Chapter – 4

Preparation and Optimization of Liposomes

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Liposomal preparations that are successful in seeing the light of market are mainly those of anti cancer agents. It has been established that small and stable liposomes can passively target tumours. Hence, to achieve this aim of cancer targeting, liposomes of different sizes have been prepared and classified accordingly as small unilamellar vesicles (SUV, single, bilayer, 20-200 nm), large unilamellar vesciles(LUV, single layered,100 nm-1 μm), multilamellar vesicles (MLVs, bilayers, 100 nm-20 μm), oligolamellar vesicles (OLVs, size:0.1-1 μm). Different methods can be adopted for preparing the different types of liposomes. Various procedures employed in liposome preparation chiefly comprise of entrapping water soluble or hydrophilic materials by using aqueous solution of these materials as hydrating fluid or by addition of drug solution at a particular stage during manufacturing (Ostro, 1987, New, 1989). The lipid soluble materials are solubilized in the organic solution of constitutive lipids and then evaporated to dryness followed by subsequent hydration. These methods involve loading of entrapped agents before or during manufacturing procedure (Passive loading). The most commonly employed method are lipid film hydration also referred as thin layer evaporation method (THF) (Bangham et al., 1965), reverse phase evaporation technique (REV) (Sozoka and PapBUDadjopoules, 1978), rehydration-dehydration technique (Shew and Deamer, 1985), ethanol injection method (Batzri and Korn, 1975), ether infusion method (Deamer et al 1976), French press technique (Barenholz et al, 1979) and detergent dialysis technique (Kagawa and Racker, 1971). Selection of method largely depends on the nature and physic chemical nature of bioactive to be entrapped. From pharmaceutical point of view, a method has to be selected on basis of drug entrapment and retention efficiency, stability and drug:lipid ratio. (Betagiri, 1993).

Thin Film Hydration (TFH) cuts the edge over other methods of liposome preparation in terms of feasibility on laboratory scale, convenience and high entrapment efficiency particularly in case of hydrophobic or poorly water soluble drugs like Etoposide and Docetaxel.

Encapsulation or entrapment efficiency is one of the crucial parameters to select the method for liposomes preparation.TFH technique when employed for preparing liposomes of hydrophobic or poorly water soluble drugs can result in nearly 80 to 100% drug entrapment efficiency. The unentrapped drug has to be separated from liposomally encapsulated drug by a suitable

technique. Separation of unentrapped drug (Betagiri et al, 1993): Separation of unincorporated drug from liposomes can be achieved either by 'gel filtration' (mini-column centrifugation), ultra centrifugation, protamine aggregation, dialysis or controlled centrifugation at low speed. Literature survey enlists the use of number of methods to separate the free drug by various methods; however, all of them suffer from one or the other limitation. The free drug separation procedures such as dialysis, gel filtration etc. suffered from their own sets of drawbacks as discussed subsequently. Passage through exclusion columns for removal of unentrapped drug are often time-consuming, tedious, expensive, and make recovery of unentrapped drug difficult. Gel filtration was found to be very tedious method with limited capacity and was not feasible for the entire formulation purification. Dialysis method was time consuming and wad observed that drug leaks during the dialysis period. Protamine aggregation was destructive approach and its use is restricted for the determination of the drug entrapment and could not be used for the separation of the liposomal dispersion. Hence, controlled centrifugation under cooling conditions was used in this investigation due to easy and faster method suitable for separation of unentrapped drug.

In spite of availability of wide variety of lipoidal materials for liposome preparation, stability and cost effectiveness are major issues to be addressed for commercially feasible manufacturing of liposomes. Acidic (negatively charged) lipids such as phosphatidylserine, cardiolipin and phosphatidic acid are not preferred components as compared to phosphatidylcholine due to high costs and the often labile nature. Similarly, the use of unsaturated lipids, such as soya phosphatidylcholine or naturally occurring lipids, phosphatidylethanolamine and cardiolipin should be avoided due to its susceptibility towards oxidation. Hydrogenated Soya Phosphatidyl Choline (HSPC) was selected as one of the lipids. HSPC exhibits reasonably fair stability owing to its saturated structure in contrast to Soya Phosphatidyl Choline that shows higher degree of unsaturation. Dipalmitoyl Phosphatidyl ethanolamine (DPPE) was the second lipid used. This lipid was chosen so as to ease the grafting of ligand (Hyaluronic acid-HA) to the formed liposomes by carbodiimide chemistry. Cholesterol (Chol) was incorporated to rigidize the vesicular phase.(Papahadjopolous et al, 1973, Kirby and Gregoriadis, 1980, New, 1989).Thus, given similar loading and retention characteristics, liposomal systems composed of hydrogenated varieties of egg or soya phosphotidylcholine are pharmaceutically more preferred. Though,

efficacy and toxicity of the preparation will dictate the optimum drug/lipid ratio, high drug:lipid ratios are feasible and cost effective from pharmaceutical point of view.

In nutshell, the TFH technique employed for preparation of liposomes in the current investigation can successfully address issues such as drug entrapment efficiency(>90%), stability (due to use of relatively stable lipids like HSPC) and cost effectiveness (High Drug;lipd ratio and relative ease of procedure and comparatively inexpensive lipids used).

Apart from above aspects that determine the selection of procedure employed for liposome synthesis, selection of the appropriate method is also dependent on applications of the liposomes. The method should also ensure sufficient stability during administration of liposomes in a biological system. Lyophilization or Spray Drying techniques are usually employed for liposome stabilization. Some of the pre requisites for stabilization by aforementioned procedures are: sufficient rigidity of liposomal membrane to withstand drying, minimum drug leakage of entrapped drug and liposomal size should preferentially be below 5 μm. Nanoliposomes are ideal for pulmonary administration and their optimal uptake and subsequent retention in leaky vasculature of lung cancer and other tumours.

Conventionally, pharmaceutical formulations are developed by trial and error method whereby one crucial parameter is changed, keeping the other constant. The method is tedious and lacks the provision of developing an ideal formulation since it is not possible to consider the combined effects of the independent variables and mutually interactive effects of these variables. It is therefore necessary to understand the complexity existing in pharmaceutical formulations by using statistical tools such as factorial design. The number of experiments required for these studies depends upon the number of independent variables selected. Factorial design and contour plots are used to study the main and interaction effects of the variables on the PDE (Fannin et al, 1981; Deshayes, 1980; Matthews et al, 1981). The optimization procedure based on factorial design comprises of experimental designs, multiple regression analysis for seeking the optimum formulation. Since, theoretical relationships between the response variables and casual factors are not clear, multiple regression analysis can be applied to prediction of response variables on the basis of a second order polynomial equation. In the present study, drug:lipid ratio, speed of

rotation during film drying and hydration, solvents(Chloroform:Methanol volume ratio) were selected as independent variables whereas Percentage Drug Entrapment (PDE) was selected as dependent variable.

This chapter delineates the detailed preparation methodology of liposomes, optimization by mathematical 2^3 factorial design followed by grafting with HA. Liposomes of Etoposide (ETP), and Docetaxel (DOC) were prepared using TFH technique with membrane composition consisting of lipids such as HSPC, DPPE, and cholesterol. Prepared liposomes were characterized for size and size distribution, zeta potential, and percent drug Entrapment. (PDE) Optimization was carried out by selection of various formulations and process variables using 2^3 factorial design. The liposomes optimized by characterization in terms of particle size, percentage drug entrapment and zeta potential were subjected to surface modification by grafting with HA by carbodiimide coupling chemistry using EDC as coupling agent.

4.1 MATERIALS AND EQUIPMENTS

Material	Source/Supplier
Etoposide (ETP)	Gift sample from Cadila Pharmaceuticals Ltd., Ahmedabad, India.
Docetaxel (DOC)	Gift sample from Sun Pharmaceutical and Advanced Research Centre (SPARC), Vadodara, India.
Hyaluronic acid (HA)	Sigma Aldrich Corporation, Mumbai, India.
EDC	HiMedia, Mumbai, India
Hydrogenated Phosphatidylcholine (HSPC), Dipalmitoyl	Gift samples from Lipoid GmbH,

Phosphatdiylethanolamine(DPPE)	Germany
Cholesterol	S.D.Fine Chemicals, Mumbai,India
6-Coumarin	Gift sample from Neelikon Dyes, Mumbai,India.
Bichinconinic acid (BCA) protein Assay Kit	Bengaluru Genei, India.
Potassium dihydrogen phosphate, disodium hydrogen phosphate, boric acid, borax, sodium chloride, chloroform, methanol	S.D.Fine Chemicals Ltd., Mumbai, India.
Nuclepore Polycarbonate membrane 2 µm	Whatman, USA.
Equipments	**Source**
Calibrated pipettes of 1.o ml, 5.0 ml and 10.0 ml, volumetric flasks of 10 ml, 25 ml, 50 ml and 100 ml capacity, beakers (250 ml) and other necessary glassware	Schott and Corning Ltd., Mumbai.
Dialysis membrane (Molecular weight cut off: 12000 Dalton; thickness: 200µm and porosity 0.45 µm)	Sigma Aldrich Corporation(India) Ltd., Mumbai, India,
Analytical Balance	Precisa 205A SCS, Switzerland
pH meter	Systronics 335, India
Rotary Flash Evaporator	Superfit Equipments, Mumbai,India.
Vortex Mixer	Sphinx, Mumbai
Magnetic stirrers and heating mantle	Remi, Mumbai
Cyclomixer, three blade stirrer	Remi, Mumbai

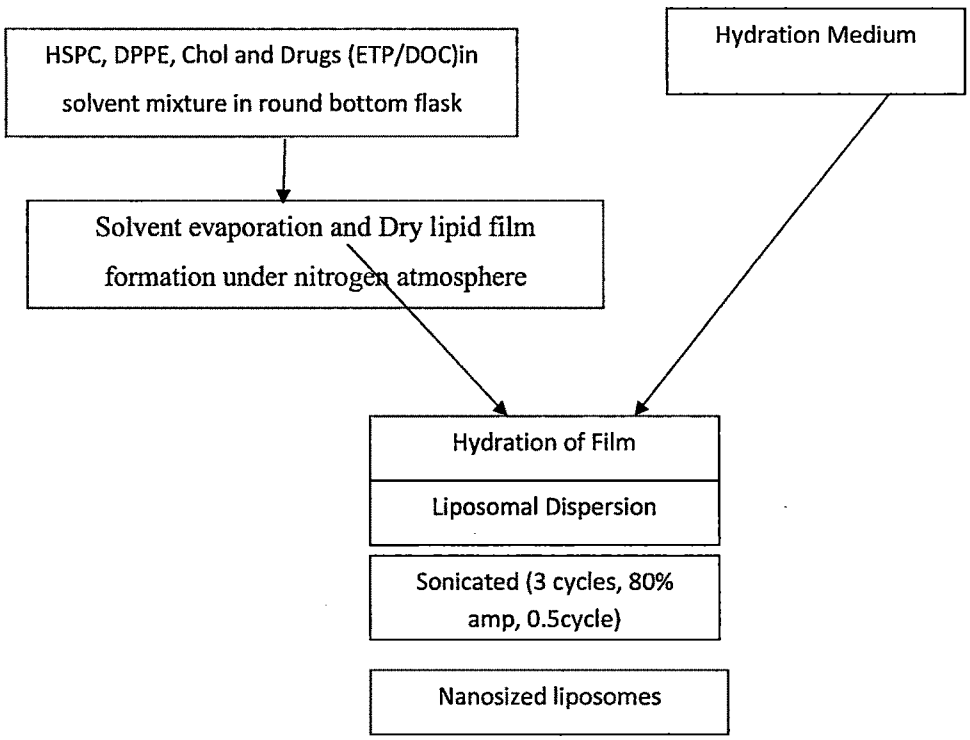
Sigma cooling centrifuge	3k 30, Sigma Laboratory Centrifuge, Osterode, GmbH
Lyophilizer, DW1, 0-60E	Heto Drywinner, Denmark
UV Visible Spectrophotometer	Shimadzu Uv-1601, Japan.
Vacuum Pumps F16	Bharat Vacuum Pumps, Bengaluru, India.
Bath Sonicator	INCO, Ambala, India.
Probe Sonicator	Ralsonics, Mumbai,India.
Optical microscope with polarizer (BX 40)	Olympus Optical Co. Ltd., Japan
Malvern Particle Size Analyser	Malvern Master Sizer 2000 SM, U.K.
Malvern Zetasizer analyzer	Malvern Zetasizer 3000 HS, U.K.
Differential Scanning Calorimeter	Mettler DSC 20, Mettler Toledo, Switzerland
Scanning electron microscope	JSM-840 SEM, Jeol,Japan.

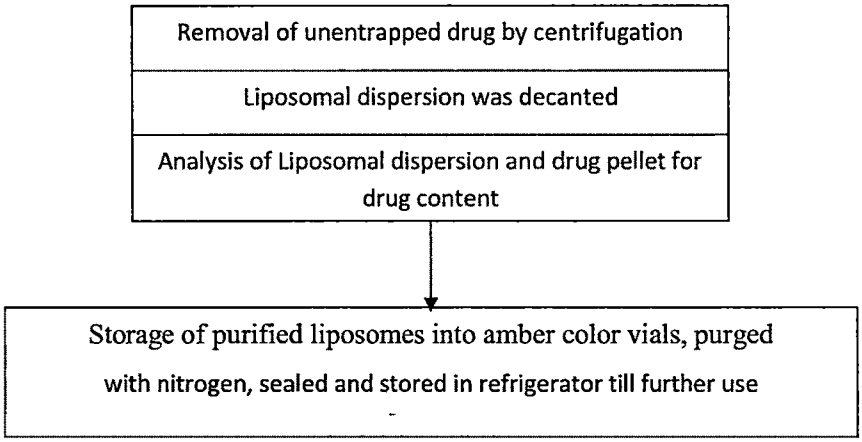
4.2 PREPARATION OF LIPOSOMES OF ETOPOSIDE (ETPLIP) AND DOCETAXEL (DOCLIP)

4.2.1 Preparation of ETP liposomes by TFH method.

Liposomes of ETP consisting of HSPC, DPPE and CHOL were prepared by TFH technique (New, 1990). Briefly, ETP and lipids in drug:lipid ratio of 1:10 were dissolved in a mixture of chloroform and methanol (ratio 2:1 v/v) in a 250 ml round bottom flask in different molar ratios

(Table 4.1 and Table 4.2). The solvent was evaporated in the rotary flash evaporator. The thin dry lipid film thus formed was hydrated using distilled water at 65°C for 60 minutes. The formed liposomal dispersion was sonicated (3 cycles, 80% amp, 0.5cycle) in probe sonicator using ice bath to prevent temperature induced distortion of liposomes. Resultant Liposomes were subjected to centrifugation at 25,000 rpm, 4°C for 20 minutes using ultracentrifuge (Sigma cooling centrifuge, Osterode GmbH, Germany). Liposomal suspension was decanted and drug pellet was separated. Liposomal suspension was then characterized for vesicle size and percent drug entrapment (PDE). Mass balance was calculated by measuring unentrapped drug in pellet. A flowchart depicting the process is shown in scheme 4.1. The liposomal compositions and process parameters were optimized to achieve maximum drug entrapment.





Scheme 4.1: TFH process stages in the preparation of Drug (ETP/DOC) loaded liposomes

4.2.2 Preparation of DOC liposomes by TFH method.

Liposomes of DOC consisting of HSPC, DPPE and CHOL were prepared by TFH technique (New, 1990). Briefly, the DOC and lipids in drug:lipid ratio (1:15) were dissolved in a mixture of chloroform and methanol (ratio 2:1 v/v) in a 250ml round bottom flask in different molar ratios. The solvent was evaporated in the rotary flash evaporator. The thin dry lipid film thus formed was hydrated using aqueous hydrating medium distilled water at 65°C. The formed liposomal dispersion was sonicated (3 cycles, 80% amp, 0.5cycle) in probe sonicator using ice bath to prevent temperature induced distortion of liposomes. Resultant liposomes were subjected to centrifugation at 25,000 rpm, 4°C for 20 minutes using ultracentrifuge (Sigma cooling centrifuge, Osterode GmbH, Germany). Liposomal suspension was decanted and drug pellet was separated. Liposomal suspension was then characterized for vesicle size and percent drug entrapment (PDE). Mass balance was calculated by measuring unentrapped drug in pellet. The liposomal compositions and process parameters were optimized to achieve maximum drug entrapment.

4.2.3 Preparation of 6-Coumarin loaded liposomes by TFH method.

Liposomes of 6-coumarin consisting of HSPC, DPPE and CHOL were prepared by TFH technique (New, 1990). Briefly, the lipids in drug:lipid ratio (1:10) were dissolved in a mixture of chloroform and methanol (ratio 2:1 v/v) in a 250ml round bottom flask. The solvent was evaporated in the rotary flash evaporator. The thin dry lipid film thus formed was hydrated using aqueous hydrating medium distilled water at 65°C. (Procedure of preparation of 6-coumarin loaded liposomes was exactly similar to the one adopted for preparation of drug loaded liposomes) The formed liposomal dispersion was sonicated (3 cycles, 80% amp, 0.5cycle) in probe sonicator using ice bath to prevent temperature induced distortion of liposomes. Liposomal suspension was then characterized for vesicle size and zeta potential. Results were recorded in Table 4.14.

4.3 Optimization of Liposomal formulation using 2^3 Factorial design

4.3.1 Optimization of formulation components for Drug (ETP/DOC) loaded liposomes

A prior knowledge and understanding of process and formulation variables under investigation are necessary to achieve a more realistic model. Based on the results obtained in preliminary experiments, drug:lipid ratio, speed of rotation during film drying and hydration, solvents(Chloroform:methanol) volume ratios were selected as independent variables to optimize for highest percentage drug entrapment (PDE) using 2^3 factorial design and contour plots whereas PDE was selected as dependent variable. The values of these selected variables along with their transformed values are shown in Table 4.1 and Table 4.2. The prepared batches were evaluated for PDE- a dependent variable and the results are recorded in Table 4.3 and Table 4.4.

Table 4.1: Coded values for formulation parameters for preparation of Etoposide liposomes (ETPLIP)

Coded Values	Actual Values		
	X_1	X_2	X_3
-1	1:5	100	2:1
1	1:10	120	3:1

X_1-Drug: Lipid ratio

X_2-Speed of rotation during film drying and hydration.

X_3-Chloroform: Methanol (Solvent volume ratio)

Table 4.2: Coded values for formulation parameters for preparation of Docetaxel (DOCLIP) liposomes

Coded Values	Actual Values		
	X_1	X_2	X_3
-1	1:10	100	2:1
1	1:15	120	3:1

X_1-Drug: Lipid ratio

X_2-Speed of rotation during film drying and hydration.

X_3-Chloroform: Methanol (Solvent volume ratio)

Table 4.3: 2^3 Full Factorial Design for Etoposide Liposomes (ETPLIP)

Batch No.	x1	x2	x3	PDE (%)
1	-1	-1	-1	62
2	-1	-1	1	62
3	-1	1	-1	53
4	1	-1	-1	80
5	-1	1	1	53
6	1	-1	1	79.7
7	1	1	-1	68
8	1	1	1	67.4

Table 4.4: 2^3Full Factorial Design for Docetaxel Liposomes (DOCLIP)

Batch No.	x1	x2	x3	PDE
1	-1	-1	-1	52
2	-1	-1	1	52
3	-1	1	-1	46
4	1	-1	-1	70
5	-1	1	1	43
6	1	-1	1	69.9
7	1	1	-1	58.2
8	1	1	1	57

Mathematical modeling was carried out using to derive a polynomial equation as under:

$$Y=b_0+b_1X_1+b_2X_2+b_3X_3+b_{12}X_1X_2+b_{23}X_2X_3+b_{13}X_1X_3 \quad (4.1)$$

Where Y is the dependent variable (PDE) while b_0 is the intercept, b_i (b_1, b_2 and b_3), b_{ij} (b_{12}, b_{23} and b_{13}) represent the regression coefficient for second order polynomial and X_i represents the levels of independent formulation variables. A full model for ETPLIP (Equation 4.2) and DOCLIP (Equation 4.3) was established after substituting the values of regression coefficients in equation 4.1. The predicted values for ETPLIP and DOCLIP were determined using the

mathematical model derived from the coefficients of the model as shown in Table 4.5 and Table 4.6 respectively and the predicted values along with their observed values are shown in Table 4.7 and Table 4.8 respectively

$$Y=65.6375+8.137X_1-5.287X_2-0.112X_3-0.787X_1X_2-0.112X_2X_3-0.037X_1X_3 \quad (4.2)$$

$$Y=56.012+7.762X_1-4.96X_2-0.53X_3-1.21X_1X_2+0.212X_2X_3-0.512X_1X_3 \quad (4.3)$$

Table 4.5: Model coefficients estimated by Multiple Linear Regression for ETPLIP.

Regr. Coefficients; Var.:PDE; R-sqr=.99999; Adj:.9999 (Spreadsheet1) 2**(3-0) design; MS Residual=.01125 DV: PDE						
	Regressn	**Std.Err.**	**t(1)**	**P**	**-95.%**	**+95.%**
Mean/Interc.	65.63750	0.037500	1750.333	0.000364	65.16102	66.11398
(1)x1	8.13750	0.037500	217.000	0.002934	7.66102	8.61398
(2)x2	-5.28750	0.037500	-141.000	0.004515	-5.76398	-4.81102
(3)x3	-0.11250	0.037500	-3.000	0.204833	-0.58898	0.36398
1 by 2	-0.78750	0.037500	-21.000	0.030292	-1.26398	-0.31102
1 by 3	-0.11250	0.037500	-3.000	0.204833	-0.58898	0.36398
2 by 3	-0.03750	0.037500	-1.000	0.500000	-0.51398	0.43898

Table 4.6 Model coefficients estimated by Multiple Linear Regression for DOCLIP

Regr. Coefficients; Var.:PDE; R-sqr=.99935; Adj:.99546 (Spreadsheet1) 2**(3-0) design; MS Residual=.45125 DV: PDE

	Regressn	Std.Err.	t(1)	p	-95.%	+95.%
Mean/Interc.	56.01250	0.237500	235.8421	0.002699	52.99478	59.03022
(1)x1	7.76250	0.237500	32.6842	0.019472	4.74478	10.78022
(2)x2	-4.96250	0.237500	-20.8947	0.030445	-7.98022	-1.94478
(3)x3	-0.53750	0.237500	-2.2632	0.264875	-3.55522	2.48022
1 by 2	-1.21250	0.237500	-5.1053	0.123140	-4.23022	1.80522
1 by 3	0.21250	0.237500	0.8947	0.535331	-2.80522	3.23022
2 by 3	-0.51250	0.237500	-2.1579	0.276263	-3.53022	2.50522

Table 4.7: Observed Responses and Theoretically predicted Responses for ETPLIP.

Observed, Predicted, and Residual Values (Spreadsheet1) 2**(3-0) design; MS Residual=.01125 DV: PDE			
	Observed	**Predicted**	**Residuals**
1	62.00000	61.96250	0.037500
2	62.00000	62.03750	-0.037500
3	53.00000	53.03750	-0.037500
4	80.00000	80.03750	-0.037500
5	53.00000	52.96250	0.037500
6	79.70000	79.66250	0.037500
7	68.00000	67.96250	0.037500
8	67.40000	67.43750	-0.037500

Table 4.8: Observed Responses and Theoretically predicted Responses for DOCLIP

Observed, Predicted, and Residual Values (Spreadsheet1) 2**(3-0) design; MS Residual=.45125 DV: PDE

	Observed	Predictd	Resids
1	52.00000	52.23750	-0.237500
2	52.00000	51.76250	0.237500
3	46.00000	45.76250	0.237500
4	70.00000	69.76250	0.237500
5	43.00000	43.23750	-0.237500
6	69.90000	70.13750	-0.237500
7	58.20000	58.43750	-0.237500
8	57.00000	56.76250	0.237500

Analysis of Variance (ANOVA) of full model was carried out and F statistic was applied to check whether non significant terms could be omitted or not. The results for ETPLIP are shown in Table 4.9 and that of DOCLIP are shown in Table 4.10.

Table 4.9: ANOVA (Analysis of Variance) of Second Order Polynomial equation for ETPLIP.

ANOVA; Var.:PDE; R-sqr=.99999; Adj:.9999 (Spreadsheet1) 2**(3-0) design; MS Residual=.01125 DV: PDE

SS	Df	MS	F	P
529.7513	1	529.7513	47089.00	0.002934
223.6613	1	223.6613	19881.00	0.004515
0.1012	1	0.1012	9.00	0.204833
4.9612	1	4.9612	441.00	0.030292
0.1012	1	0.1012	9.00	0.204833
0.0112	1	0.0112	1.00	0.500000
0.0113	1	0.0113		
758.5988	7			

Table 4.10: ANOVA (Analysis of Variance) of Second Order Polynomial equation for DOCLIP

ANOVA; Var.:PDE; R-sqr=.99935; Adj:.99546 (Spreadsheet1) 2**(3-0) design; MS Residual=.45125 DV: PDE					
	SS	**Df**	**MS**	**F**	**P**
(1)x1	482.0512	1	482.0512	1068.258	0.019472
(2)x2	197.0112	1	197.0112	436.590	0.030445
(3)x3	2.3112	1	2.3112	5.122	0.264875
1 by 2	11.7613	1	11.7613	26.064	0.123140
1 by 3	0.3613	1	0.3613	0.801	0.535331
2 by 3	2.1013	1	2.1013	4.657	0.276263
Error	0.4513	1	0.4513		
Total SS	696.0488	7			

4.3.1.1 CONTOUR PLOTS:

Two dimensional contour plots for ETPLIP and DOCLIP were established using full model polynomial equation. (Fig 4.1 and Fig. 4.2 respectively).

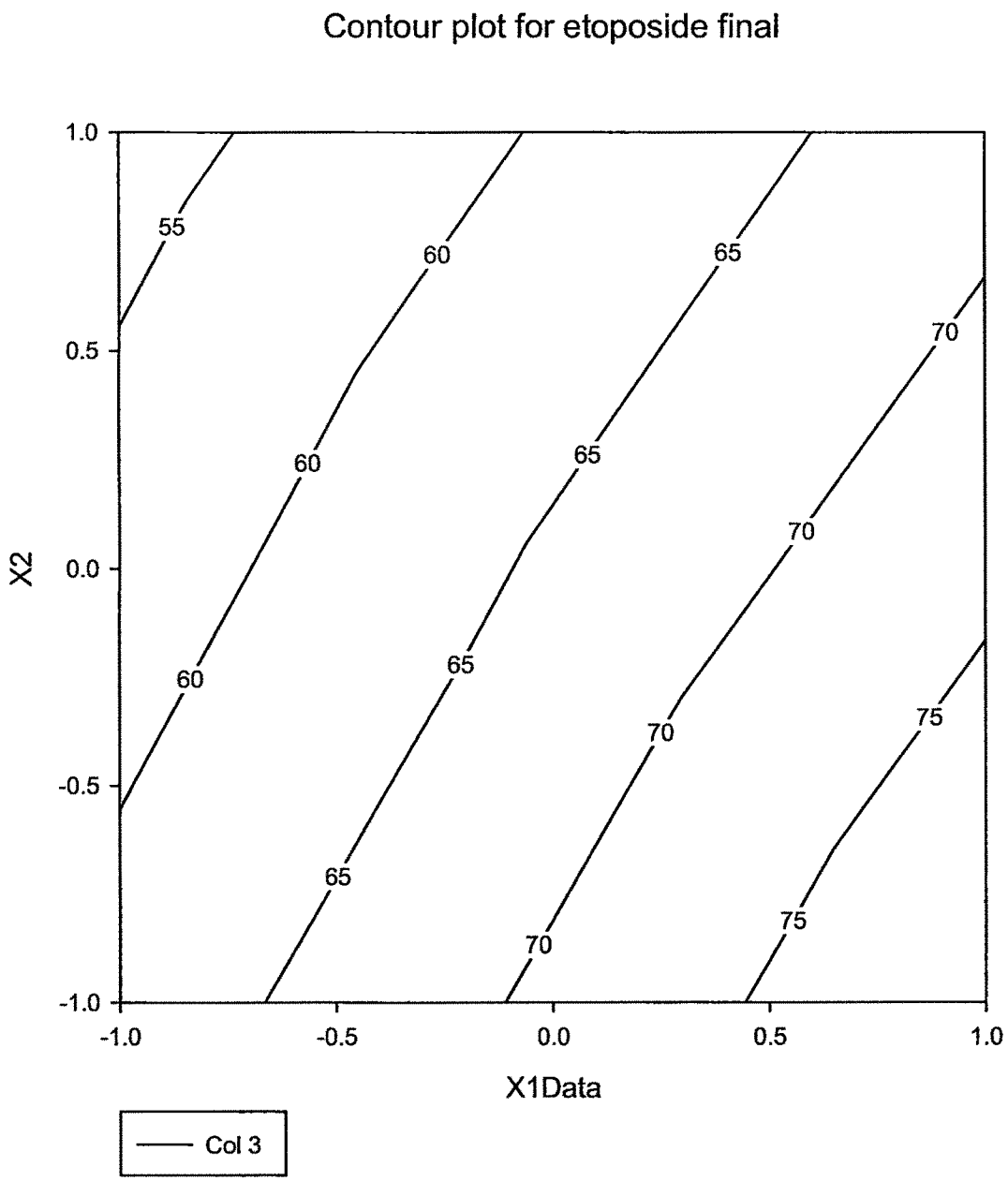


Fig 4.1: Contour Plot for Etoposide Liposomes

At -1 level of Variable X_3

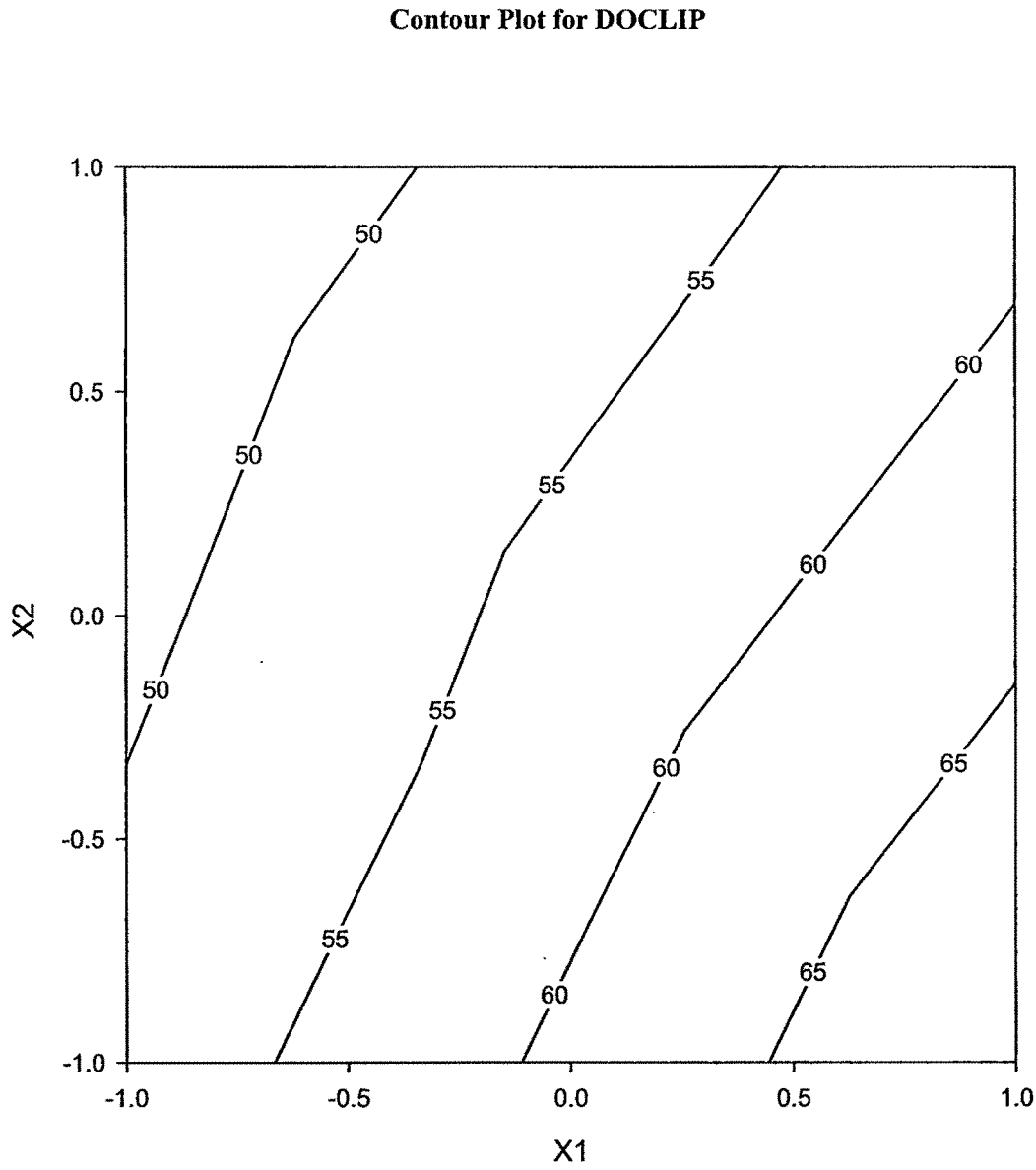


Fig 4.2 : Contour Plot for Docetaxel Liposomes (DOCLIP)

At -1 level of Variable X_3

4.3.1.2 CHECKPOINT ANALYSIS:

A checkpoint analysis was performed to confirm the applicability of established contour plots and second order polynomial equation in preparation and optimization of Etoposide and Dcetaxel liposomes. Values of independent variables (X_1 and X_2) were taken from three check points each on contour plots plotted at fixed levels of -1 and +1 of X_3 and values of PDE were calculated by placing the values in derived second order polynomial equation. Etoposide and Docetaxel liposomes were prepared practically by taking the amounts of the independent variables (X_1 and X_2) on same check points. Each batch was prepared three times and mean values of ETPLIP were determined as shown in table 4.11 and those for DOCLIP are shown in Table 4.12. Difference of theoretically calculated value of PDE and mean values of experimentally obtained PDE was compared using student t test method.

Table 4. 11: Checkpoint Analysis for Etoposide Liposomes (ETPLIP)

Chloroform:Methanol solvent Volume ratio (X_3)	Values from Contour plot		Calculated PDE (%)	Experimentally Obtained PDE*± SEM (%)
	X_1(Drug:lipid ratio)	X_2(Speed of rotation in rpm)		
2:1	1:10	100	80.03	80**(0.437)
3:1	1:10	120	67.43	67.4**(0.566)

*n=3

**=Difference from calculated PDE was found to be insignificant ($p>0.05$).

Table 4.12: Checkpoint Analysis for Docetaxel Liposomes (DOCLIP)

Chloroform:Methanol solvent Volume ratio (X_3)	Values from Contour plot		Calculated PDE (%)	Experimentally Obtained PDE*± SEM (%)
	X_1(Drug:lipid ratio)	X_2(Speed of rotation in rpm)		
-1 (2:1)	1:15	100	70.00	69.46**(0.437)
1 (3:1)	1:15	120	57.00	55.96**(0.566)

*n=3,

**=Difference from calculated PDE was found to be insignificant ($p>0.05$).

4.4 PARTICLE SIZE REDUCTION AND SEPARATION OF UNENTRAPPED DRUG.

Techniques that are adopted for achieving required liposomal size mainly comprise of sonication using bath or probe sonicator, membrane extrusion and high pressure homogenization. In the current studies liposomes were subjected to sonication using a probe sonicator in order to achieve the desired nanometric size range in case of liposomes. The unentrapped drug from liposomes was separated by controlled centrifugation. Briefly, the size of liposomal dispersion was reduced by sonicating liposomal dispersion in ice bath to prevent liposomal distortion due to temperature rise that is likely during sonication using probe sonicator at (3 cycles, 80% amplitude, 0.5 cycle) in Probe sonicator (RR-120, Ralsonics, Mumbai).

The unentrapped drug was removed from the liposomal suspension by centrifugation at 25,000 rpm for 10 min at 4°C temperature. The liposomal dispersion was decanted and analyzed for entrapped drug content. Drug pellet was dissolved and analyzed for un-entrapped drug content. The liposomal dispersion of ETP and DOC thus obtained was filled in amber colored vial under

nitrogen atmosphere, sealed and stored in refrigerator until required for further experiments. The liposomal compositions and process parameters were optimized to achieve maximum drug entrapment.

4.5 CHARACTERIZATION OF LIPOSOMES.

The liposomes of ETP and DOC were characterized for the following physico-chemical properties.

4.5.1 Particle Size Measurement

The sizes of prepared Etoposide Liposomes (ETPLIP), Docetaxel liposomes (DOCLIP) and 6-coumarin loaded liposomes were measured by dynamic light scattering with a Malvern Zetasizer 3000 HS (Malvern Instruments, Malvern, UK). Diluted liposomal suspension was transferred to the sample cuvette followed by placing the sample cuvette in Zetasizer. Particle size was measured after stabilizing the sample for two minutes. The average particle size was measured after performing the experiment in triplicate. Results for ETPLIP and DOCLIP were recorded in Table 4.13 and those of 6-coumarin loaded liposomes in Table 4.14.

4.5.2 Zeta Potential Determination

The zeta potential of developed Etoposide liposomes (ETPLIP), Docetaxel liposomes (DOCLIP) and 6-coumarin loaded liposomes was determined using Malvern Zetasizer 3000 HS (Malvern Instruments, Malvern, UK). The zeta potential was calculated by Helmholtz Smoluchowski's equation from the electrophoretic mobility of liposomes at 25 °C (Mu and Feng 2001). Results for ETPLIP and DOCLIP were recorded in Table 4.13 and those of 6-coumarin loaded liposomes in Table 4.14.

4.5.3 Percent Drug Entrapment

To determine percent drug entrapment (PDE), free and entrapped drug was measured. The free ETP and DOC (un-entrapped) in the liposomal dispersion were separated by ultracentrifugation at low speed method as described by (New, 1990a). Briefly, the liposomal dispersion was centrifuged at 25,000 rpm, 4°C for 20 minutes using sigma centrifuge and the liposomal

dispersion was removed without disturbing the drug pellet. The drug pellet was dissolved in methanol: chloroform (9:1) mixture and estimated for un-entrapped drug content. Fixed volume of liposomal suspension was withdrawn and dissolved in methanol: chloroform (9:1) mixture and estimated for entrapped drug content. Results for ETPLIP and DOCLIP were recorded in Table 4.13 and those of 6-coumarin loaded liposomes in Table 4.14.

Table 4.13: Size, Zeta Potential and PDE of optimized ETP and DOC Liposomal Formulations

Formulation Code	Particle Size (nm) *	Zeta Potential (mV)*	Percentage Entrapment *
ETPLIP	190 ± 3.7	-10.7 ± 1.9	80.1 ± 3.4
DOCLIP	195 ± 3.0	-8.8 ± 1.2	70.1 ± 2.8

***Mean ± S.D. (n=3)**

Table 4.14: Size and Zeta potential of 6-coumarin loaded liposomes

Formulation name	**Particle size (nm)**	**Zeta Potential (mV)**
Coumarin loaded liposomes	**198 ±2.5**	**-10.1±1.1**

4.6 DISCUSSION

Liposomes of ETP and DOC were prepared by the selected TFH method using HSPC, DPPE and CHOL, that were optimized to achieve maximum PDE and desired size range. 2^3 factorial design was adopted to optimize liposomal preparation. Using 2^3 factorial design, eight batches of ETPLIP (Table 4.1) and DOCLIP (Table 4.2) were prepared by thin film hydration technique varying three independent variables viz. Drug:Lipid (molar ratio-X_1), Speed of rotation during film drying and hydration (X_2) and solvent(Chloroform:Methanol volume ratio-X_3). PDE (Percentage drug Entrapment) which was taken as a dependent variable was determined and results for ETPLIP and DOCLIP were recorded. (Table 4.3 and Table 4.4 respectively) A

strikingly higher PDE achieved in liposomes prepared by thin film hydration technique was 80% at 1 level of X_1 (1:10),-1 level of X_2 (100 rpm) and -1 level of X_3(2:1 Chloroform:Methanol volume ratio). Similarly, significantly higher PDE achieved in liposomes prepared by thin film hydration technique was 70% at 1 level of X_1 (1:15),-1 level of X_2 (100 rpm) and -1 level of X_3(2:1 Chloroform:Methanol volume ratio).

The PDE (dependent variable) obtained at various levels of three independent variables (X_1, X_2 and X_3) were subjected to multiple regression to obtain a second order polynomial equation. The main effects of X_1, X_2 and X_3 represent the average result of changing one variable at a time from its low to high value. The interactions (X_1X_2, X_2X_3 and X_1X_3) show the effect on PDE when two or more variables are changed simultaneously. The PDE values for eight batches showed significant variation from 53 to 80% and 43 to 70 % for ETPLIP and DOCLIP respectively. (Table 4.3 and Table 4.4). Small values of X_3 and X_1X_3 can be considered as least contributing factors in optimization of liposomes.

The significance of each coefficient of equation 4.2 and equation 4.3 was determined by student "t"test and p-value listed in Table 4.5 and Table 4.6. Larger the magnitude of t value and smaller the p-value, more significant is the corresponding coefficient (Adinarayan et al, 2002).From this one can arrive on a conclusion that effects of Drug:lipid ratio and speed of rotation maintained during drying of film and hydration are significant on PDE as indicated from their p-values. From p-values one can say that X_1, X_2 and X_1X_2were found to be very significant.

Each of the observed values, $Y_f(O)$ is compared with $Y_f(P)$ from the model and listed in Table 4.7 and Table 4.8 for ETPLIP and DOCLIP respectively. The ANOVA results for ETPLIP and DOCLIP are recorded in Table 4.9 and Table 4.10 respectively. When the coefficients of three independent variables in equations 4.2 and 4.3 were compared, the value for variable $X_1(b_1=8.137)$ for ETPLIP and $X_1(b_1=7.762)$ for DOCLIP were found to be maximum among all the three and hence the variable X_1 was considered to be a key contributing variable for PDE of ETPLIP and DOCLIP respectively. The goodness of fit of the model was checked by determination coefficient (R^2). In this case, values of determination coefficients ($R^2=0.9999$ for ETPLIP and $R^2=0.9993$ for DOCLIP, Table 4.9 and Table 4.10 respectively) indicated that over

99% of the total variations can be explained using this model. The values of adjusted determination coefficient are also nearly same indicating acceptable significance of this model.

Fig 4.1 shows the contour plot for ETPLIP drawn at -1 level of X_3(2:1 Chloroform:Methanol volume ratio), the plots were found to be linear for 70 % and 75 %, but curvilinear for 55%, 60% and 65% PDE indicating non linear relationship between X_1 and X_2 variables. It was observed from the contour plot that PDE > 70% could be obtained with X_1 between -0.11 To 1.0 and X_2 level of -1 to 0.6625. Similarly, Fig 4.2 shows the contour plot of DOCLIP drawn at -1 level of X_3(2:1 Chloroform:Methanol volume ratio), the plots were found to be linear for 60 % and 65 %, but curvilinear for 50%, and 55% PDE indicating non linear relationship between X_1 and X_2 variables. It was observed from the contour plot that PDE > 65% could be obtained with X_1 between 0.465 To 1.0 and X_2 level of -0.15 to -1.

It can be concluded from observed results that higher amount of lipid would be necessary to entrap the drug within liposomes and speed of rotation of film drying and hydration should be kept at 100 rpm only.

Interpretation of contour plot showed that PDE was directly related to drug:lipid ratio where increase in amount of lipid used resulted in corresponding increase in PDE while increase in speed of rotation (120 rpm) during film drying and hydration resulted in reduced PDE.

The full model combination obtained by using solver function of Microsoft Excel 2003 showed that maximum PDE for ETPLIP (80.03%) and for DOCLIP (69.46 %) could be obtained at 1,-1,-1 values of X_1, X_2 and X_3 variables. At fixed values of -1 and +1 of independent variable X_3, three checkpoints were selected on three plotted contours. (Table 4.11). ETPLIP and DOCLIP at these three checkpoints were prepared practically using thin film hydration technique (discussed under title: Materials and Methods) and keeping the other process variables as constant with the amounts of X_1 and X_2 at selected checkpoints. The experiment was repeated three times and experimentally obtained PDE values for ETPLIP and DOCLIP were shown in Table 4.11 and Table 4.12 respectively. Non significant difference was observed among theoretically predicted and practically observed mean PDE values confirming the validity and significance of second

order polynomial equation and contour plots in preparation of etoposide and docetaxel liposomes with desired PDE.

Etoposide and Docetaxel being lipophilic drugs entrapment in to liposomes involved co-evaporation of the lipid and drug from the solvent system in a round bottom flask. Selection and optimization and selection of various process and formulation variables were carried out followed by the selection of suitable method for the optimum percentage drug entrapment (PDE). The results are summarized and discussed in the following sections.

Process variables, such as vacuum conditions for dry film formation, hydration time, and speed of rotation of flask were optimized for desired results. The effect of one variable was studied at a time keeping other variables constant. The results are recorded in Table 4.3 from which the following conclusions are drawn:

1.Drug:lipid ratio: The drug:lipid ratio for ETP was varied from 1:5 to 1:10. It was found that lower drug:lipid ratio resulted in poor PDE while increase in drug:lipid ratio to 1:10 resulted in optimum PDE for ETP.

The drug:lipid ratio for DOC was varied from 1:5 to 1:15. It was found that lower drug:lipid ratio resulted in poor PDE while increase in drug:lipid ratio to 1:15 resulted in optimum PDE for DOC. Increase in drug:lipid ratio resulted in corresponding increase in PDE. (Schneider et al, 1994).

2. Speed of rotation: The speed of rotation of flask was increased from 50 rpm to 120 rpm. Rotation conducted at 50 rpm resulted in thick, incompletely dried film and presence of residual solvents. While at 120-rpm speed, a dry film with variable thickness with poor PDE was produced. A speed of 100 rpm was found to be adequate to give thin, uniform and completely dry film. Hence, 100-rpm speed of rotation of flask was selected to be optimum for liposomal preparations.

3. Solvent ratio: Volume ratio of Chloroform:Methanol was selected as independent variable affecting PDE. Solvent volume ratios tried were: 3:1 and 2:1. Solvent volume ratios were found to be not significantly affecting PDE and there was practically no significant difference of PDE

observed with either of the solvent volume ratio and hence, 2:1 volume ratio was selected to render the preparation process cost effective. It was observed that at solvent volume ratio (1:1) lesser than the one selected (2:1) during study resulted in inadequate film drying and improper hydration. Hence, 2:1 was an optimum volume ratio.

In spite of availability of large number of methods of separation of unentrapped drug, all these methods suffer from one or the other drawback. E.g. gel filtration is highly tedious method; dialysis method suffers from limitation of being time consuming and also leads to drug leakage. Hence, ultracentrifugation was preferred for separation of unentrapped drug from the entrapped one followed by subsequent estimation of their PDE.

6- coumarin loaded liposomes were found to have particle size and zeta potential similar to ETPLIP and DOCLIP.

Etoposide (ETP) and Docetaxel (DOC) liposomes optimized in terms of particle size, zeta potential and percentage drug entrapment were selected for ligand grafting and all further studies.

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