5.1. Introduction

Quality by Design (QbD) driven approach was used to optimize the formulation. The UDNVs formulation was designed and engineered for uterine targeting via intravaginal route. The experiments were scientifically designed for screening and optimization of the formulation using Design of Experiments (DoE). The systematic QbD approach and use of various statistical tools enabled exhaustive evaluation of the impact of material attributes and process parameters on the critical formulation attributes.

5.2. Materials and Equipment

Paclitaxel (PTX) was obtained as a gift sample from Sun Pharma Advanced Research Centre, Vadodara, India. 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) and L-α-phosphatidylcholine, Egg (EggPC) was obtained from Avanti Polar Lipids, Inc Alabaster, Alabama. Sodium Deoxycholate was purchased from Loba Chemie Pvt Ltd, Mumbai, India. HPLC grade methanol (MeOH) and acetonitrile (ACN) were procured from Fisher Scientific (Vadodara, Gujarat) to carry out chromatographic analysis. Double distilled water used in the study was filtered using 0.22 micron nylon filter, Nylon N66 membrane filters 47 mm, Rankem, India. All other reagents were purchased from S.D. finechem Ltd, India and were of analytical grade.

Equipment

- Electronic weighing balance (ATX 224, Shimadzu, Japan)
- Vortex Mixer (Spinix-Vortex Shaker, Tarsons, India)
- Ultrasonic Bath Sonicator (Ultrasonics Selec, Vetra, Italy)
- Rotary evaporator (IKA RV10, Karnataka, India)
- Probe Sonicator (LabsonicM, Sartorius Ltd, Mumbai, India)
- Zeta sizer (Nano ZS Malvern Instruments, UK)

5.3. Preparation and Optimization of PTX loaded UDNVs

5.3.1. Methods

5.3.1.1. Preparation of PTX loaded UDNVs

The UDNVs were prepared by Thin Film Hydration method. Briefly, PTX, Lipids (DSPC and EggPC; total lipids 40 mg) and Surfactant in different proportions were taken in Round Bottom Flask and dissolved in Methanol: Chloroform (1:9) mixture. The organic solvents were evaporated using rotary flask evaporator under vacuum on a thermostatic water bath at 50 ± 2°C temperature and the speed of 100 RPM. After evaporation of all the organic solvents, the RBF was kept in desiccator overnight to ensure complete removal of organic solvent. The thin film was hydrated by 2.5ml aliquots of triple distilled water. Hydration media, previously heated at 50°C was added followed by shaking RBF manually for 2-3 minutes after each addition of aliquot into RBF, maintained at same temperature on thermostatic water bath. Hydration of film was done for 10 min. Probe sonication method was used for size reduction of MLVs prepared by thin film hydration method. Sonication was performed at 80% amplitude with 0.8 second pulse rate for 20 second exposure time to obtain desired size range and visually clear dispersion.

The similar method was used for preparation of conventional liposomes by replacing edge activator with cholesterol keeping identical proportion of lipids which was further used as control for comparison purpose.

5.3.1.2. Quality Target Product Profile (QTPP) of PTX loaded UDNVs and Identification of CQAs

The template for target product profile (TPP) has been provided by United States Food and Drug Administration (USFDA) guidance that portrays the parts of TPP for new drug applications. The target product quality profile is enlisted as the quality properties that a drug product ought to possess so as to fulfill the objectives set in TPP as quantitative attributes. The International conference of harmonization (ICH) Q8 (R2) recapitulates them as QTPP. The QTPP lays the foundation of design criteria for the product and ought to embody patient relevant product performance characteristics. It should furnish a quantitative surrogate to ascertain the aspects of clinical safety and efficacy.

Thus it ought to form the basis for determining the critical quality attributes (CQAs), critical material attributes, critical process parameters, and control strategy.

The primary step in defining QTPP is to decide the type of dosage form, what is the purpose of your product, its key desired quality attributes, manufacturing methodology, etc. Based on the scientific, therapeutic, industrial and regulatory aspects, quality target product profile (QTPP) for PTX loaded UDNVs were established. The ICH working definition of CQA was stated as: "A CQA is a quality attribute (a physical, chemical, biological or microbiological property or characteristic) that must be controlled (directly or indirectly) to ensure the product meets its intended stability, safety, efficacy and performance". The CQAs relies on the type of formulation, dosage form designed, manufacturing or production methodology, etc. employed and selected amongst many possible options. Consequently, formulation and process development typically rely on empirical prior knowledge and small scale feasibility studies. The identification of a CQA from the QTPP was based on the severity of harm caused by the product falling outside the acceptable range for that attribute. Based on the prior knowledge, literature review and experiment trials, three response variables viz., % Entrapment Efficiency, vesicle size and Deformability Index (DI) were selected as critical quality attributes (CQA) for PTX loaded UDNVs.

5.3.1.3. Qualitative Risk assessment and Identification of Independent variables (factors)

Risk based compliance is an imperative FDA initiative for current Good Manufacturing Practice in the 21st century. ICH Q9 guidance document introduced the concept of quality risk management for evaluating, communicating, controlling and reviewing risks to the quality of drugs across product life cycle. After careful observation and cerebration of the development process of the PTX loaded UDNVs formulation, variables/factors involved were qualitatively categorized as "low, medium and high risk" based on their anticipated impact on CQA as described in **Table 5-1**.

Table 5-1: Measures of qualitative risk assessment

Low Risk	Factors with wide range of acceptability. No investigation required
Medium Risk	Acceptable risk. No adverse effect on product quality on small changes.
High Risk	Unacceptable risk. Acceptable range need to be investigated

The risk associated with the medium and low risk factors was mitigated by making them constant based on scientific knowledge gained during preliminary trials and literatures.

5.3.1.4. Quantitative risk assessment: Factors Screening Design

Screening Design refers to an experimental plan that is intended to find the few significant factors from a list of many potential ones. Even when the experimental objective is to finally fit a response surface model (an RSM Model), the primary experiment run should be a screening design when there are many factors to study. Here, the factors at High risk were screened using 2-level Plackett-Burman design to statistically detect important factors and utilize them in main-optimization design to define the control limits (the design-space). The non-critical factors were also specified with their constant levels using the Screening design. Minitab® 16.1.1 was used to generate a Plackett-Burman design based on which experimental batches were prepared and the effect of the high risk factors on CQA were evaluated. Software generated Pareto charts were utilized to determine critical factors while the main effects charts were utilized to decide the optimum levels of non-critical factors. Following methods used for the estimation of response variables.

5.3.1.4.1. Entrapment Efficiency (%EE)

PTX loaded UDNVs formulation was centrifuged at 5000 RPM for 10 min to separate un-entrapped drug in sediment. The supernatant was separated and methanol was added to it in order to dissolve the lipids and to extract drug in solution. The amount of PTX was estimated by high performance liquid chromatography technique at 227 nm as described in Chapter 3 [1].

The entrapment efficiency was calculated using following formula-

$$\%\ Entrapment\ Efficiency\ = \frac{Estimated\ Entrapped\ drug}{Total\ drug\ added\ to\ formulation} \times 100$$

5.3.1.4.2. Vesicular size and size distribution

50 µl of the vesicular formulation was taken and added to 2 ml of distilled water in order to obtain proper vesicle density in the final dispersion for measurement of size of the vesicles. The dispersion thus prepared was filled in clear disposable sizing cuvettes and the globule size was measured using ZetaSizer (Nano ZS, Malvern Instruments, UK) equipped with a He-Ne laser at 633 nm and scattered light detector at an angle of 90°.

5.3.1.4.3. Deformability evaluation

For the measurement of deformability of the nano-vesicles, formulations were extruded at constant pressure through the 25 mm diameter Isopore polycarbonate filter membrane having a pore diameter of 100 nm. The amount of vesicle dispersion that was extruded during 5 min was measured, and the vesicle was monitored before and after extrusion. The deformability index of the nano-vesicles was measured using following equation [2]:

$$DI = J \times \left(\frac{R_v}{R_p}\right)^2$$

Where, *DI*= Deformability Index;

J= Amount of dispersion extruded (ml);

R_v= Vesicles size after extrusion (nm); and

R_p= Pore size of the barrier (nm)

5.3.1.5. Formulation optimization using Box–Behnken Response Surface Design

Box-Behnken design is a spherical, revolving response surface methodology (RSM) design that consists of a central and middle points on the edges of the cube circumscribed on the sphere. RSM is a useful method for studying the effect of several variables influencing the responses; this method varies the variables simultaneously and carries out a limited number of experiments have reported that statistical methods

are effective and powerful approaches for screening key factors rapidly from a multivariable system for the optimization of a particular process. Box–Behnken statistical design with 3-factors, 3- levels, and 17 runs was specifically employed for the optimization study using Design-Expert software (Design-Expert 7, State- Ease Inc., Minneapolis, USA). The suitable model for the experimental data set was suggested by the software. The polynomial equation was generated using software and the significant model terms were decided based on ANOVA and F-test. The insignificant model terms from the polynomial equation were later removed in order to simplify the equation for estimation of the CQAs. 3D response surface plots were generated in order to understand and explore the effect of variations in the independent factors on the response variables. Desirability criteria was defined based on the set QTPP and the design space was also created in order to define range of the independent variables to achieve desired characteristics in the optimized formulation.

5.3.2. Results and Discussion

5.3.2.1. Quality Target Product Profile (QTPP) of PTX loaded UDNVs and Identification of CQAs

The parameters that will be focused in our study were chosen and enlisted as QTPP for PTX loaded UDNVs. QTPP for PTX loaded UDNVs is tabulated in **Table 5-2**. The depicted QTPP laid down the basis for determining CQA. % Entrapment Efficiency, vesicle size and DI were identified as critical factors governing the response variables.

Table 5-2: QTPP for PTX loaded UDNVs

QTPP Element	Target	Justification
Dosage form	Nano-vesicle formulation	Due to their small size and large surface area, nano-vesicles show enhanced bioavailability and additional ability to cross the biological membranes. Furthermore, in cancer therapy, nano-vesicles deliver the drug into the tumor tissue and avoid normal tissues and organs by accumulation in tumors by a passive targeting, furnishing

			higher therapeutic efficiency and less side effects
Formulation Design		Targeted Delivery	Vesicles accumulate in tumours by a passive targeting leading to higher therapeutic efficiency and less side effects
Route of administration		Intravaginal	Achieve passive targeting to uterus by first uterine pass effect (FUPE)
Quality attributes of the formulation	Vesicle size	Minimize	Most important factor influencing biodistribution and cellular uptake
	Zeta potential	>±30mV	Better colloidal stability of the dispersion
	Drug Entrapment	Maximize	A higher percentage of drug entrapment and Loading could reduce the manufacturing cost and increase drug concentration in the final formulation allowing greater flexibility in dosing. Higher drug concentration can result in increased dosing intervals and hence improved patient compliance
	Drug Loading	Maximize	
	Vesicle Deformability	Optimum	To enhance transmembrane permeability of vesicles
	Surface characteristics	Smooth and spherical	To achieve enhanced permeation
	In-vitro Drug release	Prolonged release	To ensure controlled drug release for desired duration
	Biocompatibility	Minimal Haemolytic Activity	Incompatibility with blood components can results into complex clinical and/or pathological conditions
Ex vivo permeation		Maximum Transmembrane flux	Enhanced drug concentration at target site, Minimum vaginal tissue deposition
Stability		NLT 3 month at suitable storage conditions	Minimum time period (at least 3 months initially) decided to ensure stability of final formulation

5.3.2.2. Qualitative Risk assessment and Identification of Independent variables (factors)

Based on QTPP, CQA were identified. An overall risk assessment of the drug product formulation components was performed to determine which formulation components have a high risk of impacting the drug product attributes. Following table describes risk assessment of PTX loaded UDNVs.

Table 5-3: Qualitative Risk Assessment

Factors	Process step	Impact on CQA	Constant levels
API Source	Raw Materials Selection and Specifications	Low risk	Authentic Source
API Storage		Low risk	Stored at 2–8 °C and protected from the light as per recommended storage conditions
Selection of Lipid-Type I		Low risk	DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine)
Selection of Lipid-Type II		Low risk	EggPC (L-α-phosphatidylcholine)
Lipid Source		Low risk	Authentic Source
Lipid Storage		Low risk	Recommended storage condition
Edge Activator Selection		Low risk	Sodium deoxycholate
Edge Activator Source		Low risk	Authentic Source
Edge Activator Storage		Low risk	Recommended storage condition
Organic Solvent- Type I		Medium risk	Methanol
Organic Solvent- Type II		Medium risk	Chloroform
Source of Organic Solvents		Low risk	Authentic Source
Hydration Media		Low risk	Water
Source of Hydration Media		Low risk	In House
Hydration Media Standards		Low risk	Double distilled; filtered through 0.2 µ
Dispensing area Temp/RH	Dispensing	Low risk	25 ± 3°C at RH NMT 45%
Weighing balance type		Low risk	Digital weighing balance

Sensitivity and Calibration of Weighing balance		Low risk	Calibrated with 0.1 mg least count
Manufacturing Vessels	Manufacturing Setup	Low risk	Type I Borosilicate glass vessels
Temp/RH of area		Low risk	25±3°C, Ambient RH
Amount of Drug	Organic Phase Preparation	High Risk	To be optimized
Lipid: Surfactant Ratio		High Risk	To be optimized
DSPC : EggPC Ratio		High Risk	To be optimized
Evaporation Temperature	Evaporation of Organic Solvent and Thin film preparation	Low risk	45± 2°C (in thermostatic water bath)
Evaporation Time		Medium risk	2 hr
Speed of Rotation		Medium risk	100 RPM
Hydration Temperature	Aqueous Phase Preparation	High Risk	To be optimized
Hydration Time		High Risk	To be optimized
Hydration Volume		High Risk	To be optimized
Type and MOC of the centrifuge tube	Removal of free drug	Low risk	Screw cap conical bottom standing centrifuge tube
Centrifugation Temperature		Low risk	4°C
Centrifugation Time		Medium risk	10 min
Centrifugation Speed		Medium risk	5000 RPM
Analytical instruments	Analytical Setup and Storage	Low risk	Calibrated
Analytical methods		Low risk	Validated
Analytical Reagents/Solvents		Low risk	Analytical Grade
Formulation Storage vessel		Medium risk	20 ml glass vials with screw cap
Formulation Storage Temp.		Low risk	Refrigerated Conditions
Formulator	Personnel	Low risk	Common for all experiments and analysis
Analyst		Low risk	

5.3.2.3. Quantitative risk assessment: Factors Screening Design

Factors with high risk were evaluated further using quantitative risk assessment. The statistical evaluation was done by 2-level Plackett-Burman screening design. High (+1) and low (-1) levels of the independent variables were determined based on the preliminary trials conducted as well as the literatures available. The experimental matrix was generated using Minitab® 16.1.1 statistical software. The data obtained by experiments conducted as per the matrix design were fed in the software to process for the results in terms of Pareto charts, Normal and main effect plot for all the CQA considering p-value < 0.05 as the level of significance.

Table 5-4: Independent variables and levels for Plackett-Burman screening design

Independent variables		Units	Levels	
			Low (-1)	High (+1)
A:	Amount of Drug	mg	3	7
B:	Lipid : Surfactant Ratio	By weight	75:25	95:5
C:	DSPC : EggPC Ratio	By weight	30:70	70:30
D:	Hydration Temperature	°C	30	60
E:	Hydration Time	min	5	25
F:	Hydration Volume	ml	2	10

Table 5-5: Plackett-Burman design experimental matrix and results

Batch no.	Run order	Independent Variables						Response Variables		
		A	B	C	D	E	F	% EE	Size (nm)	DI
1	3	7	75	70	30	5	2	63.76	255.2	15.15
2	6	7	95	30	60	5	2	64.56	228.5	13.92
3	5	3	95	70	30	25	2	78.37	262.2	13.45
4	1	7	75	70	60	5	10	65.34	232.4	15.20
5	8	7	95	30	60	25	2	65.12	300.2	14.86
6	13	7	95	70	30	25	10	66.17	284.7	13.35
7	11	3	95	70	60	5	10	80.59	298.1	13.95
8	12	3	75	70	60	25	2	70.01	266.9	17.03
9	9	3	75	30	60	25	10	65.60	225.2	17.90
10	10	7	75	30	30	25	10	47.69	269.4	15.58

11	7	3	95	30	30	5	10	71.46	226.1	15.08
12	4	3	75	30	30	5	2	64.75	243.1	18.37
13	15	5	85	50	45	15	6	68.50	273.1	16.55
14	2	5	85	50	45	15	6	67.08	259.5	17.52
15	14	5	85	50	45	15	6	64.42	286.7	16.79

Pareto charts and Normal Plot charts were generated to screen the factors that significantly influence the response variables. The Pareto chart shows the absolute values of the standardized effects from the largest effect to the smallest effect. The chart also plots a reference line to indicate which effects are statistically significant. The reference line for statistical significance depends on the significance level (denoted by α or alpha). The magnitude of factors if crosses the reference line signifies its effect on the response. The normal plot of the standardised effect also depicts significantly affecting factors.

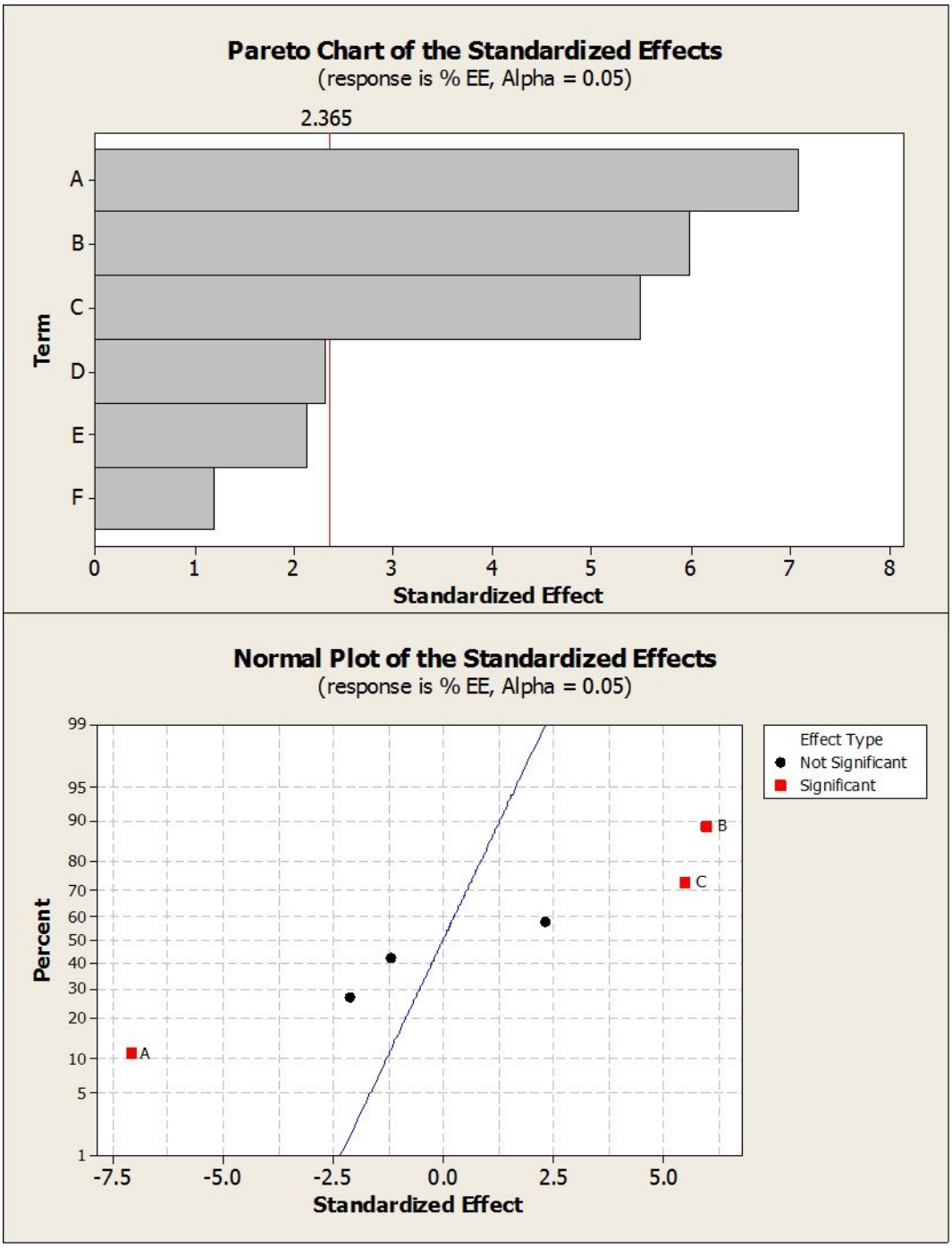

Fig. 5-1: Pareto and Normal plots for %EE

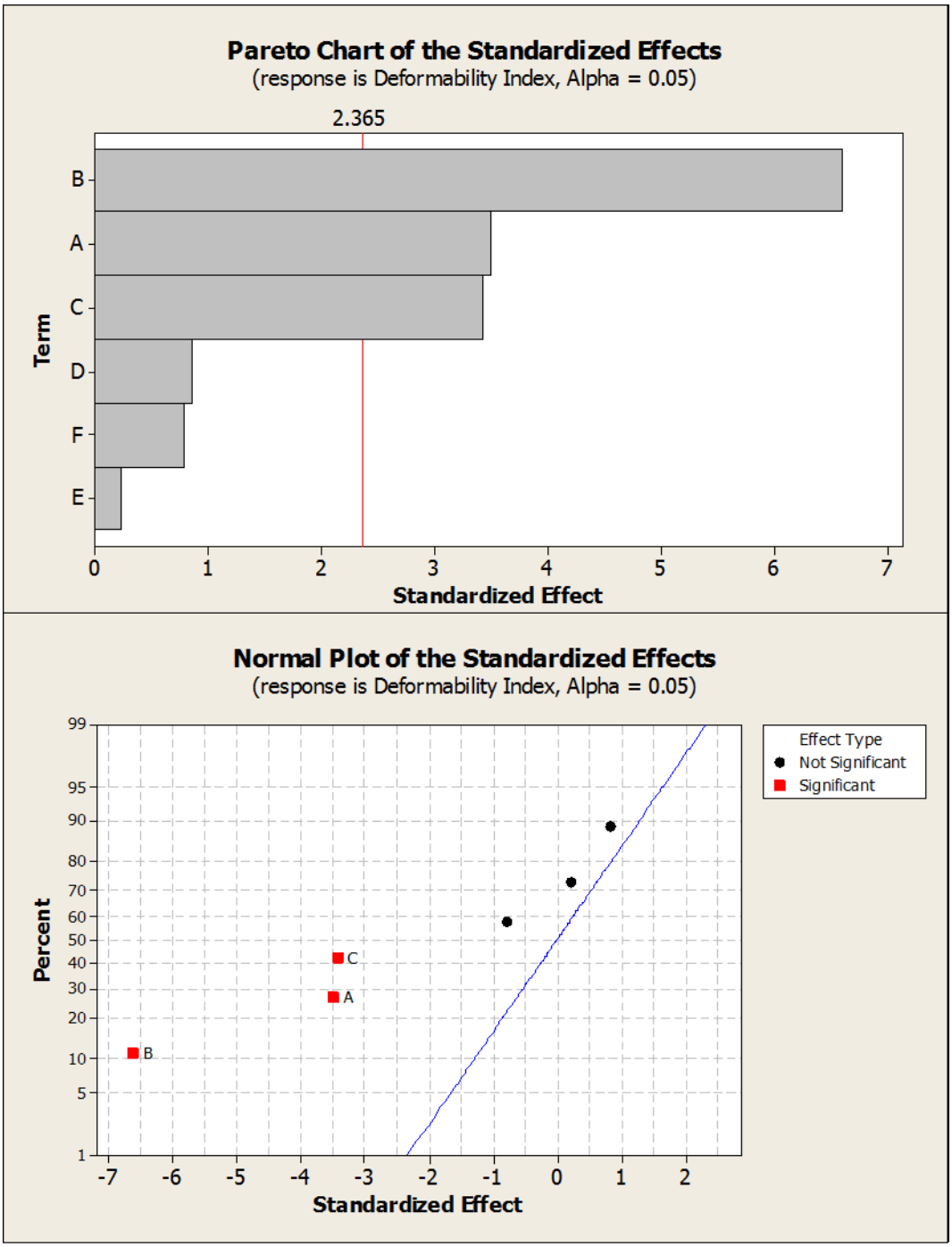

Fig. 5-2: Pareto and Normal plots for DI

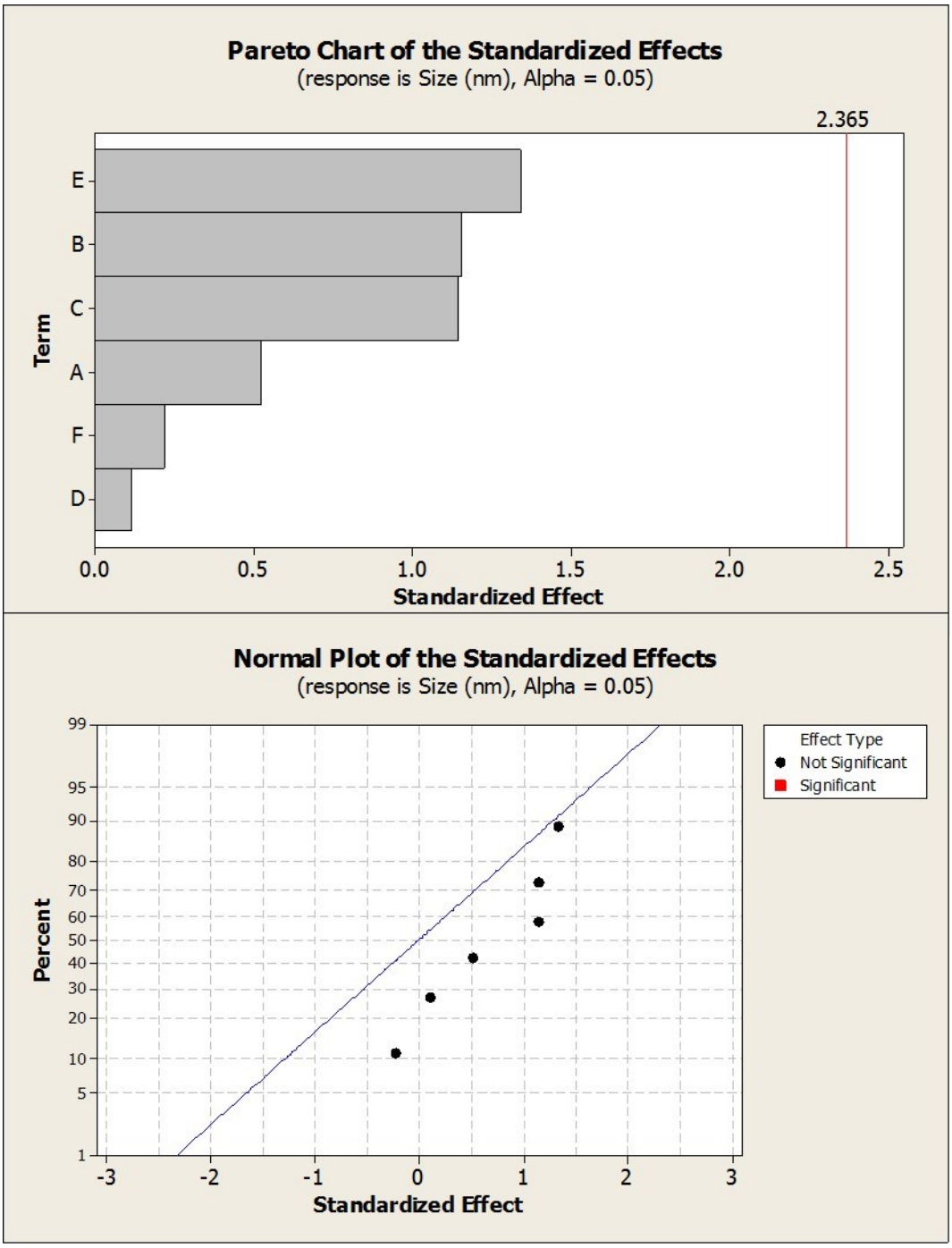

Fig. 5-3: Pareto and Normal plots for Vesicle Size

%EE was found to be significantly affected by drug amount, lipid/surfactant ratio and DSPC/EggPC ratio. The above fact can be evidenced from the Pareto Chart and Normal plot. Hydration temperature (50 ± 2 °C) and hydration media aliquot volume (2.5 mL) were fixed at the level at which they found to achieve maximum %EE in the preliminary trials. Hydration time was not found significantly affecting any of the CQAs and it was

fixed at 10 min based on the results of preliminary trials. The vesicle size of all the experimental trials was not significantly affected by any of the independent factors in the screening design and hence dropped for the evaluation in final optimization design. The results as depicted in the pareto charts and the normal plot showed that the deformability of the lipid bilayer was significantly affected by lipid/surfactant ratio, drug amount and DSPC/EggPC ratio. Hence all the three material attributes were further considered for the evaluation by final optimization design.

5.3.2.4. Formulation optimization using Box–Behnken Response Surface Design

As suggested by the results of the screening design/preliminary trials, three critical material attributes were considered for the evaluation of their effects on critical quality attributes i.e. %EE and DI. The independent factors i.e. Amount of Drug (mg), Lipid:Surfactant Ratio and DSPC:EggPC Ratio were exhaustively studied using Box–Behnken Response surface statistical design. Selected independent variables and respective values were set at low (-1), medium (0) and high (+1) levels.

Table 5-6: Various critical material attributes along with their levels for optimization by Box-Behnken design

Independent variables (CMAs)		Units	Levels		
			Low (-1)	Medium (0)	High (+1)
A:	Amount of Drug	mg	3	5	7
B:	Lipid : Surfactant Ratio	By weight	75:25	85:15	95:5
C:	DSPC : EggPC Ratio	By weight	30:70	50:50	70:30

The 3-factor 3-level Box-Behnken experimental design matrix was generated using Design-Expert software. The batches presented in the design matrix were prepared and evaluated for CQA. The resulting values of the CQAs were used to analyse the respective experimental models. Analysis of variance (ANOVA) was used to establish the statistical validation of the polynomial equations generated by Design Expert software. All the responses observed were simultaneously fitted to linear (first order), second order, and quadratic models. Various feasibilities were conducted over the

experimental domain to find the compositions of the optimized formulation. 3D response surface plots were generated by the software, whereby intensive grid search performed over the whole experimental region. Checkpoint formulations were selected to validate the chosen experimental domain. Design space was created in order to identify area within which the deviations made in the independent factors will result into desired outcome. The resultant experimental values of the responses were quantitatively compared to that of the predicted values.

Table 5-7: Box-Behnken design experimental matrix and results

Batch no.	Run order	Independent Variables			CQA	
		A	B	C	% EE	DI
1	13	3	75	50	62.77	22.73
2	16	7	75	50	76.16	14.77
3	2	3	95	50	84.37	26.76
4	7	7	95	50	86.82	17.88
5	8	3	85	30	71.84	24.41
6	14	7	85	30	75.37	16.95
7	5	3	85	70	72.46	23.91
8	4	7	85	70	83.94	15.11
9	6	5	75	30	70.61	19.09
10	12	5	95	30	84.35	25.14
11	17	5	75	70	77.28	18.34
12	1	5	95	70	89.68	24.57
13	10	5	85	50	74.42	22.52
14	11	5	85	50	75.96	22.7
15	9	5	85	50	74.27	21.44
16	3	5	85	50	76.63	22.83
17	15	5	85	50	75.55	21.07

ANOVA- a multivariate analysis was applied to the results and the full as well as reduced models for %EE and DI were presented in below tables. ANOVA suggested a quadratic model as the best fit for the available data set since there were curvilinear relationships amongst the multiple variables. The lack of fit was insignificant for the models selected which clearly indicates that there is existence of the good fit for the models selected.

Table 5-8: ANOVA of full as well as reduced quadratic model for %EE

Source	Full model					Backward Elimination Reduced model (α out - 0.1)*				
	DF	Adj SS	Adj MS	F-Value	P-Value	DF	Adj SS	Adj MS	F-Value	P-Value
Model	9	714.60	79.40	56.51	< 0.0001	8	714.15	89.27	69.44	< 0.0001
A-Amt of Drug	1	118.97	118.97	84.67	< 0.0001	1	118.97	118.97	92.54	< 0.0001
B-Lipid:EA	1	426.32	426.32	303.42	< 0.0001	1	426.32	426.32	331.63	< 0.0001
C-DSPC:EggPC	1	56.13	56.13	39.95	0.0004	1	56.13	56.13	43.66	0.0002
AB	1	29.92	29.92	21.30	0.0024	1	29.92	29.92	23.27	0.0013
AC	1	15.80	15.80	11.25	0.0122	1	15.80	15.80	12.29	0.0080
BC	1	0.45	0.45	0.32	0.5896					
A^2	1	6.13	6.13	4.36	0.0751	1	6.13	6.13	4.77	0.0605
B^2	1	47.84	47.84	34.05	0.0006	1	47.84	47.84	37.21	0.0003
C^2	1	12.80	12.80	9.11	0.0194	1	12.80	12.80	9.95	0.0135
Residual	7	9.84	1.41			8	10.28	1.29		
Lack of Fit	3	5.75	1.92	1.88	0.2739	4	6.20	1.55	1.52	0.3474
Pure Error	4	4.08	1.02			4	4.08	1.02		
Total	16	724.44				16	724.44			

* Shaded rows represent insignificant model terms removed during model reduction by backward elimination technique.

The ANOVA of %EE indicates that there are significant main effects of amount of drug, Lipid:EA ratio and DSPC:EggPC ratio with p-values significantly lower than 0.05 at 5% level of significance. Moreover, interaction effects amongst all three independent variables found significant in full model. The reduced model was developed using backward elimination technique at alpha out level of 0.1 that selectively removed factor effects which were not found to be useful contributor for the prediction of the response. The polynomial equations were generated and represented below:

Full model equation:

$$\%EE = 75.366 + 3.86A + 7.3B + 2.65C - 2.73AB + 1.99AC - 0.33BC - 1.21A^2 + 3.37B^2 + 1.74C^2$$

Reduced model equation:

$$R1 = 75.366 + 3.86A + 7.3B + 2.65C - 2.73AB + 1.99AC - 1.21A^2 + 3.37B^2 + 1.74C^2$$

The number associated with the individual term indicates the magnitude of the effect contributed by that variable and the positive or negative sign indicative of the direct or inverse relationship of the model term.

Table 5-9: ANOVA of full as well as reduced quadratic model for DI

Source	Full model					Backward Elimination Reduced model (α out - 0.1)*				
	DF	Adj SS	Adj MS	F-Value	P-Value	DF	Adj SS	Adj MS	F-Value	P-Value
Model	9	198.62	22.07	25.58	0.0002	3	195.65	65.22	94.12	< 0.0001
A-Amt of Drug	1	136.95	136.95	158.73	< 0.0001	1	136.95	136.95	197.65	< 0.0001
B-Lipid:EA	1	47.14	47.14	54.64	0.0002	1	47.14	47.14	68.04	< 0.0001
C-DSPC:EggPC	1	1.67	1.67	1.94	0.2062					
AB	1	0.21	0.21	0.25	0.6356					
AC	1	0.45	0.45	0.52	0.4941					
BC	1	0.01	0.01	0.01	0.9255					
A^2	1	11.24	11.24	13.02	0.0086	1	11.55	11.55	16.68	0.0013
B^2	1	0.01	0.01	0.02	0.9042					
C^2	1	0.62	0.62	0.72	0.4249					
Residual	7	6.04	0.86			13	9.01	0.69		
Lack of Fit	3	3.47	1.16	1.81	0.2857	9	6.44	0.72	1.12	0.4951
Pure Error	4	2.57	0.64			4	2.57	0.64		
Total	16	204.65				16	204.65			

* Shaded rows represent insignificant model terms removed during model reduction by backward elimination technique.

ANOVA was also applied to the results obtained for the DI of the vesicles. The significant effects were indicated by the p-values lower than 0.05 at 5% level of significance. In full model, two of the main effects i.e. amount of drug and Lipid:EA ratio were found significant while DSPC:EggPC ratio was found to be insignificant. The reduced model was generated using backward elimination technique at alpha out level of 0.1 that selectively removed those model terms which were not found significantly affecting the final response there by improving prediction power the model. Polynomial equations generated for full model as well as reduced model were presented below:

Full model equation:

$R2 = 22.112 - 4.137A + 2.427B - 0.457C - 0.23AB - 0.335AC + 0.045BC - 1.633A^2 + 0.056B^2 - 0.384C^2$

Reduced model equation:

$R2 = 21.967 - 4.138A + 2.428B - 1.652A^2$

Table 5-10: Model terms summary for full as well as reduced model of %EE and DI

Terms	%EE		DI	
	Full Model	Reduced Model	Full Model	Reduced Model
R-Squared	0.99	0.99	0.97	0.96
Adj R-Squared	0.97	0.97	0.93	0.95
Pred R-Squared	0.86	0.91	0.71	0.92
Adeq Precision	29.10	32.47	18.43	32.52
Std. Dev.	1.19	1.13	0.93	0.83
Mean	77.20	77.20	21.19	21.19
C.V. %	1.54	1.47	4.38	3.93
PRESS	98.45	66.24	59.60	16.13

The difference between adjusted R-Squared and predicted R-Squared was considerably decreased in reduced model. Also the value of predicted R^2 was increased in reduced model which clearly indicates that the prediction power of the reduced model was improved by model reduction. Model R^2 was not significantly affected by the backward elimination of the model terms. It was found near to 1 before and after model reduction. Moreover, standard deviation for both the response variable was reduced by model reduction which shows data consistency improvement after model reduction.

Table 5-11: Coded coefficients of full as well as reduced model for %EE

Factor	Coefficient Estimate	VIF	Full Model			Reduced Model		
			Standard Error	95% CI		Standard Error	95% CI	
				Low	High		Low	High
A-Amt of Drug	3.86	1	0.42	2.87	4.85	0.40	2.93	4.78
B-Lipid:EA	7.30	1	0.42	6.31	8.29	0.40	6.38	8.22
C-DSPC:EggPC	2.65	1	0.42	1.66	3.64	0.40	1.72	3.57
AB	-2.74	1	0.59	-4.14	-1.33	0.57	-4.04	-1.43
AC	1.99	1	0.59	0.59	3.39	0.57	0.68	3.29
BC	-0.34	1	0.59	-1.74	1.07			
A^2	-1.21	1.006	0.58	-2.57	0.16	0.55	-2.48	0.07
B^2	3.37	1.006	0.58	2.00	4.74	0.55	2.10	4.64
C^2	1.74	1.006	0.58	0.38	3.11	0.55	0.47	3.02

Table 5-12: Coded coefficients of full as well as reduced model for DI

Factor	Coefficient Estimate	VIF	Full Model			Reduced Model		
			Std Error	95% CI		Std Error	95% CI	
				Low	High		Low	High
A-Amt of Drug	-4.14	1	0.33	-4.91	-3.36	0.29	-4.77	-3.50
B-Lipid:EA	2.43	1	0.33	1.65	3.20	0.29	1.79	3.06
C-DSPC:EggPC	-0.46	1	0.33	-1.23	0.32			
AB	-0.23	1	0.46	-1.33	0.87			
AC	-0.34	1	0.46	-1.43	0.76			
BC	0.05	1	0.46	-1.05	1.14			
A^2	-1.63	1.006	0.45	-2.70	-0.56	0.40	-2.53	-0.78
B^2	0.06	1.006	0.45	-1.01	1.13			
C^2	-0.38	1.006	0.45	-1.45	0.69			

The values confidence interval ranges for both the response variable for full and reduced model at 95% level tabulated above. The reduced model ranges for confidence interval showed that the range was narrow than the full model. Narrowed ranges at same level of confidence interval indicates that the prediction of the developed model in more precise and accurate. The variance inflation factor (VIF) quantifies the extent of correlation between one predictor and the other predictors in a model. It is used for diagnosing co-llinearity / multi-co-llinearity. Higher values signify that it is difficult to assess accurately the contribution of predictors to a model. The higher the VIF, the more the standard error is inflated, and the larger the confidence interval and the smaller the chance that a coefficient is determined to be statistically significant. The value of the VIF was found 1 and near to 1 for all the model terms for both the response variables indicates that the predictor is not correlated with other variables [3].

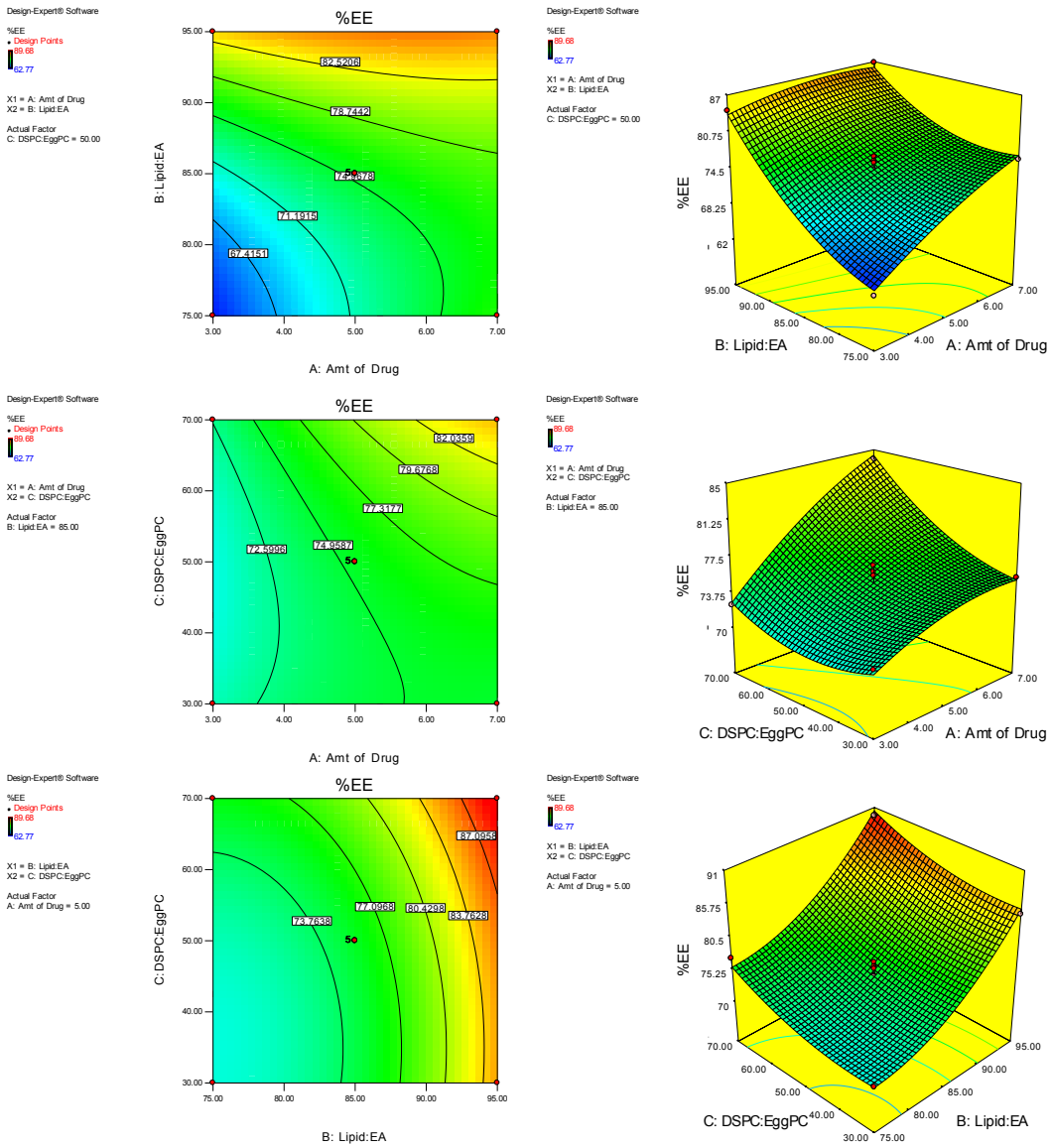

Fig. 5-4: Contour Plot and response surface plots for %EE

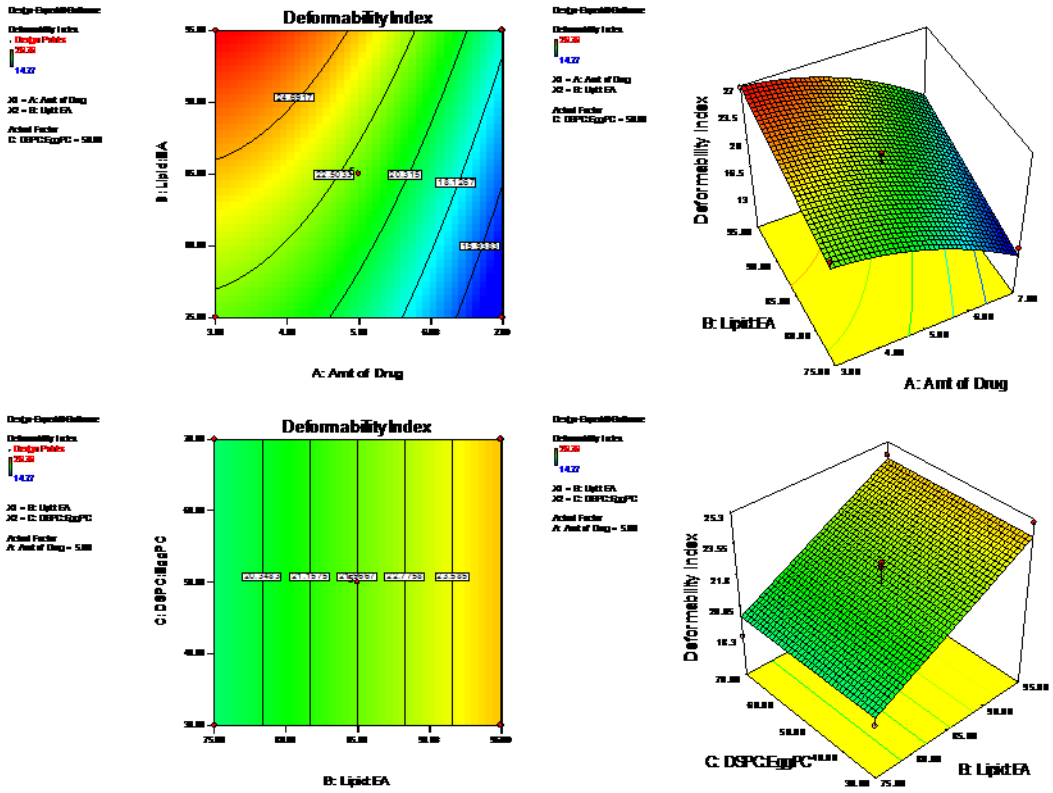

Fig. 5-5: Contour Plot and response surface plots for DI

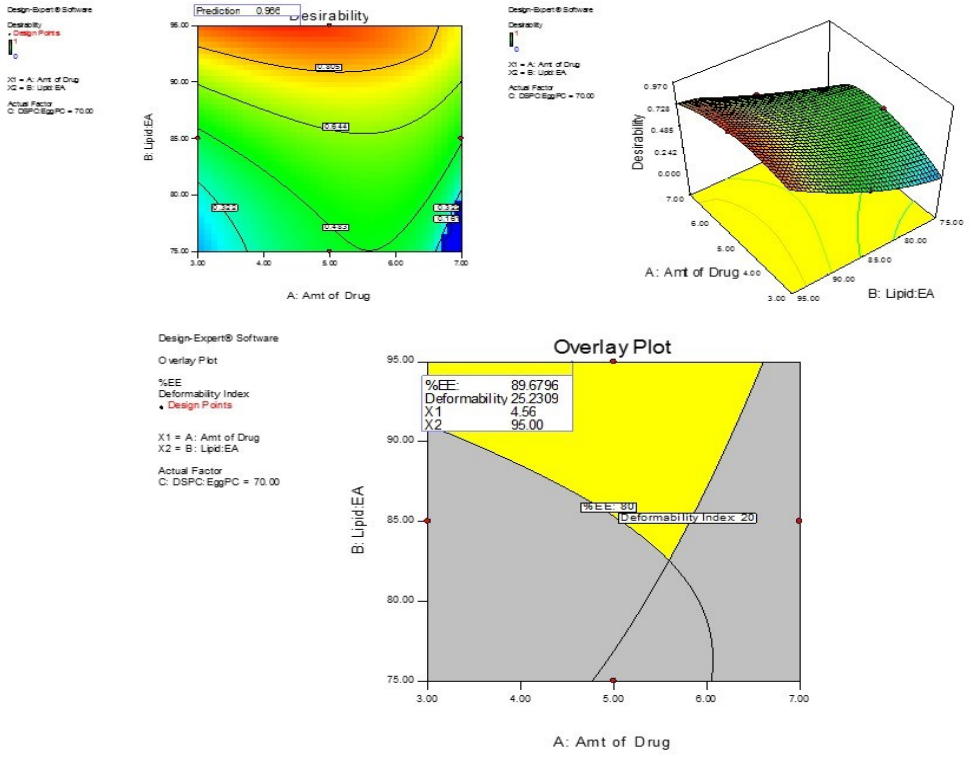

Fig. 5-6: Desirability Plot and Overlay plot for the model

Numerical optimization was performed by the software for defined optimization criteria. The software was programmed to provide the optimization solution with maximum %EE and DI while keeping all the CMA within experimental range.

Table 5-13: Criteria for optimization of the PTX-UDNVs

Constraints name	Goal	Lower	Upper
Amount of Drug	in range	3	7
Lipid:EA	in range	75	95
DSPC:EggPC	in range	30	70
%EE	maximize	62.77	89.68
DI	maximize	14.77	26.76

The optimization process using design space resulted into a composite desirability of 0.966 for the solution provided by the software.

Table 5-14: Summary of the optimization solution

Multiple Response Prediction

Variable	**Setting**
Amt of Drug	4.56
Lipid:EA	95.00
DSPC:EggPC	70.00

Responses	**Fit**	**SE Fit**	**95% Confidence interval**		**95% Prediction interval**	
			Lower	**Upper**	**Lower**	**Upper**
%EE	89.68	0.82	87.80	91.56	86.46	92.90
DI	25.23	0.40	24.36	26.10	23.23	27.23

Table 5-15: Results of verification trials

Responses	**95% Prediction interval**		**Results**			
	Lower	**Upper**	**Batch-1**	**Batch-2**	**Batch-3**	**Average**
%EE	86.46	92.90	89.96	91.37	92.47	**91.27**
DI	23.23	27.23	25.11	24.76	26.95	**25.61**

The mean of both the quality attributes of the formulation found to fall within 95% confidence interval. The results are indicative of the statistical validity of the model.

5.4. Preparation of PTX-UDNVs loaded Intravaginal Rod Inserts

The past ten years has witnessed unprecedented advances in vaginal ring technology for the delivery of drugs, driven almost exclusively by the development of practical, long-acting and user-friendly devices [4]. Various innovative technologies for the designing of IVR have been studied by different scientists to suit desired formulation properties. The rod insert type of IVR has been studied here.

The rod inserts were prepared by lyophilisation of the PTX-UDNVs loaded gel matrix containing silicon tubing. The lyophilized matrix swells in contact with vaginal fluid and release the formulation. Briefly, PTX-UDNVs dispersion was evaporated to 1 mL using a rotary vacuum evaporator at 500 mmHg vacuum and mixed with 15 % w/v of mannitol and 3.5 % w/v of gelatin and allowed to hydrate overnight at 2 to 8 °C to obtain a gel like consistency. This was then mixed properly and inserted into medical grade silicon tubing (3 mm ID, VWR International, UK) using a syringe. The tubing was then frozen at -20 C for 12 hr and then cut into 1 cm segments to obtain rods. These rods were kept for lyophilisation of the gel matrix in freeze dryer under ramping to -30 °C and hold for 6 h, followed by primary drying at -20 °C for 20 h and ramping to +20°C over 60 min and holding for 10 h [5, 6]. The freeze dried rods were removed from the lyophilizer and stored at refrigerated conditions (2-8 °C) in moisture protective coverings until used. The PTX-UDNVs were further characterized for physical stability in IVR and in vitro drug release. The rods were used to study the efficacy of the developed formulations in endometrial cancer induced rabbit model.

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