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Development and Optimization of Stealth Liposomal Drug Delivery System Using Box–Behnken Design

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ABSTRACT

The aim of the present study was to develop and optimize a stealth liposomal drug delivery system of Eribulin mesylate using Box–Behnken design and desirability function by numerical optimization. The first step is standardizing Eribulin mesylate by evaluating parameters like solubility, melting point, identification by FT-IR, identification by HPLC, water content, specific optical rotation and assay. Compatibility studies was performed by FT-IR and DSC. Stealth liposomes were prepared by the thin-film hydration method and optimized using Box – Behnken design to study the effect of amount of Egg Phosphatidylcholine, amount of Cholesterol, and amount of DSPE-PEG 2000 on entrapment efficiency and *in-vitro* drug release. By using the desirability function with numerical optimization by assigning specific goal to each response the optimized formulation was fabricated by using the composition calculated by the Design Expert® software. The formulation was evaluated for percentage drug entrapment, percentage cumulative drug release. The obtained results were compared with software predicted vales and percentage bias was calculated. The optimized formulation was further evaluated by sterility test, bacterial endotoxin test, assay, surface charge, particle size, shape and texture. Stability study was performed by storing at 5°C±3°C and 25°C±2°C/60% RH± 5% RH for 6 months. Forced degradation studies performed by exposing the formulation to various stress conditions. From the obtained results, it can be concluded that stealth liposomes of Eribulin mesylate was successfully developed and optimized using desirability function by three-factor, three level Box–Behnken design using numerical optimization.

INTRODUCTION:

A reasonable experimental design is very important, especially when complex formulations need to be developed, as it can save time, money and reduce experimental errors to obtain reliable experimental data. In particular, the multivariate strategy of experimental design allows simultaneous investigation of the effects of several variables, their actual significance on the considered response, and the possible interrelationship among them. This approach yields maximum information with a small number of experiments [1]. Response surface methodology (RSM) explores the relationships between several independent variables and one or more response variables. RSM includes central composite design (CCD), Box-Behnken design (BBD), and Doehlert design (DM). The present study used BBD since it has a high fitting correlation coefficient, good predictability, and high precision and has been considered to be a cost-effective technique compared to the other usual processes of formulation and optimization because it requires fewer experimental runs and therefore saves time [2].

Liposomes are spherical vesicles of different sizes consisting of a lipid bilayer and aqueous center compartment that is generated in-vitro [3]. These are popular in terms of biocompatibility, biodegradability, low toxicity, and can control the biodistribution of the drug by altering the size, composition of lipids, and hence the characteristics [4]. These are the carriers that are suitable for encapsulation of drugs with different lipophilicities, such as strongly lipophilic drugs, strongly hydrophilic drugs, and drugs with intermediate log P. Liposomes can protect the encapsulated drug or drugs and can target the organ or tissue passively [5]. But it was found that conventional liposomes suffer with two major drawbacks as sustained as well as targeted release system for drugs in vivo. First one is its attraction toward the reticuloendothelial system (RES), which will cause the removal of drug from the bloodstream as well as will result in adverse effects on the host defense system [6] and will decrease the availability of entrapped drug to the other tissues. The next is recognition of conventional liposomes by RES leads to nonlinear pharmacokinetics for the carrier, which makes calculating the amount of entrapped drug required to attain therapeutic drug dose difficult [7][8][9][10]. In addition, conventional liposome formulations containing saturated phospholipids and cholesterol are more prone to the influence of plasma proteins and other biologic fluids in vivo, which leads to rapid removal of drug contents [11][12]. To avoid the above mentioned difficulties, especially to avoid the RES uptake of the vesicles it is

necessary to have previous administration of empty liposomes. Moreover, small unilamellar vesicles have the drawback of low aqueous entrapment volume; the use of charged liposomes could be toxic. Thus, mechanical or electrostatic stabilization cannot improve the long circulation of liposomes in biological systems. Further attempts to alter the biodistribution of liposomes resulted in the generation of new liposomal formulations called stealth liposomes (SLs), which have considerably reduced RES uptake, and remain in circulation for long period [13][14] with dose-independent pharmacokinetics and have reduced susceptibility to protein-induced leakage [15][16].

The rationale for the development of Eribulin mesylate in the form of stealth liposomes is to reduce drug toxicity, with maintaining the efficacy of the drug for a maximum period of time. Stealth liposomal drug delivery system increases the circulation time of the drug in the body by avoiding the reticuloendothelial system and achieves linear pharmacokinetics. This study aimed to optimize the formulation of Stealth containing Eribulin Mesylate prepared by the lipid film hydration method. An experimental design has been used to evaluate the influence of three formulation parameters, i.e. amount Egg phosphatidylcholine, amount of Cholesterol, and amount of DSPE-PEG 2000, on encapsulation efficiency and the cumulative drug release. Secondly, the experimental design was used to optimize the preparation formula to obtain maximum encapsulation efficiency and cumulative drug release. The optimized formulation was fabricated and evaluated by sterility test, bacterial endotoxin test, assay, surface charge, particle size, shape and texture. Stability study for the optimized formulation was performed by storing at 5°C ± 3°C and 25°C ± 2°C / 60% RH ± 5% RH for 6 months. Forced degradation studies performed by exposing the formulation to various stress conditions like acid stress, base stress, oxidation stress, hydrolytic stress, metallic stress, thermal stress, humid stress and photolytic stress.

MATERIALS AND METHODS

Materials

Eribulin Mesylate was a kind gift sample from Natco Pharma Limited, Hyderabad, whereas Egg Phosphatidylcholine from Vav Life Sciences, Mumbai. DSPE-PEG 2000 from Niram Chemicals, Mumbai, and Cholesterol from S.D. Fine Chemicals Limited, Mumbai. All other reagents were of analytical grade.

Drug Standardization

1. Solubility:

To choose an appropriate solvent system to dissolve the drug, a solubility analysis was conducted. Solubility of the Eribilun mesylate was tested in water, in methanol, in ethanol and in Dimethyl sulfoxide.

2. Melting Point

The melting point of the drug sample was determined by capillary method. One sided closed capillary filled with drug was kept in melting point apparatus to determine temperature range within which solid drug melts.

3. Identification by Fourier-transform infrared spectroscopy (FT-IR)

Weighed about 2 mg of Eribulin mesylate sample and 200 mg of IR grade Potassium bromide in to a dry and mortar. Mixed and grinded with the help of pestle. Using the KBr die set and hydraulic press, the grinded mixture was compressed into pellet form under the pressure of about 8-10 tons. 16 scans of background measurement were performed using the empty pellet holder followed by the recording of sample spectrum using the pellet made. The obtained spectrum was verified peak by peak for the presence of characteristic bands corresponds to the Eribulin mesylate in the test sample.

4. Identification by High Performance Liquid Chromatography

Accurately weighed and transferred about 10 mg of Standard into 20 mL volumetric flask dissolved and made up to the volume with diluent. 10 mL of this solution was transferred into 25 mL volumetric flask and made up to the volume with diluent and filtered through 0.22 µm PVDF filter.

Sample preparation was done as per same procedure as in standard preparation with accurately weighed 10 mg of test sample. Gradient elution method was used as per the Table 1 and the Chromatographic parameters as per Table 2. The major peak's retention time in the test solution's chromatogram was compared to the major peak's retention time in the chromatogram of the standard solution.

Table No. 1. Gradient table for identification of Eribulin mesylate

Time in Minutes	Mobile Phase-A (V/V) (Buffer pH 5.00± 0.05)	Mobile Phase-B (V/V) (Acetonitrile)
0	67	33
15	67	33
25	45	55
35	45	55
37	67	33
45	67	33

Table No. 2. Chromatographic parameters for Eribulin mesylate identification

Column	Kromasil 100-C18, (250 × 4.6 mm), 3.5µm
Wavelength	UV – 200 nm
Flow rate	0.8 mL/min
Injection Volume	20 µL
Run time	45 Minutes
Elution mode	Gradient

5. Water Content (% W/W by Coulometer)

Water content in the sample was estimated with three blank determinations in Metrohm Karl fisher Coulometer. 10 mg of sample was accurately weighed under nitrogen atmosphere in "Atmos Bag" which is previously evacuated and filled with nitrogen. Moisture content in the weighed sample was determined.

6. Specific Optical Rotation

Weighed accurately 50 mg of Eribulin mesylate into a 10.0 mL volumetric flask. Dissolved completely and diluted to the volume with Dimethyl sulfoxide. Specific optical rotation of blank and sample was measured at 20°C using wavelength of 365 nm. A single measurement corrected for solvent blank shall be made followed by five sample measurements. The average of five sample measurements taken as result. The following formula was used to determine the specific optical rotation on an anhydrous basis.

7. Assay by High Performance Liquid Chromatography

Standard ad Sample preparations, mobiles phases and chromatographic conditions are same as described in "Identification by High Performance Liquid Chromatography" section. 20 µL of standard solution and sample solutions are injected into chromatographic system and assay (%W/W) was calculated.

COMPATIBILITY STUDIES

1. Compatibility study by Fourier-transform infrared spectroscopy (FT-IR)

Drug alone and with the physical mixtures of Egg phosphatidylcholine, Cholesterol, and DSPE-PEG 2000 was first solubilized in chloroform-methanol solvent mixture and dried to form a pellet. These pellets were kept for one month at room temperature that is 25°C±2°C and 60%±5% relative humidity for complete interaction between the drug and polymer. The drug and drug-polymer samples were dried in a hot air oven at 60°C for 30 minutes for the removal of moisture. These samples were scanned from 4000 to 400 cm^{-1} wavenumbers. Spectra obtained were compared with standard spectra of Eribulin mesylate, for changes in the peaks if any interaction transpires.

2. Compatibility study by Differential Scanning Calorimetry (DSC)

The compatibility between drug and excipients was analyzed by Differential Scanning Calorimetry. Here the samples were weighed and placed in sealed aluminum pans. The purge gas was nitrogen and the flow rate was kept at 20 mL/min. The samples were scanned at 10ºC/min from 20°C-350°C. DSC thermograms of pure Eribulin mesylate and physical mixture of Eribulin mesylate with excipients were recorded.

Identification of Optimized formulation using desirability function

A Box-Behnken optimization design was applied using three independent variables to optimize the conditions and to analyze the sensitivity of responses to the changes made in the settings of experimental design [17][18][19]. A total of 15 experiments were performed out of which the center point was repeated three times. The center points improve the evaluation of the response surface curvature and simplify the estimation of the model error. Using Design Expert software, experimental runs were generated and evaluated. The major response factors used to evaluate the liposome formulation, including Entrapment Efficiency (Y1), and

Cumulative drug release (Y2), were determined. The selected factors with the actual and coded levels according to the design are represented in Table 3.

Table No. 3. Process parameters for Experimental design

Process Parameters	**Levels**		
Independent Variables	**(-1)**	**(0)**	**(+1)**
Egg phosphatidyl choline (µM)	50	100	150
Cholesterol (µM)	50	100	150
DSPE-PEG 2000 (µM)	0.5	1	1.5
Dependent Variables	**Goal for dependent variables**		
(Y1) Entrapment Efficiency	Maximize		
(Y2) Cumulative drug release	Maximize		

The obtained responses were concurrently optimized by a desirability function that uses the numerical optimization method introduced by Derringer and Suich in the Design Expert software [20]. In this approach, a specific goal was assigned to each response. A partial desirability function is associated with an individual response, where value 0 is assigned to an undesired/unacceptable response while for an acceptable response, the value lies between 0 and 1. The value between 0 and 1 indicates the closeness of the response to its target value (i.e., minimum to most desirable. In our study, Design Expert was utilized to conclude the maximum desirability value after assigning desired goals to the responses.

EVALUATION OF OPTIMIZED FORMULATION

1. Sterility testing

Sterility of prepared and optimized formulation was evaluated by sterility test as per Indian pharmacopoeia. The method selected for testing is Method-I i.e Membrane Filtration Method. The vials of liposomal formulation were opened under aseptic condition provided by laminar air flow work station. All precaution and preventive measures were taken to avoid contamination by the process or by the analyst. The formulation was then passed through 0.45µm sterile membrane lodged on a membrane holder assembly. After passing through the solution, the membrane was rinsed three times with 100 ml of sterile peptone (Fluid-A). The membrane was cut into two halves using sterile scissors. One half of the filter paper was inoculated into the tube with SCDM and the other half into the FTGM tube. SCDM tubes

were then incubated at 22.5±2.5°C and FTGM tubes at 32.5±2.5°C. The tubes were observed for turbidity or appearance of growth of microorganisms for 14 days. Positive control and negative control tests were done to validate the sterility testing procedure.

2. Bacterial endotoxin test

Bacterial Endotoxin Test was performed to detect the presence of Endotoxins that may be present in the formulation. The test was performed as per Indian Pharmacopoeia by using Gel-Clot (Qualitative Method) technique. In this method each tube is interpreted as either positive or negative. A positive test is one in which a firm gel forms that is able to maintain its integrity after the test tube is inverted 180 degrees. The absence of gel or the formation of a viscous mass that does not hold when the tube is inverted up to 180 degrees is indicative of a negative test. There should be no gel formation in Negative Control and Test and should be positive in Positive Control and Positive Product Controls.

3. Surface Charge – Zeta potential

The zeta potential is an indication of the stability of the colloidal systems and indicates charge present on the colloidal systems. Highly positive or highly negative surface charge on liposomes indicates higher stability because anticipated surface repulsion between similar charged particles inhibits aggregation of the particles. Zeta potential of optimized formulation was determined using Malvern Zetasizer. Results were recorded by placing the sample into transparent disposable zeta cells.

4. Particle size and Size distribution

Malvern Zetasizer used the laser light scattering technique to determine the particle size and size distribution of liposomes. To measure the particle size and particle size distribution, a Malvern laser light scattering Zetasizer fitted with an argon laser was used. Based on intensity, volume, and bimodal distribution with spherical particle assumptions, the mean droplet size was estimated.

5. Scanning and Transmission Electron Microscopy

Scanning electron microscopy (SEM) and Transmission Electron Microscopy (TEM) of the optimized liposomal formulation was performed for determining the surface morphology & texture, size and shape of formulation and to observe the aggregation property of liposomes.

6. Assay

The assay of the Eribulin mesylate in the optimized formulation was determined by high performance liquid chromatography with the parameters that are described in "Identification by High Performance Liquid Chromatography" section.

7. Stability Study

Stability studies were carried out on optimized formulations according to ICH guidelines. An accelerated stability study of the optimized liposomal formulation was performed to investigate the physical appearance and leak out of the drug from liposomes during storage. Optimized Liposomal suspension was sealed in glass vials and stored at refrigeration 5°C ± 3°C temperature and 25°C ± 2°C / 60% RH ± 5% RH for 6 months. Samples were withdrawn at definite time intervals (0, 3, and 6 months) and observed for Visual appearances like colour, sedimentation, creaming, and extent of leakage. To calculate the extent of leakage, the percentage entrapment efficiency after storage was calculated at regular intervals and then correlated with the extent of leakage. The extent of leakage is estimated from the following formula;

$$\text{Extent of leakage (\%)} = \frac{\text{Initial entrapment efficiency (\%)}}{\text{Amountof drud retained after storage (\%)}} \times 100$$

8. Forced degradation study

The optimized batch is subjected to forced degradations study as per the stress conditions mentioned in Table 4. The formulation after exposing to different modes of degradation conditions, analysed for assay and related substances of Eribulin mesylate, and for presence of degradation products [21]. The mass balance was calculated using the following formula;

$$\text{Mass balance} = \frac{\text{Assay} + \text{Impurities in Stressed sample (\%)}}{\text{Assay} + \text{Impurities in Control sample (\%)}} \times 100$$

Table No. 4. Conditions for Forced Degradation Study

Sr. No	Mode of Degradation	Study Condition
1	Acid Stress (Treatment with Acid)	Treatment with 0.5N HCl at 60°C for 3 Hours.
2	Base Stress (Treatment with Base)	Treatment with 0.2N NaOH at 60°C for 24 Hours.
3	Oxidizing Stress (Treatment with Oxidizing agent)	Treatment with 5% H2O2 at 60°C for 1 Hour.
4	Hydrolytic Stress (Treatment with water)	Treatment with water at 60°C for 24 Hours.
5	Metallic Stress (Treatment with metal ion (Fe3+) solution	Treatment with 100 ppm solution at 60°C for 24 hours.
6	Thermal Stress (Exposure to heat)	At 60°C for 7 days.
7	Humid Stress (Exposure to Humidity)	At 85% RH for 7 days.
8	Photolytic Stress (Exposure to Light)	200 Watt hours/m2 of near UV light and 1.2 million Lux hours.

RESULTS AND DISCUSSION

Drug Standardization

1. Solubility

From the solubility analysis, it was observed that the Eribulin mesylate was freely soluble in Water, Methanol, Ethanol and Dimethyl sulfoxide.

2. Melting Point

The melting point of obtained sample was found to be 160.2°C which is close to the reference value of 160°C. It complies with the standards thus indicating that, the Eribulin mesylate sample was pure.

3. Identification by Fourier-transform infrared spectroscopy (FT-IR)

The FT-IR spectrum of pure Eribulin mesylate was recorded using FT-IR spectrometer is shown in the Figure 1, which were compared with the standard functional group frequencies of Eribulin mesylate as mentioned in Table 5.

Table No. 5. Reported and observed IR frequencies of Eribulin mesylate

Functional Group	**Reported frequencies (in cm^{-1})**	**Observed frequencies (in cm^{-1})**
O-H/N-H Stretch	3400-3600	3429.49
Aliphatic C-H deformation	2900-2950	2934.74
C=O Stretch	1715-1725	1718.60
N-H Stretch	1630-1660	1653.99
S=O Stretch	1180-1200	1192.99
C-O Stretch	1130-1140	1135.13
	1070-1090	1080.16

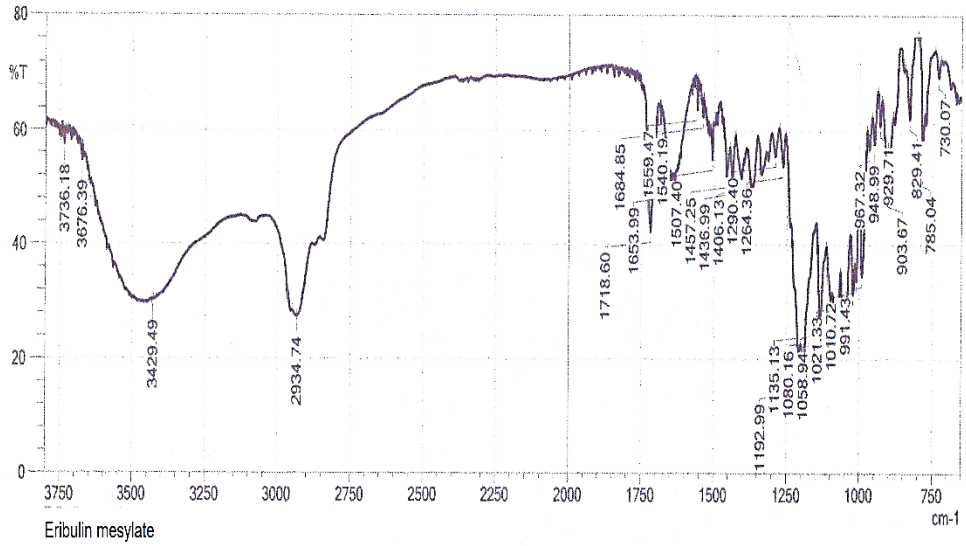

Figure No. 1. FT-IR Spectra of Eribulin mesylate

4. Identification by High Performance Liquid Chromatography

The retention time of the major peak in the chromatogram of the test solution was compared to retention time of the major peak in the standard chromatogram as shown in Figure 2 and Figure 3. It was observed that the retention times of reference standard, test sample are similar and the given drug is identified as Eribulin mesylate.

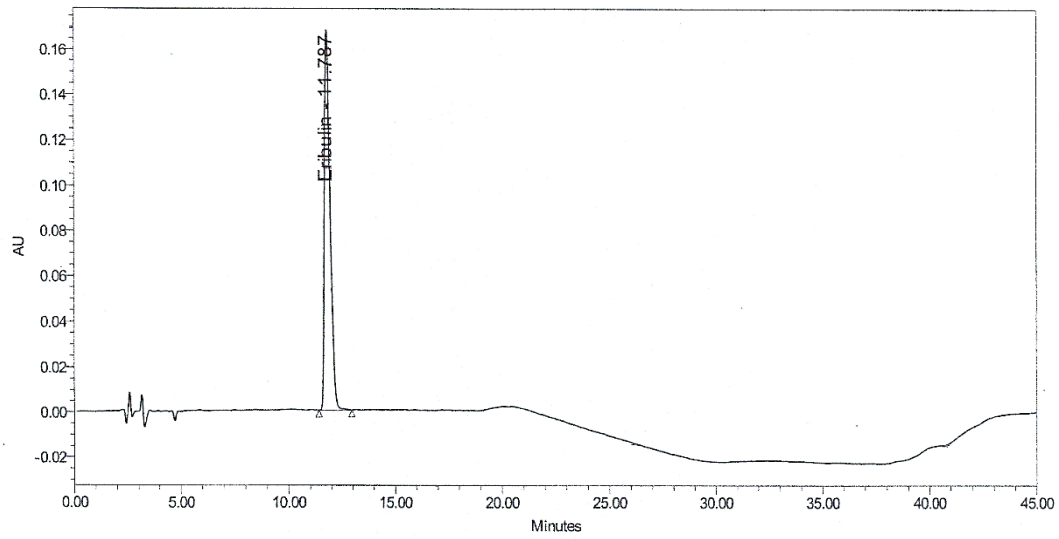

Figure No. 2. HPLC Chromatogram of Eribulin mesylate reference standard

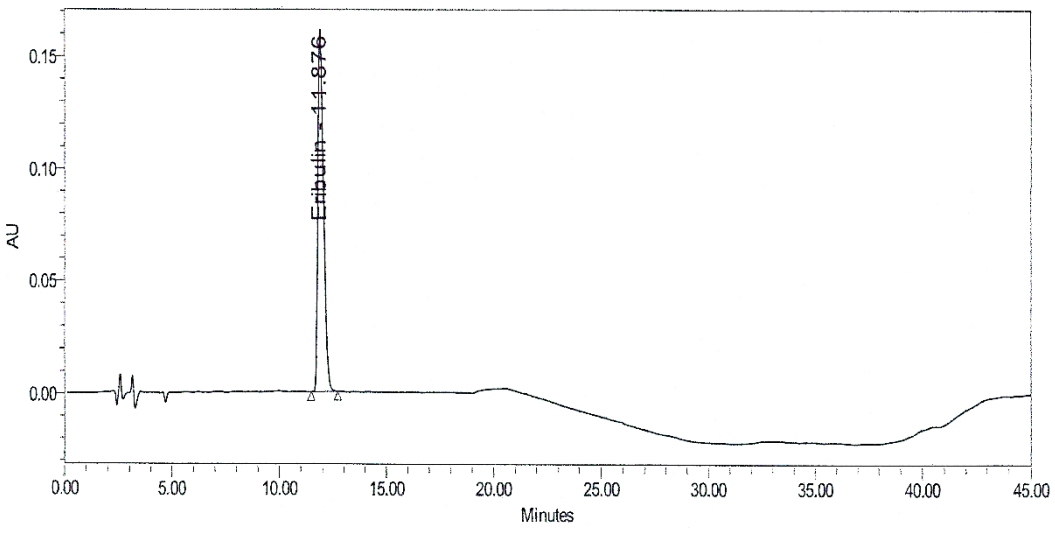

Figure No. 3. HPLC Chromatogram of Eribulin mesylate test sample

5. Water Content (% W/W by Coulometer)

Water content in the Eribulin mesylate sample was determined in %W/W by coulometer and the water content was found to be 1.91% i.e equivalent to 190.84 µg as shown in Figure 4.

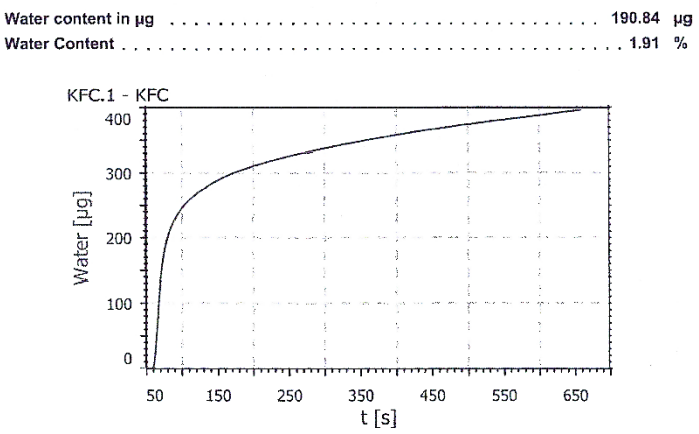

Figure No. 4. Titration curve for water content

6. Specific optical rotation

The specific optical rotation of the test sample was found to be -182° which is within the specification range of -170° and -185°.

7. Assay by High Performance Liquid Chromatography

The Eribulin mesylate assay in the given sample was done by high performance liquid chromatography using gradient elution method with Kromasil 100-C18, (250 × 4.6 mm), 3.5µm column. From the results it was observed that the assay value was found to be 99.93% W/W which indicates that the give drug sample was pure.

COMPATIBILITY STUDIES

1. Compatibility study by Fourier-transform infrared spectroscopy (FT-IR)

The FT-IR spectra of the drug alone and with the physical mixtures of Egg phosphatidylcholine, Cholesterol, and DSPE-PEG 2000 indicate no interaction between the drug and the excipients when compared with the infrared spectrum of the pure drug as all functional group frequencies are present. Figures 5 to 9 shows the FT-IR spectra of the drug alone and the physical mixture of the drug with the excipients. Overlay spectra as in Figure

10 shows that the peaks of pure Eribulin mesylate are identical with the peaks of Eribulin mesylate with the excipients.

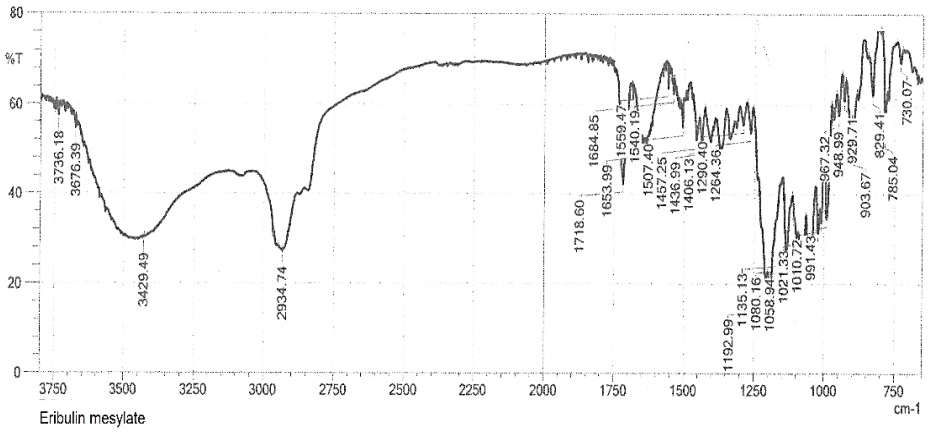

Figure No. 5. FT-IR Spectra of Eribulin mesylate

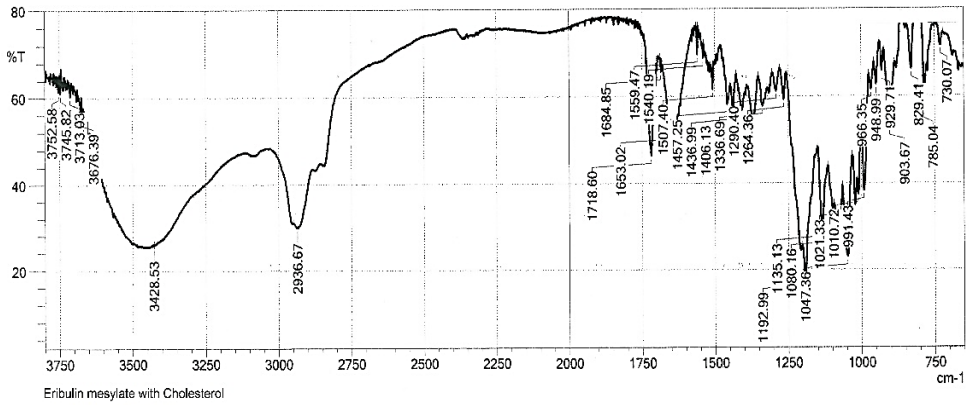

Figure No. 6. FT-IR Spectra of Eribulin mesylate with Cholesterol

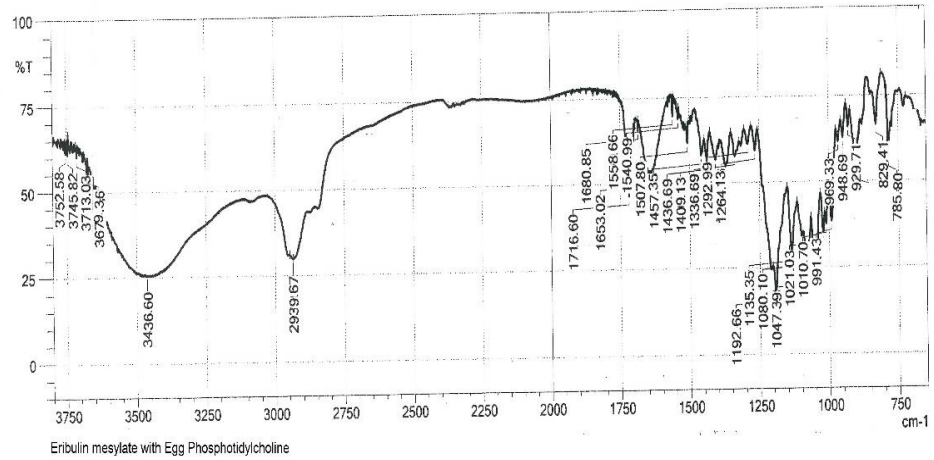

Figure No. 7. FT-IR Spectra of Eribulin mesylate with Egg Phosphatidylcholine

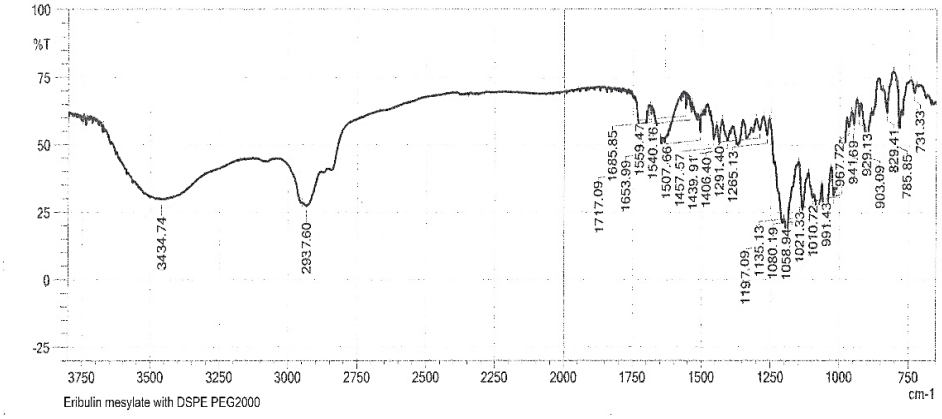

Figure No. 8. FT-IR Spectra of Eribulin mesylate with DSPE PEG 2000

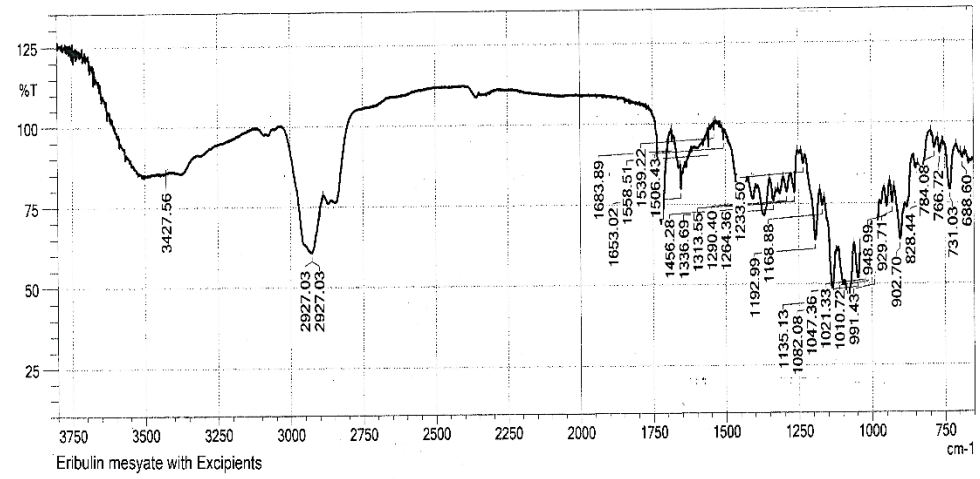

Figure No. 9. FT-IR Spectra of Eribulin mesylate with all excipients

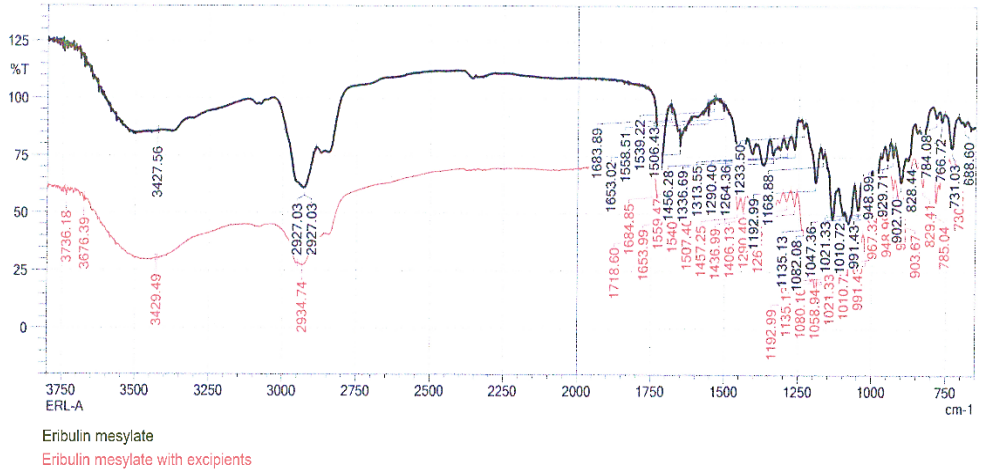

Figure No. 10. FT-IR Overlay spectra

2. Compatibility study by Differential Scanning Calorimetry (DSC)

The DSC thermograms of individual drug and along with excipients were compared with the thermogram of standard thermogram. On comparison, it was observed that the peaks were at the same temperature as those in standard thermograms. Thus, it was proved that there were no significant changes in thermograms hence the excipients were compatible with the drug.

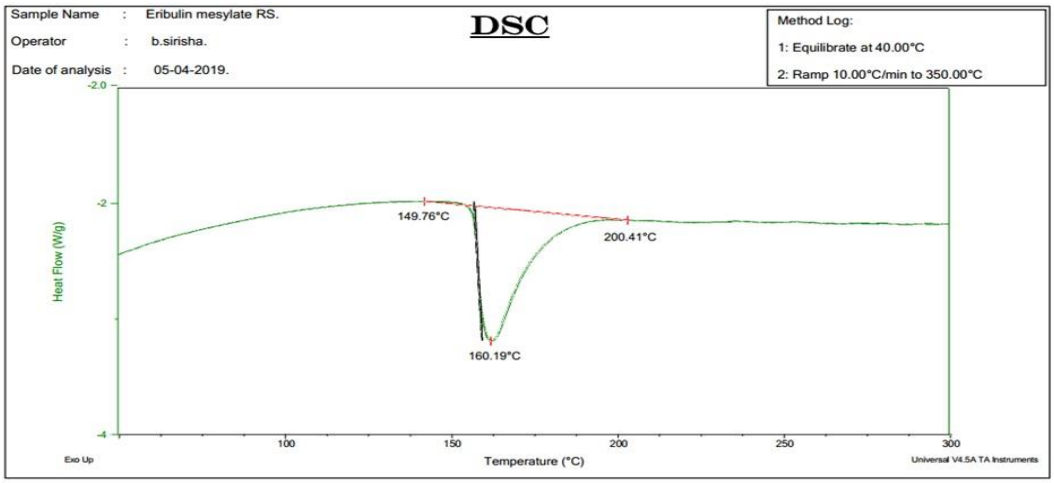

Figure No. 11. DSC thermogram of Eribulin mesylate reference standard

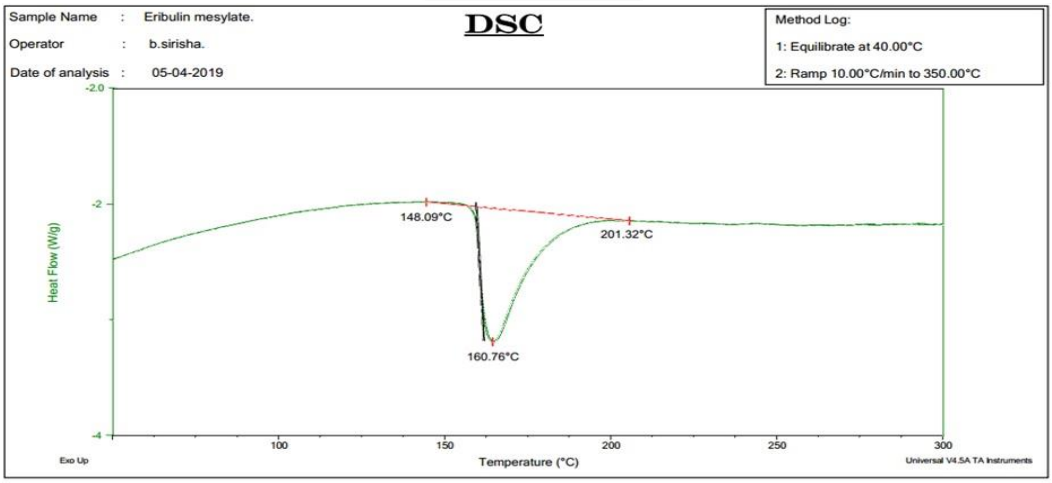

Figure No. 12. DSC thermogram of Eribulin mesylate test sample

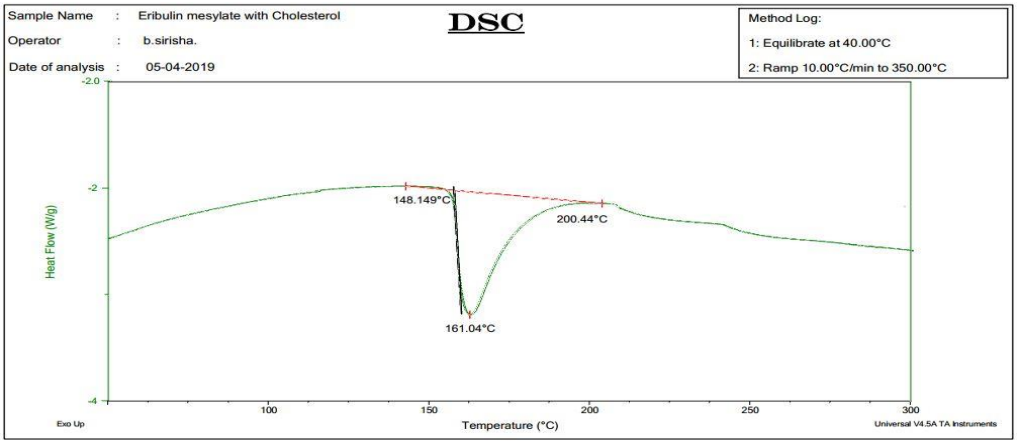

Figure No. 13. DSC thermogram of Eribulin mesylate with Cholesterol

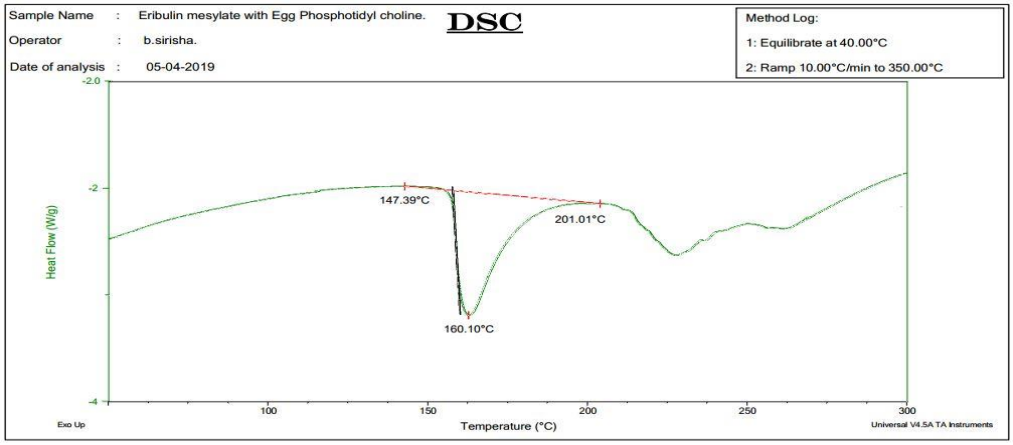

Figure No. 14. DSC thermogram of Eribulin mesylate with Egg phosphatidylcholine

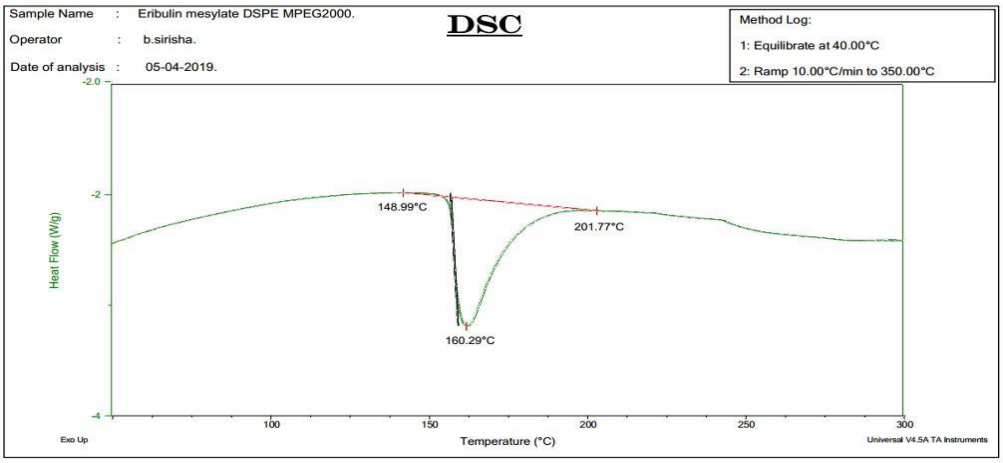

Figure No. 15 DSC thermogram of Eribulin mesylate with DSPE PEG2000

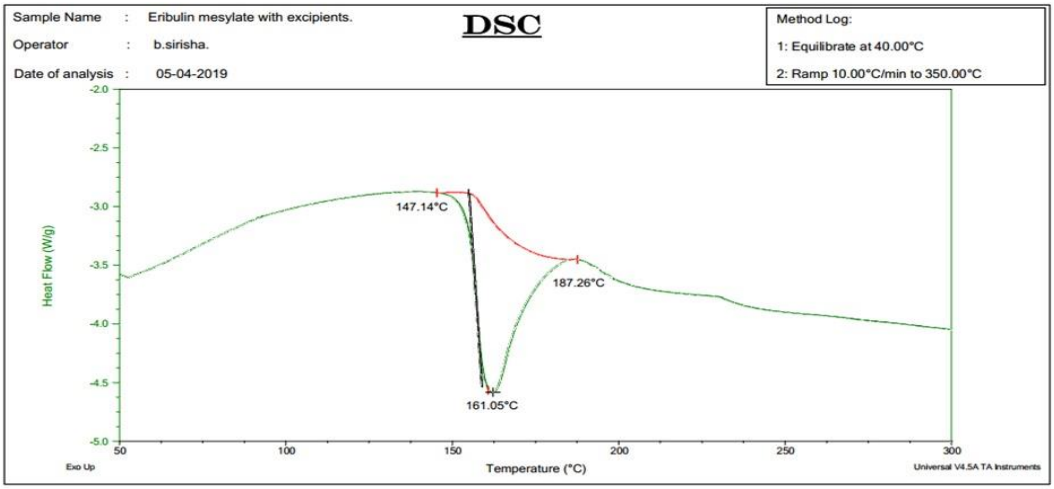

Figure No. 16. DSC thermogram of Eribulin mesylate with all excipients

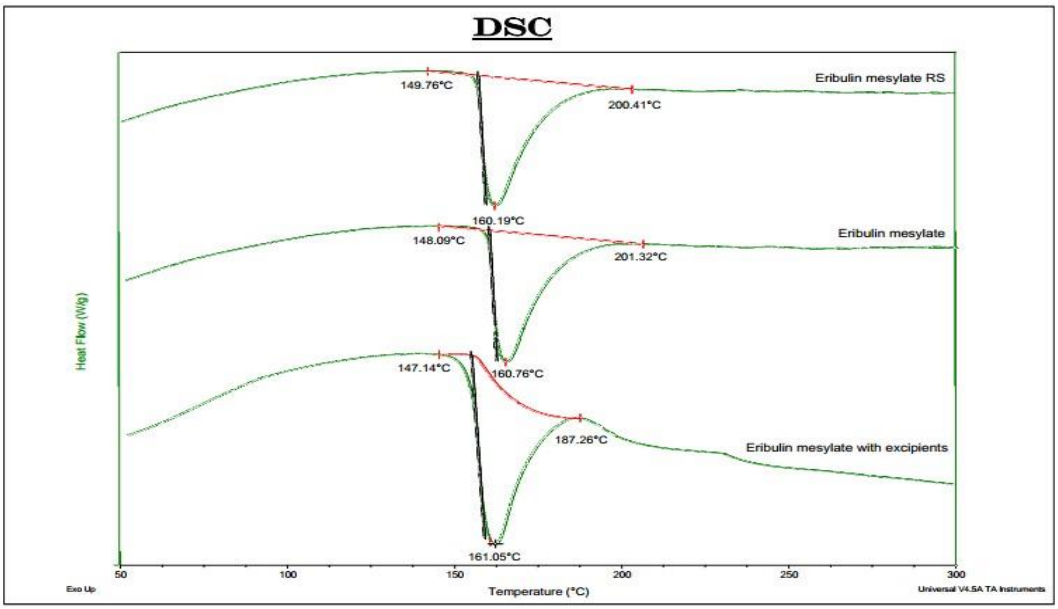

Figure No. 17. DSC Overlay thermogram

Identification of Optimized formulation

An experimental design of fifteen runs was generated for three factors at three levels to identify the optimum levels of different independent process parameters according to Box – Behnken design. Table 6 shows the observed responses along with the predicted values for designed formulations. The observed values for entrapment efficiency, and drug release range from 56.35 to 84.25%, and 62.38 to 94.26%, respectively. As per the optimization design, a positive value shows favorable optimization, whereas a negative value shows an inverse relationship between the factor and the response. It is evident that all the three independent

variables, namely the amount of Egg phosphatidylcholine, Cholesterol, DSPE-PEG 2000, have interactive effects on the responses drug entrapment efficiency and cumulative drug release.

Table No. 6. Observed and predicted values of responses of Box – Behnken DesignFormulation Code	**Dependent Variables**			
	Actual		**Predicted**	
	(Drug Entrapment Efficiency**)**	**(**Cumulative Drug Release**)**	**(**Drug Entrapment Efficiency**)**	**(**Cumulative Drug Release**)**
F01	68.05	71.34	67.91	71.30
F02	77.46	84.18	77.91	83.89
F03	84.25	82.27	84.27	82.59
F04	71.65	70.79	71.85	70.29
F05	60.12	91.05	60.26	91.09
F06	63.27	94.26	63.11	93.90
F07	69.76	62.38	69.54	62.56
F08	78.36	83.26	77.91	83.89
F09	77.92	84.22	77.91	83.89
F10	81.11	89.67	81.05	90.21
F11	66.18	62.39	66.34	62.75
F12	76.23	86.88	76.21	86.56
F13	76.57	79.39	76.63	78.85
F14	58.02	97.88	58.24	97.70
F15	56.35	93.56	56.15	94.06

The desirability function approach was applied in the present study using Design Expert. The constraints were set for all the responses. The responses, Y1 and Y2 were set to be maximized. Equal weight (1) and importance (+++) were given to all responses (weight and importance are the constraints of the software used where 3 pluses (+++) is a default setting that indicates equal importance of all responses). In the desirability function approach, the individual desirability function is calculated which is required for combining all the responses in one measurement. This will help in forecasting the optimum levels for the independent

factors. Optimized formulation with the best desirability function, fulfilling the maximum requirement of response variables was selected. The selected optimized formulation composition was shown in table 6. 18, and the overall desirability was found to be 0.839. The predicted value of the optimized formulation for the response Y1 and Y2 was 80.19 and 91.64 % respectively. To confirm and validate the optimization, an optimized formulation was prepared. All the responses were evaluated for each formulation as observed values. The comparison of the observed and predicted value is shown in Table 7. The percent biased range is between - 0.33 and + 1.66%.

Table No. 7. Predicted and Observed values of Optimized Formulation

Response Variables	Predicted Value	Observed Value	% Bias
Entrapment Efficiency (Y1)	80.19	80.46 ± 2.07	- 0.33
In-vitro drug release (Y2)	91.64	90.11 ± 2.56	+ 1.66

EVALUATION OF OPTIMIZED FORMULATION

1. Sterility test

Media controls (SCDM & FTGM) did not show any growth up to the completion of 14 days incubation period. Negative control (0.1% Peptone) did not show any growth up to the completion of 14 days incubation period and observations are mentioned in Table 8. Test control (Optimized formulation) did not show any growth up to the completion of 14 days incubation period. The bacterial culture showed comparable growth within 5 days of incubation in positive control.

Table No. 8. Observations of Sterility Test of Optimized Formulation

Days	Observations							
	Media Control		Negative Control		Test Control		Positive Control	
	SCDM	FTGM	SCDM	FTGM	SCDM	FTGM	SCDM	FTGM
Day 1	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve	+Ve	+Ve
Day 2	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve	+Ve	+Ve
Day 3	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve	+Ve	+Ve
Day 4	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve	+Ve	+Ve
Day 5	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve	+Ve	+Ve
Day 6	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve	Observation discontinued	
Day 7	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve		
Day 8	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve		
Day 9	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve		
Day 10	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve		
Day 11	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve		
Day 12	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve		
Day 13	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve		
Day 14	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve		

Note: -Ve indicates No turbidity (No growth) and +Ve indicates presence of Turbidity (Growth).

The observations complies to the acceptance criteria and the absence of turbidity in the Test controls indicates the optimized formulation passes the sterility test and the optimized formulation is free from aerobic and or anaerobic and fungal contamination.

2. Bacterial endotoxin test

Bacterial Endotoxin Test was performed to detect the presence of endotoxins that may be present in the formulation. The test was performed as per Indian Pharmacopoeia by using Gel-Clot (Qualitative Method) technique and the observations are mentioned in Table 9.

Table No. 9. Observations of Bacterial endotoxin test of Optimized formulation

Description	Observations			
	Expected		Observed	
	Tube - 1	Tube - 2	Tube - 1	Tube - 2
Negative Control	-Ve	-Ve	-Ve	-Ve
Positive Control	+Ve	+Ve	+Ve	+Ve
Product Control	-Ve	-Ve	-Ve	-Ve
Positive Product Control	+Ve	+Ve	+Ve	+Ve

Note: -Ve indicates absence of Gel and + Ve indicates presence of Gel.

From the obtained results, it was observed that there is no gel formation in the product control tubes which indicates the absence of endotoxins in the formulation. So, the optimized formulation passes the Bacterial endotoxin test.

3. Surface Charge – Zeta potential

The Zeta potential is an indication of the stability of the colloidal systems and indicates charge present on the colloidal systems. The zeta potential of stealth liposomes was found to be -24.88 mV as shown in Figure 18 which indicates high negative surface charge on stealth liposomes which in turn indicates high stability because of the anticipated surface repulsion between similar charged particles, hence inhibiting aggregation of the colloidal liposomal particles.

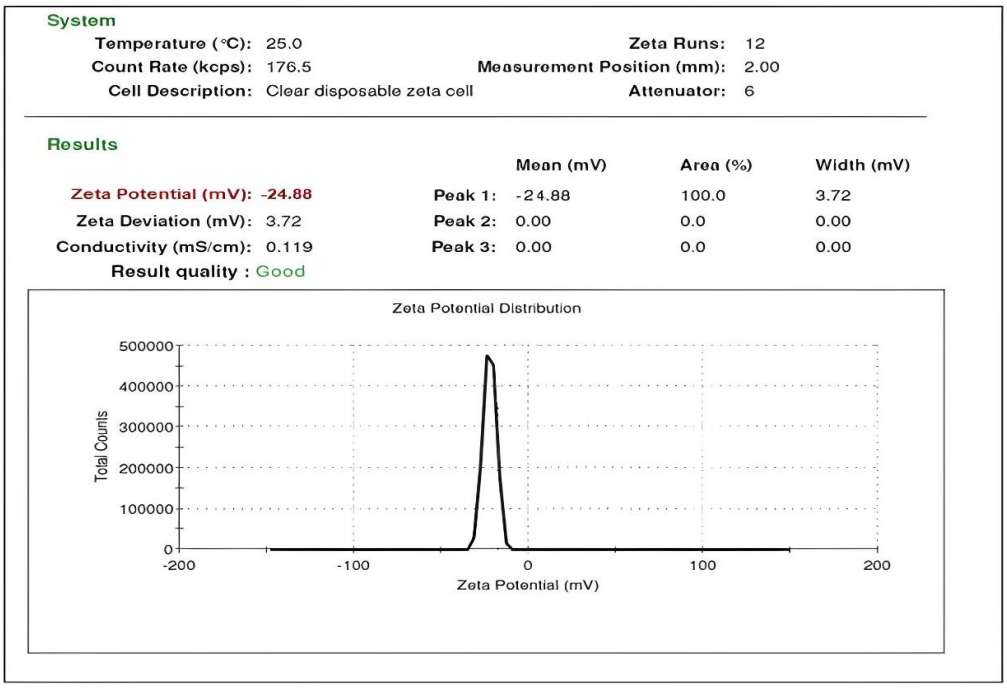

Figure No. 18 Surface charge-Zeta Potential graph of Optimized Formulation

4. Particle size and Size distribution

The vesicle size of optimized formulation was determined by laser diffraction using Malvern zeta sizer. Particle size of vesicles obtained for optimized formulation of stealth liposomes was 139.6 nm which is having desired particle size range (<0.2 µm) as shown in the Figure 19.

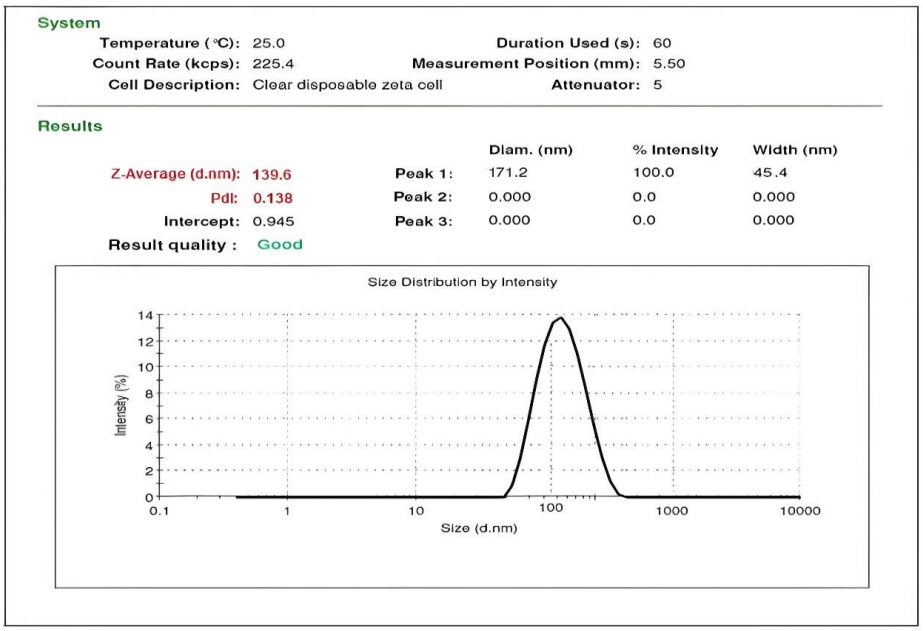

Figure No. 19. Particle Size and Size Distribution curve of Optimized Formulation

5. Scanning and Transmission Electron Microscopy

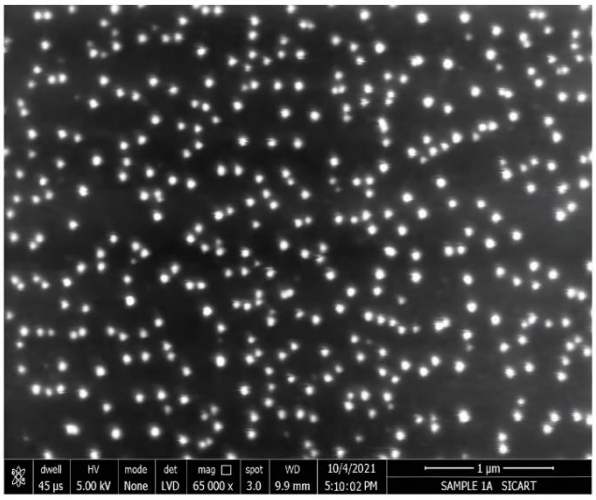

Figure No. 20(A). SEM

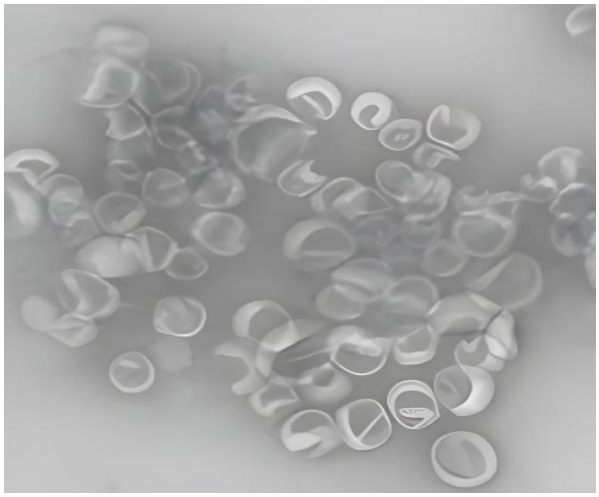

Figure No. 20(B). TEM

Scanning Electron Microgram of optimized formulation was shown in the Figure 20(a). From the figure it was observed that the vesicles are spherical in shape with smooth surface. From the Transmission Electron Microscopy images of the optimized formulation, it was observed that the vesicles are intact and circular as shown in Figure 20(b).

The Eribulin mesylate assay in the optimized formulation was done by high performance liquid chromatography using gradient elution method with Kromasil 100-C18, (250 × 4.6 mm), 3.5µm column. From the results it was observed that the total amount of drug in the stealth liposomal suspension is around 98.36%.

6. Stability Study

The optimized batch was observed for colour, sedimentation, creaming, and extent of leakage during the stability study. The results were mentioned in Table 10. At refrigeration temperature, the formulation was found to be stable and no changes in the physical appearance was observed in all the time intervals. The maximum extent of leakage observed was 2.85% at 6 months. When stored at temperature 25 ± 2°C and 60% ± 5% RH, there is a change observed in the physical attributes of the formulation at 3 and 6 months. The maximum extent of leakage was observed as 93.66%. From the results, it was observed that liposomes remained more stable at refrigeration temperature.

Table No. 10. Stability Study results of Optimized formulation

Time (Months)	Colour	Sedimentation	Creaming	Extent of leakage (%)
At Refrigeration Temperature (5°C ± 3°C)				
0	Off-White	No	No	0
3	Off-White	No	No	1.23
6	Off-White	No	No	2.85
At temperature (25±2°C) (60%± 5% RH)				
0	Off-White	No	No	0
3	Off-White with white sediment	Slight	Slight	69.54
6	Off-White with pale yellow sediment	Prominent	Intense	93.66

7. Forced degradation study

The stress conditions engaged for degradation studies and preferred conditions includes acid stress, base stress, oxidation stress, hydrolytic stress, metallic stress, thermal stress, humid stress and photolytic stress. The acid stress was performed with 0.5 N HCl at 60°C for 3 hours and further dilution was done to analyte concentration for the quantification of Eribulin mesylate and its degradants. The base stress was performed with 0.2N NaOH at 60°C for for 24 hours. The oxidation stress was done using 5% hydrogen peroxide for 1 hour at 60 °C. Water hydrolysis was performed for 24 hours at 60 °C. Metallic stress was performed with 100 ppm solution of (Fe+3) at 60°C for 24 hours. The thermal stress was done at 60 °C for 7 days. Humidity stress was performed at 85% RH for 7 days. The photolytic stressed studies were performed with available photo stability chamber at 200 Watt hours/m2 of near UV light and 1.2 million Lux hours. All the stressed samples were quantified for Eribulin mesylate and the impurities and compared with the control samples. Peak purity of stressed samples of Eribulin mesylate were checked by HPLC instrument. The summary of the forced degradation was captured in table 11 and 12.

Table No. 11. Control sample results for Forced Degradation Study

Sample Details	Assay (%)	Total Impurities (%)	Assay + Impurities (%)
Control Sample – 01 (For Photolytic Stress)	102.2	0.32	102.52
Control Sample – 02 (For Metallic Stress)	98.67	0.36	99.03
Control Sample – 03 (For Acid Stress, Hydrolytic Stress, Base Stress & Oxidation Stress)	100.43	0.22	100.65
Control Sample – 04 (For Thermal Stress & Humidity Stress)	101.98	0.27	102.25

Table No. 12. Forced degradation results for Optimized Formulation

Stress Condition	Assay (%)	Total Impurities (%)	Assay + Impurities (%)	Mass Balance (%)
Acid Stress	84.36	20.31	104.67	104.0
Base Stress	100.39	0.85	101.24	100.6
Oxidation Stress	97.06	1.30	98.36	97.7
Hydrolytic Stress	101.64	1.45	103.09	102.4
Metallic Stress	97.23	1.28	98.51	99.5
Thermal Stress	95.62	2.97	98.59	96.4
Humidity Stress	100.43	0.50	100.93	98.7
Photolytic Stress	100.7	0.38	101.08	98.6

Degradation was not observed in stressed conditions when the analyte was subjected to photolytic stress, thermal stress, humid stress, base stress and hydrolytic stress. The degradation of drug substance was observed only under acid stress. The assay of Eribulin mesylate is unaffected and the mass balance was close to 100%.

CONCLUSION

The Stealth Liposomes of Eribulin mesylate were prepared using the thin-film hydration technique. The formulation was developed and optimized using Box-Behnken design by assessing the response of dependent variables, percentage drug entrapment, and percentage cumulative drug release using the independent variables, amount of Egg phosphatidylcholine, amount of Cholesterol, and the amount of DSPE-PEG 2000. The formulation was optimized by desirability function using numerical optimization. Percentage bias between the observed and predicted results of the quantitative responses of percentage drug entrapment and percentage cumulative drug release of optimum formulation was found relatively less.

The optimized formulation was further evaluated by sterility test, bacterial endotoxin test, surface charge, particle size, Scanning and Transmission Electron Microscopy studies. All the evaluation results were found within the desired limits. Stability studies indicate that the liposomes are stable at refrigeration temperature. Forced degradation studies reveals that the degradation occurred only in acidic stress conditions. Therefore, it can be concluded that a Stealth Liposomal drug delivery system for Eribulin mesylate was developed using a three-factor, three-level Box – Behnken design and optimized by desirability function using numerical optimization.

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