**RESEARCH PAPAER**

# Development of a Lyophilized Phosphatidylcholine-Based Liposomal Formulation of Voriconazole: Optimization, Stability and *In Vivo* Pharmacokinetic Evaluation

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## ABSTRACT

Voriconazole is a first-line broad-spectrum triazole for invasive fungal infections, but its intravenous formulation relies on a sulfobutylether-β-cyclodextrin vehicle that accumulates in renal impairment and the drug undergoes rapid, saturable hepatic metabolism that produces highly variable systemic exposure. This research reports the quality-by-design development of a lyophilized, phosphatidylcholine-based liposomal formulation of voriconazole intended to prolong systemic circulation and improve pharmacokinetics after intravenous administration. Liposomes composed of hydrogenated soy phosphatidylcholine, cholesterol and 1,2-dipalmitoyl-sn-glycero-3-phospho-rac-glycerol sodium salt (3:1:1) were manufactured by spray-drying of the drug-lipid composite followed by aqueous hydration and microfluidization and were subsequently lyophilized with sucrose as cryoprotectant. Formulation variables were first screened with a Plackett-Burman design. Total lipid content, homogenization pressure and sucrose content were then optimized using a three-factor, three-level Box-Behnken design against entrapment efficiency, particle size and polydispersity index. The optimized formulation (400 mg total lipid, 15,000 psi, 125 mg sucrose per vial) yielded a reconstituted vesicle size of 147.5 nm (PDI 0.157), entrapment efficiency of 95.9%, zeta potential of -15.7 mV and a rapidly reconstituting elegant cake with residual moisture below 3.2%. The product remained within specification over three months at 2-8 °C and 25 °C/60% RH. In rats, the liposomes markedly altered voriconazole disposition relative to free drug and the marketed formulation, prolonging the terminal half-life from <1 h to ~22.9 h, delaying $T_{max}$, and substantially increasing systemic exposure and mean residence time. A lyophilized phosphatidylcholine liposomal platform is therefore a promising, cyclodextrin-free alternative for parenteral voriconazole delivery.

**Keywords:** Voriconazole; Liposome; Box-Behnken design; Quality by design; Lyophilization; Phosphatidylcholine; Pharmacokinetics; Invasive fungal infection.

**How to site this article:** Kiran Jani, Gayatri Patel,"Development of a Lyophilized Phosphatidylcholine-Based Liposomal Formulation of Voriconazole: Optimization, Stability and *In Vivo* Pharmacokinetic Evaluation"Int J Drug Deliv Technol. 2026;16(90s): 165-181; DOI: 10.25258/ijddt.16.90s.17

## 1. INTRODUCTION

Invasive fungal infections have emerged as a major and under-recognized cause of global morbidity and mortality with recent estimates attributing several million deaths each year to severe fungal disease [1, 2]. In 2022, the World Health Organization (WHO) released its first fungal priority pathogens list placing *Aspergillus fumigatus* and several *Candida* species including *Candida auris* in the important group and explicitly highlighting the paucity of antifungal agents and delivery strategies as an urgent unmet need. Populations at greatest risk, such as haematology and transplant recipients, patients in intensive care and those receiving immunosuppressive therapy, continue to expand, and mortality from invasive *aspergillosis* remains high despite the availability of active drugs [3].

Voriconazole is a second generation triazole derivative which is recommended as first-line therapy for invasive *aspergillosis* and is active against a broad range of *Candida*, *Scedosporium* and *Fusarium* species. It acts by inhibiting the fungal cytochrome-P450-dependent enzyme lanosterol 14-α-demethylase (CYP51) thereby depleting ergosterol and disrupting the fungal cell membrane [4-7]. Despite its clinical value, voriconazole presents pronounced pharmaceutical and pharmacokinetic challenges. The molecule is insoluble in water, so the intravenous product is solubilized with sulfobutylether-β-cyclodextrin (SBECD). Because this excipient is cleared renally and accumulates in patients with impaired renal function, the intravenous route carries a specific safety caution in renal impairment [8, 9]. In addition, voriconazole displays non-linear, saturable hepatic metabolism mediated by CYP2C19, CYP2C9 and CYP3A4, a short half-life of only 1-2 h, high inter individual variability driven in part by CYP2C19 polymorphism, and dose-limiting hepatotoxicity and visual disturbances. Clinically, efficacy and safety are tied to a narrow trough concentration window of approximately 1.0-5.0 μg/mL, so that sub-

therapeutic exposure risks treatment failure while excess exposure drives toxicity, and therapeutic drug monitoring is routinely recommended. Together these factors complicate the maintenance of therapeutic exposure and motivate the search for a delivery system that reduces reliance on cyclodextrin, prolongs circulation and moderate metabolism (Figure 1).

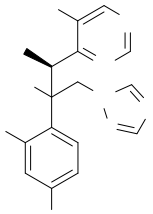


**Figure 1.** Structure of voriconazole

Liposomes are among the most clinically validated nanocarriers. They are biodegradable, biocompatible and composed of physiological phospholipids, they can entrap both lipophilic and hydrophilic molecules and they can profoundly alter the pharmacokinetics and tissue distribution of an encapsulated drug while lowering the toxicity associated with the free molecule or its solubilizing vehicle [10, 11]. Particularly, for antimicrobial and antifungal agents, liposomal encapsulation can sustain circulating concentrations, shield the drug from premature metabolism and favour accumulation at sites of infection [12-14]. The clinical results of liposomal amphotericin B provides direct precedent for a phospholipid carrier in antifungal therapy and an intravenous liposomal voriconazole has been shown pre-clinically to reduce clearance, increase exposure and enhance antifungal activity relative to the marketed product [15, 16].

Rational liposome development, however, must contend with obstacles such as limited long-term physical and chemical stability of aqueous dispersions, batch-to-batch variability, control of vesicle size and size distribution, and scalability [17-19]. A quality-by-design (QbD) framework, in which a quality target product profile and critical quality attributes guide risk assessment and a structured design of experiments, provides an efficient route through this multidimensional problem [20-23], while lyophilization of the optimized dispersion converts a metastable suspension into a dry, storage-stable powder that is reconstituted immediately before use.

The present study describes the end-to-end development of a lyophilized, phosphatidylcholine-based liposomal formulation of voriconazole for intravenous use. A validated chromatographic method and pre-formulation studies underpinned the selection of lipids and manufacturing route. A Plackett-Burman screening design identified the variables most critical to the product's critical quality attributes and a Box-Behnken response-surface design was used to optimize total lipid content, homogenization pressure and cryoprotectant level against entrapment efficiency, particle size and polydispersity. The optimized formulation was characterized in a lyophilized, subjected to a three-month stability study under refrigerated and accelerated conditions, and evaluated *in vivo* in rats using a plasma bioanalytical method, with free drug and the marketed formulation as comparators. The objective was to demonstrate that a cyclodextrin-free liposomal platform can be developed reproducibly by QbD and can meaningfully improve the systemic disposition of voriconazole.

## 2. MATERIAL AND METHOD

### 2.1 Materials

Voriconazole was obtained from Medichem S.A. (Barcelona, Spain). Hydrogenated soy phosphatidylcholine (HSPC) and 1,2-dipalmitoyl-sn-glycero-3-phospho-rac-glycerol sodium salt (DPPG-Na) were supplied by Lipoid GmbH (Ludwigshafen, Germany). Cholesterol was obtained from Dishman Carbogen Amcis (Ahmedabad, India). Low-endotoxin sucrose and analytical-grade chloroform and methanol were from Merck (Darmstadt, Germany), and sodium succinate hexahydrate from Sigma-Aldrich (St. Louis, MO, USA). The marketed intravenous voriconazole comparator (Vorier, 200 mg/vial) was procured commercially. Water was in-house purified water of injectable quality. All other reagents were of analytical or HPLC grade.

### 2.2 Pre-formulation studies

The equilibrium solubility of voriconazole was determined in a solvent such as water, methanol, ethanol, dichloromethane, acetone, isopropyl alcohol, chloroform, diethyl ether, toluene and dimethyl sulfoxide by adding an excess of drug (200 mg) to 10 mL of each solvent and stirring at 350 rpm for 60 min at controlled room temperature in closed vials. The suspensions were centrifuged at 500 rpm for 15 min and the supernatants were filtered through a 0.22 µm membrane and the dissolved drug was quantified by HPLC against a nominal claim of 20 mg/mL. Solid-state identity and crystallinity of the drug were confirmed by Fourier-transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC) and X-ray powder diffraction (XRD).

Because liposome formation requires the lipids to be dissolved in an organic phase and converted to a dry composite, the solubility of HSPC, cholesterol and DPPG-Na was screened in the same solvents above 50 °C using 500 mg of each lipid, and a chloroform: methanol (60:40, v/v) mixture was selected on the combined basis of complete lipid solubility, ease of removal and operator safety [24]. An

apparent partition study was then used to fix the lipid ratio: 500 mg of lipid mixture and 10 mg of drug were dissolved in chloroform: methanol, dried, hydrated with 10 mL of distilled water and stirred for 30 min at room temperature, after which the aqueous phase was separated, centrifuged and assayed so that the drug partitioned into the solid-lipid phase could be calculated by difference. The HSPC content was varied while cholesterol and DPPG-Na were held constant (mole ratios 3:1:1, 2:1:1 and 1:1:1) and the composition giving the greatest association of drug with the lipid phase was selected [25].

### 2.3 Preparation of voriconazole liposomes

Two manufacturing routes including thin-film hydration and spray-drying followed by hydration were evaluated on a common composition [26-29]. In thin-film hydration, drug and lipids were dissolved in the chloroform: methanol mixture, transferred to a round bottom flask and the organic solvent was removed under reduced pressure and gentle heating on a rotary evaporator to deposit a thin lipid film on the wall of the flask. The film was then hydrated with the aqueous medium under rotation. This route was assessed in three trials for entrapment efficiency, particle size and polydispersity. In the spray-drying route, voriconazole and the lipid mixture (HSPC: cholesterol: DPPG-Na, 3:1:1) were dissolved together in chloroform: methanol (60:40, v/v) with gentle heating to give a clear organic phase, which was atomized and dried in a laboratory spray-drier to a free-flowing drug-lipid composite powder that was collected and stored at -20 °C [26, 27]. Four spray-drying trials were performed in which inlet, plate and product temperatures, aspirator flow rate and feed-pump flow were varied, and the resulting powders were evaluated for drug entrapment, particle size, residual solvent and water content so as to define a robust set of drying conditions.

The dry composite was hydrated in an aqueous solution of sodium succinate hexahydrate (0.54% w/v) at 55-60 °C, a temperature maintained above the phase transition temperature of the HSPC-based bilayer to permit ordered vesicle formation with stirring at 250 rpm for 60 min under a nitrogen blanket to mitigate lipid oxidation. The resulting coarse multilamellar dispersion was size-reduced by high pressure micro fluidization, in which the suspension is forced at high velocity through a z-shaped interaction chamber so that shear, impact and cavitation progressively reduce vesicle size and lamellarity to yield small, comparatively uniform vesicles [30, 31]. Size reduction was carried out at 55 °C and the homogenization pressure evaluated at 5,000, 10,000 and 15,000 psi in preliminary work and subsequently treated as a design variable was found to be the dominant determinant of the final vesicle size.

### 2.4 Quality-by-design (QbD) enabled product development

#### *2.4.1 Identification of QTPP and CQA of the liposomal formulation*

The QbD approach was founded on defining the quality target product profile (QTPP), which summarizes the quality characteristics an intravenous liposomal voriconazole product should possess and deriving from it the critical quality attributes (CQAs) which is the physicochemical properties that must be controlled within appropriate ranges to ensure safety and efficacy [20, 22]. The QTPP and CQAs, with justifications, are presented in Table 1.

**Table 1.** Quality target product profile (QTPP) and critical quality attributes (CQA) of the voriconazole liposomal formulation.

| Product attribute | Target profile | CQA | Justification |
|---|---|---|---|
| **Route of administration** | Intravenous | — | Pharmaceutical requirement |
| **Dosage form** | Lyophilized liposome for injection | — | Sterile powder for reconstitution |
| **Reconstitution medium** | Water for injection / saline | — | Facile reconstitution |
| **Entrapment efficiency** | ≥ 90% | ✓ | Dose delivery and maximized efficacy |
| **Particle size** | ≤ 150 nm | ✓ | Prolonged circulation; suitability for IV use |
| **Polydispersity index** | < 0.5 | ✓ | Homogeneous, monodisperse system |
| **Zeta potential** | -30 to +30 mV | ✓ | Physical stability of the dispersion |
| **Assay of voriconazole** | 90-110% | ✓ | Compendial quality and efficacy |
| **Free drug** | ≤ 10% | ✓ | Encapsulation quality |
| **Water content** | ≤ 5% | ✓ | Cake integrity and shelf-life |
| **pH** | 6.0-7.5 | ✓ | Biocompatibility with blood plasma |

| | | | |
|---|---|---|---|
| **Osmolality** | 280-350 mOsm $kg^{-1}$ | ✓ | Avoid irritation and haemolysis |
| **Reconstitution time** | ≤ 5 min | ✓ | Clinical usability |
| **Residual solvents** | ≤ ICH limits | ✓ | Patient safety |
| **Storage / shelf-life** | 2-8 °C; ≥ 24 months | — | Product life and distribution |

### *2.4.2 Qualitative and quantitative risk assessment*

Quality risk management was used to identify the critical material attributes (CMAs) and critical process parameters (CPPs) whose variation could adversely affect the CQAs of the product. Unit operations such as dispensing, spray-drying, hydration, size reduction, lyophilization and packaging were rated qualitatively as low, medium or high risk against each CQA, focusing subsequent screening on the highest-risk variables [21].

## 2.5 Experimental design for preparation

Design generation and statistical analysis were performed in Design-Expert 11 (Stat-Ease Inc., Minneapolis, MN, USA), with runs randomized to guard against experimental error [20, 32]. The factors and levels studied in the screening and optimization designs are summarized in Table 2.

**Table 2.** Screening (Plackett–Burman) and optimization (Box–Behnken) design factors with levels.

| Code | Factor (unit) | Low (−1) | Mid (0) | High (+1) |
|---|---|---|---|---|
| **Plackett–Burman screening factors** | | | | |
| **A** | Organic phase mixing time (min) | 30 | — | 60 |
| **B** | Hydration temperature (°C) | 60 | — | 90 |
| **C** | Stirring speed during hydration (rpm) | 200 | — | 250 |
| **D** | Hydration time (min) | 60 | — | 90 |
| **E** | Homogenization temperature (°C) | 50 | — | 60 |
| **F** | Homogenization pressure (psi) | 5000 | — | 15000 |
| **G** | Total lipid content (mg) | 100 | — | 400 |
| **H** | Sucrose content (mg) | 50 | — | 200 |
| **I** | Inlet temperature (°C) | 30 | — | 40 |
| **J** | Atomization pressure (bar) | 0.5 | — | 2 |
| **K** | Feed pump flow rate (rpm) | 10 | — | 20 |
| **Box–Behnken optimization factors** | | | | |
| **A** | Total lipid content (mg) | 100 | 250 | 400 |
| **B** | Homogenization pressure (psi) | 5000 | 10000 | 15000 |
| **C** | Sucrose content (mg) | 50 | 125 | 200 |

*Responses (both designs): entrapment efficiency (% EE), particle size (nm) and polydispersity index (PDI).*

### *2.5.1 Screening design model*

Eleven process and formulation variables were screened in a 12-run Plackett-Burman design against particle size, polydispersity index and entrapment efficiency with the aim of identifying the factors most strongly influencing the responses while economizing on the number of experiments [32-34]. The Plackett-Burman design is a two-level, resolution limited screening design in which main effects are, in general, heavily confounded with two-factor interactions. It is therefore not used to model interactions but to distinguish, efficiently and unambiguously, the few factors with large main effects from the many with negligible ones. The levels of each factor were set from the preceding pre-formulation and preliminary trials and from the literature. The significance of each factor was assessed on Pareto analysis against two reference lines including the t-value limit, above which an effect is likely to be significant, and the Bonferroni limit, above which it is almost certainly significant and corroborated by analysis of variance. Factors

exceeding these thresholds for a given response were retained as critical for that response.

### *2.5.2 Optimization design modelling by response surface methodology*

The three variables emerging as critical parameters including total lipid content (A), homogenization pressure (B) and sucrose content (C) were optimized in a three-factor, three-level Box-Behnken design comprising 15 runs including three replicated centre points [35]. The Box-Behnken design was chosen in preference to a central-composite design because it requires only three levels of each factor, places all experimental points at the midpoints of the edges of the factor space and at the centre rather than at extreme corner combinations and is rotatable making it efficient and practical for a formulation in which extreme combinations of all factors might be difficult or wasteful to prepare. Entrapment efficiency ($Y_1$), particle size ($Y_2$) and polydispersity index ($Y_3$) were fitted to second-order polynomial models and analysed by analysis of variance. The adequacy of each model was judged on the model F-value and p-value, the significance and non-significance respectively of the model terms and the lack-of-fit, the coefficient of determination ($R^2$) together with its adjusted and predicted forms and the adequate precision signal-to-noise ratio, for which a value greater than 4 is desirable. Three-dimensional response surfaces and two-dimensional contour plots were generated from the coded polynomial equations to visualize the effect of each factor and their interactions on the responses.

### *2.5.3 Validation of design based on desirability function*

The design space was numerically optimized for maximum entrapment and minimum size and polydispersity using the desirability function. The solution of highest desirability was prepared and its observed responses were compared with the model predictions to authenticate the design.

## 2.6 Lyophilization of voriconazole liposomes

The selection of a cryoprotectant and the design of the freeze-drying cycle were treated as integral parts of product development rather than as a terminal drying step. Freshly prepared liposomes were mixed with suitable cryoprotectants such as sucrose, trehalose or mannitol at a fixed concentration and lyophilized and the reconstituted products were compared for the change in vesicle size and polydispersity relative to the pre-lyophilization dispersion, on the principle that an effective lyoprotectant vitrifies the freeze concentrate and H-bonds to the phospholipid head groups thereby preserving bilayer integrity through the freezing and dehydration stresses [17, 19]. The disaccharide giving the smallest change in size and polydispersity was carried forward, and its concentration was subsequently optimized as a formulation factor in the experimental design.

For lyophilization, a pre-specified volume of the size-reduced dispersion containing the selected cryoprotectant was filled into clear glass vials and half-stoppered with double-slotted lyophilization stoppers. The freeze-drying cycle comprised three stages: a freezing stage with staged ramps and holds to approximately -45 °C to ensure complete solidification and an appropriate ice structure, a primary drying stage under reduced pressure (of the order of 100 mtorr) with shelf temperatures held below the collapse temperature of the freeze concentrate to sublime the ice and a secondary drying stage with the shelf ramped to 25 °C to desorb residual bound water. The cycle was refined over successive engineering runs in which endpoints such as melt-back, cake collapse, micro-collapse with prolonged reconstitution and elevated residual moisture were diagnosed and corrected until an intact cake was obtained that reconstituted in under 5 min to a dispersion of unchanged size and gave a residual moisture below 5% and the optimized cycle was verified for reproducibility on repeat manufacture.

## 2.7 Characterization of liposomal formulations

### *2.7.1 HPLC-PDA method*

Voriconazole was quantified on a Shimadzu Prominence-iLC2010 HPLC equipped with an isocratic pump, autosampler and photodiode-array detector, using a method adapted from the USP monograph and published, validated procedures [36, 37]. Separation used a Hypersil BDS C18 column (200 × 4.0 mm, 5 µm) at 35 °C, an isocratic mobile phase of buffer: acetonitrile: methanol (60:30:10, v/v/v), a flow rate of 1.0 ml/min, an injection volume of 20 µL and detection at 256 nm. The retention time was approximately 8.2 min and the method was linear from 0.5 to 40 µg/mL.

### *2.7.2 Entrapment efficiency (% EE) and assay determination*

Entrapment efficiency was determined by an indirect method after separation of unentrapped drug on a Sephadex G-50 gel-filtration column. Liposome containing fractions were collected, the vesicles were lysed with absolute alcohol and sonicated for 20 min and the released drug was quantified by HPLC. Entrapment efficiency was calculated as % EE = (total drug - free drug)/total drug × 100. Assay was determined after disruption of the liposomes and dilution in mobile phase, quantified by the same HPLC method.

### *2.7.3 Particle size (PS), PDI and zeta potential (ZP) determination*

Vesicle size (Z-average), polydispersity index and zeta potential were measured by dynamic and electrophoretic light scattering (Malvern Zetasizer 3000, Malvern Instruments, UK) on the reconstituted product, with the scattering angle set to 90°. Samples were diluted appropriately and equilibrated before measurement and results are reported as the mean of three determinations.

### *2.7.4 Physicochemical identification (DSC, XRD, FTIR)*

Solid state identity of voriconazole was confirmed by DSC, XRD and FTIR, and drug excipient compatibility was

assessed by infrared spectroscopy. Additional characterization and reconstituted product tests included description, pH, osmolality, assay of drug and of each lipid, water content (Karl Fischer), reconstitution time and residual solvents.

### 2.8 Bioanalytical method for voriconazole in plasma

Voriconazole in plasma was determined by HPLC hyphenated with mass spectrometric detection [38, 39]. Plasma was separated by centrifugation (5,000 rpm, 15 min) and stored at -70 °C until analysis. A 250 µL aliquot was subjected to protein precipitation with organic solvent, vortex mixing, centrifugation (10,000 rpm, 30 min) and filtration before injection. Matrix-matched calibrators were prepared and the method was linear across the calibration range with a correlation coefficient of approximately 0.9878 and a limit of quantification of 0.2 mg/L per European Pharmacopoeia criteria. Mass-spectrometric parameters (vaporizer temperature 450 °C; sheath, auxiliary and sweep gas flows 50, 5 and 5 arbitrary units; capillary temperature 275 °C) provided the required selectivity and sensitivity.

### 2.9 Stability study of voriconazole liposomes

The lyophilized optimized product was placed on stability in its final container closure system at 2-8 °C and at 25 °C/60% relative humidity in accordance with ICH principles [40]. Samples were withdrawn initially and at 1, 2 and 3 months and tested for description, assay, free drug, particle size, osmolarity, pH and water content against pre-defined acceptance criteria.

### 2.10 *In vivo* pharmacokinetic study

The animal study was conducted after approval by the Institutional Animal Ethics Committee (protocol no. RPCP/IAEC/2022-23/R9) and in accordance with applicable national guidelines for the care and use of laboratory animals. Rats were acclimatized under standard housing conditions with free access to food and water and were randomized into three treatment groups. Each group received, by the intravenous route, an equivalent dose of drug as one of three preparations: free voriconazole, the reconstituted lyophilized liposomal formulation, or the marketed formulation (Vorier). This three-arm design allowed the liposomal formulation to be benchmarked simultaneously against the unformulated drug and against the current standard-of-care intravenous product, isolating the contribution of the liposomal carrier itself.

At pre-determined time points after dosing, blood samples were collected into anticoagulant tubes, plasma was separated by centrifugation and stored at -70 °C until analysis and voriconazole was quantified by the plasma bioanalytical method described above [41]. As is common for liposomal pharmacokinetic studies that do not employ an on-line separation of encapsulated and free drug, the freeze-thawing of samples and the organic protein precipitation step disrupt the liposomal membranes, so that the concentration measured represents total (free plus liposome-associated) voriconazole. This is stated explicitly because it bears on the interpretation of the exposure parameters. Plasma concentration time profiles were constructed for each group and the pharmacokinetic parameters states maximum plasma concentration ($C_{max}$), time to maximum concentration ($T_{max}$), area under the concentration time curve (AUC), terminal elimination half-life ($t_{1/2}$), clearance and mean residence time (MRT) were derived from the profiles. Results are expressed as mean ± standard deviation and the DoE responses were evaluated by analysis of variance with a probability level of $p < 0.05$ regarded as statistically significant.

## 3. RESULTS AND DISCUSSION

### 3.1 Mapping elements of QbD: identifying QTPP and CQA

The liposomal product was developed and optimized using the QbD technique beginning with the definition of a quality target product profile. The QTPP is a prospective summary of the quality characteristics that the drug product should possess to deliver its intended therapeutic performance safely and it forms the basis of design for the entire development programme [20]. For an intravenous liposomal voriconazole, the considerations informing the QTPP included the route of administration (intravenous), the mode of administration (infusion of a reconstituted product), the dosage form (a sterile lyophilized powder for injection) and its physical nature, the reconstitution medium and time, the target physicochemical attributes of the reconstituted dispersion and the storage conditions and shelf life required for distribution [22].

From the QTPP, the critical quality attributes those measurable properties that must be maintained within an appropriate range to assure the desired quality were derived. Entrapment efficiency was designated a CQA because it governs the delivered dose and the efficiency of drug utilization. Particle size and polydispersity index because they control the intravenous injectability, the circulation behaviour and the physical homogeneity of the product. Zeta potential because it underlies the colloidal stability of the dispersion and assay, free drug, water content, pH, osmolality, reconstitution time and residual solvents because each bears directly on the identity, strength, purity, safety or usability of the finished product. Attributes such as sterility, bacterial endotoxin and particulate matter, though essential to a commercial parenteral, were not investigated at this laboratory development stage, as strict aseptic processing was outside the scope of the study. They are noted here as attributes to be addressed in subsequent development. This structured mapping of QTPP to CQAs established the quality objectives against which the formulation and process were subsequently designed and judged [20, 22].

### 3.2 Assessing qualitative and quantitative risks in fabrication

With the CQAs defined, quality risk management was used to identify the critical material attributes and critical process parameters whose variation could adversely affect them. Each unit operation of the manufacturing train dispensing,

spray-drying, hydration, size reduction, lyophilization and packaging was assessed against each CQA and assigned a qualitative risk level of low, medium or high, so that development effort could be concentrated where it mattered most [21]. This exercise highlighted a small number of high-risk relationships: the assay and content of the drug were most at risk during hydration and packaging. Particle size was at high risk during size reduction and lyophilization. Free drug and *in vitro* release were sensitive to the size reduction step, related substances were sensitive to the thermal stress of spray-drying and water content was governed chiefly by the lyophilization cycle.

Translating these qualitative judgements into experimental factors, entrapment efficiency was anticipated to be influenced most strongly, directly and indirectly, by the total lipid content and by the intensity of the size reduction step, whereas particle size and polydispersity were expected to be governed mainly by the homogenization pressure and by the cryoprotectant level introduced for lyophilization [21]. The lipid composition and ratio (HSPC: cholesterol: DPPG-Na, 3:1:1) had already been established as a well-defined parameter during pre-formulation and was therefore not varied further and several process settings judged low risk were fixed at convenient values. The remaining higher risk material and process variables eleven in total were carried forward into a formal screening design so that their influence could be quantified rather than assumed, in keeping with the risk-based philosophy of QbD.

### 3.3 Formulation using design of experiment (DoE)

#### *3.3.1 Pre-formulation and lipid selection*

The reversed-phase HPLC-PDA assay gave a symmetrical voriconazole peak at approximately 8.2 min and a linear response over the calibration range with a coefficient of determination of 0.999, and served throughout for assay, entrapment and free-drug determinations. Pre-formulation confirmed the identity and solid-state form of the drug DSC showed a single sharp melting endotherm at 134.96 °C in comparison with the reported melting point and indicating a crystalline, anhydrous material of high purity. XRD displayed the sharp, intense reflections characteristic of a crystalline solid and the IR reproduced the characteristic functional-group bands of voriconazole with no evidence of interaction that would contraindicate the chosen excipients.

Solubility screening quantified the strong lipophilic character of voriconazole. The drug was practically insoluble in water but dissolved readily in chlorinated and polar aprotic organic solvents about 19-20 mg/ml in dichloromethane, dimethyl sulfoxide and chloroform and 18-19 mg/ml in ethanol and methanol consistent with a log P of approximately 1.8. This solubility pattern, unfavourable for a simple aqueous injection but favourable for partitioning into a lipid bilayer, provides the physicochemical rationale for a lipid-based carrier and explains the reliance of the marketed product on a solubilizing cyclodextrin. The solubility of the lipids themselves guided the choice of organic phase: HSPC, cholesterol and DPPG-Na were all freely soluble above 50 °C in chloroform and methanol, and a 60:40 chloroform: methanol mixture was selected as offering complete solubilization with acceptable ease of removal and handling safety [24]. The partition study fixed the lipid ratio. As the proportion of HSPC was increased, progressively more drug associated with the solid-lipid phase and less remained in the aqueous phase. At a HSPC: cholesterol: DPPG-Na mole ratio of 3:1:1 the drug was almost quantitatively lipid-associated, compared with 9914.28 µg and 9870.80 µg in lipid at the 2:1:1 and 1:1:1 ratio respectively, so the 3:1:1 ratio was selected to maximize entrapment and minimize drug wastage [25]. This composition is also rational from a biophysical standpoint: HSPC provides a high gel-to-liquid-crystalline phase-transition temperature and therefore a rigid, low-permeability bilayer that resists drug leakage at body temperature; cholesterol, at one-third the phospholipid content, fills packing defects and modulates membrane fluidity and permeability without disrupting the ordered bilayer; and the anionic DPPG-Na imparts a negative surface charge that stabilizes the vesicles electrostatically against aggregation and fusion.

#### *3.3.2 Selection and optimization of the manufacturing route*

The two manufacturing routes gave markedly different products. Thin-film hydration produced very large, highly polydisperse vesicles across three trials (Z-average 2,249-2,530 nm, polydispersity index up to 1.06) with entrapment of 83.0-86.4%, and was additionally hampered by non-uniformity and by difficulty in completely recovering the product from the flask. Spray drying of the drug-lipid composite followed by hydration, in contrast, produced smaller and considerably more uniform particles (≈1,194-1,345 nm before size reduction; polydispersity index 0.35-0.51) with higher entrapment of 90.2-93.0% [26, 28]. Beyond these analytical advantages, the spray drying route offered decisive practical benefits for development and scale-up: the drying step could be scheduled and decoupled from hydration, the dry composite could be stored at -20 °C for later processing and the approach avoided the volume limitation inherent to a rotary flask. Spray drying was therefore selected for further optimization [27, 42].

Because the temperature of spray drying can affect the level of process related degradation products and the particle size depends on atomization and feed conditions, the key drying parameters were optimized in four trials in which inlet, plate and product temperatures, aspirator flow rate and feed-pump flow were varied. Across these trials drug entrapment remained high (84.6-90.0%), particle size of the intermediate powder ranged from 584 to 1,257 µm, residual chloroform was low to undetectable and residual methanol was reduced with appropriate conditions and water content was held near or below 5%. The condition set giving the best combination of entrapment, controllable particle size, low residual solvent and acceptable moisture was fixed for subsequent work [42]. Micro-fluidization of the hydrated dispersion then reduced the vesicles to the target sub-150 nm range with size

decreasing steeply as pressure increased with 324, 251 and 132 nm at 5,000, 10,000 and 15,000 psi, respectively, a monotonic relationship that identified homogenization pressure as the principal lever for size control and justified its inclusion as a factor in the formal experimental design [30, 31].

### *3.3.3 Screening model design*

Eleven material and process variables including organic-phase mixing time, hydration temperature, stirring speed, hydration time, homogenization temperature, homogenization pressure, total lipid content, sucrose content, spray-drier inlet temperature, atomization pressure and feed-pump flow rate were screened in a 12-run Plackett-Burman design. In which main effects are estimated with a minimal number of runs so that the vital few factors can be separated from the trivial many before committing resources to optimization [32, 34]. Across the design the responses spanned a wide range entrapment efficiency 81-95%, particle size 109-200 nm and polydispersity index 0.10-0.90 confirming that the chosen factors did indeed exert a meaningful influence over the design region and that the ranges studied were appropriately wide.

The regression models for all three responses were statistically significant, with F-values ranging from about 20 to 572 ($p < 0.05$) and coefficients of determination between roughly 0.6 and 0.9, and the significance of individual factors was judged on Pareto analysis against the Bonferroni and t-value limits. The analysis returned an unusually clean separation of effects: total lipid content was for entrapment efficiency ($F = 20.4$, $p = 0.0011$), homogenization pressure was the determinant of particle size ($F = 572$, $p < 0.0001$), and sucrose content was the principal driver of polydispersity index ($F = 29.1$, $p = 0.0003$), while the remaining eight variables fell below the significance thresholds for all responses [33, 34]. Each of the three critical responses was thus governed principally by a single, distinct factor, a convenient outcome that both simplified the subsequent optimization and provided independent mechanistic corroboration of the trends seen in the preliminary trials. The eight non-significant variables were fixed at practical set-points, and total lipid content, homogenization pressure and sucrose content were carried forward as the factors for response-surface optimization.

### *3.3.4 Optimization by response surface methodology*

The Box-Behnken design matrix and responses are given in Table 3 and the model diagnostics in Table 4. Entrapment efficiency followed a significant quadratic model ($F = 13.22$, $p = 0.0055$) with adequate precision of 12.9. Total lipid content was the strongest positive term, raising entrapment by providing greater bilayer capacity for the lipophilic drug. Particle size was represented by a significant reduced linear model ($F = 13.72$, $p = 0.0005$) dominated by homogenization pressure, whose negative coefficient reflects more efficient vesicle fragmentation at higher pressure. Polydispersity was governed principally by sucrose content ($p = 0.0004$), the lowest values coinciding with lower sucrose. The coded model equations were:

**% EE** = $92.67 + 2.00A + 0.75B + 0.75C - 2.00AB + 1.00AC + 0.67A^2 + 1.67B^2 - 1.83C^2$

**Particle size** (nm) = $152.53 + 9.62A - 21.50B + 9.88C$

**PDI** = $0.2513 + 0.0379A - 0.0349B + 0.1285C - 0.0300AB + 0.0393AC - 0.0218BC + 0.0353A^2 - 0.0174C^2$

where A, B and C are the coded levels of total lipid content, homogenization pressure and sucrose content, and the sign and magnitude of each coefficient express the direction and relative strength of that term's effect. For all three models the predicted $R^2$ was in reasonable agreement with the adjusted $R^2$ (differences below 0.2) and the adequate-precision ratio comfortably exceeded the threshold value of 4, indicating an adequate signal-to-noise ratio and confirming that each model could be used to navigate the design space rather than merely to describe the observed data [22, 35].

The behaviour of entrapment efficiency is readily rationalized. Its positive dependence on total lipid content reflects the greater bilayer volume and surface area available to accommodate the lipophilic drug as more phospholipid is provided, an effect widely reported for the encapsulation of lipophilic molecules in liposomes and consistent with the near-quantitative lipid partitioning seen in pre-formulation [12, 25]. The significant negative lipid-pressure interaction (AB) indicates that the entrapment gain from added lipid is partly offset at the highest homogenization pressures, where the intense shear that reduces vesicle size can also transiently disrupt bilayers and expel a fraction of loosely associated drug. The practical consequence is that the highest entrapment is obtained by combining a high lipid content with a pressure high enough to meet the size target but not so high as to compromise retention. Entrapment remained high (89-98%) throughout the design, so that this response, while important, was less discriminating than particle size and polydispersity.

Particle size, by contrast, was governed overwhelmingly by homogenization pressure, whose large negative coefficient captures the progressive reduction in vesicle size as the number and intensity of high-shear passages through the interaction chamber increase. Total lipid content and sucrose exerted weaker positive effects, the former because a larger lipid mass tends to form somewhat larger or more numerous lamellae and the latter through a modest increase in the viscosity and solute load of the hydration medium [30, 31]. The monotonic size reduction observed in the preliminary micro fluidization trials and is consistent with the screening design, in which pressure was by far the most significant term for size. Polydispersity was controlled principally by sucrose content: at high cryoprotectant levels the size distribution broadened, most plausibly because the increased solute concentration promotes heterogeneous nucleation and a wider range of vesicle sizes on hydration, whereas the narrowest, most monodisperse distributions were obtained at lower sucrose levels. This finding is central to the product,

because it places the requirements for a low polydispersity (favouring low sucrose) and for effective lyoprotection (favouring adequate sucrose) in tension, and the optimization was therefore required to identify a sucrose level that satisfied both.

Numerical optimization for maximum entrapment and minimum size and polydispersity, using the desirability function, resolved this trade-off and converged at a desirability of 1.0 on 400 mg total lipid, 15,000 psi and 125 mg sucrose per vial, predicting 95.75% entrapment, 140.7 nm and a polydispersity index of 0.260. The selected sucrose level of 125 mg represents an intermediate value that preserves an acceptably narrow distribution while providing sufficient cryoprotection for the subsequent lyophilization, exemplifying the way in which a response-surface design reconciles competing quality attributes within a single, defensible design space [35]. The response surfaces and contour plots (Fig. **2**) visualize these relationships, showing the ascent of entrapment toward high lipid content, the steep fall of particle size with increasing pressure, and the minimization of polydispersity at low sucrose.

**Table 3.** Box–Behnken design matrix and measured responses for voriconazole liposomes (mean ± SD).

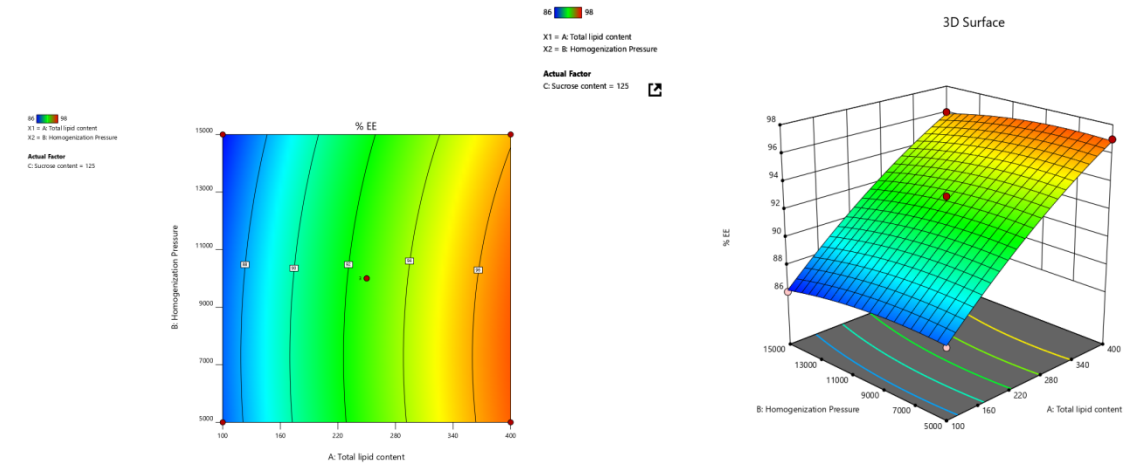
| Run | A: Lipid (mg) | B: Pressure (psi) | C: Sucrose (mg) | % EE | Size (nm) | PDI |
|---|---|---|---|---|---|---|
| 1 | 100 | 10000 | 200 | 89 ± 1.0 | 154 ± 2.0 | 0.351 ± 0.07 |
| 2 | 400 | 10000 | 200 | 91 ± 0.8 | 180 ± 1.1 | 0.461 ± 0.10 |
| 3 | 250 | 10000 | 125 | 92 ± 0.6 | 160 ± 1.1 | 0.302 ± 0.11 |
| 4 | 250 | 15000 | 50 | 92 ± 1.2 | 121 ± 0.1 | 0.114 ± 0.05 |
| 5 | 400 | 5000 | 125 | 98 ± 0.7 | 189 ± 1.0 | 0.425 ± 0.07 |
| 6 | 250 | 15000 | 200 | 94 ± 0.7 | 152 ± 2.0 | 0.311 ± 0.09 |
| 7 | 100 | 5000 | 125 | 90 ± 0.9 | 174 ± 1.6 | 0.245 ± 0.11 |
| 8 | 400 | 10000 | 50 | 92 ± 0.6 | 168 ± 0.8 | 0.109 ± 0.10 |
| 9 | 250 | 10000 | 125 | 94 ± 1.1 | 147 ± 0.7 | 0.226 ± 0.06 |
| 10 | 100 | 15000 | 125 | 96 ± 1.0 | 122 ± 0.9 | 0.209 ± 0.08 |
| 11 | 100 | 10000 | 50 | 90 ± 0.8 | 131 ± 0.6 | 0.156 ± 0.04 |
| 12 | 250 | 10000 | 125 | 92 ± 0.9 | 144 ± 1.1 | 0.226 ± 0.11 |
| 13 | 400 | 15000 | 125 | 96 ± 0.6 | 121 ± 1.0 | 0.269 ± 0.08 |
| 14 | 250 | 5000 | 200 | 93 ± 1.1 | 169 ± 1.0 | 0.398 ± 0.11 |
| 15 | 250 | 5000 | 50 | 91 ± 0.8 | 156 ± 0.8 | 0.114 ± 0.06 |

**Table 4.** Summary of the fitted response-surface models (ANOVA and diagnostics).

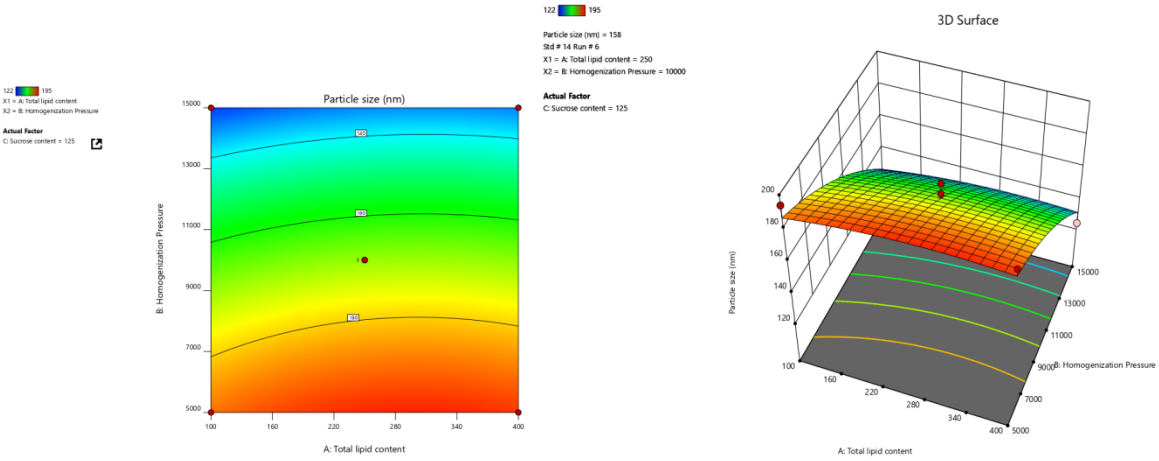
| Statistic | % EE | Particle size | PDI |
|---|---|---|---|
| **Model type** | Quadratic | Reduced linear | Quadratic |
| **Model F-value** | 13.22 | 13.72 | 9.81 |
| **Model p-value** | 0.0055 | 0.0005 | 0.0108 |
| **Significant term(s)** | A, AB, $B^2$, $C^2$ | B (A, C) | C |
| **Lack-of-fit p-value** | 0.8576 (ns) | 0.3891 (ns) | 0.5322 (ns) |
| **$R^2$** | 0.9597 | 0.7892 | 0.9464 |
| **Adjusted $R^2$** | 0.8871 | 0.7316 | 0.8499 |
| **Predicted $R^2$** | 0.7581 | 0.5724 | 0.4353 |
| **Adequate precision** | 12.87 | 10.79 | 10.63 |

| | | | |
|---|---|---|---|
| **C.V. (%)** | 0.92 | 7.38 | 16.86 |

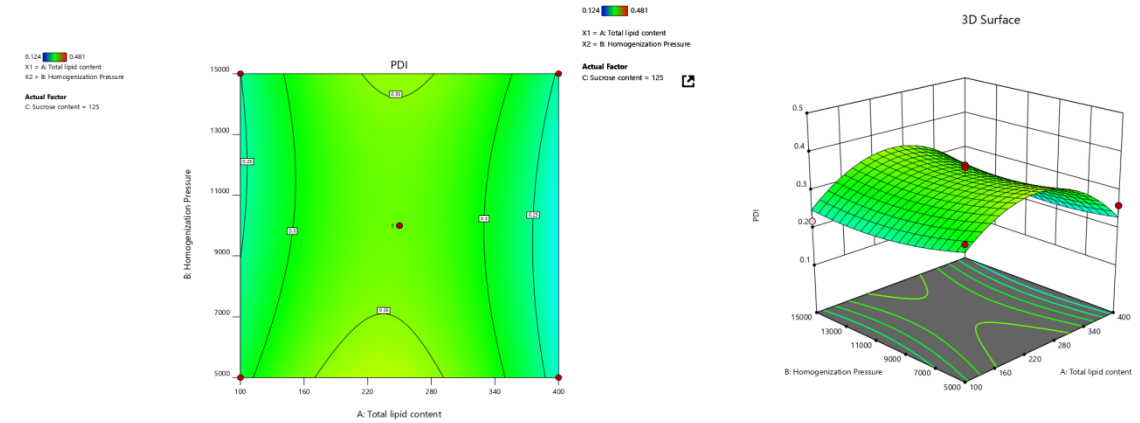
*A = total lipid content; B = homogenization pressure; C = sucrose content; ns = not significant.*




**(A)**




**(B)**




**(C)**

**Figure 2.** Box-Behnken response surfaces (top) and contour plots (bottom) for (a) entrapment efficiency and (b) particle size, and (c) polydispersity index versus total lipid and sucrose content, generated from the fitted second-order models.

### *3.3.5 Validation of the optimized formulation*

A confirmatory batch prepared at the optimized settings closely matched the model predictions, verifying the design space (predicted vs. observed: entrapment 95.75% vs. 95.9%; size 140.7 vs. 147.5 nm; polydispersity index 0.260 vs. 0.157). The reconstituted product was a white, cake reconstituting in 2 min 27 s to a 147.5 nm dispersion (PDI 0.157) with an entrapment efficiency of 95.9%, free drug of 2.8%, zeta potential of -15.7 mV, osmolality of 311 mOsm/kg, pH 6.78 and water content of 3.1%. Residual chloroform and methanol were below detection and the assays of the drug and of each of the three lipids fell within the 70-120% specification (Table 5). The final composition per 50 mg vial is given in Table 6.

**Table 5.** Certificate of analysis of the optimized voriconazole liposomal batch, and predicted vs. observed responses.

| Quality attribute | Acceptance criterion | Result |
|---|---|---|
| **Description** | White powder | White powder |
| **pH** | 6.0 – 7.5 | 6.78 |
| **Assay of voriconazole** | 90.0 – 110.0% | 97.8% |
| **Entrapment efficiency** | ≥ 90.0% | 95.9% |
| **Free drug** | ≤ 10.0% | 2.80% |
| **Particle size, Z-average** | ≤ 150 nm | 147.5 nm |
| **Polydispersity index** | < 0.5 | 0.157 |
| **Zeta potential** | −30 to +30 mV | −15.67 mV |
| **Osmolality** | 280 – 350 mOsm $kg^{-1}$ | 311 |
| **Water content** | ≤ 5.0% | 3.11% |
| **Reconstitution time** | ≤ 5 min | 2 min 27 s |
| **Residual chloroform / methanol** | ≤ 60 / ≤ 3000 ppm | ND / ND |
| **Lipid assay (HSPC / DPPG-Na / cholesterol)** | 70.0 – 120.0% | 98.8 / 97.4 / 98.9% |

*Predicted vs. observed at the optimum: entrapment 95.75% vs. 95.9%; particle size 140.7 nm vs. 147.5 nm; PDI 0.260 vs. 0.157 — confirming the model within the design space. ND = not detected.*

**Table 6.** Composition of the optimized lyophilized voriconazole liposomal formulation (per 50 mg vial).

| Ingredient | Function | Quantity per vial |
|---|---|---|
| **Voriconazole** | Active pharmaceutical ingredient | 50 mg |
| **HSPC** | Bilayer-forming phospholipid | 240 mg |
| **Cholesterol** | Membrane stabilizer / fluidity modulator | 80 mg |
| **DPPG-Na** | Anionic charge-conferring lipid (stabilizer) | 80 mg |
| **Sucrose (low-endotoxin)** | Cryoprotectant / lyoprotectant | 125 mg |
| **Sodium succinate hexahydrate** | Buffering agent (hydration medium) | 54 mg |
| **Chloroform** | Organic phase (removed on drying) | 6 mL |

| | | |
|---|---|---|
| **Methanol** | Organic phase (removed on drying) | 4 mL |
| **Water for injection** | Vehicle | q.s. |

*HSPC:cholesterol:DPPG-Na at a 3:1:1 ratio; total lipid content 400 mg per vial.*

Several of these attributes have direct implications for the clinical usability and stability of the product. The Z-average diameter of about 147 nm, together with a polydispersity index well below 0.2, describes a small, narrowly distributed and essentially monodisperse population of vesicles. A size in this range is generally regarded as well suited to intravenous administration, being large enough to be retained in the circulation yet small enough to pass safely through the microvasculature and to avoid rapid mechanical filtration, and a low polydispersity is important both for reproducible *in vivo* behaviour and for consistent manufacture [12, 26]. The moderately negative zeta potential of -15.7 mV, conferred by the anionic DPPG-Na, provides a repulsive electrostatic barrier that opposes vesicle aggregation and fusion and thus contributes to the physical stability of the reconstituted dispersion. Although the absolute value is below the ±30 mV sometimes cited as a benchmark for electrostatically stabilized colloids, it lies within the target specification and is complemented by the steric and matrix stabilization afforded by the rigid HSPC/cholesterol bilayer and in the dried state, by the vitrified sucrose. The high entrapment and low free drug confirm that the drug is almost entirely vesicle-associated, minimizing the burst of free drug on administration, while the rapid reconstitution, low residual moisture and undetectable residual solvents attest to a well-controlled lyophilization and a product suitable for bedside preparation.

### 3.4 Lyophilization

The cryoprotectant screening clearly differentiated the three disaccharide and polyol agents. Sucrose best preserved the vesicles, limiting the post-lyophilization size increase to approximately 142-146 nm from an initial value of about 130 nm at essentially unchanged polydispersity. Trehalose gave a larger increase roughly 159-168 nm with broader distributions, and mannitol performed worst yielding particles of 238-251 nm and high polydispersity [17, 19]. The superior performance of sucrose is consistent with the water replacement and vitrification hypotheses of lyoprotection as an amorphous, glass forming sugar it H-bonds to the phosphate and carbonyl groups of the phospholipid head groups in place of the water removed during drying and it immobilizes the vesicles within a rigid glassy matrix that resists fusion and mechanical damage. Mannitol, which tends to crystallize on freezing and cannot form such a protective amorphous phase and can even nucleate ice damage explain the marked size growth observed. Sucrose was therefore selected and its level optimized within the Box-Behnken design.

Development of the freeze-drying cycle itself required iterative engineering. Early cycles failed in instructive ways such as product melt-back in the vial, collapse of the cake, micro-collapse manifesting as a reconstitution time exceeding ten minutes, and cakes of acceptable appearance but excessive or non-uniform residual moisture, each of which was traced to a shelf-temperature or pressure setting that allowed the product to exceed its collapse temperature during primary drying or that left bound water inadequately desorbed in secondary drying. Progressive adjustment of the freezing profile, the primary drying shelf temperature and duration, and the secondary drying ramp ultimately delivered a robust, pharmaceutically elegant, intact cake that reconstituted in about two min to a dispersion of unchanged size, with water content reliably below 5%, and this optimized cycle reproduced the same result on repeat manufacture. Lyophilization thus achieved its central purpose of converting a thermodynamically metastable aqueous dispersion into a dry, low-mobility solid in which the physical and chemical degradation pathways that limit liposome shelf life are strongly suppressed [17, 18].

### 3.5 Stability study

The three-month stability study conducted in the final container-closure system under refrigerated at 2-8 °C and accelerated to 25 °C/60% RH conditions in line with ICH principles, demonstrated that the lyophilized product remained within all pre-defined acceptance criteria throughout (Table 7) [40]. The assay of voriconazole stayed between 95.7% and 99.5% of label at all time points and under both conditions within the 90-110% specification and free drug remained below 2.0% against a limit of 10% indicating that neither chemical degradation of the drug nor leakage from the vesicles occurred to any significant extent. Water content remained below 4% throughout confirming that the container-closure system maintained the low residual moisture achieved at the end of the lyophilization cycle and pH 6.2-6.9 and osmolality 291-318 mOsm/kg were essentially unchanged, so that the reconstituted product would remain physiologically compatible over the study period.

**Table 7.** Stability of the lyophilized voriconazole liposomes over three months (mean ± SD).

| Parameter | Criterion | Initial | 2–8 °C 1M | 2–8 °C 2M | 2–8 °C 3M | 25 °C 1M | 25 °C 2M | 25 °C 3M |
|---|---|---|---|---|---|---|---|---|
| **Assay (%)** | 90–110 | 98.7 | 97.2 | 96.8 | 99.5 | 98.4 | 96.5 | 95.7 |
| **Free drug (%)** | ≤ 10 | 1.9 | 2.0 | 1.5 | 1.5 | 1.2 | 1.5 | 2.0 |
| **Size (nm)** | ≤ 150 | 135.5 | 140.8 | 141.1 | 137.8 | 139.4 | 146.1 | 148.7 |
| **Osmolality** | 280–350 | 310 | 308 | 308 | 295 | 318 | 301 | 291 |
| **pH** | 6.0–7.5 | 6.54 | 6.21 | 6.35 | 6.87 | 6.46 | 6.38 | 6.54 |
| **Water (%)** | ≤ 5 | 3.8 | 3.5 | 3.9 | 3.7 | 3.2 | 3.5 | 2.8 |

*Accelerated condition: 25 °C/60% RH. Description and reconstitution behavior were unchanged at all time points.*

The physical stability of the vesicles was equally reassuring. Under refrigeration the reconstituted particle size drifted only marginally from 135.5 nm initially to 137.8 nm at three months while under the accelerated condition it increased modestly to 148.7 nm still below the 150 nm limit. The good growth at 25 °C is the expected consequence of increased molecular mobility and a greater tendency toward vesicle aggregation and fusion at higher temperature and humidity and mirrors the general observation that lyophilized liposomes are markedly more stable than their aqueous counterparts, which in comparable studies degrade substantially within weeks to months [18]. The retention of size, assay, low free-drug content and moisture is attributable to the combined effect of the vitrified sucrose matrix which immobilizes the vesicles and excludes water, and the rigid, saturated HSPC/cholesterol bilayer which resists both chemical hydrolysis/oxidation and physical rearrangement. Taken together, the data support storage of the finished product under refrigeration with the accelerated results indicating tolerance of short-term temperature excursions during handling and distribution. A full-length stability study to establish the definitive shelf life is a logical continuation of this work.

### 3.6 Bioanalytical method

A reliable plasma bioanalytical method is a prerequisite for a meaningful pharmacokinetic comparison and the method developed here met that requirement. The plasma assay was linear across the calibration range with a correlation coefficient of approximately 0.9878 (Fig. **3**) and a limit of quantification of 0.2 mg/L defined per European Pharmacopoeia criteria, sensitive enough to characterize the terminal phase of the profiles [38, 39]. The hyphenation of HPLC with mass-spectrometric detection was central to this performance: relative to ultraviolet detection alone, tandem chromatographic separation and mass-selective detection confer far greater selectivity against endogenous plasma constituents and far greater sensitivity, which is why liquid chromatography-mass spectrometry has become the reference approach for the quantification of drugs in biological matrices for pharmacokinetic study. Sample preparation by organic protein precipitation provided a simple, rapid and robust clean-up that was well suited to the throughput of the pharmacokinetic sampling schedule and the optimized mass-spectrometric source parameters ensured stable, reproducible ionization across the analytical run.

### 3.7 *In vivo* pharmacokinetics

*In vivo* pharmacokinetic profile of pure voriconazole, lyophilized voriconazole loaded liposomes and Vorier was assessed based on plasma voriconazole concentration during the study. The concentration-time profile of plasma is shown in Fig. **4** and the main pharmacokinetic parameters are summarized in Table 8. There was a significant difference in systemic distribution after incorporation into the liposomal formulation with voriconazole. The $T_{max}$ of the pure voriconazole and Vorier was 0.017 h and 0.015 h, respectively, with a progressive decrease in plasma concentration of the pure molecule after $T_{max}$. The $C_{max}$ for pure voriconazole, liposomal voriconazole and Vorier were 95.7, 105.9 and 88.5 µg/mL respectively. The lyophilized formulation of voriconazole-containing liposomes showed a prolonged plasma concentration curve compared to the two comparator formulations with $T_{max}$ reported at 24 h.

**Table 8.** Pharmacokinetic parameters of voriconazole in rats after intravenous administration (three-animal group average).

| Parameter | Unit | Pure drug | Liposome | Marketed (Vorier) |
|---|---|---|---|---|
| **Maximum concentration (Cmax)** | µg $mL^{-1}$ | 95.7 | 105.9 | 88.5 |
| **Time to Cmax (Tmax)** | h | 0.017 | 24 | 0.015 |
| **Total AUC** | µg $L^{-1}$·h | 82,466 | 5,096,000 | 136,653 |

| | | | | |
|---|---|---|---|---|
| **Terminal half-life ($t_{1/2}$)** | h | 0.66 | 22.89 | 1.23 |
| **Clearance** | L $h^{-1}$ | 0.05 | 0.00078 | 0.036 |
| **Mean residence time (MRT)** | h | 0.95 | 44.5 | 1.77 |

*Values represent total (free plus liposome-encapsulated) voriconazole. The magnitude of the exposure increase should be confirmed in an expanded, fully validated pharmacokinetic study.*

This extended systemic residence of the liposomal formulation was also turned up by its terminal half-life and mean residence time. The half-life of pure voriconazole was 0.6572 h while the half-life of Vorier was 1.23 h. The lyophilized liposomal formulation, on the other hand, was shown to have a distinctly prolonged $t_{1/2}$ of 22.89 h. In a similar way, the MRT for the pure voriconazole was 0.95 h and for Vorier was 1.77 h while for the liposomal formulation was 44.5 h. The apparent clearance was also considerably lower (0.05 L/h for the original voriconazole formulation and 0.036 L/h for Vorier) than that of the liposomal formulation (0.00078 L/h). Incorporation into the liposomal system made of phosphatidylcholine significantly extended the persistence of voriconazole in the systemic circulation [16].

There was also a significant difference in overall systemic exposure. The AUC values for pure voriconazole and Vorier were 82,466.3 µg/L·h and 136,653 µg/L·h, respectively. The lyophilized liposomal formulation was reported to have a much higher systemic exposure with an AUC of 5,096,000 µg/L·h in the present study. Therefore, the combination of longer half-life, higher MRT, faster reduction in apparent clearance and higher AUC indicates that the liposomal carrier significantly affected the disposition of voriconazole drug compared to the free drug and marketed formulation.

The pharmacokinetic profile observed could be linked to the incorporation of voriconazole into the lipid bilayer (phatidylcholine-based). Encapsulation may change the immediate availability and/or distribution of the drug and may allow for a slow release of voriconazole from the liposomal vesicles. This would enable the drug to be bound longer and, consequently systemic exposure to be higher than by the rapidly cleared unbound drug. This is an altered disposition pattern that resulted in a significantly greater MRT and long terminal half-life for the liposomal formulation. The current study reported plasma voriconazole after the samples were processed and did not separately quantify the free portion and the liposome-bound portion of voriconazole, therefore, measured concentrations should be assumed as total measurable voriconazole and not only the pharmacologically free portion.

The *in vivo* pharmacokinetic profile of the developed lyophilized phosphatidylcholine-based voriconazole liposomal formulation significantly affected the plasma disposition of voriconazole, resulting in a prolonged systemic persistence and higher plasma exposure level than the pure voriconazole and Vorier. The study findings indicate that the formulation developed with liposomal system could become a useful long-circulating form for voriconazole. However, the reported AUC for the liposomal formulation is significantly higher than the AUCs of the formulations and, as reported, the $T_{max}$ is reached at 24 h before any definite quantitative conclusions can be drawn, these parameters should be checked using the individual animal concentration-time data and validated with non-compartmental pharmacokinetic analysis.

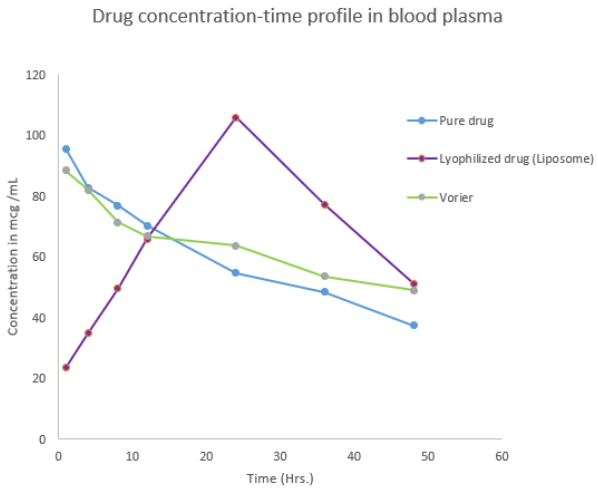



**Figure 4**. Plasma concentration-time profiles of voriconazole following intravenous administration of pure voriconazole, lyophilized voriconazole-loaded liposomes, and Vorier in the experimental animal model.

## 4. Conclusion

A lyophilized, phosphatidylcholine-based liposomal formulation of voriconazole was developed through a

systematic, end-to-end QbD. Beginning from a defined quality target product profile and a risk-based identification of the critical quality attributes, pre-formulation and process screening established the lipid composition (HSPC: cholesterol: DPPG-Na, 3:1:1) and the spray drying manufacturing route. A Plackett-Burman design isolated total lipid content, homogenization pressure and sucrose level as the three critical variables from among eleven candidates and a Box-Behnken response surface design modelled and reconciled their competing effects on entrapment efficiency, particle size and polydispersity, locating an optimum that was confirmed experimentally. The optimized product comprised sub-150 nm vesicles with an entrapment efficiency approaching 96%, a low polydispersity index and a stabilizing negative zeta potential, and met all of its target quality attributes.

Sucrose-based lyophilization, developed alongside the formulation rather than appended to it, produced a pharmaceutically elegant, rapidly reconstituting cake that remained within specification for three months under both refrigerated and accelerated conditions, thereby overcoming the physical and chemical stability limitations that constrain conventional aqueous liposome dispersions. In rats, the formulation markedly transformed the disposition of voriconazole prolonging the circulating half-life from under one hour to approximately 23 hours and substantially increasing systemic exposure and mean residence time relative to both the free drug and the marketed cyclodextrin-based product, without the need for a sulfobutylether-β-cyclodextrin vehicle.

Collectively, these findings establish the liposomal platform as a promising, cyclodextrin-free alternative for parenteral voriconazole delivery and demonstrate the power of a QbD approach to deliver a robust, well-understood product within a defined design space. As with any spontaneously assembling colloidal system, the behaviour of these vesicles is governed by the interplay of their many components and process parameters, and the present work has deliberately quantified those relationships rather than relying on empirical formulation. The study nevertheless represents a preliminary foundation: the quantitative magnitude of the pharmacokinetic enhancement, the partitioning of free and encapsulated drug, the *in vitro* release behaviour, and the antifungal efficacy and repeat-dose safety of the formulation all warrant dedicated investigation. The authors accordingly look forward to advancing this cyclodextrin-free liposomal voriconazole through such studies toward eventual clinical translation.

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