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OPTIMIZATION OF FORMULATION PARAMETERS FOR PREPARATION OF DOCETAXEL LOADED NANOSTRUCTURED LIPID CARRIERS

H. A. Nair* and D. M. Soni

Department of Pharmaceutics, Bombay College of Pharmacy, Kalina, Santacruz (East), Mumbai, 400098 India

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Correspondence to Author:
H. A. Nair

Asst. Professor of Pharmacy, Bombay College of Pharmacy, Kalina, Santacruz (East), Mumbai, India 400098.

E-mail: hemajit@rediffmail.com

ABSTRACT: Present paper explores the preparation of Nanostructured Lipid Carriers (NLCs) loaded with the anticancer agent docetaxel trihydrate, with potential for targeted anticancer drug delivery. NLCs were prepared using Melt Emulsification Solvent Evaporation – Sonication method. The procedure adopted for preparation was optimized for obtaining NLCs with high entrapment efficiency for the drug and particle size in suitable range, using Quality by Design (QbD) approach employing Design-Expert® software. NLCs were prepared using solid lipids (stearylamine and glycerylmonostearate) and liquid lipids (tocopheryl acetate) with the aid of PhospholiponS100® as emulsifier and LutrolF68® as stabilizer. Independent variables selected were {total lipid to drug ratio} (A) and {solid lipids to liquid lipid ratio} (B). Total lipid to drug ratio (A) was fixed at four levels whereas solid lipid to liquid lipid ratio (B) was fixed at three levels. Optimization batches were prepared in duplicates and entrapment efficiency and mean particle size of each batch was estimated and data analyzed for statistical significance through ANOVA and by response surface and contour plots. The optimized batch identified was found to have desirability value of 0.955 and total lipid to drug ratio and solid to liquid lipid ratio of 20:1 and 2:1 respectively. Resultant particles were found to have average size of 175-180nm with low polydispersity, zeta potential in the range of 55-65mV and had 4.7-4.9%w/w of loaded drug which corresponded to an entrapment efficiency of 98-99%w/w. DSC and SEM studies confirmed amorphization and molecular solubilization of docetaxel in NLC and nearly spherical shape of NLCs respectively.

INTRODUCTION: Solid Lipid Nanoparticles (SLNs) and Nanostructured Lipid Carriers (NLCs) are promising delivery vehicles for lipophilic drugs[1]. SLN have proved to be beneficial as drug delivery carrier. However, its drawbacks including limited drug-loading capacity and drug expulsion during storage led to the invention of second generation smarter drug carrier systems, the Nanostructured lipid carriers (NLCs). [2]

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NLCs are mainly composed of biodegradable and biocompatible solid lipids and liquid lipids constituting solid matrix[3] and surfactants [4] which are widely approved by regulatory authorities.[5] The NLC or oil-loaded SLN contain lipid droplets that are partially crystallized and have a less-ordered crystalline structure or an amorphous solid structure, which helps in overcoming limitations of SLN such as expulsion of drug due to crystallization of lipid.[6]

NLCs also exhibit advantages over other colloidal carriers viz., nanoemulsions, polymeric nanoparticles, liposomes etc. owing to an entire set of unique advantages such as enhanced drug loading capacity, prevention of drug expulsion, greater flexibility in modulating drug release[7]

which in turn make NLCs a very versatile delivery system for drug delivery via various routes of administration.[8, 9]

Docetaxel is a highly efficacious anti-neoplastic agent which acts through induction of hyper-polymerization of mitotic spindle fibers thus inducing apoptosis and cell cycle arrest.[10] Docetaxel belongs to taxane group of compounds, and is a semi-synthetic analogue of paclitaxel (Taxol), an extract from the bark of the rare Pacific yew tree *Taxusbrevifolia*.[11] It has negligible aqueous solubility hence Taxotere® the – the commercial parenteral formulation of docetaxel, is formulated into a solution of polysorbate 80 and has shown dramatic toxicities related to polysorbate 80. Whereas several new formulations for paclitaxel have recently appeared such as Abraxane and others currently in various phases of clinical study, [12,13] there is limited progress in case of docetaxel, except BIND-014®, a polymeric nanoparticle based system which recently entered phase I clinical testing.[14]

Taxotere's systemic toxicity, hypersensitivities and other side effects are significant problems that are needed to be addressed.[15] Docetaxel is indicated for different cancers including head and neck carcinoma, breast, gastric, prostate and small cell lung cancers.[16]

NLCs are suitable for delivery of anticancer molecules because of ability to load adequate quantities of both hydrophobic and hydrophilic molecules.[17] In the present study NLCs loaded with docetaxel were developed and optimized using QbD approach for efficient docetaxel loading and particle size for parenteral delivery. The focus of this concept bases on the concept of quality. [18, 19]

Quality should be built into a product with an aim of understanding the product and process by which it is developed and manufactured along with the emphasis of the risks involved in manufacturing of the product. [20, 21, 22] The QbD initiative,[23] attempts to provide guidance on pharmaceutical development to facilitate design of products and processes that maximizes the product's efficacy and safety profile while enhancing product manufacturability.[24, 25] The objective of the present study was to optimize conditions for the preparation of docetaxel loaded nanostructured lipid carriers using Quality by Design (QbD) approach with the help of Design Expert software.

Two independent factors viz. total lipid to drug ratio and solid lipid to liquid lipid ratios were fixed into four and three levels respectively and then the dependent variables viz. entrapment efficiency and particle size were quantified and evaluated using various analytical tools viz. response surface methodology and contour plots for identification of the optimum conditions for preparation of the NLCs.[26]

MATERIALS:

The following materials were received as gift samples from corresponding generous organizations: Docetaxeltrihydrate from Mac-Chem Labs Pvt. Ltd, Mumbai; Oleic acid and alpha tocopherol from Sun Pharmaceuticals Ltd, Mumbai; Glycerylmonostearate from Croda chemicals (India) Pvt. Ltd.; Stearylamine from Presol Chemicals Ltd, Mumbai; Phospholipon S100from Lipoid, Switzerland and Poloxamer 188 (Lutrol F 68) from BASF, Mumbai; All other reagents and chemicals used for the present study were of analytical grade.

Experimental:

Formulation Optimization Studies:

A Factorial design was adopted to optimize the formula for preparation of docetaxel loaded NLCs.

Preparation of optimization batches:

NLCs were prepared using Melt Emulsification Solvent Evaporation - Sonication method. Matrix lipids consisting of glycerylmonostearate (GMS), stearylamine (SN), alpha-tocopherol along with phospholipon S100 (soyalecithin) employed as an emulsifier were dissolved in 5 ml of organic phase (chloroform: methanol mixture 3:2) at 65^0C. A 3%w/v solution of Lutrol F68 (poloxamer 188) served as the aqueous phase. The aqueous phase was also heated to 65^0C and was contained in a beaker equipped with a magnetic needle. Organic phase was added drop-wise to aqueous phase using glass syringe and then the resultant crude emulsion was stirred under heating at 500 rpm for 15 minutes. Crude suspension thus obtained was kept for stirring at higher speed (1000 rpm) using digital

magnetic stirrer (Remi, Mumbai) for the duration of 1 hr at ambient temperature for further reduction in particle size. The crude suspension was then probe sonicated (Sonics Vibra Cell™, USA) at a pulse rate of 3 cycles per second to enable further size reduction of NLCs. Formulation batches were optimized for percentage drug entrapment and particle size as dependent variables and total lipid to drug ratio and solid lipid to liquid lipid ratio as independent variables.

Batches prepared using different ratios of total lipid to drug and solid lipids to liquid lipids are represented in **Table 1.** The NLC dispersion obtained at the end of the sonication procedure was referred to as docetaxel loaded NLC suspension.

During optimization, the independent factor total lipid to drug ratio (A) was fixed at four levels (20:1, 15:1, 10:1, 5:1) and represented as I, II, III and IV respectively in the formulation codes **Table 1**. The second independent factor solid lipid to liquid lipid ratio (B) was fixed at three levels 4:1, 3:1 and 2:1 and represented as fA, fB and fC respectively in the formulation codes **Table 1** As represented in the factorial layout designed by Design Expert Software **Fig. 1** total lipid to drug ratio was converted into percentage of total lipids in total lipids plus drug as {95.23% (20:1), 93.75% (15:1), 90.90% (10:1) and 83.33 % (5:1)} and solid lipid to liquid lipid ratios were converted into the percentage of solid lipids in total lipids as {80 % (4:1), 75% (3:1) and 66.66% (2:1)}. Optimization batches were prepared in duplicates. The dependent variables viz. percentage drug entrapment and particle size were quantified for each batch as per the method described in the following section and the data obtained was fed to the optimization software Design-Expert® for identification of the optimal batch.

TABLE 1: FULL FACTORIAL DESIGN LAYOUT OF NLCs

Runs	Batch	Factor A Ratio of total lipid: drug (mg : mg)	Factor B Ratio of solid lipid: liquid lipid (mg : mg)
1.	FAI	20:1(200:10)	2:1(66.66:33.33)
2.	FAII	15:1(150:10)	2:1 (100:50)
3.	FAIII	10:1(120:10)	2:1(80:40)
4.	FAIV	5:1(90:18)	2:1(60:30)
5.	FBI	20:1(160:8)	3:1(120:40)
6.	FBII	15:1 (160:10.65)	3:1(120:40)
7.	FBIII	20:1 (120:10)	3:1 (120:40)
8.	FBIV	5:1 (80:16)	3:1(60:20)
9.	FCI	20:1 (100:5)	4:1 (80:20)
10.	FCII	15:1 (75:5)	4:1 (60:15)
11.	FCIII	10:1 (100:10)	4:1 (80:20)
12.	FCIV	5:1 (50:10)	4:1 (40:10)

Characterization of docetaxel loaded NLCs (optimization batches):

Docetaxel loaded NLC batches prepared during optimization studies were characterized for quantification of dependent variables viz. percentage entrapment efficiency of docetaxel and particle size.

Percentage entrapment efficiency and loading:

The percentage entrapment efficiency (EE) for drug which corresponds to the amount of drug encapsulated within and adsorbed onto the NLCs was determined indirectly by measuring the concentration of free drug in the dispersion medium.

Docetaxel loaded NLC suspension (1mL) was ultra-centrifuged (Beckman coulter, USA) at 80,000 rpm for 1hr. Pellet was obtained at the top of the ultra-centrifuge tube due to lower density of lipids. The solution beneath was then sufficiently diluted and analyzed for quantification of docetaxel (represented as c_1) by developed HPLC method (pump: Jasco HPLC system, detector: Jasco UV detector, column: Hypersil, C_{18} Length 25cm, Inner diameter: 5µ, mobile phase: Methanol: Buffer (0.3%v/v orthophosphoric acid pH adjusted to 3.22). Pellet was carefully recovered and then redispersed with 1mL of a 15% v/v solution of glycofurol in PBS pH 7.4 by cyclomixing (CM-101, Remi, Mumbai), sonicated (Ultrasonic

System, Mumbai) and again ultra-centrifuged at 80,000 rpm for 1 hour. Pellet obtained at the top of the ultra-centrifuge tube was discarded and solution beneath was sufficiently diluted and analyzed by HPLC method as detailed above for quantification of docetaxel (represented as c_2). The amount of un-entrapped docetaxel was then calculated by the summation of c_1 and c_2.

% Entrapment efficiency and drug loading was calculated as follows:

$$\% \text{ Entrapment Efficiency (E.E)} = \frac{W_T - W_A}{W_T} \times 100$$

Wherein,

W_T stands for total amount of docetaxel added to the NLC system.

W_A stands for total amount of un-entrapped docetaxel ($c_1 + c_2$) quantified by developed HPLC method.

$$\% \text{ Drug loading (D.L)} = \frac{\text{Total amount of drug entrapped } (W_T - W_A)}{\text{Total amount of lipids and drug added}} \times 100$$

Particle size:

NLC suspension (0.5mL) was redispersed in 5mL of filtered distilled water (10 folds dilution) and the diluted suspension was analyzed for determination of particle size distribution and polydispersity index using Zetasizer (Malvern, USA) under ambient conditions. The instrument was programmed so that each output was derived from a total of 10 scans with 3 runs in each scan for the estimation of particle size distribution.

Multiple Regression Analysis:

Multiple regression analysis in factorial designs generates polynomial equations comprised of interacting terms and regression coefficients, which together assist in evaluating the responses. The Design Expert software generates two basic models, particularly full model in which non-significant terms are included and reduced model in which non-significant terms are excluded. The responses in the full model study were analysed using the quadratic equation below:

$$Y = b_0 + b_1A + b_2B + b_{12}AB + b_{11}A^2 + b_{22}B^2$$

Where Y is the response evaluated, b_0 is the arithmetic mean response of 24 runs; b_i is the estimated coefficient of independent variables.

The main effects (A and B) represent the average result of changing one factor at a time from its low to high value. The interaction term (AB) shows how the response changes when both factors are simultaneously changed. The polynomial terms (A^2 and B^2) were included to investigate non-linearity.

Statistical analysis of data:

The selected responses obtained from the various systems were tested for significant differences. Statistical analysis of data was carried out using analysis of variance (ANOVA). The statistical analysis was conducted using **Design-Expert®** version 8.0.7.1 trial (Stat-Ease Inc., Minneapolis, MN, USA).The software performs response surface methodology (RSM) which consists of multiple regression analysis (MRA), ANOVA and statistical optimization. Using Design-Expert®, analysis of all the responses was carried out using the appropriate models and then the necessary statistical transformation was obtained.

The software performs the individual analysis of responses and calculates the sum of squares (SS), mean square (MS), Fischer's ratio (F statistics) and P value. The F statistics and P value give the significance level of each term considering null hypothesis (H_0) is true. P value less than 0.05 is considered significant at a level of significance $\alpha = 0.05$.When the F value obtained is greater than the critical F value from the F distribution table, the factor becomes significant and the null hypothesis is rejected.

The dependent responses measured viz. percentage entrapment efficiency and particle size were used for statistical analysis.

Statistical relationship in the form of equations was obtained which shows the effect of varying A and B on the dependent variables, Y1 and Y2. In addition, contour and 3D surface plots were obtained by Design-Expert (trial version), to represent the effect of the independent variables graphically.

Identification of optimal batch:
To determine the optimal batch, the fitted mathematical model was evaluated. The two equations derived i.e. for percentage drug entrapment efficiency (Y1) and particle size Y2 in terms of X1 and X2 were sequentially employed. First various values of X1 and X2 (between the values evaluated) were used in fitted equation and theoretical values for Y2 (particle size) were extrapolated. Thus, we get predicted values for Y2 for all combinations of X1 and X2.

The combinations which full fill the criteria for optimal release were selected and again used in fitted equation for Y1 (Drug Entrapment efficiency). The predicted values for Y1 were then computed and batches which fulfilled the criteria of drug entrapment efficiency were selected. Thus, from these theoretical computations, formulae for NLCs which showed values for Y1 and Y2 as required were identified and these batches were also prepared and characterized. The formulation with the desirability value closer to unity is chosen as the optimized formulation.

Characterization of optimized docetaxel loaded NLC suspension:
Optimized docetaxel loaded NLC suspension was further characterized for zeta potential, Differential scanning calorimetry and Scanning electron microscopy.

Zeta potential:
For zeta potential measurements, the optimized docetaxel loaded NLC suspension (0.5mL) was diluted up to 5mL (10fold) with filtered distilled water and then analyzed. Each measurement was derived from a total of 30 scans using Zeta sizer (Malvern, USA).

Differential scanning calorimetry:
Thermal characterization of docetaxeltrihydrate (DTX), physical mixtures of formulation components viz. Docetaxel, GMS, SN, α-tocopherol and vacuum dried docetaxel loaded NLCs were performed by DSC instrument (Mettler, USA.).Samples (10mg) were sealed in aluminium pans for analysis. The DSC thermograms were recorded from -20-0-300^{0}C at a heating rate of 10^{0}C/minute. An empty pan was used as a reference. A nitrogen flow rate of 50mL/minute was used for each DSC run.

Scanning electron microscopy (SEM):
The SEM analysis of the samples was performed to investigate the surface morphology and homogeneity of the particles in the formulations. Optimized docetaxel loaded NLCs were vacuum dried and then particles were sputter-coated with gold at room temperature before examination to render the surface of particles electro conductive. The SEM analysis of the samples was carried out using Scanning electron microscope (Zeiss, France).

RESULTS AND DISCUSSION:
Formulation Optimization of docetaxel loaded Nanostructured lipid carriers (NLCs):
In the process of formulation development, optimization of the method with respect to quantities of ingredients and process parameter settings plays a vital role in order to obtain the desired characteristics of the product and also to ensure smooth transfer of the experimental batches to large scale processing.[27, 28] Statistical models are very useful as they assist formulation scientists with the understanding theoretical formulation and target processing parameters such as the range of excipient concentrations to be employed in the formulation.[29, 30] Optimization techniques provide both in-depth understanding and an ability to explore and define ranges of formulation and processing factors.[31, 32, 33] Thus, optimization may be defined as the process of identifying the experimental conditions which leads to the best value of the desired response.

a. Factorial design of batches for evaluation of critical formulation variables:
For the present study, Quality by design (QbD) approach was applied to develop the formulation and understand the effect of various critical parameters in replicates. Response surface method (RSM) was chosen, in which User Defined Design (UDD) was used to optimize the formulation.3D response surface plots give a representation of the variations in each response when the two factors are simultaneously changed from lower to higher level. It gives a three dimensional curvature of the change in response at different factor levels.

Moreover, it also provides variation in design points from the predicted response value. 24 runs were suggested by Design-Expert®. These suggested batches were experimentally prepared as per the method of preparation of NLCs by varying 3 levels of solid lipid to liquid lipid ratio (percentage of solid lipids) and 4 levels of total lipid to drug ratio (percentage of total lipids). After entrapment efficiency and particle size analysis, the responses were calculated and put into the Design layout. **Fig. 1.**

b. Influence of formulation variables on characteristics of DTX Loaded NLCs:

Entrapment Efficiency:

The influence of different lipids and their internal ratios in percentage drug entrapment of NLCs was found to be statistically significant. The entrapment efficiency of the formulations suggested by Design expert software was found to vary from 75.15 to 97.763% w/w and the effect of the independent variables on it was found to be statistically significant at $p<0.05$. The entrapment efficiency of various formulations batches is shown in **Fig. 1.**

FIG. 1: SCREEN CAPTURE IMAGE OF THE FACTORIAL DESIGN RUNS DESIGNED BY DESIGN-EXPERT® WITH RESPONSES.

Select	Std	Run	Factor 1 A:TOTAL LIPI... %	Factor 2 B:SOLID LIPI... %	Response 1 PEE %	Response 2 PS nm
	9	1	83.33	80.00	75.15	161.9
	4	2	95.23	66.66	95.154	179
	2	3	90.90	66.66	86.07	229.2
	12	4	95.23	80.00	92.48	102.9
	5	5	83.33	75.00	85.02	204.3
	6	6	90.90	75.00	93	175.3
	11	7	93.75	80.00	84.58	274.8
	10	8	90.90	80.00	84.448	161.6
	1	9	83.33	66.66	81.07	283.21
	8	10	95.23	75.00	94.5	173.1
	3	11	93.75	66.66	93.77	173.4
	7	12	93.75	75.00	94.297	169.5
	13	13	83.33	66.66	81.07	274.7
	14	14	90.90	66.66	83.43	212.8
	15	15	93.75	66.66	94.88	173
	16	16	95.23	66.66	97.763	177
	17	17	83.33	75.00	89.46	236.9
	18	18	90.90	75.00	91.11	181.3
	19	19	93.75	75.00	95.91	169.5
	20	20	95.23	75.00	96.525	153.8
	21	21	83.33	80.00	84.75	168
	22	22	90.90	80.00	84.58	165.2
	23	23	93.75	80.00	89.912	289.1
	24	24	95.23	80.00	94.16	183.3

Statistical Analysis:

ANOVA for percententrapment efficiency (EE) of DTX loaded NLCs:

ANOVA for response surface quadratic model was generated by Design-Expert® software. The ANOVA for % Entrapment Efficiency of DTX loaded NLCs is shown in **Table 2**. These results indicate that both solid lipid: liquid lipid ratio and total lipid: drug ratio were significant terms as the F values were above the critical F values at p less than 0.05 (threshold level). Hence, the null hypothesis H_0 was rejected and the alternate hypothesis was accepted i.e. both factors (A, B) used significantly influence the entrapment efficiency of DTX loaded NLCs.

Coefficient of correlation:

The predicted R-Squared value of 0.6737 is in reasonable agreement with adjusted R-Square value of 0.7909 indicating good fit of the model **Table 3** Data indicated that the % entrapment efficiency values were strongly dependent on the selected independent variables. Polynomial equations can be used to draw conclusions after considering the magnitude of coefficient and the mathematical sign it carries (i.e. positive or negative).

Fig. 2 represents the effects of individual and combined variables on %E. E of DTX loaded NLCs. The equation suggested that the factor A has positive effect and B has negative effect on % entrapment efficiency.

Negative values of coefficients of B factor indicated that percentage drug entrapment increased with decrease in the solid lipid to liquid lipid ratio where as positive signs of coefficients of A terms indicated that the higher level of total lipid: drug ratio favoured increase in % E.E of docetaxel (DTX) in DTX loaded NLCs.

When the coefficient values of two independent key variables (A and B) were compared, the value of variable AB (-1.11) was found to be maximum than B value alone (-1.16) and hence both the factors were considered to be major contributing variables responsible for efficiency of entrapment of DTX in NLCs. **Fig. 2** and **3** shows the effect of significant interaction terms on Entrapment efficiency.

TABLE 2: RESULT OF ANALYSIS OF VARIANCE (ANOVA) FOR MEASURED RESPONSE: % ENTRAPMENT EFFICIENCY

Source	Sum of squares	dF	Mean sqaure	F value	p- value (Prob>F)	Inference
Model	705.63	5	141.13	18.40	<0.0001	Significant
A	543.46	1	543.46	70.85	<0.0001	Significant
B	19.50	1	19.50	2.54	0.1282	
AB	12.00	1	12.00	1.56	0.2271	
A^2	47.43	1	47.43	6.18	0.0229	
B^2	137.79	1	137.79	17.96	0.0005	
Residual	138.08	18	7.67	-	0.3364	Not significant
Lack of Fit	53.86	06	8.98	1.28		
Pure error	84.21	12	7.02			

Factorial equation for Percentage entrapment efficiency: The polynomial equation derived from the coefficients of estimate in terms of coded factors A and B is as follows:

% Percentage entrapment efficiency = 89.09 + (6.33) A + (-1.16) B + (-1.11) AB + 3.75 A^2 + (-5.48) B^2 - (**Equation 1**)

Coefficient of correlation:
The predicted R-Squared value of 0.6737 is in reasonable agreement with adjusted R-Square value of 0.7909 indicating good fit of the model (**Table 3**).

Data indicated that the % entrapment efficiency values were strongly dependent on the selected independent variables. Polynomial equations can be used to draw conclusions after considering the magnitude of coefficient and the mathematical sign it carries (i.e. positive or negative).

Fig. 2 represents the effects of individual and combined variables on %E.E of DTX loaded NLCs. The equation suggested that the factor A has positive effect and B has negative effect on % entrapment efficiency. Negative values of coefficients of B factor indicated that percentage drug entrapment increased with decrease in the solid lipid to liquid lipid ratio where as positive signs of coefficients of A terms indicated that the higher level of total lipid: drug ratio favoured increase in % E.E of docetaxel (DTX) in DTX loaded NLCs. When the coefficient values of two independent key variables (A and B) were compared, the value of variable AB (-1.11) was found to be maximum than B value alone (-1.16) and hence both the factors were considered to be major contributing variables responsible for efficiency of entrapment of DTX in NLCs. **Fig.2** and **3** shows the effect of significant interaction terms on Entrapment efficiency.

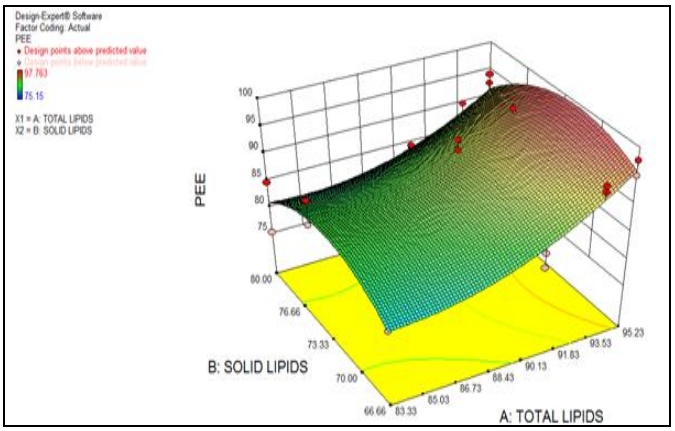

FIG. 2: SCREEN CAPTURE IMAGE OF 3D SURFACE RESPONSE PLOT FOR % ENTRAPMENT EFFICIENCY

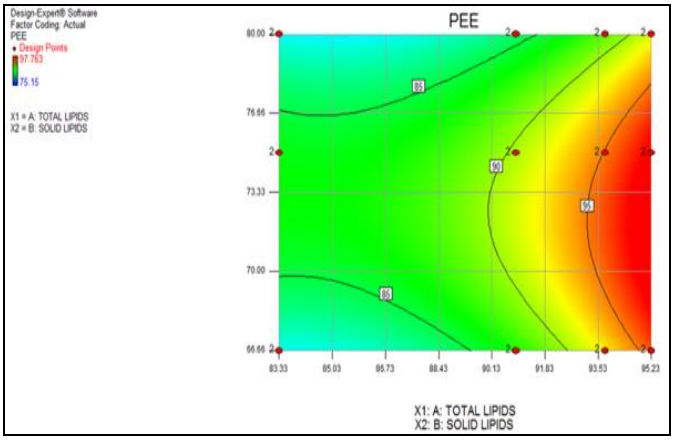

FIG. 3: SCREEN CAPTURE IMAGE OF CONTOUR PLOT FOR % ENTRAPMENT EFFICIENCY

Particle size:
The influence of different lipids and their internal ratios on particle sizes of NLCs was found to be statistically significant. Particle sizes of various formulations are shown in **Fig. 1.**

Statistical Analysis:

ANOVA for Particle size (PS) of DTX loaded NLCs: ANOVA for response surface quadratic model for particle size analysis of DTX loaded NLCs was been generated by Design-Expert® software and is shown in **Table 4.** The results indicate that both solid lipid: liquid lipid ratio and total lipid: drug ratio are significant terms as the F values are above the critical F values at P values less than 0.05 (threshold level). Thus, the null hypothesis H_0 was rejected and the alternate hypothesis was accepted: both the independent factors significantly influenced the particle sizes of DTX loaded NLCs.

TABLE 3: FULL MODEL FOR EFFECT OF INDEPENDENT VARIABLES ON % ENTRAPMENT EFFICIENCY

Term	Coefficient Estimate	dF	Standard Error	95% CI Low	95% CI High
Intercept	89.90	1	1.49	85.95	92.23
A	6.33	1	0.75	4.75	7.91
B	-1.16	1	0.73	-2.69	0.37
AB	-1.11	1	0.89	-2.98	0.76
A^2	3.75	1	1.51	0.58	6.92
B^2	-5.48	1	1.29	-8.20	-2.76

TABLE 4: RESULT OF ANALYSIS OF VARIANCE (ANOVA) FOR MEASURED RESPONSE: PARTICLE SIZE

Source	Sum of squares	dF	Mean sqaure	F value	p- value (Prob>F)	Inference
Model	22357.42	5	4471.48	2.84	0.0463	Significant
A	8749.73	1	8749.73	5.55	0.0300	
B	6350.12	1	6350.12	4.03	0.0599	
AB	11349.00	1	11349.00	7.20	0.0152	
A^2	199.02	1	199.02	0.13	0.7264	
B^2	1105.58	1	1105.58	0.70	0.4132	
Residual	28356.35	13	1575.35	11.29	0.0002	Significant
Lack of Fit	24088.55	06	4014.76			
Pure error	4267.81	12	355.65			

Factorial equation for particle sizes: The polynomial equation derived from the coefficients of estimate in terms of coded factor A and B is stated as under –

Particle size= $196.62 + (-25.39)\,A + (-20.97)\,B + (34.20)\,AB + (-7.69)\,A^2 + (15.52)\,B^2$ **(Equation-2**

Coefficient of correlation:

The predicted R-Squared value of 0.1032 is in reasonable agreement with adjusted R-Square value of 0.2855 indicating good fit of the model.

Data obtained indicated that the particle size values were strongly dependent on the selected independent variables. The fitted equation (for full model) relating the response (particle size) to the transformed factor is shown by **equation-2**.

Table 5 represents the effects of individual and combined variables on particle size of DTX loaded NLCs. The **polynomial equation-2** suggested that both the factors A and B have negative effects on particle sizes of NLCs. Negative value coefficients of B factor indicated that particle size decreased with decrease in the solid lipid to liquid lipid ratio which was likely due to increase in flexibility and fluidity provided by the increase in liquid lipid content in NLC matrix.[25]

In addition to this, negative signs of coefficients of A terms indicated different levels of total lipid: drug ratio caused decrease in particle size of DTX loaded NLCs. Similarly, prob > F and less than 0.05 indicated that total lipid: drug ratio and solid lipid: liquid lipid ratio had significant effect on particle size of DTX. **Fig. 4** and **5** demonstrate the effect of significant interaction terms on particle sizes of NLCs.

TABLE 5: FULL MODEL FOR EFFECT OF INDEPENDENT VARIABLES ON PARTICLE SIZE ANALYSIS

Term	Coefficient Estimate	dF	Standard Error	95% CI Low	95% CI High
Intercept	196.62	1	21.40	151.65	241.58
A	-25.39	1	10.77	-48.02	-2.76
B	-20.97	1	10.44	-42.91	0.97
AB	34.20	1	12.74	7.43	60.93
A^2	-7.69	1	21.63	-53.13	37.76
B^2	15.52	1	18.63	-23.40	54.44

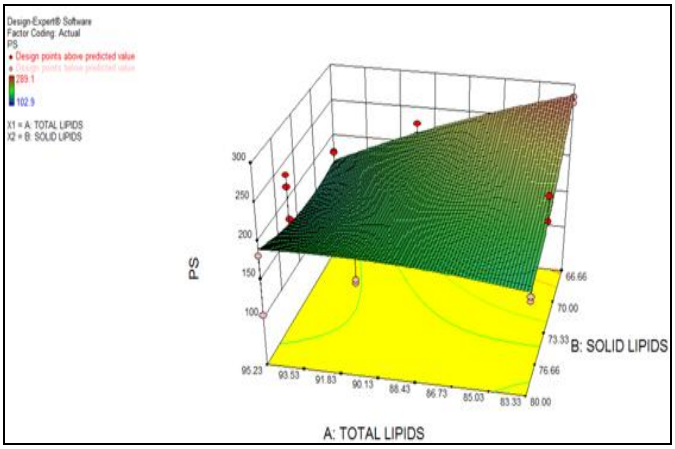

FIG. 4: SCREEN CAPTURE IMAGE OF 3D SURFACE RESPONSE PLOT FOR PARTICLE SIZE

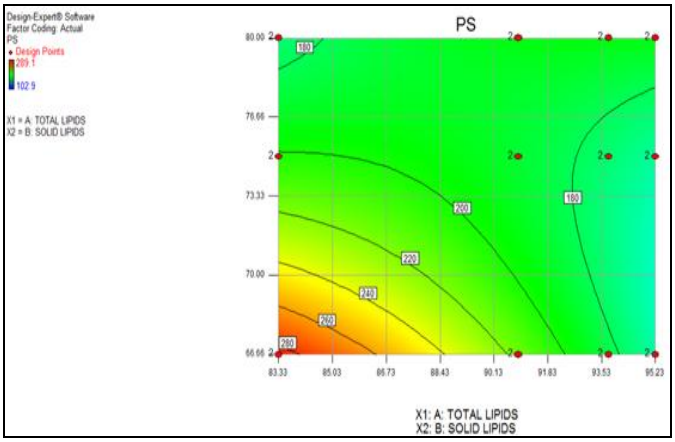

FIG .5: SCREEN CAPTURE IMAGE OF CONTOUR PLOT FOR PARTICLE SIZE

Optimized Batch of DTX loaded NLCs:

For the present study, the criteria decided for optimum batch was the one which showed at least 97% entrapment efficiency and particle size of about 175nm. Nanoparticles with sizes above 200nm are reported to be easily taken up by circulating macrophages and may form emboli.[34] Also particles with sizes below 200nm are reported to be extravagated into the neoplasms by EPR effect.[35] As obvious from the data in **Fig. 1**, the optimum batch lies in the region between the experimental points studied. Hence the different values for X1 and X2 in between the levels studied were put in **equation 2** for particle size quantification. This gave us predicted values for Y2 for all the combinations of X1 and X2. The combinations which give Y2 about 175nm were selected. There were number of such combinations. All these combinations of values were then put in **equation 1** for Y1. This gave predicted values for percentage entrapment efficiency. From these, only three combinations gave satisfactory percentage entrapment which is being presented in the **Fig. 8**.

Based on the response surface quadratic model designed batches and their response graphs, two solutions were suggested by Design-Expert® in **Fig. 8.** From the **Fig. 9**, it was clear that the desirability value of batch fA1 {(Total lipid: drug ratio as 20:1 (95.23%) and solid lipid: liquid lipid ratio as 2:1 (66.66%)} was closer to unity. Hence, the formulation **fAI** was considered the optimized formulation.Also, no statistical significant difference was observed in the measured parameters between the optimization batches prepared in duplicates as P value was greater than 0.05 at 95% confidence interval.

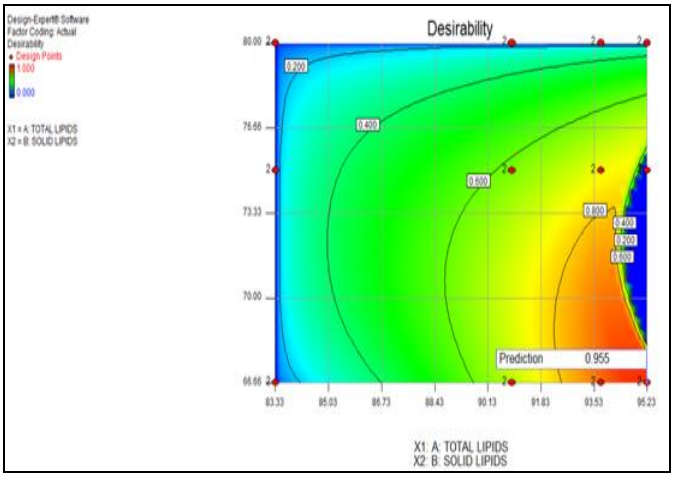

FIG. 6: SCREEN CAPTURE IMAGE OF CONTOUR PLOT OF THE OPTIMIZED BATCH

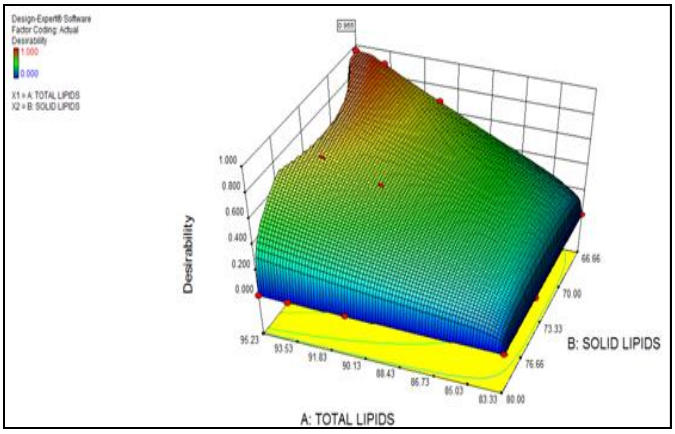

FIG.7: SCREEN CAPTURE IMAGE OF 3D OF SURFACE RESPONSE CURVE OF OPTIMIZED BATCH

Constraints

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
A:TOTAL LIPID	maximize	83.33	95.23	1	1	3
B:SOLID LIPIDS	minimize	66.66	80	1	1	3
PEE	is target = 97	75.15	97.763	1	1	3
PS	is target = 175	102.9	289.1	1	1	3

Solutions

Number	TOTAL LIPIDS	SOLID LIPIDS	PEE	PS	Desirability	
1	95.23	66.66	95.9635	165.836	0.955	Selected
2	94.75	66.66	94.7789	171.851	0.953	

2 Solutions found

FIG.8: SCREEN CAPTURE IMAGE OF 2 SOLUTIONS FOR OPTIMIZED BATCH WERE SUGGESTED BY DESIGN-EXPERT®

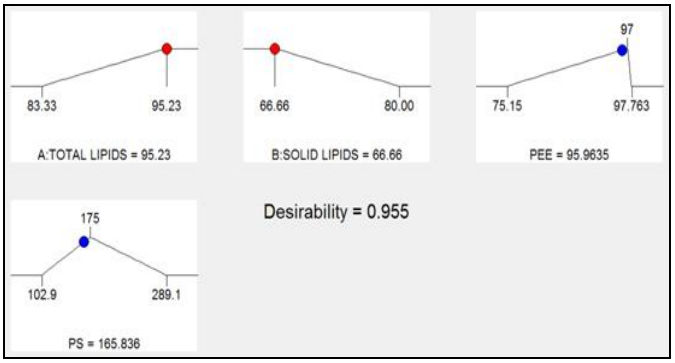

FIG.9: FORMULA FOR THE OPTIMIZED DTX LOADED NLCS

Characterization of optimized docetaxel loaded NLC suspension batch–FAI:

The zeta potential of the docetaxel loaded NLCs suspension was measured as 57.56 ± 0.108 mV, indicating good stability of the dispersion.

DSC thermogram of docetaxeltrihydrate, physical mixtures of docetaxeltrihydrate, GMS, SN, alpha tocopherol and vacuum dried docetaxeltrihydrate loaded optimized formulations are shown in **Fig. 10 (i.), 10(ii.)** and **10 (iii.)** respectively.

DSC thermogram of docetaxeltrihydrate showed two sharp endotherms between 57.7 to 89.8^{0}C which was likely attributed to the presence of water molecule of crystallization in docetaxel and 161.4 to 174.3^{0}C which was the characteristic melting endotherm of docetaxel**{Fig. 10(i.)}.** Physical mixtures of formulation containing docetaxeland other lipids (GMS, SN and alpha tocopherol) showed similar endothermic peak for docetaxel between 55.2 to 68.8^{0}C with other endothermic peaks of the lipids **Fig. 10(ii.).** However, docetaxel loaded vacuum dried optimized NLCs showed decrease in endothermic peak intensity for docetaxel indicating amorphization (molecular solubilization) of docetaxel trihydrate in vacuum dried NLCs **Figs. 10(iii.).**

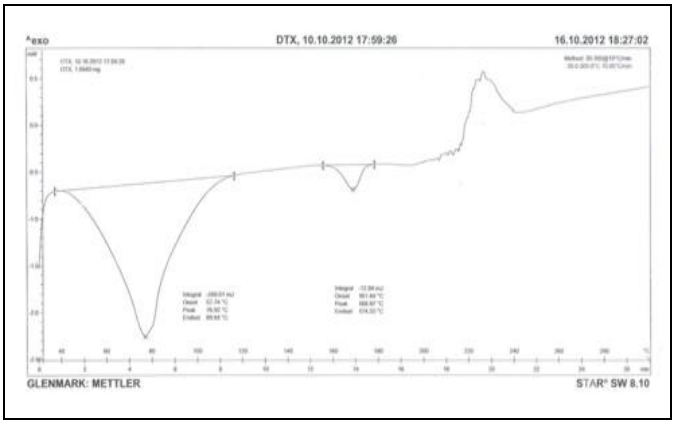

FIG.10: (i) DSC THERMOGRAM OF DOCETAXELTRIHYDRATE

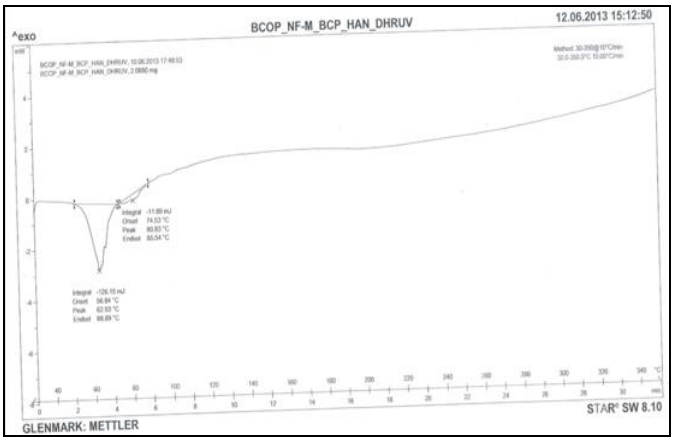

FIG. 10: (II) DSC THERMOGRAM OF PHYSICAL MIXTURE OF LIPIDS

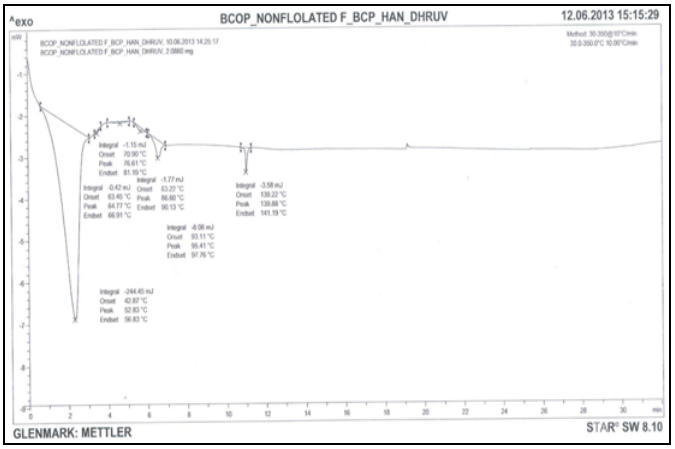

FIG. 10: (iii) DSC THERMOGRAM OF DOCETAXEL OPTIMIZED NLCs

Scanning electron microscopy (SEM):

SEM photomicrograph of docetaxel loaded NLCs suspension revealed near spherical to elliptical morphology as shown in **Fig. 11.**

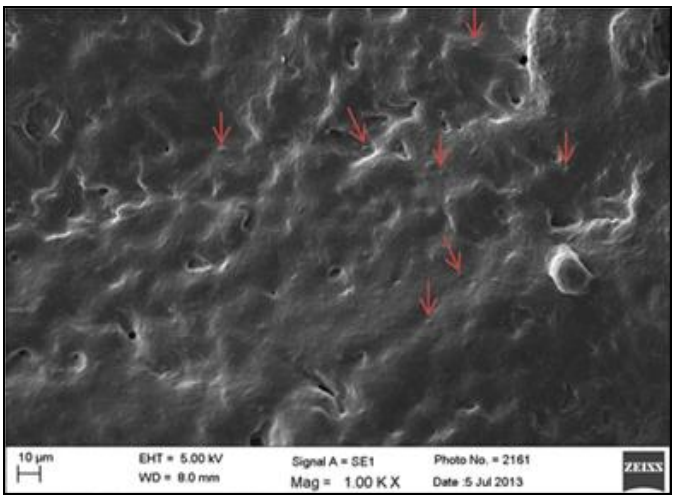

FIG. 11: SEM IMAGE OF OPTIMIZED DOCETAXEL LOADED NLC SUSPENSION

CONCLUSION: In conclusion, docetaxel loaded NLCs were successfully optimized using QbD approach. Optimized batch suggested by Design Expert software was further characterized for zeta potential, DSC and SEM studies. Optimized batch (FAI) showed highest entrapment efficiency (upto 99% w/w) and loading (upto 5%w/w) with the desired particle sizes of 175-180nm. NLCs encapsulated docetaxel molecularly into its matrix, the phenomenon which was been confirmed by DSC studies. NLCs were found to be roughly spherical to elliptical in shape as confirmed from SEM studies. This observation was desirable for efficient uptake of NLCs into the neoplastic cells due to higher surface area provided by NLCs. Thus, the optimized NLC system seemed to be suitable for docetaxel delivery.

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