APPLICATION OF RESPONSE SURFACE METHODOLOGY IN OPTIMIZATION OF PACLITAXEL LIPOSOMES PREPARED BY THIN FILM HYDRATION TECHNIQUE

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ORIGINAL ARTICLE

Objective: The present investigation was aimed to optimize the formula of paclitaxel-loaded liposomes (PTL) by using the application of response surface methodology (RSM).

Methods: Paclitaxel-loaded liposome (PTL) was optimized by response surface methodology based on two parameters, namely, percent entrapment efficiency (% EE) and percent in vitro drug release at 12 h (% DR). The liposome formula was prepared using 3² factorial design, and the selected independent variables were, phospholipid (phospholipon 90G) and cholesterol (CH) concentrations. Nine formulas of paclitaxel-loaded liposome were prepared by thin film hydration technique (THF). The entrapment efficiency, in vitro release studies and drug content, were evaluated using UV-visible spectrophotometer at λmax=230 nm. The developed PTL formulation revealed morphology, particle size, polydispersity index (PDI) and zeta potential (ζ) were evaluated by Motic digital microscope and Malvern zetasizer respectively.

Results: Using response surface methodology the estimated coefficient values obtained for independent variables in the regression equations, exhibited that the phospholipid (PL90G) and cholesterol (CH) molar concentration was observed to be highly influencing variables in optimizing % EE (86.67±0.67) and % DR (63.49±1.21). In the prediction of % EE and % DR values, the percent relative errors (PRE) was found to be low (–0.290%) and (0.058%) respectively. This suggests that design-developed model was found to be suitable for PTL formulations and thus, validate the model.

Conclusion: Experimental results show that the observed responses were in close agreement with the predicted values and this demonstrates the reliability of the RSM in an optimization of % EE and % DR in paclitaxel liposomal (PTL) formulations.

Keywords: Response surface methodology, Paclitaxel, Liposomes, Thin film hydration technique

INTRODUCTION

Paceltaxel (PT) is a chemical compound isolated from the bark of Taxus brevifolia (northwest Pacific Yew Tree), empirical formula (C27H46NO13) and on the basis of characterization named it as Taxol [1]. It has the potential anticancer drug based on previous reports, PT shows anticancer activities towards breast cancer [2], ovarian cancer [3], lung cancer [4] and pancreatic cancer [5]. However, PT exhibits poor aqueous solubility and permeability owing to biopharmaceutical classification system (BCS) class IV drug, which directs it to low bioavailability. Therefore, by consideration of these problems, there is a need to develop a novel formulation of such effective and efficient anticancer drug.

For improving poor aqueous solubility and permeability of PT, a number of formulation strategies have been developed and used. Some of them were modified, due to some excipient-drug interactions. For improving the solubility, PT dissolved in a mixture of polyoxyethylated castor oil (Cremophore EL):dehydrated ethanol (1:1) ratio as a delivery vehicle. The formulation produced hypersensitivity and non-linear pharmacokinetic behavior after intravenous administration. The hyperresitivity reaction at the site of administration could be due to an inclusion of Cremophore EL [6-7]. After that, the delivery vehicle was replaced with the addition of tween 80 alone or combination of tween 80: dehydrated alcohol, and diluted with aqueous media. The diluted formulation showed the precipitation of PT from solution due to low solubility [8]. These attempted techniques, with persistent low solubility problem, has been overcome by creating novel formulation with the aim of improving aqueous solubility, permeability, and bioavailability of PT. It includes novel oral formulation [9], novel PT self-emulsifying drug delivery system (SEDDS) [10], novel ligands based PT targeting formulation [11], micellar formulation [12], liposomal formulation [13], bioconjugates [14], dendrimers [15] and nanocarrier systems [16]. In all these formulation techniques the problem associated with PT was shown to be improved significantly.

The liposome is emerging techniques for specialized drug delivery [17] and best suitable for lipophilic drug due to its biocompatibility and reducing drug toxicity, with maintaining efficacy of the anticancer drug for a maximum period of time. Some previous studies include asulacrine [18], docetaxel [19] and tamoxifen [20] with these approaches, their poor aqueous solubility and bioavailability were found to be improved. So, need to develop and optimize the paclitaxel-loaded liposomes (PTL) for effective anticancer treatment. In pharmaceutical technology, in the development and optimization of different pharmaceutical dosage forms, there are a high number of factors which influence the product characteristics. Therefore, complex, expensive and time-consuming formulation studies are often necessary for the development of a product with required and desired properties. The experimental design methodology is a strategy to use a smaller number of experiments and to avoid unnecessary experiments [21-22].

Experiments were designed to determine the effect of the independent variables (factor) on the dependent variable (parameter/response) of a process or formulation. RSM, one of the designs of experiments, is a powerful tool for determining the relationship between a response and a set of quantitative involved factors. RSM is a technique used to find the optimum response by using the quadratic polynomial model [23]. The advantage of RSM is the reduced amount of experiments required, thereby reducing the cost of expensive analysis methods. The application of RSM is useful for understanding or mapping a region of the response surface, finding the variable level of optimum response, and selecting the process condition or formula to meet the specifications [24]. This research was carried out to optimize PTL formula with independent variables such as phospholipid (phospholipon 90G) concentration and cholesterol (CH) concentration. The optimum formula was obtained from RSM using 3² full factorial design. The optimization approach was applied to obtain desired % EE and % DR for PTL.
MATERIALS AND METHODS

Materials

Paclitaxel (PT), (purity>90%) was received as a gift sample from MAC-CHEM Products (India) Pvt. Ltd. Bharosa, Thane, India. The phospholipids samples viz., Phospholipon 90G® (PL90G), Phospholipon 80H® (PL80H) and Phospholipon 90H® (PL90H) with purity >90%, was obtained as a free gift sample from Lipoid GmbH, Ludwigshafen, Germany. The solvents namely chloroform and methanol were purchased from Merck Ltd. Mumbai, India. Cholesterol (CH), potassium dihydrogen phosphate and sodium hydroxide pellets were obtained from Sigma Chemicals, Sigma Aldrich Corporation, St. Louis, MO. Chemical used in this work were of analytical grade (AR).

Experimental design (3² full factorial design)

To reduce the number of trials and attain the highest amount of information on product properties, the screening was done by applying full factorial design (3²), systematically study the joint influence of the independent variables on the dependent variables. So, in this study two factors were evaluated, each at three levels and experimental trials was performed at all nine possible combinations. Amount of phospholipid (PL90G-1, 2 and 3 moles) was taken as the first independent variable (X₁, w) and amount of cholesterol (CH-1, 2 and 3 moles) was selected as the second independent variables (X₂, w) for liposomes. These variables varied at three levels, low level (-1), medium level (0), and high level (+1).

Design Expert software. Among them, PRESS indicates how well the model fits the data and for the chosen model it should be small relative to the other models under consideration. Level of significance was considered at p<0.05. Mathematical relationships in the form of polynomial equations are generated using multiple linear regression analysis (MLRA). A second-order polynomial equation that describes the effect of independent factors on the response is expressed in the following forms:

\[
Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_12 X_1X_2 + \beta_11 X_1^2 + \beta_22 X_2^2
\]

(1)

Where Y is the dependent variable; \(\beta_0\), \(\beta_1\), \(\beta_2\), \(\beta_12\), \(\beta_11\), and \(\beta_22\) are the coefficients for the linear, interaction and quadratic terms, respectively. The main effects \(X_1\) and \(X_2\) represent the average change in the response variable for a unit change in the corresponding factor. The interaction term \(X_1X_2\) describes the effect of changing one factor at a time from its low to high value.

The relative error (PRE) by the following equation (4).

\[
\% \text{ Relative error} = \frac{|\text{Actual value} - \text{Predicted value}|}{\text{Actual value}} 
\]

(4)

Table 1: 3² full factorial design: factors, factor levels and responses for PTL formulation

| Factors (Independent variables) | Factor levels used | Amount (mole) of phospholipid (PL90G) (X₁, w) | Amount (mole) of cholesterol (CH) (X₂, w) |
|---------------------------------|---------------------|---------------------------------------------|-------------------------------------------|
| Low (-1)                        | Medium (0)          | High (+1)                                   |                                           |
| 1                               | 2                   | 3                                           |                                           |
| 1                               | 2                   | 3                                           |                                           |

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\[
\text{Statistical analysis and optimization of formulation using RSM}
\]

Response surface modeling and evaluation of the quality fit of the model for the current study were performed employing Design Expert® DX 10.0.7.0 license version software [23-26, 30]. Polynomial models including linear, interaction and quadratic terms were generated for all the response variables using multiple linear regression analysis (MLRA). A second-order polynomial equation that describes the effect of independent factors on the response is expressed in the following forms:

\[
Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_12 X_1X_2 + \beta_11 X_1^2 + \beta_22 X_2^2
\]

(2)

The 10 µM PT (mol wt., 853.9) constant for all batches and the required quantities of phospholipid (PL90G) (mol. wt., 758.07) and cholesterol (CH) (mol. wt., 386.67) were taken in a 100 ml round bottom flask and dissolved in 10 ml chloroform. All the batches were prepared according to the experimental design in table 1. Choloroform was evaporated using rotary vacuum evaporator (Model: PBV–7D, Vertical condenser, rotavap, superfit™ continental Pvt. Ltd., Mumbai, India) and kept overnight under vacuum. Then it was hydrated by 15 ml of phosphate buffer pH 7.4 for 1 h with 10 min of extensive vortexing. The suspension of liposomes was sonicated in the water bath at 60 °C to reduce the size of liposomes. Non-incorporated PT was separated by ultracentrifuge at 10,000 rpm for 30 min at 4 °C. The supernatant was then discarded and PT loaded liposomes in the precipitate were redispersed in required volume of phosphate buffer pH 7.4. This was transferred to vials and stored at 4°C [27].

Preparation of paclitaxel-loaded liposomes (PTL)

Liposomes were prepared by the thin film hydration method (TFH). The 10 µM PT (mol wt., 853.9) constant for all batches and the required quantities of phospholipid (PL90G) (mol. wt., 758.07) and cholesterol (CH) (mol. wt., 386.67) were taken in a 100 ml round bottom flask and dissolved in 10 ml chloroform. All the batches were prepared according to the experimental design in table 1. Choloroform was evaporated using rotary vacuum evaporator (Model: PBV–7D, Vertical condenser, rotavap, superfit™ continental Pvt. Ltd., Mumbai, India) and kept overnight under vacuum. Then it was hydrated by 15 ml of phosphate buffer pH 7.4 for 1 h with 10 min of extensive vortexing. The suspension of liposomes was sonicated in the water bath at 60 °C to reduce the size of liposomes. Non-incorporated PT was separated by ultracentrifuge at 10,000 rpm for 30 min at 4 °C. The supernatant was then discarded and PT loaded liposomes in the precipitate were redispersed in required volume of phosphate buffer pH 7.4. This was transferred to vials and stored at 4°C [27].

Determination of percent drug release (% DR)

In vitro drug release

All the calculations were done at milligram level. Amount of PT (10 µM) and final formulation volume 15 ml was kept constant. Percent entrapment efficiency (% EE) (Y₁) and percent in vitro drug release at 12 h (% DR) (Y₂) were selected as dependent variables. Values of variables and batch codes are shown in table 1 and 2. Design Expert® DX 10.0.7.0 license version software was used for the generation and evaluation of statistical experimental design [25-26].

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Purification of PTL formulation was done by the ultracentrifugation method [26]. To quantify the amount of entrapped PT, 2 ml of the vesicular dispersion was centrifuged at 10,000 rpm for 1 h at the...
controlled temperature of 4 °C (Remi cooling centrifuge, Remi Elektrotechnik limited, India). Supernatant contains unentrapped drug was withdrawn and measured UV spectrophotometrically (at \( \lambda_{\text{max}} = 230 \text{ nm} \)) (Model: SPECTRO 2060 PLUS, Analytical Technologies Ltd., Gujarat, India) against 30:70 ratio of methanol: phosphate buffer solution (PBS) (pH 7.4). All the determinations were made in triplicate. A calibration plot was produced by diluting stock solutions of PT with 30:70 ratio of methanol and PBS (pH 7.4).

% EE was calculated and expressed as a percent of the available stock solutions of PT with 30:70 ratio of methanol and PBS (pH 7.4). All the determinations were carried out as per the procedure described by Utreja [29] with little modifications. In brief, the Franz diffusion cell apparatus was employed for this study. The apparatus is consisted of donor and receptor compartment, with an effective surface area for dissolution of (2.303 cm\(^2\)). A dialysis membrane (LA395, Dialysis Membrane– Elektrotechnik limited, India). Supernatant contains unentrapped solute actually encapsulated. The amount of drug entrapped in liposomes was determined by equation 1.

\[
\text{% entrapment efficiency (}\% \text{ EE}) = \frac{\text{Amount of drug in liposomes}}{\text{Total amount of drug}} \times 100
\]  

Percent in vitro drug release study (% DR) at 12 h.

The in vitro drug release study for PT from different PTL formulation was carried out as per the procedure described by Utreja [29] with little modifications. In brief, the Franz diffusion cell apparatus was employed for this study. The apparatus is consisted of donor and receptor compartment, with an effective surface area for dissolution of (2.303 cm\(^2\)). The dialysis membrane (LA395, Dialysis Membrane– Elektrotechnik limited, India). Supernatant contains unentrapped solute actually encapsulated. The amount of drug entrapped in liposomes was determined by equation 1.

\[
\text{Percent in vitro drug release (}\% \text{ DR}) = \frac{\text{Percent drug content in liposomes}}{\text{Percent drug content in stock solution}} \times 100
\]  

###RESULTS AND DISCUSSION

####Experimental design and data acquiring (3\(^{rd}\) full factorial design)

Full factorial design (3\(^{rd}\)) was applied to optimize the PTL formulation. All nine batches of PTL were prepared according to the formulation variables as shown in Table 2. Liposomes were obtained by the TFH method. RSM was exploited to estimate the influence of the molar ratio of PL90G and CH as independent variables and their interactions on the investigated responses (dependent variables: % EE and % DR). This experiment was aimed to identify considerable factor effect influencing the formulation performance and to set up to their excellent levels for the desirability of responses shown in Table 2.

####Statistical analysis and optimization of formulation using RSM

To evaluate the quantitative effects of factors (X1 and X2) and their levels low (-1), middle (0), and high (+1) on the preferred responses, the experimental values of the flux were analyzed by Design Expert\(^{\text{®}}\) DX 10.0.7.0 license version software and mathematical models obtained for each response [25-26, 30-31]. The mathematical relationship generated using multiple linear regression analysis (MLRA) for the studied response variables (% EE and % DR at 12 h.) that were related different response and independent variables are expressed as following polynomial equations (quadratic model).

\[
\text{Y}_1 (\% \text{ EE}) = 76.63+3.10X_1+9.44X_2+3.18X_1X_2+0.35X_1^2+0.91X_2^2  
\]

\[
\text{Y}_2 (\% \text{ DR12 h.}) = 64.74+6.79X_1-4.63X_2-1.38X_1X_2-15.29X_1^2-5.82X_2^2  
\]

The above equations expose the quantifiable effect of the independent variables, a molar ratio of PL90G and CH on the responses such as % EE (Y1) and in vitro % DR at 12 h (Y2) as dependent variables. The fitted polynomial equation (quadratic model) related to % EE and percent in vitro % DR used to draw a conclusion after considering the coefficient and the mathematical sign it carries, i.e. positive and negative. The correlation coefficient (r) of the quadratic model (0.9736) for response Y1 (% EE) and (0.9779) for response Y2 (% DR) was found to be significant.

####Response 1 (Percent entrapment efficiency) (% EE)

Regression analysis of above equation (6) of response Y1 (% EE) revealed that the coefficient of \( X_1 \) was positive and \( X_2 \) was negative, this indicated that as PL90G (X1) increased the % EE increased but as we further increased the PL90G (X1) to higher level the % EE decreased and on increasing cholesterol (X2) the % EE decreased. The higher concentration of cholesterol leads to rigidity in the vesicles [26] which in turn decreased the % EE. The % EE of different liposomal batches was in a range of 51.68 % to 86.67 %.

###Table 2: Composition 3\(^{rd}\) full factorial design with measured responses of PTL formulation

| Batches | X1 | X2 | Phospholipid (PL90G) in moles (X0, W) | Cholesterol (CH) in moles (X0, W) | Percent entrapment efficiency* (% EE)±SD | Percent In vitro drug release* (12 h) (% DR)±SD |
|---------|----|----|-------------------------------------|-------------------------------------|-------------------------------------------|-----------------------------------------------|
| L1      | -1 | -1 | 1                                   | 1                                   | 75.65±1.48                                | 40.28±1.58                                   |
| L2      | -1 | 0  | 1                                   | 2                                   | 64.2±0.82                                 | 43.6±0.98                                    |
| L3      | -1 | +1 | 1                                   | 3                                   | 51.68±0.75                                | 9.32±1.02                                    |
| L4      | 0  | -1 | 2                                   | 1                                   | 86.67±0.67                                | 63.4±1.21                                    |
| L5      | 0  | 0  | 2                                   | 2                                   | 78.69±1.61                                | 62.1±0.63                                    |
| L6      | 0  | +1 | 2                                   | 3                                   | 65.2±1.17                                 | 56.9±1.33                                    |
| L7      | +1 | -1 | 3                                   | 1                                   | 77.1±1.53                                 | 56.3±1.42                                    |
| L8      | +1 | 0  | 3                                   | 2                                   | 67.1±0.91                                 | 57.8±0.98                                    |
| L9      | +1 | +1 | 3                                   | 3                                   | 65.8±1.32                                 | 42.9±1.19                                    |

*Values represented as mean±SD, n = 3, All batches contain drug 10 µM and 15 ml phosphate buffer (pH 7.4) for hydration.
Table 3: Analysis of variance (ANOVA) table of % EE

| Source            | Sum of squares | df | Mean squares | F Value | p-value | Prob > F |
|-------------------|----------------|----|--------------|---------|---------|----------|
| Model             | 829.57         | 5  | 165.91       | 22.14   | 0.0142  | Significant |
| X₁-Phospholipid (PL90G) | 57.72       | 1  | 57.72        | 7.70    | 0.0692  |           |
| X₂-Cholesterol (CH) | 534.68       | 1  | 534.68       | 71.36   | 0.0035  |           |
| X₁X₂              | 40.58          | 1  | 40.58        | 5.42    | 0.1024  |           |
| X₁²               | 196.35         | 1  | 196.35       | 26.20   | 0.0144  |           |
| X₂²               | 0.24           | 1  | 0.24         | 0.032   | 0.8693  |           |
| Residual          | 22.48          | 3  | 7.49         |         |         |           |
| Cor-total         | 852.05         | 8  |              |         |         |           |

For estimation of the significance of the model, the analysis of variance (ANOVA) was executed, from the ANOVA data, the model F-value of response (Y₁) (22.14) indicated that the model is significant shown in table 3. There is only a 1.42% chance that an F-value this large could occur due to noise. Values of “prob>F” less than 0.0500 indicate model terms are significant. In these case X₂, X₁X₂ are significant model terms. Values greater than 0.1000 indicate that model terms are not significant.

Table 4: Parameter of selected quadratic model of % EE

| Std. dev. | 2.74 |
|-----------|------|
| Mean      | 70.26|
| C. V. %   | 3.90 |
| PRESS     | 228.77|

The predicted R-squared value of 0.7315 is in reasonable agreement with the adjusted R-squared of 0.9296; i.e. the difference is less than 0.2. Adequate precision measures the signal to noise ratio. A ratio greater than 4 is desirable and the result of adequate precision was 15.693 indicates an adequate signal. So, this model can use to navigate the design space.

Response 2 (Percent drug release at 12 h) (% DR)

The effect on drug release at 12 h (% DR) (Y₂) was observed to be significant (P<0.05) by ANOVA and the polynomial equation (7) revealed that the coefficient of β₁ was positive and β₂ was negative, this indicated that as PL90G (X₁) increased the % DR increased and on increasing cholesterol (X₂) the % DR decreased. The % DR increased with increased concentration of lipid and at a certain level the percent release is retarded above that and the release was decreased at higher levels of cholesterol. This is because cholesterol at higher levels makes the lipid bilayers more rigid and retards the release of the drug. This was evident by the higher cholesterol concentration of vesicles showed around 50 % of the release except for (L6) formulations. The L4 formulation found to have 63.43 % DR at 12 h (table 2) with the composition of PL90G:CH (2:1 molar ratio) (0,-1). At lower concentration of phospholipid and cholesterol, the drug release was very less due to the formation of stagnant layer [26].
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**Fig. 2:** Response surface plot showing the effect of phospholipid (PL90G) ($X_1$) and cholesterol (CH) ($X_2$) on % EE ($Y_1$) of PTL

**Table 5:** Analysis of variance (ANOVA) table of % DR at 12 h

| Source          | Sum of squares | df | Mean squares | F Value | p-value | prob > F |
|-----------------|----------------|----|--------------|---------|---------|----------|
| Model           | 948.33         | 5  | 189.67       | 26.53   | 0.0109  | significant |
| A - Phospholipid| 276.62         | 1  | 276.62       | 38.70   | 0.0084  |          |
| B - Cholesterol | 128.81         | 1  | 128.81       | 18.02   | 0.0239  |          |
| $A^2$           | 467.57         | 1  | 467.57       | 65.41   | 0.0040  |          |
| $B^2$           | 67.74          | 1  | 67.74        | 9.48    | 0.0542  |          |
| Residual        | 21.44          | 3  | 7.15         |         |         |          |
| Cor Total       | 969.78         | 8  |              |         |         |          |

From the ANOVA data, the model F-value of response ($Y_2$) (26.53) indicated that the model is significantly shown in table 5. There is only a 1.09% chance that a model F-value this large could occur due to noise. Values of “prob>F” less than 0.0500 indicate model terms are significant. In these case, $X_1$, $X_2$, and $X_1^2$ are significant model terms. Values greater than 0.1000 indicate that model terms are not significant.

**Table 6:** Parameter of selected quadratic model of % DR at 12h

| Std. Dev. | 2.67 | R-squared ($r^2$) | 0.9779 |
|-----------|------|-------------------|--------|
| Mean      | 50.67| Adjusted R-Squared| 0.9410 |
| C. V. %   | 5.28 | Predicted R-Squared| 0.8063 |
| PRESS     | 187.85| Adequate Precision | 14.272 |

The predicted R-squared value of 0.8063 is in reasonable agreement with the adjusted R-squared of 0.9410; i.e. the difference is less than 0.2. Adequate precision measures the signal to noise ratio. A ratio greater than 4 is desirable and result of adequate precision was 14.272 indicates an adequate signal. So, this model can be used to navigate the design space.
To envisage the effect of an independent factor on the response ($Y_2$) the contour plot (fig. 3) and 3D-response surface plots (fig. 4) of % DR at 12 h can be obtained for a combination middle level of $X_1$ and low level of $X_2$ factors.

**Desirability and overlay plot**

The aim of pharmaceutical formulation optimization is generally to find the levels of the variable that affect the chosen responses and determine the levels of the variable from which a robust product with high-quality characteristics may be produced. All the measured responses that may affect the quality of the product were taken into consideration during the optimization procedure. The % EE and % DR at 12 h were set out in the maximum criteria. Each response criterion was combined (overlay plot) to obtain the optimum value (fig. 5). The optimization results of this research can be seen in table 7.

**Validation of RSM results**

In order to evaluate the optimization capability of models generated according to the results of the RSM (3^2 factorial design), PTL formulation was prepared using the optimal process variables settings that $X_1$ and $X_2$ were equal to 2:1. The response $Y_1$ (% EE) and $Y_2$ (% DR at 12 h) obtained with predicted models and the experimental model were shown in table 8. The percent relative error was obtained using equation 4. The percent relative error (PRE) for response $Y_1$ (% EE) and $Y_2$ (% DR at 12 h) were found to be (–0.290) and (0.058) respectively. The maximum PRE value was (–0.290). However, the values were found to be<2 % and hence it confirmed the suitability of experimental design. The results showed good agreement on preparation properties with theoretical properties.

**Table 7: Characteristics of optimum formula**

| Objects | Phospholipid (PL90G) in moles ($X_1$, W) | Cholesterol (CH) in moles ($X_2$, W) | % EE ($Y_1$, %) | In vitro % DR at 12 h ($Y_2$, %) | Desirability |
|---------|----------------------------------------|-----------------------------------|----------------|----------------------------------|--------------|
| Predicted | 1.996 | 1.000 | 86.419 | 63.527 | 0.996 | Selected |
| Actual (L4) | 2 | 1 | 86.67±0.67 | 63.49±0.21 | |

The optimization parameter of desirability was determined by regulating the optimum input variables to obtain one or more optimal parameters. The desirability value ranged between 0 and 1, where a value of 1 is perfect, i.e., the ideal parameter value [30]. The PTL desirability plot was shown in fig. 6. The optimizing desirability of PTL formulation was 0.996. This value was near to ideal value (1), meaning that the predicted parameters were desired parameter values. The composition of the predicted formulations was matching with L4 liposomes (table 7).

**Fig. 4: Response surface plot showing the effect of phospholipid (PL90G) ($X_1$) and cholesterol (CH) ($X_2$) on % DR at 12 h ($Y_2$) of PTL**

**Fig. 5: Overlay plot of % EE and % DR at 12 h**
Table 8: Validation of predicted and experimental PTL batch

| Response          | Experimental values | Predicted value | % Relative error (PRE) |
|-------------------|---------------------|-----------------|------------------------|
| \( Y_1 \) (% EE)  | 86.67±0.67          | 86.419          | -0.290                 |
| \( Y_2 \) (% DR at 12 h) | 63.49±1.21        | 63.527          | 0.058                  |

**Vesicle morphology, percent drug content, particle size, PDI and zeta potential (ζ) of developed PTL formulation**

Vesicle morphology of developed PTL formulation was observed by Motic Digital Microscope (type DM-1802). The liposomes were spherical in shape with a smooth surface shown in fig. 7. The developed liposomal percent drug content was found to be 98±1.0 %, (mean±SD, n = 3). Percent drug content indicated that the PT was uniformly distributed in vesicular dispersions and percent drug content near to 100 % indicated no loss of the material during the preparation.

Fig. 7: Microphotograph of PTL by motic image plus 2.0 ML software

Fig. 8: The particle size (A) and Zeta potential (B) of developed PTL formulation
The particle size (fig. 8A) and \( \zeta \) potential (fig. 8B) of developed PTL formulation was found to be 144.4 nm and (-) 22.6 mV. The particle size (fig. 8A) and \( \zeta \) zeta potential (fig. 8B) of developed piperazine-based liposomal formulations.

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AUTHORS CONTRIBUTION

This work was carried out in collaboration between all the four authors in the concept and design of the work, collection, assembly, analysis and interpretation of data, writing, critical revision and approval of the final manuscript.

CONFLICT OF INTERESTS

All authors have none to declare

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