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Research Article

# Optimization of Sterically Stabilized Liposome using Design of Experiment Approach

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ABSTRACT

Recently, a drug delivery system with controlled and targeted drug release at the tumor sites emerged as an attractive option for improving anticancer therapeutics. Advanced nanotherapeutics must not be limited to nanoscale, but should find their way to target the solid tumor via direct or indirect way. Pegylation on the surface of liposome helps to become liposome as long-circulating and indirect or passive targeting to tumors. The purpose of this study is to develop and optimize the critical process parameters, which play an important role in the quality pegylated liposome. The design of experiment (DoE) was used to study the impact of critical process variables like hydration temperature, extrusion process temperature, ethanol concentration, drug loading temperature, and drug loading time. Pegylated liposome was prepared using the ethanol injection method. Size reduction was achieved using the extrusion method. Drug encapsulation was achieved by a remote loading method using an ammonium phosphate gradient. A fractional factorial design was chosen for the optimization of process variables. Hydration temperature and extrusion process temperature directly impact on the degradation of lipids used in liposome formation. Higher temperature increases the lipid degradation during the process. The concentration of ethanol during the size reduction process inversely affects the particle size of the liposome. Higher the ethanol content lowers the particle size achieved. The temperature during drug loading process directly affects the degradation of the drug while inversely affect the encapsulation property. Stability study indicates that optimized formulation using DoE approach remains stable. The present research confirms the feasibility of developing and optimizing sterically stabilized liposome using DoE approach.

## Introduction

The efficacy of currently available anticancer agents is mainly compromised due to their drawbacks, like poor solubility, poor bioavailability, and lack of selectivity for normal and cancer cells resulted in toxicity. After entering into the body, every molecule pass through the liver and undergoes metabolism by Cytochrome P450 and other enzymes.[1] This first-pass metabolism changes the pharmacokinetic profile of the molecule by altering target specificity, solubility, and other physicochemical parameters that make the drugs inactive or with modified toxicity and bioavailability profiles. Change in pharmacokinetic profile leads to a larger dose to maintain therapeutic concentration. Similarly, lengthy infusion may require to overcome changed solubility challenges. Due to metabolism changes, organs responsible for drug clearance face more damages due to changed properties. For example, cisplatin causes severe nephrotoxicity,[2] and camptothecin regains activity in the acidic environment of the bladder to induce toxicity. Due to the non-selectivity of current chemotherapeutic agents, normal body cells that are in the growing stage, like bone marrow and digestive tissues are getting affected and causing most side effects associate with anti-cancer agents. To overcome these side effects,

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one of the alternatives is to change the molecule to its salt form or slight modification in the chemical moiety. But this approach may lead to giving additional toxicities or diminished activity.[3,4] So, different delivery platforms, like polymers, liposomes, and nanoparticles are being explored as effective methods to modulate drug activity.[5-9] Most of the large molecular carriers move drugs to the site of action, thereby limiting metabolism and reduce the toxicity. These carriers should have several favorable features, including but not limited to water solubility, long circulation times with half-lives ranging from hours to days, lack of toxicity and immunogenicity, high drug loading capacity, and biocompatibility. As the pharmacokinetic property of drugs will be decided by the carrier system, longer circulation and high drug loading capacity can be achieved by changing the different physicochemical properties of the carrier system. Further, these carrier systems offer an advantage of passive targeting of a molecule to the tumor site by the mechanism known as enhanced permeation and retention effect (EPR). Endothelial cells of the tumor have very poor vasculature due to high nutritional demand and rapid growth. There is very little or no lymphatic system available due to abnormal growth. This environment creates a favorable condition for novel nanocarriers to accumulate and target the encapsulated drugs at the site of the tumor. Normal tissue contains tight junctions between the blood capillary cells, so preventing macromolecules but small molecules can enter in normal tissue leads to poor tumor targeting and systemic toxicities.

Vincristine (VCR) is a plant alkaloid that gives marked antitumor activity.[10] As a microtubule inhibitor, at an M-phase cell-cycle specific antitumor activity, its efficacy is dependent on concentration and exposure time.[11] However, the pharmacokinetic (PK) profile reveals a rapid clearance rate and a large volume of distribution in the body.[12] This undesired PK profile and the dose-related toxicity limits its full potential.[13] Liposomal formulations are known to alter the PK profile of the drug by prolong plasma half-life and increase drug accumulation in tumor tissue with EPR effects and thus, mitigating drug toxicity.[14-17] In 2012, food and drug administration (FDA) approved the first vincristine sulfate liposome injection (VSLI; Marqibo®), which is made up of a sphingomyelin/ cholesterol liposome and by loading the drug, using pH gradient. It was approved for the patients with Philadelphia chromosome-negative (Ph-) acute lymphoblastic leukemia (ALL).[18] However, this formulation faces several challenges. The main issue is with its tedious preparation process. Due to its limited stability after drug-loaded liposome formation, to achieve a nominally stable product, the whole formulation is supplied as a three-vial kit, and its preparation needs an on-site, multi-step tedious drug loading process. The whole liposome preparation process must be done in a biological safety cabinet or by established hospital pharmacy safety procedures.[19] A big challenge for stable liposomal VCR preparation is the chemical instability of VCR. Stability data for approved formulation showed very limited stability of only 24 hours at room temperature.[20] The typical degradation route for VCR is oxidation and hydrolysis.[21] Yang *et al.*[22] attempted to reduce oxidation of VCR by incorporating antioxidants into VCR preparations. The result exhibited a positive output, yet far from long-term storage requirement. The degradation of VCR in aqueous media is mainly driven by hydrolysis.[20] The VCR is rather stable in solid-state or in an insulated oxygen atmosphere. Freeze-dried/lyophilized VCR liposomal formulation is one of the options explored by some researchers.[23] But the main problem with freeze-drying is the retention of desired formulation properties after reconstitution of the drug product apart from the other issues, like higher product costing and manufacturing time. Further, the critical quality attributes like encapsulation efficiency (EE) might be affected during the reconstitution process, and a high amount of free VCR content might be a safety concern. Apart from lyophilization, remote loading is one of the other options by which stable liposomal formulation of VCR can be prepared. Currently approved liposomal formulations of VCR encapsulate the drug but keep it in a solubilized form, which cannot avoid its degradation.[20,24,25] The ion gradient is an active loading method generally employed for amphipathic weak bases. Ammonium sulfate gradient and sucrose octasulfate triethylamine salt (TEA-SOS) gradient are some of the successful cases. Marketed drug product, Doxil®, is the first FDA approved nano-drug (1995), which employed an ammonium sulfate gradient to encapsulate the drug within the liposome, providing 18 months shelf life.[26,27] The sucrose octasulfate triethylamine salt (TEA-SOS) gradient was first reported for irinotecan liposomal formulation,[28] which gives higher loading efficiency and rather stable inner drug form with controlled drug release.[29]

In the present study, ammonium phosphate is proposed to use as a remote loading agent for efficient loading of vincristine, and it could be shown that vincristine gets precipitates not only in the presence of sulfate ions but also in the presence of phosphate ions. Vincristine could be efficiently loaded into liposomes with a transmembrane $(NH_4)(H_2PO_4)$-gradient. This turns out to be a superior alternative technique of loading vincristine into lipid vesicles. These liposomes show slow drug release rates. This may improve the applicability of vincristine liposomes at tumor sites, which exhibit a decreased pH-value compared with the non-tumor environment and help the drug to remain stable at the tumor site. Furthermore, this study contributes to the understanding of the different loading mechanisms of vincristine into liposomes. This technique might be an important alternative to other methods offering comparative therapeutic efficacy.

To optimize the process variables used during the manufacturing of vincristine liposome, fractional factorial

design was chosen for optimization. DoE is used to study the impact of critical process parameters, like hydration temperature, extrusion process temperature, ethanol concentration, drug loading temperature, and drug loading time. The selection of an optimum batch was done using a constraint-based graphical optimization technique. The optimum batch exhibited desired *in vitro* physicochemical parameters, like lipid degradation, drug degradation, encapsulation of vincristine, and particle size. The optimum batches were also tested *in vitro* testing in 50% human plasma and stability study.

## Materials and Method

### Materials

Hydrogenated soy phosphatidylcholine (HSPC) was obtained from Lipoid GmbH. Cholesterol (CH) was supplied by Dishman Netherland BV. 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (mPEG2000-DSPE) was purchased from Lipoid GmbH. VCR sulfate was purchased from Sigma. Oasis® HLB 1cc (30 mg) extraction cartridge, was obtained from the Water Inc., USA. All other chemicals were of analytical grade.

### Methods

#### *Preparation of Sterically Stabilized Liposome of Vincristine*

Sterically stabilized liposome has been prepared using the ethanol injection solvent evaporation method. Lipid excipients containing HSPC/CH/mPEG2000-DSPE (3/1/1, W/W) were dissolved in ethanol and injected into a solution containing a remote loading agent (ammonium phosphate, 250 mM solution) at predefined temperature of 65°C. Multilamellar vesicle (MLV) formed in the above step was introduced for size reduction using the extrusion technique (Lipex® by Northern Lipids). The extrusion process was carried out at 65°C using four polycarbonate membranes of 80 nm pore size at an operating pressure of 250 psi. The particle size distribution of formed small unilamellar liposomes was measured using dynamic light scattering (Malvern zeta sizer ZS-90) technique.

Liposomes were processed through an ultrafiltration assembly [with 750 Kd dialysis membrane made up of Polyethersulfone (PES) membrane]. Extra liposomal ammonium phosphate solution was replaced with a 10% sucrose solution. The ultrafiltration process was continued until the conductivity of the permeate solution became equal to that of the conductivity of a 10% sucrose solution. Upon complete replacement of outer medium (ammonium phosphate solution) with the intended medium (10% sucrose solution) for the extra liposomal environment, the ultrafiltration process was ended, and liposomal bulk was stored at 2 to 8°C, till further processing.

Empty liposome prepared in the above step contained the inner medium of ammonium phosphate, while the outer medium contained a 10% sucrose solution. To load the empty liposome with vincristine, an empty liposomal solution was kept above glass transition (Tg) temperature (58°C) under stirring. Vincristine sulfate was added in empty liposomal dispersion to get the final concentration of vincristine 0.16 mg/mL under stirring. The dispersion was incubated to load the drug inside liposome with maintaining the temperature of dispersion above Tg for a minimum of 60 minutes, followed by cooling of dispersion at a temperature between 2 to 8°C.

#### *Experimental Design*

A $2^{(5-1)}$ fractional factorial design (FFD) of experiments with three center points were selected in the optimization of the process variables. In the present investigation, lipid concentration in ethanol (X1), hydration temperature (X2), extrusion temperature (X3), drug loading heating temperature (X4), and drug loading heating time (X5) were selected as independent variables. Degradation of lipid (mg/mL), particle size (D90) (nm), drug degradation (%), and free drug (%) were selected as dependent variables to define design space. The experimental design with corresponding compositions is outlined in Tables 1 and 2. The experiment sequence was generated and randomized using Design Expert® ver.12 (Stat-Ease Inc., Minneapolis, MN 55413) software. A total of 19 experiments were designed by the software, including three center points. Table 3 lists the studied responses and their constraints.

**Table 1:** Formulation variables and their levels for fractional factorial design

| *Batch code* | *X1* | *X2* | *X3* | *X4* | *X5* |
|---|---|---|---|---|---|
| FD 1 | -1 | -1 | -1 | -1 | 1 |
| FD 2 | 1 | 1 | 1 | -1 | -1 |
| FD 3 | 1 | 1 | -1 | -1 | 1 |
| FD 4 | -1 | 1 | 1 | -1 | 1 |
| FD 5 | -1 | -1 | 1 | 1 | 1 |
| FD 6 | 1 | 1 | 1 | 1 | 1 |
| FD 7 | -1 | -1 | -1 | 1 | -1 |
| FD 8 | 1 | 1 | -1 | 1 | -1 |
| FD 9 | -1 | 1 | -1 | -1 | -1 |
| FD 10 | -1 | -1 | 1 | -1 | -1 |
| FD 11 | 0 | 0 | 0 | 0 | 0 |
| FD 12 | -1 | 1 | -1 | 1 | 1 |
| FD 13 | 1 | -1 | 1 | -1 | 1 |
| FD 14 | 1 | -1 | -1 | 1 | 1 |
| FD 15 | 0 | 0 | 0 | 0 | 0 |
| FD 16 | -1 | 1 | 1 | 1 | -1 |
| FD 17 | 1 | -1 | 1 | 1 | -1 |
| FD 18 | 0 | 0 | 0 | 0 | 0 |
| FD 19 | 1 | -1 | -1 | -1 | -1 |

**Table 2:** Translation of coded levels into actual values of independent variables

| | *Actual values* | | | | |
|---|---|---|---|---|---|
| *Coded levels* | *Lipid concentration in ethanol (X1)* | *Hydration temperature (X2)* | *Extrusion temperature (X3)* | *Drug loading heating temperature (X4)* | *Drug loading heating time (X5)* |
| -1 | 100 | 60 | 60 | 60 | 30 |
| 1 | 300 | 70 | 70 | 70 | 90 |

**Table 3:** Studied responses and their constraints

| *Responses (dependent variables)* | *Constraints (goal)* |
|---|---|
| Degradation of lipid (mg/mL) | NMT 10 mg/mL |
| Particle size (D90) (nm) | NMT 200 nm |
| Drug degradation (%) | NMT 0.2% |
| Free drug (%) | NMT 10% |

## Statistical Analysis

The statistical analysis of FFD batches was performed by Design Expert® ver.12 (Stat-Ease Inc., Minneapolis, MN 55413) software. All statistical analyses regarding DoE batches were performed using the same software. Response surface plots, overlaid contour plots were generated using the same software.

## Evaluation of Sterically Stabilized Liposome of Vincristine

### *Drug and Lipid Quantification*

Drug quantification was performed as per the reported literature.[30] Vincristine was quantified using HPLC with the ACE C18 column (150 × 4.6 mm, 5 µm) employing UV detection at 221 nm. Samples were eluted using a mixture of phosphate buffer 0.04 M, pH 3, and methanol. The separation was carried out using a gradient method, beginning at 30% methanol and increasing to 70% methanol. Flow speed was 1 mL/min, and the injection volume was 20 µL.

Lipid quantification was performed using HPLC with charged aerosol detector. All three lipids (HSPC, cholesterol, and mPEG-DSPE-2000) were quantified using a Durashell C18 (L), 150 × 4.6 mm, 5 µm, and 150 A° with a CAD detector (Dionex). The mobile phase consists of water/methanol (with ammonium acetate). Methanol was used as a diluent to prepare samples.

### *Degradation of Lipid*

Method to check the degradation of lipids was developed in the laboratory. The system included Waters HPLC (with injector loop for 200 µL injection volume) and Waters data system with refractive index (RI) detector. The column included was Inertsil ODS 3V, 250"x" 4.6 mm, 5 µ, (GL Sciences, Japan). The flow rate was 2 mL/min. The cell temperature of the detector was 45°C. 2% water in methanol was used as the mobile phase, as well as, diluent.

### *Particle Size Measurement*

Particle size distribution was measured using a dynamic light scattering technique (Nano Brook 90 Plus, Brook haven instrument). The analysis was performed by diluting samples to 10 times with water and measuring at a 90° angle.

### *Drug Degradation*

Method for the related compounds (drug degradation) of vincristine sulfate was developed at the laboratory by HPLC Methods. The analytical column was Shiseido C8 (250 × 4.6 mm, 5 µm), with a column temperature of 30°C. The mobile phase was composed of diethylamine of 15 mL with 985 mL water, adjusted pH to 7.5 by phosphate as A, methanol as B, detected by gradient elution. The detection wavelength was 297 nm, and the flow rate of 1.5 mL/min.

### *% Free Drug*

Encapsulation efficiency was measured by subtracting the free vincristine from the total vincristine. Free vincristine content in the liposomal dispersion was measured after separation. Separation of free vincristine was achieved using a solid-phase extraction cartridge [Oasis® HLB 1 cc (30 mg) extraction cartridge]. The quantification of the separated free drug was measured as described in the previous section. Encapsulated vincristine was calculated using the formula:

$$\%\ entraped\ vincristine = \frac{(\%\ content\ of\ total\ vincristine - content\ of\ \%\ free\ vincristine) \times 100}{\%\ content\ of\ total\ vincristine}$$

### *In vitro Leakage of Vincristine from Liposome*

*In vitro* drug leakage testing to characterize the physical state of the lipid bilayer and encapsulated vincristine should be investigated to support a lack of uncontrolled leakage under a range of physiological conditions and equivalent drug delivery to the tumor cells. Vincristine liposome was studied for *Vitro* drug leakage at 37°C in 50% human plasma for 24 hours. As per method, study was performed using 50% human plasma and K2EDTA was used as an anticoagulant. The analytical technique used was liquid chromatography–mass spectrometry/mass spectrometry (LC-MS/MS) with a mass spectrometer as a detection mode. Solid-phase extraction was used to determine free vincristine, while protein precipitation technique was used to determine total vincristine inside the plasma sample. In chromatographic and mass spectrometric system, hypurity $C_{18}$ (50 × 4.6 mm) 5 µm column was used with acetonitrile (pump-A):buffer (pump-B) (35:65), respectively, as mobile phase, and acetonitrile:water (80:20, v/v) as a rinsing solution. In mass parameters (API-4500 QTrap), ion source was electrospray ionization (ESI), and polarity was kept positive. % leakage of vincristine was measured using the following formula:

$$\%\ leakage\ at\ each\ time\ point = \frac{(\text{free drug at respective time point} - \text{free drug at 0 time}) \times 100}{Entrapped\ drug\ at\ 0\ time}$$

### *Cryo Transmission Electron Microscopy (TEM) Analysis of the Encapsulated Drug inside Loaded Liposomes*

Cryo TEM analysis was performed by a frozen-hydrated vitrified technique using a semi-automated system Vitrobot Mark IV. About 4 µL of the liquid sample was taken on a holey fomvar carbon film 200 mesh Cu (Cu-200HFC Pacific Grid Tech www.grid_tech.com) and transferred to a Gatan model 655 cryo holder with a cryo-transfer system, and then, cryo-transferred into the TEM goniometer, while maintaining the cold chain throughout imaging was done in a JEOL 2100 HRTEM operating at 200 KeV, while maintaining the sample holder at about -172 to -174°C, as measured by a Gatan Smart, set model 900, cold stage controller. Images were grabbed by Orius Camera Gatan make controlled by digital micrograph software.

### *Electrical Surface Potential or Surface Charge*

Surface charge or zeta potential was measured by the zeta sizer (Malvern Instrument, Nano ZS-90 model) using clear disposable zeta cells.

### *Stability Study*

The optimized formulation of vincristine liposome was filled in type 1 glass vial, capped with a rubber stopper, and kept at 2 to 8°C for stability study for three months. Various stability tests, like appearance, an assay of drug and lipid, drug and lipid degradation, free drug, and particle size were measured at different time points.

## Results

### Effect of Independent Factors on the Responses

Effect of lipid concentration in ethanol (X1), hydration temperature (X2), extrusion temperature (X3), drug loading heating temperature (X4), and drug loading heating time (X5) on various measured responses were summarized in Table 4.

### Evaluation of Dependent Variables

#### *Response 1: Effect of Independent Variables on Degradation of Lipid*

The Pareto chart for the effect of selected independent variables on degradation of lipid is shown in Fig. 1.

The selected factors were statistically analyzed, and the results of ANOVA analysis are represented in Table 5. The model F value of 65.25 implies the model is significant. There is only a 0.01% chance that an F value this large could occur due to noise.

The p values of less than 0.05 indicate model terms are significant. In this case, X2, X3, and X2 × X3 are significant model terms.

**Table 4:** Matrix of experiments of central composite design and measured responses

| *Batch Code#* | *X1* | *X2* | *X3* | *X4* | *X5* | *Degradation of lipid (mg/mL)* | *Particle size (D90) (nm)* | *Drug degradation (%)* | *Free drug (%)* |
|---|---|---|---|---|---|---|---|---|---|
| FD 1 | -1 | -1 | -1 | -1 | 1 | 0.1 | 159 | 0.16 | 4.5 |
| FD 2 | 1 | 1 | 1 | -1 | -1 | 0.9 | 156 | 0.02 | 11.2 |
| FD 3 | 1 | 1 | -1 | -1 | 1 | 0.5 | 186 | 0.15 | 5.6 |
| FD 4 | -1 | 1 | 1 | -1 | 1 | 0.9 | 139 | 0.14 | 6.1 |
| FD 5 | -1 | -1 | 1 | 1 | 1 | 0.3 | 131 | 0.19 | 0.7 |
| FD 6 | 1 | 1 | 1 | 1 | 1 | 0.8 | 159 | 0.16 | 0.8 |
| FD 7 | -1 | -1 | -1 | 1 | -1 | 0.2 | 176 | 0.13 | 4.5 |
| FD 8 | 1 | 1 | -1 | 1 | -1 | 0.3 | 181 | 0.14 | 3.7 |
| FD 9 | -1 | 1 | -1 | -1 | -1 | 0.4 | 154 | 0.16 | 12.6 |
| FD 10 | -1 | -1 | 1 | -1 | -1 | 0.3 | 145 | 0.02 | 15.1 |
| FD 11 | 0 | 0 | 0 | 0 | 0 | 0.4 | 151 | 0.1 | 1.9 |
| FD 12 | -1 | 1 | -1 | 1 | 1 | 0.5 | 165 | 0.18 | 12.4 |
| FD 13 | 1 | -1 | 1 | -1 | 1 | 0.5 | 171 | 0.14 | 4.6 |
| FD 14 | 1 | -1 | -1 | 1 | 1 | 0.2 | 179 | 0.12 | 11.9 |
| FD 15 | 0 | 0 | 0 | 0 | 0 | 0.4 | 150 | 0.12 | 2.1 |
| FD 16 | -1 | 1 | 1 | 1 | -1 | 0.9 | 136 | 0.16 | 6.1 |
| FD 17 | 1 | -1 | 1 | 1 | -1 | 0.4 | 141 | 0.15 | 5.9 |
| FD 18 | 0 | 0 | 0 | 0 | 0 | 0.5 | 149 | 0.11 | 2.3 |
| FD 19 | 1 | -1 | -1 | -1 | -1 | 0.1 | 183 | 0.03 | 14.8 |

Lipid concentration in ethanol (X1); hydration temperature (X2); extrusion temperature (X3); drug loading heating temperature (X4); drug loading heating time (X5)

**Table 5:** ANOVA analysis of response: lipid degradation

| *Source* | *Sum of squares* | *df* | *Mean square* | *F value* | *p value* | - |
|---|---|---|---|---|---|---|
| Model | 1.11 | 3 | 0.369 | 65.25 | < 0.0001 | Significant |
| B-hydration temperature | 0.6006 | 1 | 0.6006 | 106.22 | < 0.0001 | - |
| C-extrusion temperature | 0.4556 | 1 | 0.4556 | 80.57 | < 0.0001 | - |
| BC | 0.0506 | 1 | 0.0506 | 8.95 | 0.0097 | - |
| Curvature | 0.0013 | 1 | 0.0013 | 0.2346 | 0.6356 | - |
| Residual | 0.0792 | 14 | 0.0057 | - | - | - |
| Lack of fit | 0.0725 | 12 | 0.006 | 1.81 | 0.4101 | Not significant |
| Pure error | 0.0067 | 2 | 0.0033 | - | - | - |
| Cor total | 1.19 | 18 | - | - | - | - |

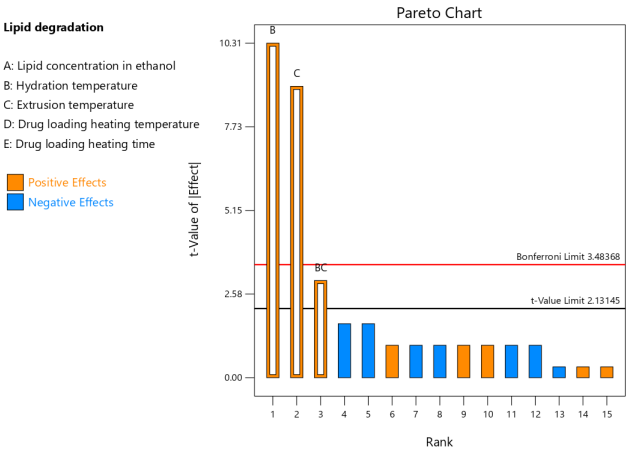


**Fig. 1:** Pareto chart for the selection of significant effects of independent variables on lipid degradation

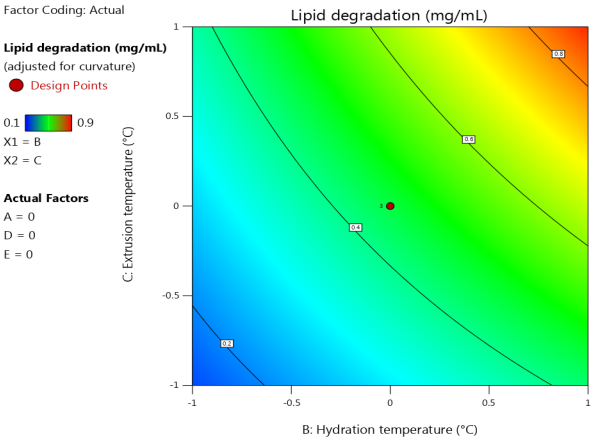


**Fig. 2:** Contour plot for the effect of an independent variable on lipid degradation

The lack of fit F value of 1.81 implies the lack of fit is not significant relative to the pure error. There is a 41.01% chance that a lack of fit F value this large could occur due to noise.

The predicted $R^2$ of 0.8787 is in reasonable agreement with the adjusted $R^2$ of 0.9189, i.e., the difference is less than 0.2. Adeq precision measures the signal to noise ratio. A ratio greater than 4 is desirable. A ratio of 18.794 indicates an adequate signal.

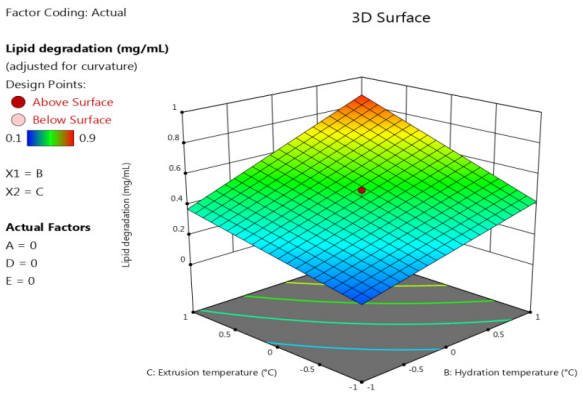


**Fig. 3:** 3D surface plot for the effect of an independent variable on lipid degradation

The response plots, including contour plots (Fig. 2) and 3D surface plots (Fig. 3) of all the significant model terms, are depicted in the succeeding section.

The results of lipid degradation were found to be directly proportional to hydration temperature and extrusion temperature.

### *Response 2: Effect of Independent Variables on Particle Size*

The Pareto chart for the effect of selected independent variables on particle size is shown in Fig. 4.

The selected factors were statistically analyzed, and the results of ANOVA analysis are represented in Table 6.

The model F value of 21.27 implies the model is significant. There is only a 0.01% chance that an F value this large could occur due to noise.

The p values less than 0.05 indicate model terms are significant. In this case, X1, X3, and X3 × X4 are significant model terms.

The lack of fit F value of 43.06 implies the lack of fit is significant. There is only a 2.29% chance that a lack of fit F value this large could occur due to noise.

The predicted $R^2$ of 0.7496 is in reasonable agreement with the adjusted $R^2$ of 0.8774, i.e., the difference is less than 0.2.

**Table 6:** ANOVA analysis of response: particle size (D90)

| *Source* | *Sum of squares* | *df* | *Mean square* | *F value* | *p value* | - |
|---|---|---|---|---|---|---|
| Model | 4,519.37 | 6 | 753.23 | 21.27 | < 0.0001 | Significant |
| A-lipid concentration in ethanol | 1,425.06 | 1 | 1,425.06 | 40.24 | < 0.0001 | - |
| C-extrusion temperature | 2,626.56 | 1 | 2,626.56 | 74.17 | < 0.0001 | - |
| D-drug loading heating temperature | 39.06 | 1 | 39.06 | 1.1 | 0.3161 | - |
| E-drug loading heating time | 18.06 | 1 | 18.06 | 0.51 | 0.49 | - |
| AE | 162.56 | 1 | 162.56 | 4.59 | 0.0554 | - |
| CD | 248.06 | 1 | 248.06 | 7 | 0.0227 | - |
| Curvature | 255.8 | 1 | 255.8 | 7.22 | 0.0211 | - |
| Residual | 389.56 | 11 | 35.41 | - | - | - |
| Lack of fit | 387.56 | 9 | 43.06 | 43.06 | 0.0229 | Significant |
| Pure error | 2 | 2 | 1 | - | - | - |
| Cor total | 5,164.74 | 18 | - | - | - | - |

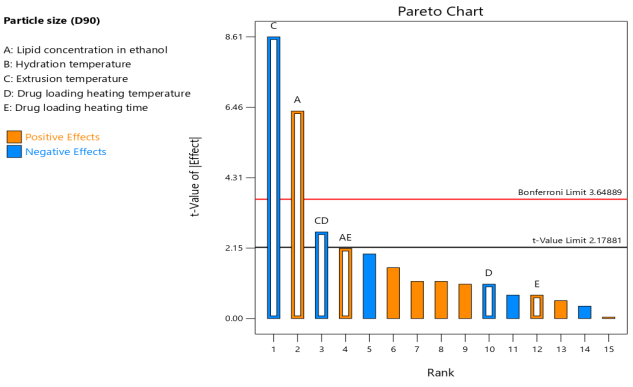


**Fig. 4:** Pareto chart for selection of significant effects of independent variables on particle size

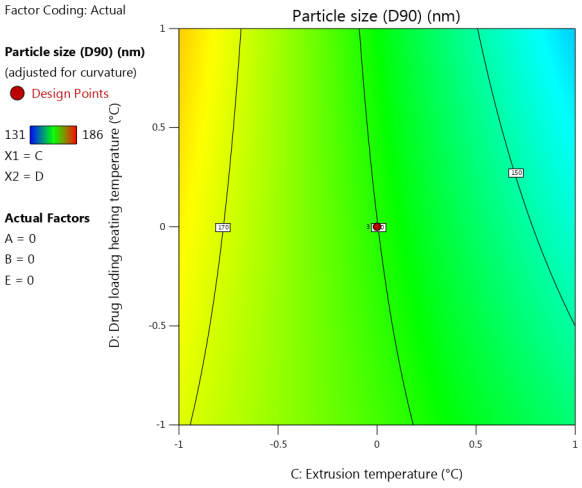


**Fig. 5:** Contour plot for the effect of the independent variable on particle size

Adeq precision measures the signal to noise ratio. A ratio greater than 4 is desirable. A ratio of 15.214 indicates an adequate signal.

The response plots, including contour plots (Fig. 5) and 3D surface plots (Fig. 6) of all the significant model terms, are depicted in the succeeding section.

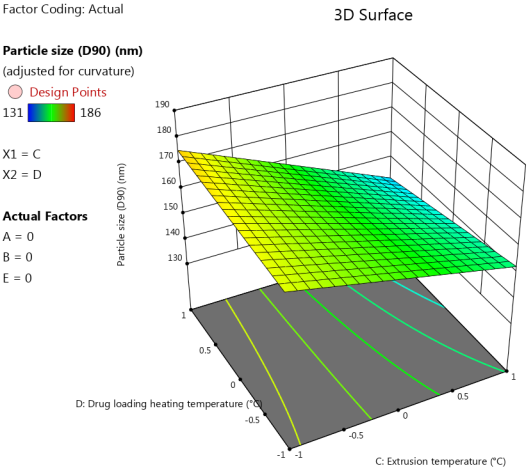


**Fig. 6:** 3D surface plot for the effect of the independent variable on particle size

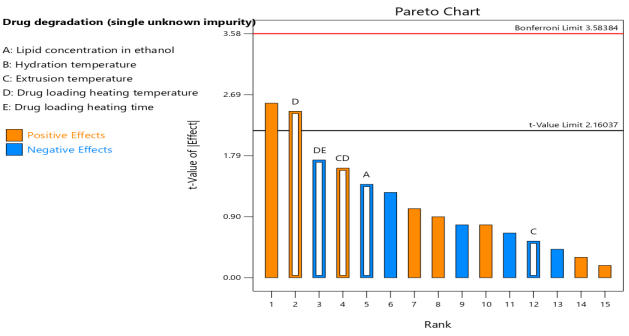


**Fig. 7:** Pareto chart for the selection of significant effects of independent variables on drug degradation

The results of particle size was inversely proportional to lipid concentration while directly proportional to extrusion temperature.

### *Response 3: Effect of Independent Variables on Drug Degradation*

The Pareto chart for the effect of selected independent variables on drug degradation is shown in Fig. 7.

**Table 7:** ANOVA analysis of response: drug degradation

| *Source* | *Sum of squares* | *df* | *Mean square* | *F value* | *p value* | - |
|---|---|---|---|---|---|---|
| Model | 0.0324 | 5 | 0.0065 | 6.04 | 0.0051 | significant |
| C-extrusion temperature | 0.0005 | 1 | 0.0005 | 0.4723 | 0.505 | - |
| D-drug loading heating temperature | 0.0105 | 1 | 0.0105 | 9.8 | 0.0087 | - |
| E-drug loading heating time | 0.0116 | 1 | 0.0116 | 10.78 | 0.0065 | - |
| CD | 0.0046 | 1 | 0.0046 | 4.25 | 0.0616 | - |
| DE | 0.0053 | 1 | 0.0053 | 4.90 | 0.0469 | - |
| Curvature | 0.0008 | 1 | 0.0008 | 0.7743 | 0.3962 | - |
| Residual | 0.0129 | 12 | 0.0011 | - | - | - |
| Lack of fit | 0.0127 | 10 | 0.0013 | 12.66 | 0.0754 | not significant |
| Pure error | 0.0002 | 2 | 0.0001 | - | - | - |
| Cor total | 0.0461 | 18 | - | - | - | - |

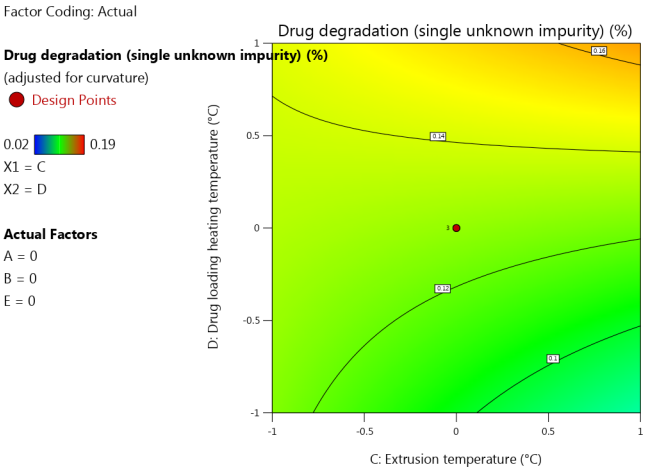


**Fig. 8:** Contour plot for the effect of an independent variable on drug degradation

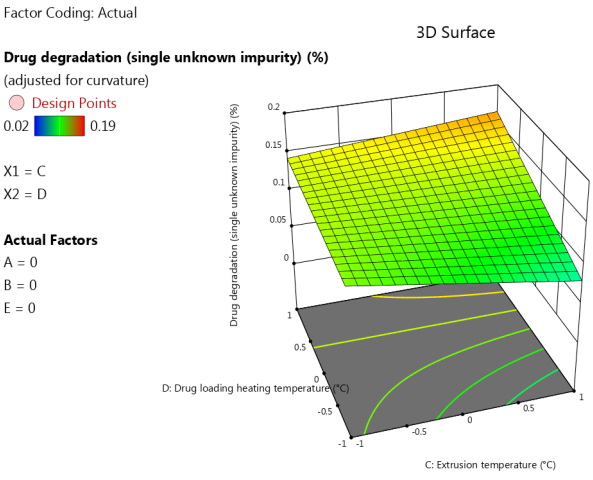


**Fig. 9:** 3D surface plot for the effect of an independent variable on drug degradation

The selected factors were statistically analyzed, and the results of ANOVA analysis are represented in Table 7.

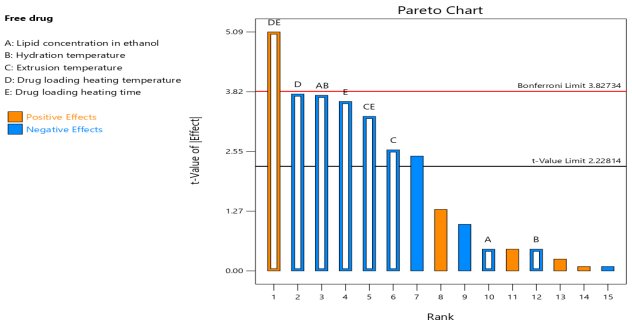


**Fig. 10:** Pareto chart for selection of significant effects of independent variables on % free drug

The model F value of 6.04 implies the model is significant. There is only a 0.51% chance that an F value this large could occur due to noise.

The p values less than 0.05 indicate model terms are significant. In this case, X4, X5, and X4 × X5 are significant model terms.

Adeq precision measures the signal to noise ratio. A ratio greater than 4 is desirable. A ratio of 6.982 indicates an adequate signal. This model can be used to navigate the design space.

The response plots, including contour plots (Fig. 8) and 3D surface plots (Fig. 9) of all the significant model terms, are depicted in the succeeding section.

The results of drug degradation were found to be directly proportional to the drug loading heating temperature.

### *Response 4: Effect of Independent Variables on % Free Drug*

The Pareto chart for the effect of selected independent variables on % free drug is shown in Fig. 10.

The selected factors were statistically analyzed, and the results of ANOVA analysis are represented in Table 8.

The model F value of 10.64 implies the model is significant. There is only a 0.09% chance that an F value this large could occur due to noise.

The p values less than 0.05 indicate model terms are significant. In this case, X3, X4, X5, X1 × X2, X3 × X4, and X4 × X5 are significant model terms.

The lack of fit F value of 114.59 implies the lack of fit is significant. There is only a 0.87% chance that a lack of fit F value this large could occur due to noise.

Adeq precision measures the signal to noise ratio. A ratio greater than 4 is desirable. A ratio of 9.761 indicates an adequate signal. This model can be used to navigate the design space.

The response plots, including contour plots (Fig. 11) and 3D surface plots (Fig. 12) of all the significant model terms, are depicted in the succeeding section.

The results of % free drug were found to be inversely proportional to drug loading heating temperature and time.

## Graphical Optimization of Measured Responses (Overlay Plot)

The design space was established using Design Expert® Ver.12 (Stat-Ease Inc., Minneapolis, MN 55413), based on given constraints for measured responses. Based on available data for the independent variable, the "overlay plot," as shown in Fig. 13, was obtained through graphical optimization. The design space is shown in yellow color. Independent factors with levels selected within design-space yield desired results within given specifications. From the data, batches FD 11, FD 15, and FD 18 [lipid concentration in ethanol: 200 mg/g (X1), hydration temperature: 65°C (X2), extrusion temperature: 65°C (X3), drug loading heating temperature: 65°C (X4), and drug loading heating time: 60 minutes (X5)] were found to be optimum to get desired results.

## Checkpoint Batches and Cross-Validation of DoE Model

Two experiments were performed at different parameters of

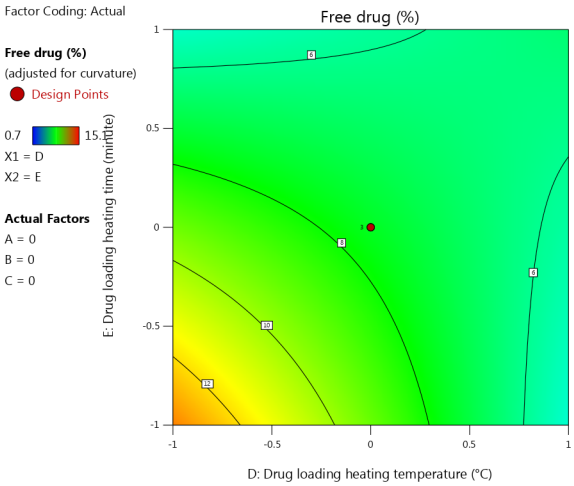


**Fig. 11:** Contour plot for the effect of an independent variable on % free drug

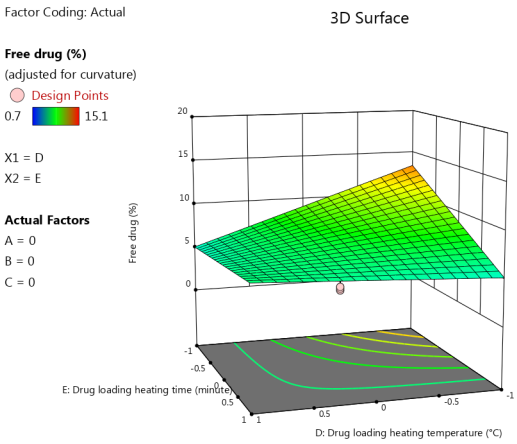


**Fig. 12:** 3D surface plot for the effect of an independent variable on % free drug

**Table 8:** ANOVA analysis of response: % free drug

| *Source* | *Sum of squares* | *df* | *Mean square* | *F value* | *p value* | - |
|---|---|---|---|---|---|---|
| Model | 304.09 | 8 | 38.01 | 10.64 | 0.0009 | Significant |
| A-lipid concentration in ethanol | 0.7656 | 1 | 0.7656 | 0.2142 | 0.6545 | - |
| B-hydration temperature | 0.7656 | 1 | 0.7656 | 0.2142 | 0.6545 | - |
| C-extrusion temperature | 23.77 | 1 | 23.77 | 6.65 | 0.0298 | - |
| D-drug loading heating temperature | 50.77 | 1 | 50.77 | 14.2 | 0.0044 | - |
| E-drug loading heating time | 46.58 | 1 | 46.58 | 13.03 | 0.0057 | - |
| AB | 50.06 | 1 | 50.06 | 14.01 | 0.0046 | - |
| CE | 38.75 | 1 | 38.75 | 10.84 | 0.0093 | - |
| DE | 92.64 | 1 | 92.64 | 25.92 | 0.0007 | - |
| Curvature | 74.52 | 1 | 74.52 | 20.85 | 0.0014 | - |
| Residual | 32.16 | 9 | 3.57 | - | - | - |
| Lack of fit | 32.08 | 7 | 4.58 | 114.59 | 0.0087 | Significant |
| Pure error | 0.0800 | 2 | 0.04 | - | - | - |
| Cor total | 410.78 | 18 | - | - | - | - |

lipid concentration in ethanol (X1), hydration temperature (X2), extrusion temperature (X3), drug loading heating temperature (X4), and drug loading heating time (X5) to check the reliability of the model at values other than those used in experimental design. Bias or % relative error was calculated for each response as per the following equation;

$$\%\ bias\ = \left[\frac{Predicted\ value - Experimental\ value}{Predicted\ value} \times\ 100\%\right]$$

From the data, it can be deduced that the equations satisfactorily demonstrate the influence of process variables on the responses of the study due to fairly good agreement between the predicted and experimental values in both checkpoint batches.

## *In vitro* Leakage of Vincristine from Liposome

*In vitro* drug leakage testing was performed to check the *in vitro* behavior of optimized batch obtained after DoE trials. Additionally, encapsulated vincristine should be investigated to support a lack of uncontrolled leakage under a range of physiological conditions and equivalent drug delivery to the tumor cells.

To evaluate the *in vivo* behavior of vincristine liposome *in vitro* drug leakage studies have been conducted at 37°C in 50% human plasma for 24 hours.

*In vitro* drug leakage at 37°C in 50%, human plasma for 24 hours is used to study the release behavior of entrapped vincristine from the sterically stabilized long circulating liposome. Free vincristine was measured from 50% human plasma at 10 times dilution at different time point till 24 hours. Based on the above parameters, % leakage was calculated and data are presented in Fig. 14.

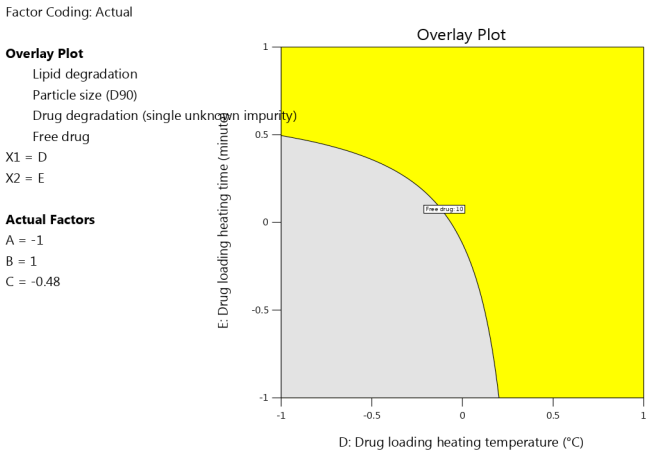


**Fig. 13:** Overlay plot showing design space for vincristine liposome

The *in vitro* leakage of vincristine in 50% human plasma, when incubated at 37°C, was studied at 200 µg/mL (10 times dilution) till 24 hours. The % leakage with 200 µg/mL (10 times dilution), was found to be ~1% till 24 hours. The results show that liposomal formulation is having long circulation and slowly release characteristic, which helps them to be long-circulating inside the body and passively targeting the tumor with desired concentration.

## Cryo TEM

The objective of the study was to evaluate the cryo TEM analysis of vincristine liposome with placebo liposome. Cryo TEM analysis of vincristine liposome and placebo liposome are discussed and presented in Tables 9 and 10.

Cryo-TEM images of vincristine liposome and placebo liposome are presented in Fig. 15.

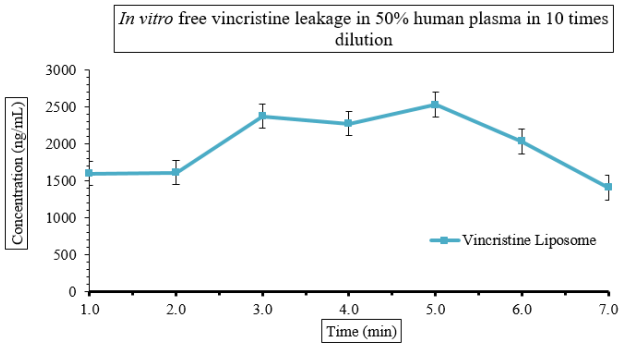


**Fig. 14:** *In vitro* free vincristine leakage in 50% human plasma in 10 times dilution

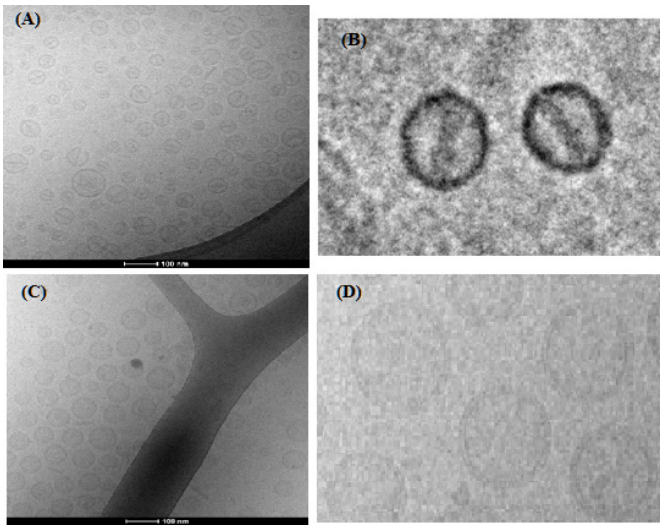


**Fig. 15:** Cryo TEM images of **(A)** Vincristine liposome-lower magnification; **(B)** Vincristine liposome-higher magnification; **(C)** Placebo liposome-lower magnification; **(D)** Placebo liposome-higher magnification

**Table 9:** Cryo TEM results for vincristine liposome and placebo liposome

| *Product name* | *Particle size* | | | *Lamellarity* | *Drug strand thickness* | *Number of liposomes observed* |
|---|---|---|---|---|---|---|
| | *Mean* | *Max* | *Min* | | | |
| Vincristine liposome | 57.3 | 93 | 33.4 | Unilamellar | 17.79 | 300 |
| Placebo liposome | 58.3 | 89.9 | 34 | Unilamellar | 17.8 | 296 |