and sonication time displayed in table 1. [14 - 15]

**Screening of significant risk factors using Placket - Burman design [16 - 17]**

- Plackett - Burman design is an efficient screening method to identify the critical factors using as few experimental runs as possible.
- Design is used for screening of independent variables: significant (critical) or non - significant (non - critical).
- It is two - level design i. e. Low ( - 1) level & High (+1) level.
- PB design gives 12 runs that may be used for an experiment containing up to 11 factors.
- The parameter level selection was based on preliminary studies and literature survey.
- In this experiment, as per the method of preparation of Invasomes, to screen critical material attributes (CQAs) and process parameters (CPPs) are conducted to run the screening design of 12 - run Plackett - Burman design utilizing Design Expert® software (Version 13.0.1) displayed in the table 2.

**Characterization of Amphotericin B Invasomal Batches**
Amphotericin B loaded Invasomes were prepared by using mechanical dispersion method which is characterized on the basis of %E. E & Vesicle size of each batch. Results were showed in the figure 3.

**Statistical Discussion**

**ANOVA interpretation of Y1 Response (%E. E.)**
The analysis of variance (ANOVA) revealed a statistical difference between the batches from the figure 4. A regression coefficient is said to be significant if p - value is less than 0.05. R2 value was 0.9482 indicating a good fit. From the result, it is evident that phospholipid conc. (0.0073) and Terpene conc. (0.6506) significantly affect the entrapment efficiency which is again confirmed by Pareto chart as both the graphs are crossing the t - value limit which is 2.776. When the higher EE is desired within selected factor range, factor X1and X2 have positive coefficients which indicate that increasing factor value increases the response which means that increasing phospholipid and terpene concentration increases the %EE of Invasomes.

**ANOVA interpretation of Y2 Response (V. S)**
The analysis of variance (ANOVA) revealed a statistical difference between the batches from the figure 5. From the result, it is evident that phospholipid concentration (0.0066), terpene conc. (0.0144) and sonication speed (0.0347) significantly affect the V. S which is again confirmed by Pareto chart. When the concentration of phospholipid and terpenes increases with rise in sonication speed, then desired vesicle size within selected range can be obtained.

Summary Critical Quality Attributes Analysis of various CMAs & CPPs based on PBD Screening Design Analysis & Pareto Chart. Based on the Characterization data and Pareto charts, following summary has been made which is depicted in table 4.

## 4. Conclusion

The study successfully identified key critical quality attributes CQAs influencing the formulation of Amphotericin B invasomes. Optimizing these factors, particularly phospholipid and terpene concentrations, as well as sonication speed, resulted in a formulation with enhanced entrapment efficiency and controlled vesicle size, demonstrating its potential for improved transdermal delivery of Amphotericin B.

**Acknowledgements:**

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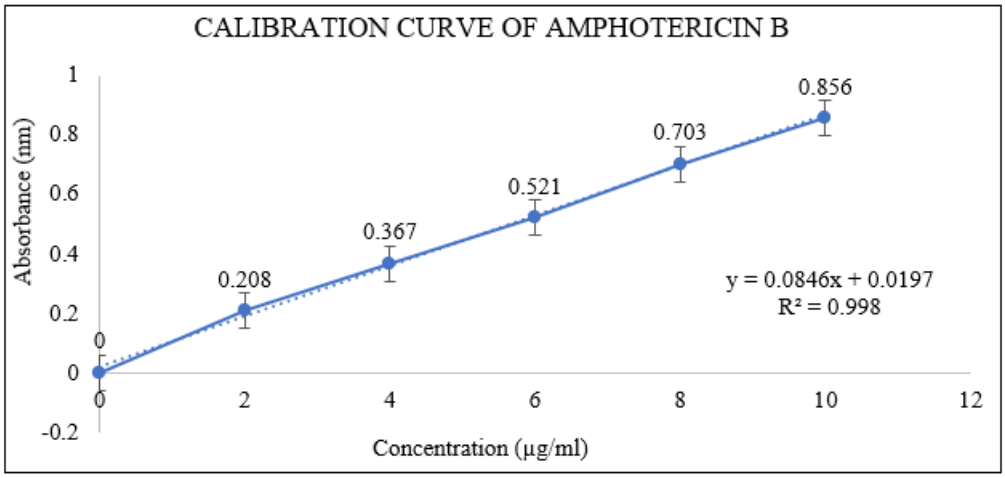


**Figure 1:** Calibration curve of Amphotericin B.

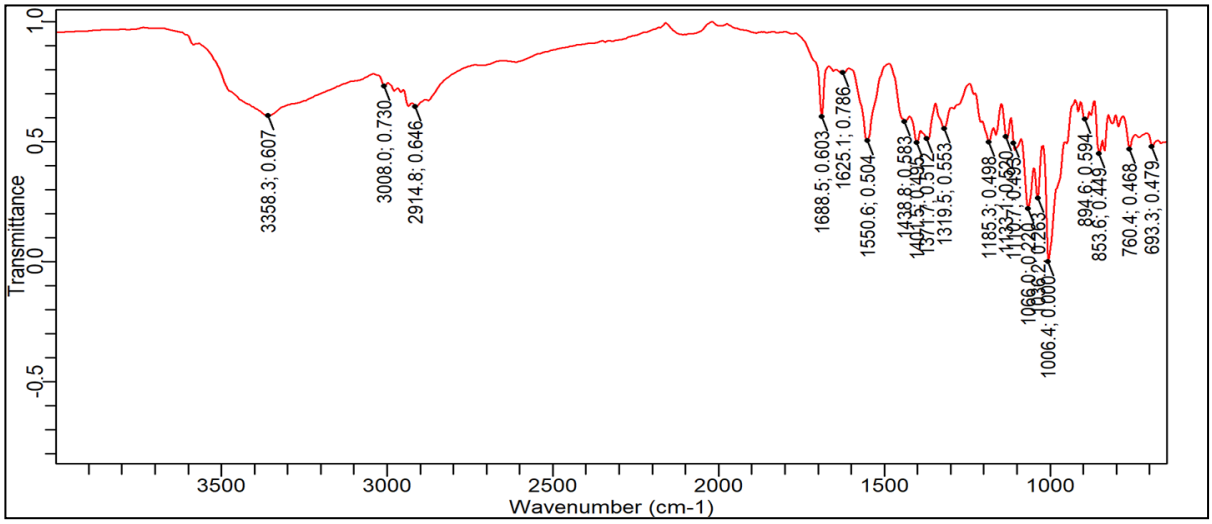


**Figure 2:** FTIR spectra of Amphotericin B.

**Table 1:** Selection of Independent Variables and reason for selection

| Independent Variables (CQAs) | Range | Reason for Selection |
|---|---|---|
| CRITICAL MATERIAL ATTRIBUTES (CMAs) | | |
| **Concentration of Phospholipid** | 100 - 200 mg | • Disintegrates on skin penetrate deep into the intercellular matrix and get mixed with the lipid bilayer of SC\<br>• changes its structural integrity, thus making it easier for active moieties to permeate deep into the skin. |
| **Type of Phospholipid** | • DMPC (Dimyristoylphosphatidylcholine),<br>• SPC (soya phosphatidylcholine) | • To form stable vesicles<br>• To prevent leakage<br>• To form solid, rigid & vesicular structure |
| **Concentration of Terpene** | 5 - 10 mL | • Permeability enhancement is<br>• their ability to disturb or break the hydrogen bonds between the components of cellular bilayer membrane. |
| **Type of Terpene** | Fenchone,<br>D - Limonene | • Terpenes with high lipophilic character act as strong boosters for sorption of lipophilic drug.<br>• Terpenes with non - polar hydrogen (e. g. limonene) are much more efficient boosters than oxygen - rich polarized terpenes. |
| **Concentration of Ethanol** | 3 - 4 %v/v | • It improves vesicular ability to penetrate the SC<br>• Provides net ( - ve) surface charge and<br>• prevents aggregation of vesicle due to electrostatic repulsion.<br>• Ethanol interacts with lipid moiety in the polar head group region,<br>• resulting in a decrease in the transition temperature of the lipids thus improved their fluidity. |
| **CRITICAL PROCESS PARAMETERS (CPPs)** | | |
| **Sonication time** | 3 - 5 min | when increasing the sonication time<br>• PDI is decreased<br>• due to decreased the VS. |
| **Sonication Speed** | 500 - 700 RPM | Particle size decreased when speed of sonication increased. |

**Table 2:** Parameters feed in Design Expert to run Plackett Burman Design

| Variable Code | Variables | UNITS | LEVELS | |
|---|---|---|---|---|
| | | | LOW (-1) | HIGH (+1) |
| Independent Variables: X - Inputs | | | | |
| X1 | Concentration of Phospholipid | mg | 100 | 200 |
| X2 | Type of Phospholipid | Categoric | DMPC | SPC |
| X3 | Concentration of Terpene | mL | 5 | 10 |
| X4 | Type of Terpene | Categoric | Fenchone | D - Limonene |
| X5 | Concentration of Ethanol | %v/v | 3 | 4 |
| X6 | Sonication time | Min | 3 | 5 |
| X7 | Sonication Speed | RPM | 500 | 700 |
| DEPENDENT VARIABLES: Y - RESPONSES | | | | |
| Y1 | Entrapment Efficiency | % | | |
| Y2 | Vesicle Size | nm | | |

**Table 3:** Compositions of Variables in decoded forms

| RUN | PL. Conc. (mg) | PL Type | Terpene Conc. (ml) | Terpene Type | Eth Conc. (%v/v) | ST (Min) | SS (RPM) |
|---|---|---|---|---|---|---|---|
| AIN 1 | 200 | SPC | 10 | fenchone | 3 | 3 | 700 |
| AIN 2 | 100 | SPC | 10 | D - limonene | 3 | 3 | 500 |
| AIN 3 | 200 | DMPC | 10 | D - limonene | 3 | 5 | 700 |
| AIN 4 | 100 | SPC | 5 | D - limonene | 4 | 3 | 700 |
| AIN 5 | 100 | DMPC | 10 | fenchone | 4 | 5 | 500 |
| AIN 6 | 100 | SPC | 10 | fenchone | 4 | 5 | 700 |
| AIN 7 | 200 | SPC | 5 | D - limonene | 4 | 5 | 500 |
| AIN 8 | 200 | DMPC | 10 | D - limonene | 4 | 3 | 500 |
| AIN 9 | 200 | DMPC | 5 | fenchone | 4 | 3 | 700 |
| AIN 10 | 100 | DMPC | 5 | D - limonene | 3 | 5 | 700 |
| AIN 11 | 200 | SPC | 5 | fenchone | 3 | 5 | 500 |
| AIN 12 | 100 | DMPC | 5 | fenchone | 3 | 3 | 500 |

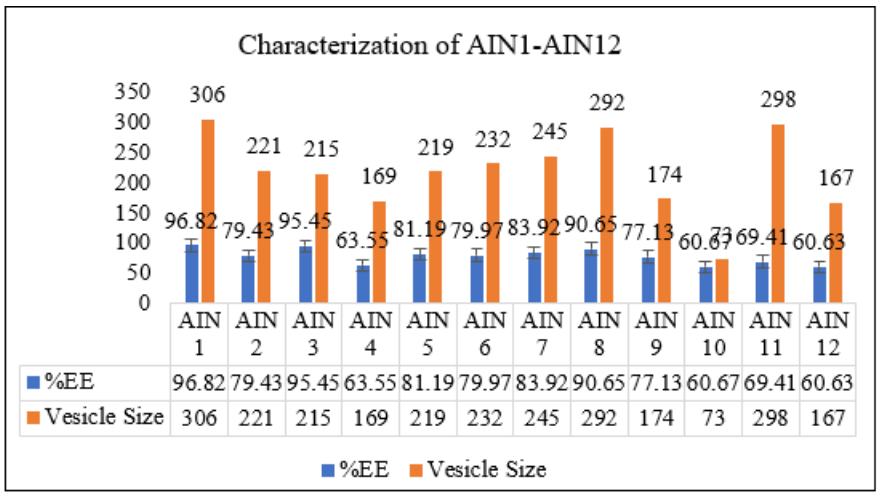


**Figure 3:** Characterization of AIN1 - AIN12

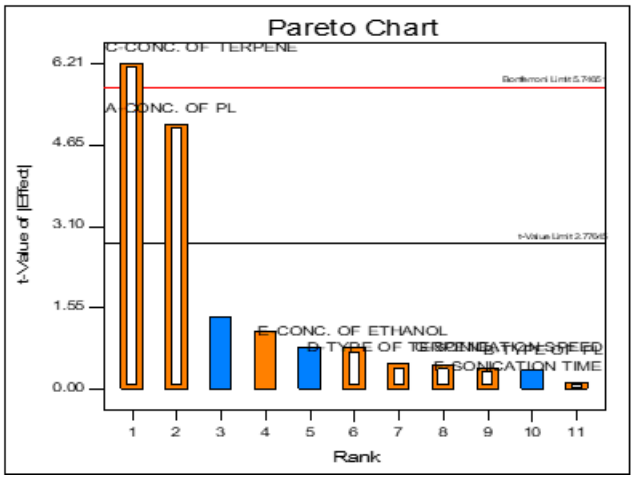


**Figure 2:** Pareto chart of Y1 Response - %E. E.

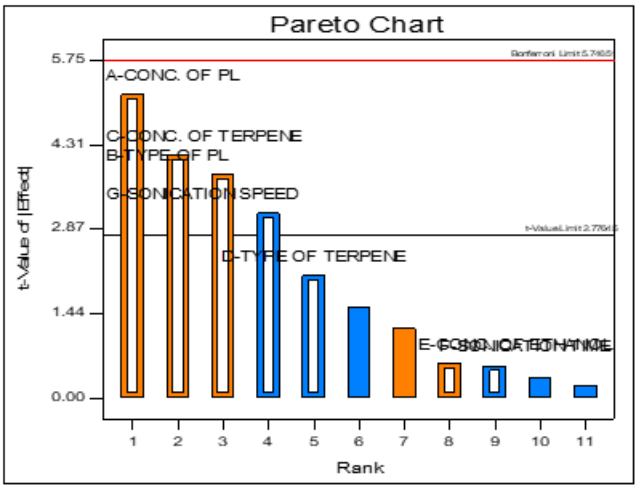


**Figure 3:** Pareto Chart of Y2 Response - Vesicular Size (nm)

**Table 4:** Critical Quality Attributes Analysis of various CMAs & CPPs based on PBD Screening Design Analysis & Pareto Chart

| Critical Quality Attributes Analysis of various CMAs & CPPs | Effect on % E. E. | Reason | Effect on Vesicular Size | Reason |
|---|---|---|---|---|
| A - CONC. OF PL | ++ | Higher the concentration of phospholipid, more the drug incorporated into it. | ++ | Changes its structural integrity, thus making it easier for active moieties to permeate |
| B - TYPE OF PL | 00 | - - | 0+ | Depends internal structure. |
| C - CONC. OF TERPENE | ++ | Higher the lipophilicity of the terpene, higher the solubilization of drug which increases space for drug incorporation. | ++ | Terpenes with high lipophilic character act as strong boosters for sorption of lipophilic drug |
| D - TYPE OF TERPENE | 00 | - | 00 | - |
| E - CONC. OF ETHANOL | +0 | Ethanol interacts with lipid moiety in the polar head group region which results in decrease in transition temperature of lipids | 00 | Higher the concentration of ethanol, higher the fluidity in vesicles. |
| F - SONICATION TIME | 00 | - | +0 | when increasing sonication time, PDI is decreased due to decreased the VS. |
| G - SONICATION SPEED | 00 | - | - - | Particle size decreased when speed of sonication increased. |

**Note:** ++: Positive Effect, +0: Moderate Effect, 00: No effect, - -: Negative Effect

**Tables and figure titles and legend:**

**Figure 1: Calibration curve of Amphotericin B.**
Each reading has been performed thrice (n=3) to obtain standard deviation plot.

**Figure 2: FTIR spectra of Amphotericin B.**

**Table 1: Selection of Independent Variables and reason for selection**

**Table 2: Parameters feed in Design Expert to run Plackett Burman Design**
Parameters feed was gathered from the design expert software version 13.0.1

**Table 3: Compositions of Variables in decoded forms**
Parameters feed was gathered from the design expert software version 13.0.1

**Figure 3: Characterization of AIN1 - AIN12**
Each reading of %E. E has been performed thrice (n=3) to obtain standard deviation plot.
%E. Vesicle size

**Figure 4: Pareto chart of Y1 Response - %E. E.**
**Figure 5: Pareto Chart of Y2 Response - Vesicular Size (nm)**

**Table 4: Critical Quality Attributes Analysis of various CMAs & CPPs based on PBD Screening Design Analysis & Pareto Chart**
++: Positive Effect, +0: Moderate Effect, 00: No effect, - -: Negative Effect