Chapter 5

Preparation of Liposomes and Microbubbles

Optimization of formulation parameters for the preparation of flutamide liposomes by 3^3 factorial 26-term logit model, **Pharmaceutical Development and Technology, 2004**, Vol 9(4), 369-377.

And

Preparation, Optimization, and Characterization of 6-Mercaptopurine liposomes, **Journal of Pharmacy and Pharmaceutical Sciences** (Under communication)

5.1 INTRODUCTION

5.1.1 General

There are a large variety of methods for preparing liposomes. From a pharmaceutical point of view, the most important factors to be evaluated before selecting the method of preparation are the method of preparation, entrapping efficiency, drug retention properties and drug/lipid ratio. The method of preparation include selection of methods which would avoid the use of organic solvents and detergents, yield well-defined and reproducible liposomes and which are rapid and amenable to scale up procedures. An optimum loading procedure would achieve trapping efficiency of 90% or more and however, if trapping efficiency is high, unentrapped drug need not be removed. Th0e. optimum drug/lipid ratio of a liposomal formulation will likely be dictated by biological efficacy and toxicity of the preparation. The high drug/lipid ratio is more economical from a pharmaceutical point of view. The optimum liposomal formulations will exhibit drug trapping efficiencies in excess of 90%, employ inexpensive and relatively saturated lipids such as egg PC and Cholesterol exhibit the highest possible drug/lipid ratio which is consistent with maintained efficacy of the preparation.

5.1.2 Drugs

Flutamide and 6-Mercaptopurine were kindly supplied as a gift sample by Coral Drugs Pvt. Ltd., New Delhi; and by Dabur Therapeutics Ltd., Ghaziabad, India, respectively.

5.1.3 Materials

Egg Phosphotidylcholine (PC), Hydorgenated Soya PC (HSPC), phosphotidyl ethanolamine (PE), Cholesterol and methoxy polyethylene glycol (M.Wt-2000) were purchased from Sigma Chemical Co., St.Louis, M.O.; DL-α-tocopherol was purchased from E.merck India Ltd,., Mumbai. Cholorform and Methanol (AR grade) were purchased from S.D.fine chemicals, Biosar, Thane. Cyanuric chloride was purchased from National Chemicals, Baroda. Petroleum ether (40°C-60°C, AR grade), diethyl ether (AR grade), acetone, triethylamine, sodium carbonate and iodine were purchased from Qualigen fine chemicals, Mumbai.

5.1.4 Apparatus

Rotary evaporator with vacuum pump and thermostatically controlled water bath (Superfit Equipments, India), probe sonicator RR-120 (Ralsonics, Mumbai), laboratory centrifuge (Sigma, 3K30), Remi heating mantle and Remi magnetic stirrer 1 MLH (Remi Equipments, Mumbai), Shimadzu UV-1601 UV-Visible spectrophotometer (Shimadzu corporation, Japan), Advance DPX 200 dual probe ^{13}C-NMR (Broker Inc., Switzerland), High-pressure homogenizer (Microfluidizer, Avestin, Canada).

5.1.5 Solutions

16.7% w/v solution of sucrose, Sodium sulphate solutions (0M to 2M)

5.2 PREPARATION OF FLUTAMIDE LIPOSOMES

5.2.1 Introduction

Flutamide (4-nitro-3-trifluoromethyl isobutyranilide: FLT; MW 276.12) is acetanilide, non-steroidal, anti-androgen drug (277) and used in treatment of prostate cancer. The currently recommended dose of Flutamide (250 mg) (278) does not appear to produce better therapeutic response and associates with a higher incidence and degree of gynaecomastia or hepatotoxicity (279) because of distribution in whole body. Liposomes have been used as drug carriers for several drugs to reduce toxicity by concentrating the drug at its target site (280). Hence, FLT liposomes are also expected to behave similarly. Thus, the preparation of FLT liposomes is an attempt to use in treatment of prostatic cancer. Many methods have been used to prepare liposomes (281). FLT is insoluble in water and soluble in chloroform, methanol, and ethyl acetate. Hence, liposomes of FLT were prepared by conventional thin film hydration technique as described by Bangham et al (282) to obtain maximum % entrapment efficiency (% EE). Experimental design techniques such as factorial design and optimization are useful tools in the characterization of pharmaceutical formulation by studying the effect of various factors affecting on it and its possible interactions economically (283). The objective of this study was to determine the effect of the volume of organic phase, the volume of aqueous phase and the Drug/PC/CHOL molar ratio, which are formulation parameters, on the % EE, to develop and to optimize FLT liposomes using 3^3 factorial 26-term logit model design.

5.2.2 Experimental Design

3^3 factorial 26-term logit model experimental design was used to determine the effect of the three independent factors: the volume of organic phase (X1), the volume of aqueous phase (X2) and Drug/PC/CHOL molar ratio (X3) on the % entrapment efficiency of liposomes. Each factor was tested at three levels designed as -1, 0, and +1. The normalized factor levels of independent variables are given in Table 5.1.

Table 5.1: Factorial $3^{3:}$ factors, their levels and transformed values

Variables	**Levels**		
	Low	**Medium**	**High**
Volume of Organic Phase ($CHCl_3$: MeOH) (1:2) (X1)	3 ml	5 ml	7 ml
Volume of Aqueous Phase (Distilled Water) (X2)	1 ml	1.5 ml	2 ml
Drug/PC/CHOL molar ratio (X3)	1:5:2	1:10:2	1:15:2
Transformed Values	-1	0	+1

5.2.3 Preparation of Liposomes

Twenty-seven batches of FLT liposomes were prepared by conventional thin film hydration technique as described by Bangham et al (282) according to experimental design and order shown in Table 5.2. The rotary vacuum evaporator (Superfit, Rotovap) was used to form the lipid film as well as to hydrate the film. Drug, phosphotidylcholine, and cholesterol were accurately weighed and dissolved in organic phase in round bottom flask (RBF). The RBF was attached to the rotary evaporator, secured in position with a clip and rotated at definite RPM at 37^0C under vacuum (250 mmHg) until uniform film was deposited in flask. The flask was rotated under vacuum for 20 min additionally. Film formed was hydrated by adding an aqueous phase in the flask. 4-5 pieces of glass-beads were added to improve hydration. The flask was attached to evaporator using clip and rotated at the same RPM and temperature for 20 min or until lipid film had completely dispersed. The dispersion was allowed to stand for 2 hours in refrigerator to complete hydration. Each batch was prepared three times at a three different days in order mentioned in Table 5.2 and evaluated for %EE.

5.2.4 Statistical Analysis

Factorial design is a useful tool in order to characterize multivariable processes (284). It gives the possibility to separate important factors from those, which are not, and indication of the possible interactions between them. In this study, 3^3 factorial 26-term logit model design and multi-linear regression analysis described by Juslin et al (285) were used to study the dependency of response variable on the three independent variables (X1, X2, X3). The regression model for the three variables can be presented in a general form:

$$Y(X1, X2, X3) = b_0 + b_1X1 + b_2X2 + b_3X3 + b_4X1X2 + b_5X1X3 + b_6X2X3 + b_7X1X2X3 + b_8X1^2 + b_9X2^2 + b_{10}X3^2 + b_{11}X2\ X1^2 + b_{12}X3X1^2 + b_{13}X3X1^2 + b_{14}X3X2^2 + b_{15}X1X3^2 + b_{16}X2X3^2 + b_{17}X1^2X2X3 + b_{18}X1X3X2^2 + b_{19}X1X2X3^2 + b_{20}X1^2X2^2 + b_{21}X1^2X3^2 + b_{22}X2^2X3^2 + b_{23}X1X2^2X3^2 + b_{24}X2X1^2X3^2 + b_{25}X3X1^2X2^2 + b_{26}X1^2X2^2X3^2$$ —(Equation 1)

Where Y is the predicted response, b_0 is the intercept and b_1-b_{27} is the regression co-efficients of the system. The main effects X1, X2, and X3 denote the volume of organic phase, the volume of aqueous phase and Drug/PC/CHOL molar ratio, respectively. The X1X2, X2X3, X3X1, X1X2X3, X2 $X1^2$, $X3X1^2$, $X3X1^2$, $X3X2^2$, $X1X3^2$, $X2X3^2$, $X1^2X2X3$, $X1X3X2^2$, $X1X2X3^2$, $X1^2X2^2$, $X1^2X3^2$, $X2^2X3^2$, $X1X2^2X3^2$, $X2X1^2X3^2$, $X3X1^2X2^2$ and $X1^2X2^2X3^2$ indicate interactions. The quadratic terms ($X1^2$, $X2^2$ and $X3^2$) account for curvature in the %EE response surface. A full model was established after including all above terms into second order polynomial equation (Equation 2). Neglecting non-significant ($p > 0.05$) terms from the full model equation, the model was re-estimated or reduced (Equation 3). The statistical analysis and the generation of regression model were performed on the basis of data given in (Table 5.3).

$$Y_{\ln[\%EE/(100-\%EE)]} = 0.54072 + 0.38885\ X1 + 0.03289\ X2 + 2.32463\ X3 - 0.13744\ X1X2 + 0.05137\ X1X3 - 0.13031X2X3 + 0.04664\ X1X2X3 - 0.56517\ X1^2 - 0.03524\ X2^2 + 1.98053\ X3^2 - 0.28614\ X2\ X1^2 - 0.79563\ X3X1^2 - 0.22401\ X3X1^2 - 1.23444\ X3X2^2 - 0.19480\ X1X3^2 - 0.02927\ X2X3^2 + 0.15999\ X1^2X2X3 - 0.05444\ X1X3X2^2 + 0.06350X1X2X3^2 - 0.08404\ X1^2X2^2 - 0.66236\ X1^2X3^2 - 1.56614\ X2^2X3^2 + 0.12319\ X1X2^2X3^2 + 0.45243\ X2X1^2X3^2 + 0.59489\ X3X1^2X2^2 + 0.89762\ X1^2X2^2X3^2$$ —(Equation 2)

Table 5.2: Factorial 33: Matrix of Experiments and results for the measured response

Std	Run	X1	X2	X3	X1	X1	X2	X1*X2	X12	X22	X32	X2	X3	X1	X3	X1	X2	X12*X2	X1*X3	X1*X2	X12	X12	X22	X1*X22	X2*X12	X3*X12	X12*X22	%EE(SEM)	Pred. Y	Pred. %EE
Order	Order				*X2	*X3	*X3	*X3				*X12	*X12	*X22	*X22	*X32	*X32	*X3	*X22	*X32	*X22	*X32	*X32	*X32	*X32	*X22	*X32			
1	1	-1	-1	-1	1	1	1	-1	1	1	1	-1	-1	-1	-1	-1	-1	1	1	1	1	1	1	-1	-1	-1	1	32.29(0.136)	-0.74	0.32
4	2	-1	0	-1	0	1	0	0	1	0	1	0	-1	0	0	-1	0	0	0	0	0	1	0	0	0	0	0	40.66(0.056)	-0.37	0.4
24	3	1	0	1	0	1	0	0	1	0	1	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	95.56(0.282)	3.06	0.95
20	4	1	-1	0	-1	0	0	0	1	1	0	-1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	60.15(0.136)	0.41	0.6
23	5	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	59.01(0.089)	0.36	0.59
12	6	0	-1	1	0	0	-1	0	0	1	1	0	0	0	1	0	-1	0	0	0	0	0	1	0	0	0	0	89.44(0.342)	2.13	0.89
15	7	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	99.22(0.106)	4.84	0.99
7	8	-1	1	-1	-1	1	-1	1	1	1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	-1	1	-1	1	44.55(0.076)	-0.21	0.44
2	9	-1	-1	0	1	0	0	0	1	1	0	-1	0	-1	0	0	0	0	0	0	1	0	0	0	0	0	0	45.20(0.136)	-0.19	0.45
21	10	1	-1	1	-1	1	-1	-1	1	1	1	-1	1	1	1	1	-1	-1	1	-1	1	1	1	1	-1	1	1	78.81(0.282)	1.31	0.78
25	11	1	1	-1	1	-1	-1	-1	1	1	1	1	-1	1	-1	1	1	-1	-1	1	1	1	1	1	1	-1	1	43.35(0.330)	-0.26	0.43
10	12	0	-1	-1	0	0	1	0	0	1	1	0	0	0	-1	0	-1	0	0	0	0	0	1	0	0	0	0	42.45(0.141)	-0.3	0.42
26	13	1	1	0	1	0	0	0	1	1	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	40.86(0.094)	-0.36	0.4
13	14	0	0	-1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	54.90(0.089)	0.19	0.54
18	15	0	1	1	0	0	1	0	0	1	1	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0	86.80(0.056)	1.88	0.86
27	16	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	83.99(0.342)	1.65	0.83
19	17	1	-1	-1	-1	-1	1	1	1	1	1	-1	-1	1	-1	1	-1	1	-1	-1	1	1	1	1	-1	-1	1	42.39(0.330)	-0.3	0.42
11	18	0	-1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	61.60(0.141)	0.47	0.61
22	19	1	0	-1	0	-1	0	0	1	0	1	0	-1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	47.69(0.056)	-0.09	0.47
6	20	-1	0	1	0	-1	0	0	1	0	1	0	1	0	0	-1	0	0	0	0	0	1	0	0	0	0	0	92.94(0.282)	2.57	0.92
14	21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	63.20(0.089)	0.54	0.63
9	22	-1	1	1	-1	-1	1	-1	1	1	1	1	1	-1	1	-1	1	1	-1	-1	1	1	1	-1	1	1	1	82.23(0.046)	1.53	0.82
16	23	0	1	-1	0	0	-1	0	0	1	1	0	0	0	-1	0	1	0	0	0	0	0	1	0	0	0	0	49.09(0.141)	-0.03	0.49
3	24	-1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	1	1	-1	-1	1	1	74.62(0.282)	1.07	0.74
8	25	-1	1	0	-1	0	0	0	1	1	0	1	0	-1	0	0	0	0	0	0	1	0	0	0	0	0	0	39.55(0.069)	-0.42	0.39
5	26	-1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	39.81(0.154)	-0.41	0.39
17	27	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	63.14(0.235)	0.53	0.63

Table 5.3: Summary of Results of Regression Analysis

Source Effect	Regression Coefficients	p value	Reduced Regression Coefficients
Average Constant	b_0= 0.54072	0.000*	0.5172
X1	b_1= 0.38885	0.000*	0.258
X2	b_2= 0.03289	>0.05	-
X3	b_3= 2.32463	0.000*	2.3246
X1*X2	b_4= -0.13744	0.025*	-0.0951
X1*X3	b_5= 0.05137	>0.05	-
X2*X3	b_6= -0.13031	>0.05	-
X1*X2*X3	b_7= 0.04664	>0.05	-
$X1^2$	b_8= -0.56517	0.000*	-0.6211
$X2^2$	b_9= -0.03524	>0.05	-
$X3^2$	b_{10}= 1.98053	0.001*	2.004
$X2*X1^2$	b_{11}= -0.28614	0.002*	-0.2532
$X3*X1^2$	b_{12}= -0.79563	0.000*	-0.7956
$X1*X2^2$	b_{13}= -0.22401	0.048*	-0.1418
$X3*X2^2$	b_{14}= -1.23444	0.000*	-1.2344
$X1*X3^2$	b_{15}= -0.19480	>0.05	-
$X2*X3^2$	b_{16}= -0.02927	>0.05	-
$X1^2*X2*X3$	b_{17}= 0.15999	>0.05	-
$X1*X3*X2^2$	b_{18}= -0.05444	>0.05	-
$X1*X2*X3^2$	b_{19}= 0.06350	>0.05	-
$X1^2*X2^2$	b_{20}= -0.08404	>0.05	-
$X1^2*X3^2$	b_{21}= -0.66236	0.001*	-0.6063
$X2^2*X3^2$	b_{22}= -1.56614	0.000*	-1.6014
$X1*X2^2*X3^2$	b_{23}= 0.12319	>0.05	-
$X2*X1^2*X3^2$	b_{24}= 0.45243	0.000*	0.4231
$X3*X1^2*X2^2$	b_{25}= 0.59489	0.001*	0.5949
$X1^2*X2^2*X3^2$	b_{26}= 0.89762	0.000*	0.8136
r (correlation coefficient)	0.982	-	-

* Statistically significant (p-value<0.05)

$$Y_{\ln [\%EE/(100-\%EE)]} = 0.517 + 0.258\ X1 + 2.32\ X3 - 0.621\ X1^2 + 2.00\ X3^2 - 0.606\ X1^2X3^2 - 0.795\ X3X1^2 - 1.23\ X3X2^2 + 0.594\ X3X1^2X2^2 - 0.253\ X2X1^2 - 1.60\ X2^2X3^2 + 0.423\ X2X1^2X3^2 + 0.813\ X1^2X2^2X3^2 - 0.095\ X1X2 - 0.141\ X1X2^2$$ --(Equation 3)

5.2.5 Characterization of Liposomes

5.2.5.1 % Encapsulation Efficiency

From each prepared batch, a definite amount of liposomal dispersion was taken and subjected to centrifugation on laboratory centrifuge (Sigma, 3K30) at 15, 000 RPM for 15 min at $0^{\circ}C$ after mixing with 50 µl protamine solution (10 mg/ml) (286) using

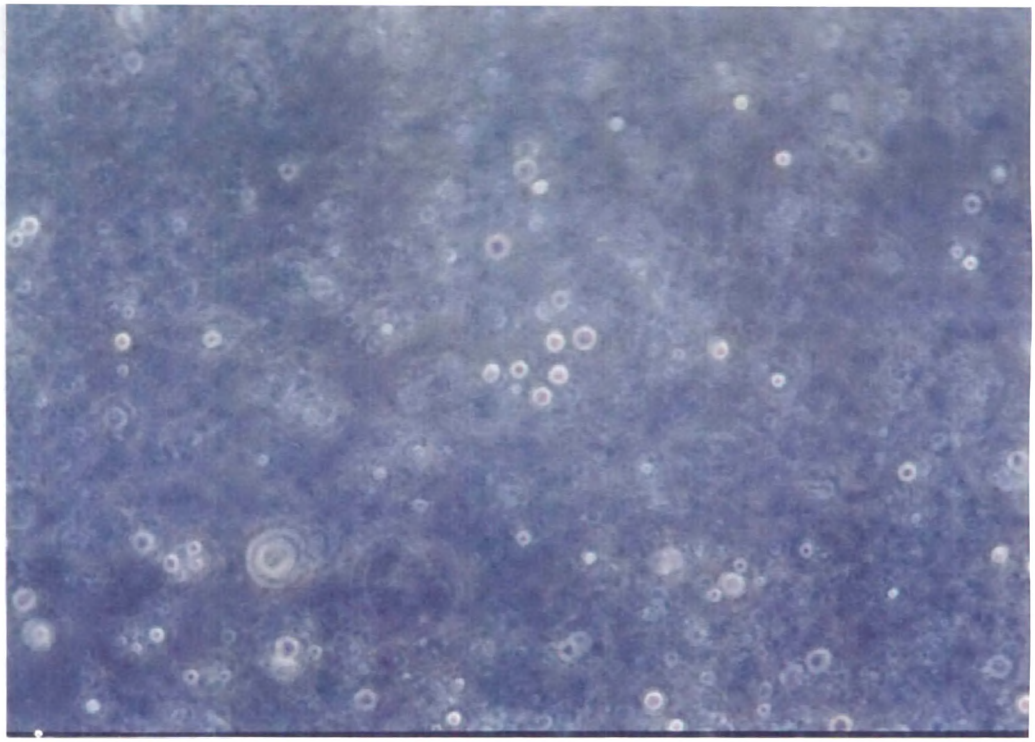


Figure 5.1(a): Flutamide conventional liposomes

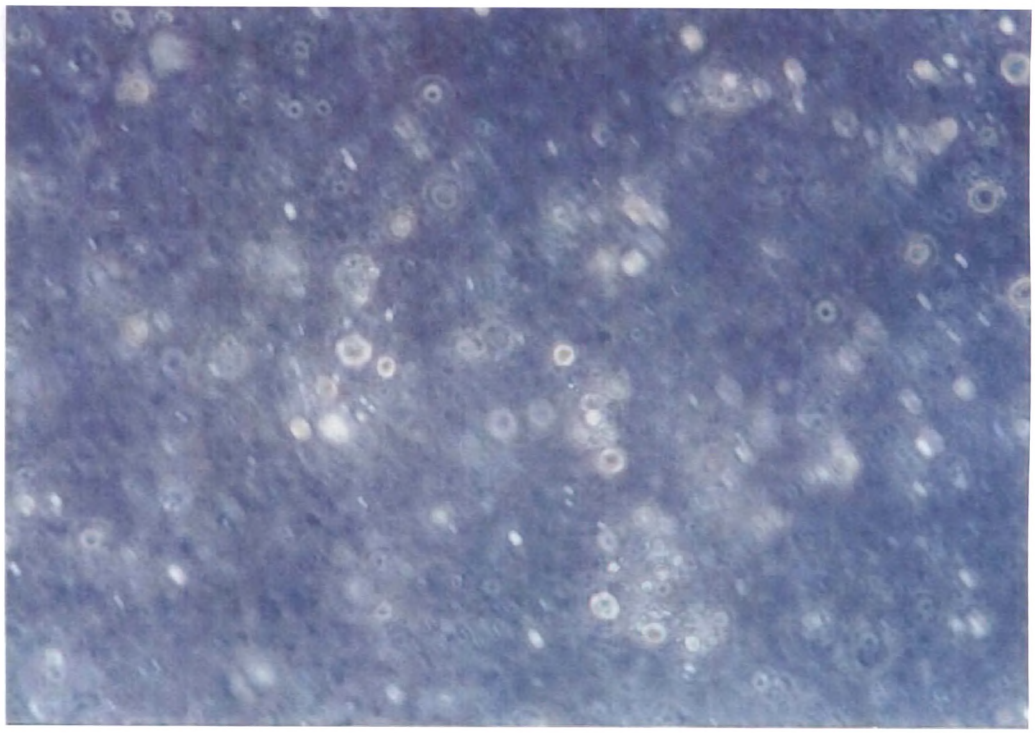


Figure 5.1 (b): Flutamide stealth liposomes

micropipette. The clear supernatant and sediment were separated. The definite amount of supernatant was diluted to 5 ml with methanol and the absorbance was recorded at 295 nm on Shimadzu 1601 UV-Visible Spectrophotometer (287). The sediment was resuspended in 1.5 ml distilled water and aliquot was diluted to 10 ml with methanol to lyse the liposomes and the absorbance was recorded at 295 nm. Each time blank containing blank liposome was treated in the same manner to account for any absorbance due to lipid components. Table 5.2 shows the % EE (Mean± SEM) of all batches.

The entrapment efficiency (% EE) was calculated as follows: (288)

$$\% EE = \frac{\text{Amount of FLT in Sediment} * 100}{\text{Total Amount of FLT added in sample}}$$

5.2.5.2 Photomicrography

All the batches of the liposomes prepared were observed under Olympus (BX 40F4, Tokyo, Japan) microscope with a polarizing attachment to study their size and lamellarity. A photomicrography under 100X magnification is shown in Figure 5.1 (a) and (b).

5.2.6 Results and Discussion

Twenty seven batches of FLT liposomes were prepared by conventional thin film hydration method using 3^3 factorial 26- term logit model design (Table 5.2) by varying three independent variables the volume of organic phase (X1), the volume of aqueous phase (X2) and Drug/PC/CHOL molar ratio (X3). The % EE (response variable) of the prepared batches was determined (Table 5.2) and the highest % EE achieved in liposomes was 99.22 % at 0 level of X1 (5 ml), 0 level of X2 (1.5 ml) and 1 level of X3 (1:15:2 molar ratio). The results were subjected to multiple regression analysis. The fitted equation related to % EE and transformed factors shows in Equation 2.

The data clearly indicated that % EE is more dependent on the Drug/PC/CHOL molar ratio and the volume of organic phase than the volume of aqueous phase. The value of correlation-coefficient (r) was found to be 0.982, indicating a good fit. The

small values of coefficients of terms X2, X1X3, X2X3, X1X2X3, $X2^2$, $X1X3^2$, $X2X3^2$, $X1^2X2X3$, $X1X2^2X3$, $X1X2X3^2$, $X1^2X2^2$, $X1X2^2X3^2$ and $X1^2X2X3^2$ (Equation 2) were least contributing in preparation of FLT liposomes ($p>0.05$). Hence, they were omitted to evolve the reduced model (Equation 3).

The regression coefficient of X2 (b_2=0.0328) was found to be minimum and regression coefficient of X3 (b_3=2.3246) was maximum. The reduced model (Equation 3) was used to plot three different two-dimension contour plots (Figures 5.2, 5.3, and 5.4 using Minitab14® Software) at fixed level of -1, 0, and +1 of X1, respectively and the values of X2 and X3 were computed between -1 and +1 at predetermined value of % EE.

Figure 5.2 represents the contour plots drawn at -1 level of X1 at predetermined % entrapment efficiency values of 32%, 35%, 40%, 50%, 60%, 70%, 80%, and 90%. Plots were found to be linear which indicated linear relationship between X2 and X3 variables. It was concluded from the linear contour that the % EE (90%) could be obtained with X2 range from -0.35 level (1.375 ml) to 0.6 level (1.8 ml) and X3 at 1 level (1:15:2 molar ratio) for both levels of X1. Secondly, the same % EE could also be found with X3 range from 0.95 level (1:14.75:2 molar ratio) to 1 level (1:15:2 molar ratio) and X2 at level of 0 level for both levels of X3. It was estimated from this contour plot results that the liposomes with 90% EE could be prepared at -1 level of X1 with in these range of X3 (1:14.75:2 to 1:15:2 molar ratio) and X2 (1.375 ml to 1.8 ml).

Figure 5.3 represents the possible contour plots plotted at 0 level of X1 at predetermined % EE values of 47%, 50%, 60%, 70%, 80%, 90%, 95% and 99%. All contour plots were found to be linear in nature. The %EE of 95% or more could be achieved with X2 range from -0.65 level (1.175 ml) to 0.6 level (1.8 ml) and X3 range from 0.9 level (1:14:2 molar ratio) to 1 level (1:15:2 molar ratio).

Figure 5.4 represents the possible contour plot plotted at 1 level of X1 for predetermined % EE values of 40%, 45%, 50%, 60%, 70%, 80%, 90% and 95%. All contour plots were found to be linear in nature. The %EE (90%) could be achieved with X2 range from -0.45 level (1.275 ml) to 0.6 level (0.8 ml) and X3 range from 0.9 level (1:14:2 molar ratio) to 1 level (1:15:2 molar ratio).

Figure 5.2. Contour Plot of % Entrapment Efficiency at X1= -1 level of Organic Phase (Chloroform: Methanol) (1:2) using 14-term logit model

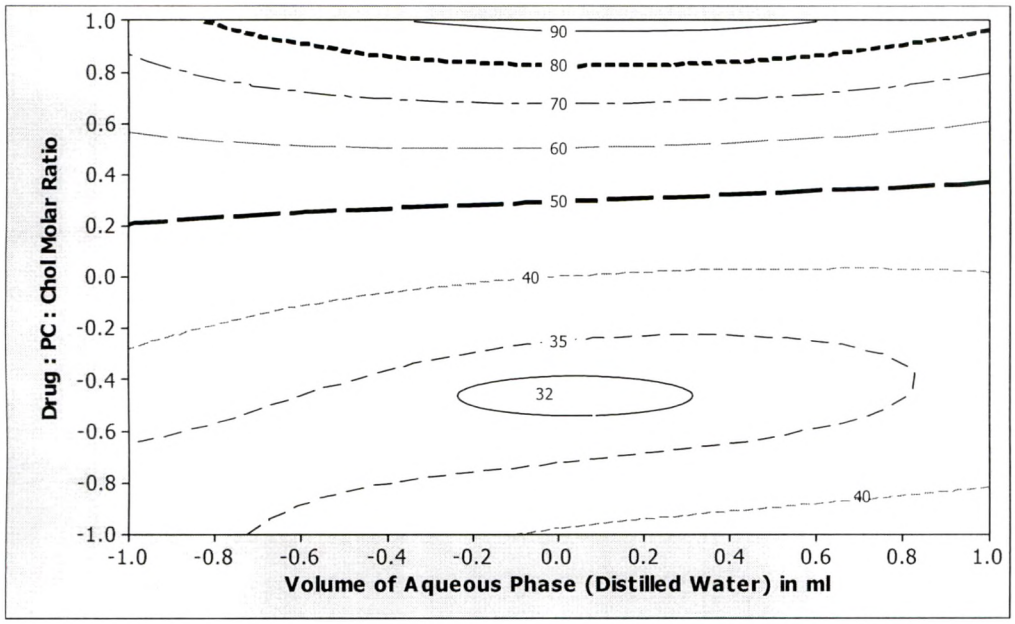


Figure 5.3. Contour Plot of % Entrapment Efficiency at X1= 0 level of Organic Phase (Chloroform: Methanol) (1:2) using 14-term logit model

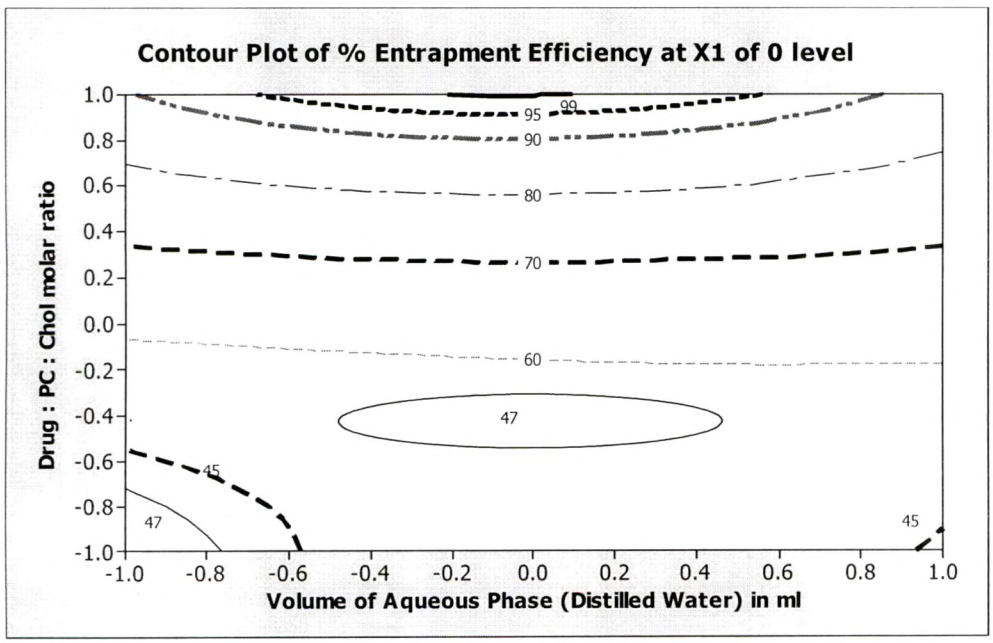
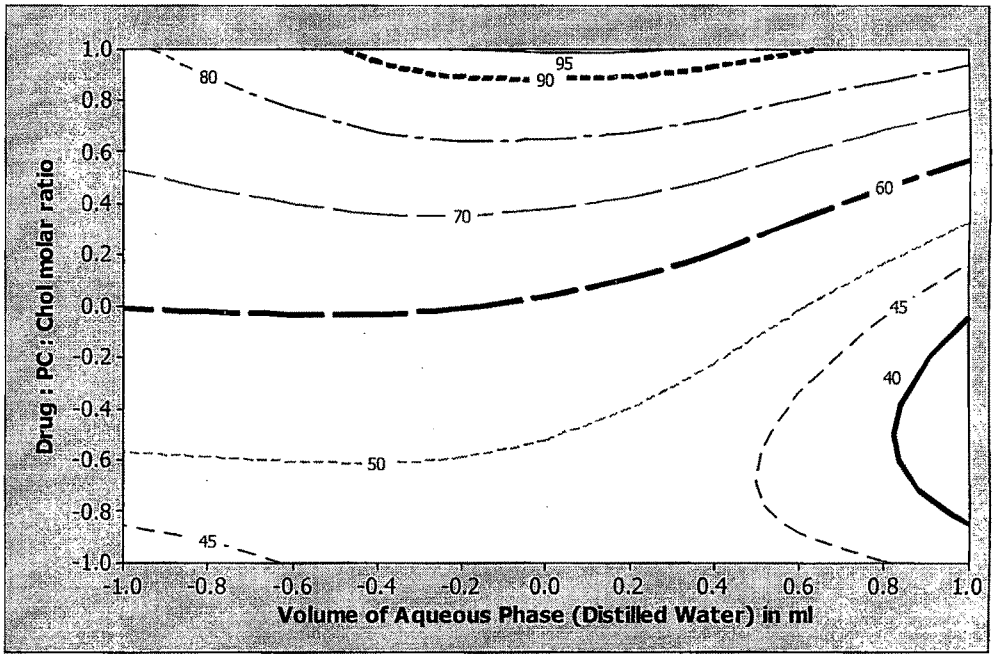


Figure 5.4. Contour Plot of % Entrapment Efficiency at X1= +1 level of Organic Phase (Chloroform: Methanol) (1:2) using 14-term logit model



From the results of contour plots, it was concluded that the range of volume of aqueous phase and the Drug/PC/CHOL molar ratio in conventional thin film hydration technique was from 1.175 ml to 1.8 ml and from 1:14:2 to 1:15:2 molar ratio, respectively at volume of organic phase 5 ml (0 level) to achieve maximum % EE.

5.2.7 Checkpoint Experiment

Three checkpoints were selected on three plotted contours at fixed levels of -1, 0, and +1 of X1. The predetermined % EE values for check point from contours at different levels of X1, X2 and X3 shown in (Table 5.4). Liposomes were prepared by conventional thin film hydration technique using the amount of X1, X2 and X3 at selected check points (Table 5.4).

Table 5.4: Check Point Table

Values from contour plots						% EE[a] (Mean ± SEM)	
X1		X2		X3			
Level	Vol	Level	Vol	Level	Molar ratio	Predeter-mined	Experiment-ally Obtained
-1	3 ml	+0.8	1.9 ml	-0.8	1:6:2	44.43	43.52(0.270)*
0	5 ml	-0.4	1.3 ml	+0.8	1:14:2	95.64	96.60(0.056)*
+1	7 ml	-0.2	1.4 ml	+0.2	1:11:2	59.51	60.36(0.182)*

[a] n=3

* Non-significant difference from predetermined % entrapment efficiency ($p>0.05$)

The experiments were repeated three times and obtained % EE (Mean ± SEM) values were 43.52%, 96.60% and 60.36% at fixed levels of -1, 0, and +1 of X1, respectively. When the student't' test was applied between predetermined and experimentally obtained % EE values, the non-significant difference was found. The value of calculated t (-0.4951) was less than the critical value of t (4.302) at the degree of freedom of 2 (Table 5.5).

Table 5.5: Student 't' test for Check-Point Experiments

Sources	Values
Number of Observations	3
Pearson Correlation	0.9996
Degree of Freedom	2
t calculated two tail	-0.4951
t critical	4.3026
p value two tail	0.6695

5.2.8 Conclusion

The present study deals with the study of the optimization of the volume of organic phase (X1), the volume of aqueous phase (X2) and the Drug: Phosphotidylcholine (PC): Cholesterol (Chol) molar ratio (X3) using 3^3 factorial 26-term logit model to maximize the flutamide absorption at the target site in the treatment of prostatic cancer by maximizing the entrapment of flutamide in the preparation of flutamide liposomes. Flutamide liposomes are expected to be an excellent carrier for flutamide

to the prostatic cancer site based on the use of liposomes with other drugs. A 3^3 factorial 26-term logit model for coded factors X1, X2 and X3 is used to develop a second order response surface regression equation for predicting percent entrapment efficiency (%EE) for flutamide. In turn, the regression equation is used to develop contour plots that show the %EE is maximized at the level of 1:15:2 of the Drug: PC: Chol molar ratio with the volume of organic phase (Chloroform: Methanol) (1:2) at 5 ml and the volume of distilled water at 1.5 ml.

5.3 PREPARATION OF FLUTAMIDE STEALTH LIPOSOMES USING POLY ETHYLENE GLYCOL DERIVATIZED PHOSPHATIDYL ETHANOLAMINE ($MPEG_{2000}$-PE)

5.3.1 Introduction

Polyethylene glycols of different chain lengths have been attached to the liposome for steric stabilization and longer circulation time in blood. Intermediate molecular weights from 1500 to 5000 Daltons at 5% to 10% mol in the bilayer give rise to the longest blood circulation times. Although many different lipids have been used, the only lipid used for attachment of longer PEG chains was phosphatidylethanolamine with different chain lengths and degrees of saturation because of the reactivity of the amino group. The reactivity of this group is further catalyzed by deprotonation by triethylamine of triethanolamine. There are three different reactions to link mPEG with PE such as succinyl chloride, cyanuric chloride and carbonate derivatives yielding ester, secondary amine and urethane linkage respectively.

5.3.2 Experimental

5.3.2.1 Synthesis of methoxy polyethylene glycol 2000 activated with cyanuric chloride ($mPEG_{2000}$-CC)

The method of Abuchowski (289) was modified for preparing methoxy polyethylene glycol 2000 ($mPEG_{2000}$) activated with cyanuric chloride. 10g of $mPEG_{2000}$, 2.75g of cyanuric chloride (molar ratio 1:3) and 5 g of anhydrous potassium carbonate were taken in a 250ml round bottomed flask. To the contents 200ml of benzene was added

and the flask was fitted with a calcium chloride guard tube. The contents were then filtered and the compound was precipitated by adding 300ml of petroleum ether (40°C -60°C) slowly with stirring. The compound was then purified by successive precipitation from benzene using petroleum ether (40°C -60°C), the process was monitored by quantitative ultraviolet spectroscopy for ascertaining the absence of impurities viz. cyanuric chloride. Absorptive scans over the ultraviolet wavelength range of methoxy polyethylene glycol 2000 and $mPEG_{2000}$-CC in methanol taken on a Shimadzu 1601 UV- Visible spectrophotometer are shown in Figures 5.5 and 5.6 respectively. The identity of $mPEG_{2000}$ activated with cyanuric chloride was ascertained by taking its mid infrared spectrum on a Shimadzu FTIR-8300 spectrophotometer (Figure 5.7).

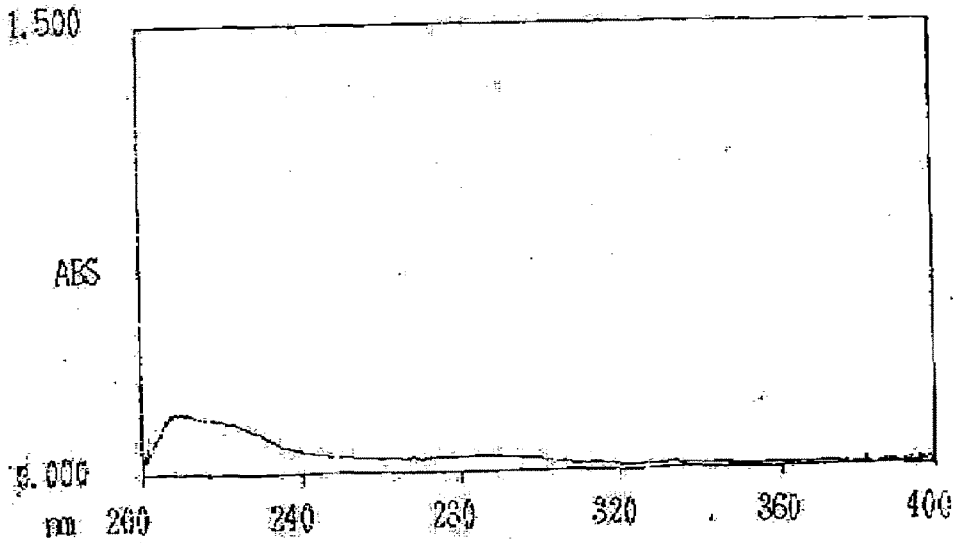


Figure 5.5: Ultraviolet Absorptivity scan of methoxy polyethylene glycol 2000 (mPEG2000) in methanol (1mg/ml)

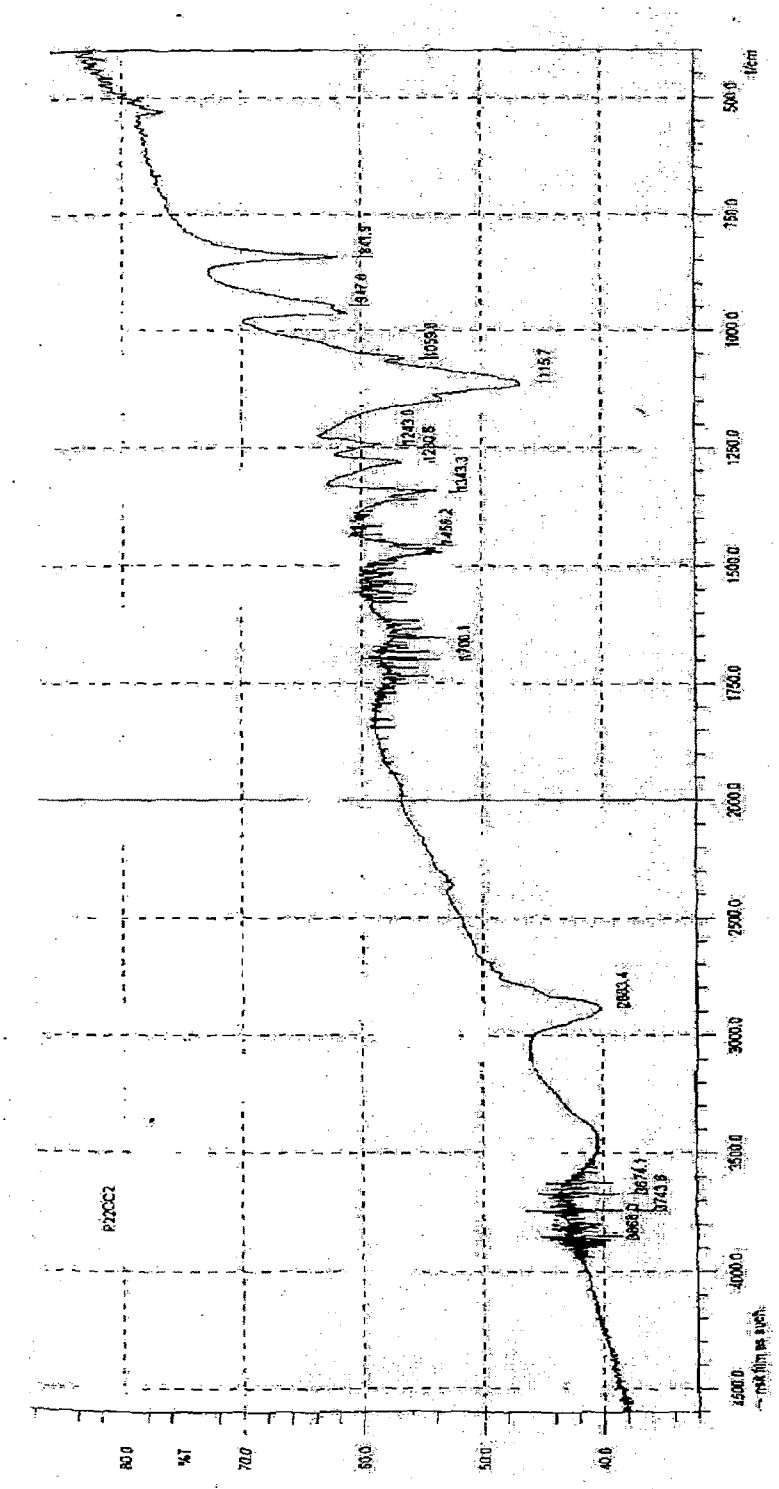


Figure 5.7: Mid infra-red spectrum of methoxy polyethylene glycol 2000 activated with cyanuric chloride-phosphatidylethanolamine conjugate ($mPEG_{2000}$-CC-PE)

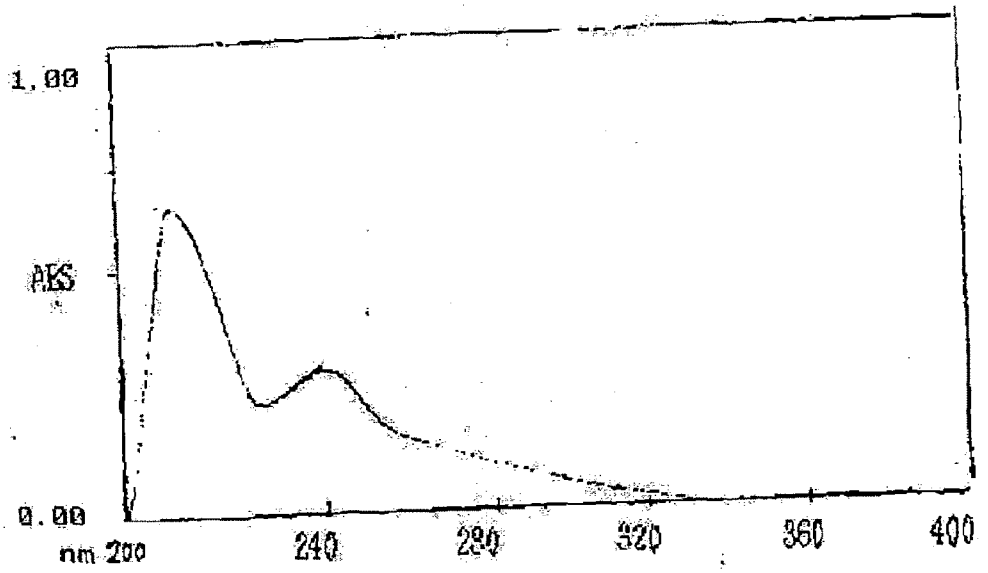
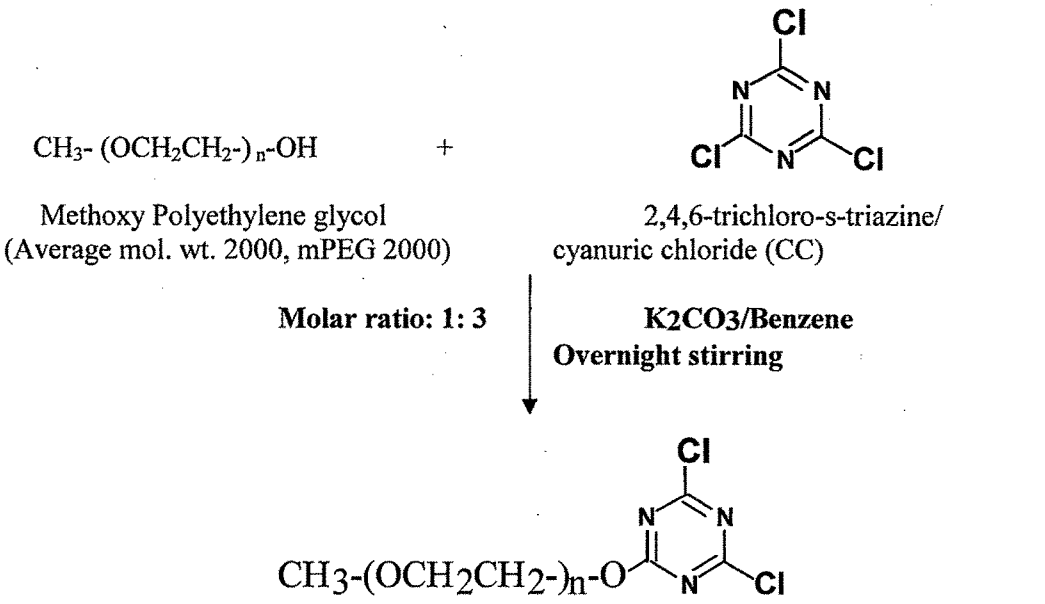


Figure 5.6: Ultraviolet Absorptivity scan of methoxy polyethylene glycol 2000 activated with cyanuric chloride ($mPEG_{2000}$-CC) in methanol (1mg/ml)

5.3.2.2 Synthesis of methoxy polyethylene glycol 2000 activated with cyanuric chloride-phosphatidylethanolamine conjugate ($mPEG_{2000}$-CC-PE)

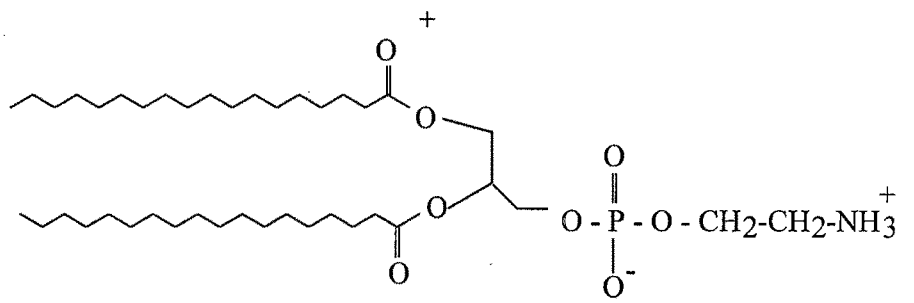
The method suggested by Blume and Cevc (290) was followed to prepare the conjugate of phosphatidylethanolamine (PE) with $mPEG_{2000}$ activated with cyanuric chloride ($mPEG_{2000}$-CC) .The entire reaction scheme is shown in Figure 5.9. PE (0.35mM) and triethylamine (1.2mM) were dissolved in 10ml of chloroform: methanol (5: 1 by volume) and added to a solution of $mPEG_{2000}$-CC (0.4mM) in 50ml of chloroform: methanol (1:5 by volume). The mixture was stirred magnetically under a calcium chloride guard tube for 5 days at room temperature. The course of the reaction was monitored by thin layer chromatography on silica gel plates using chloroform: methanol: water (65: 25: 4 v/v/v) as mobile phase. At the end of the reaction, the compound was recovered by precipitation using solvent ether, dried using a rotary flash evaporator and the solid so collected was redissolved in chloroform: methanol (2: 1 by volume) and stored at less than 0°C until further use. The ultraviolet, mid infrared region and ^{13}C-NMR spectra of the compound were taken using the same instruments are shown as Figures 5.8, 5.10 and 5.11 respectively.

Figure 5.9: Reaction scheme of the Synthesis of mPEG2000-CC PE



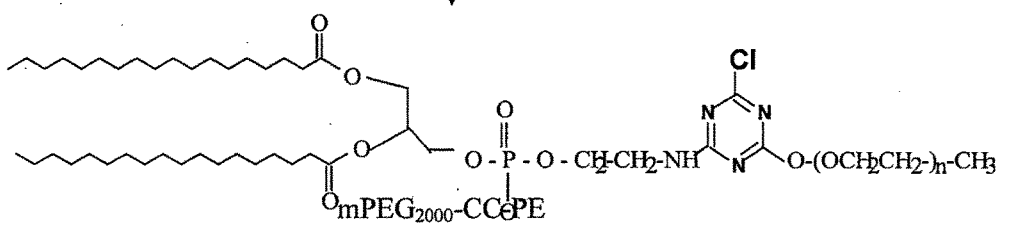
Methoxy polyethylene glycol 2000 activated with cyanuric chloride ($mPEG_{2000}$-CC)

(Precipitated using Pet ether)



Phosphatidylethanolamine

Stirred for 5 days in
Chloroform methanol mixture



(Precipitated using solvent ether)

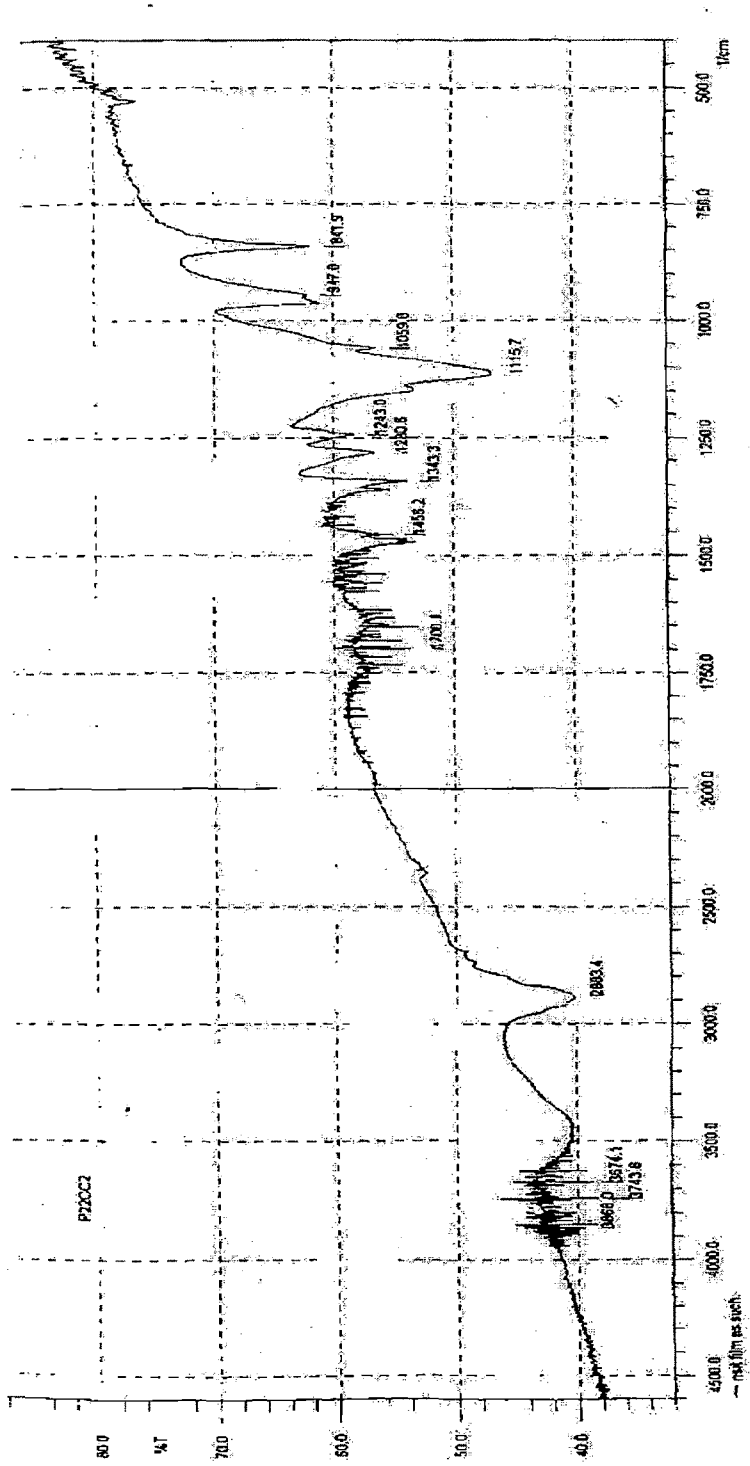


Figure 5.10: Mid infra-red spectrum of methoxy polyethylene glycol 2000 activated with cyanuric chloride-phosphatidylethanolamine conjugate ($mPEG_{2000}$-CC-PE)

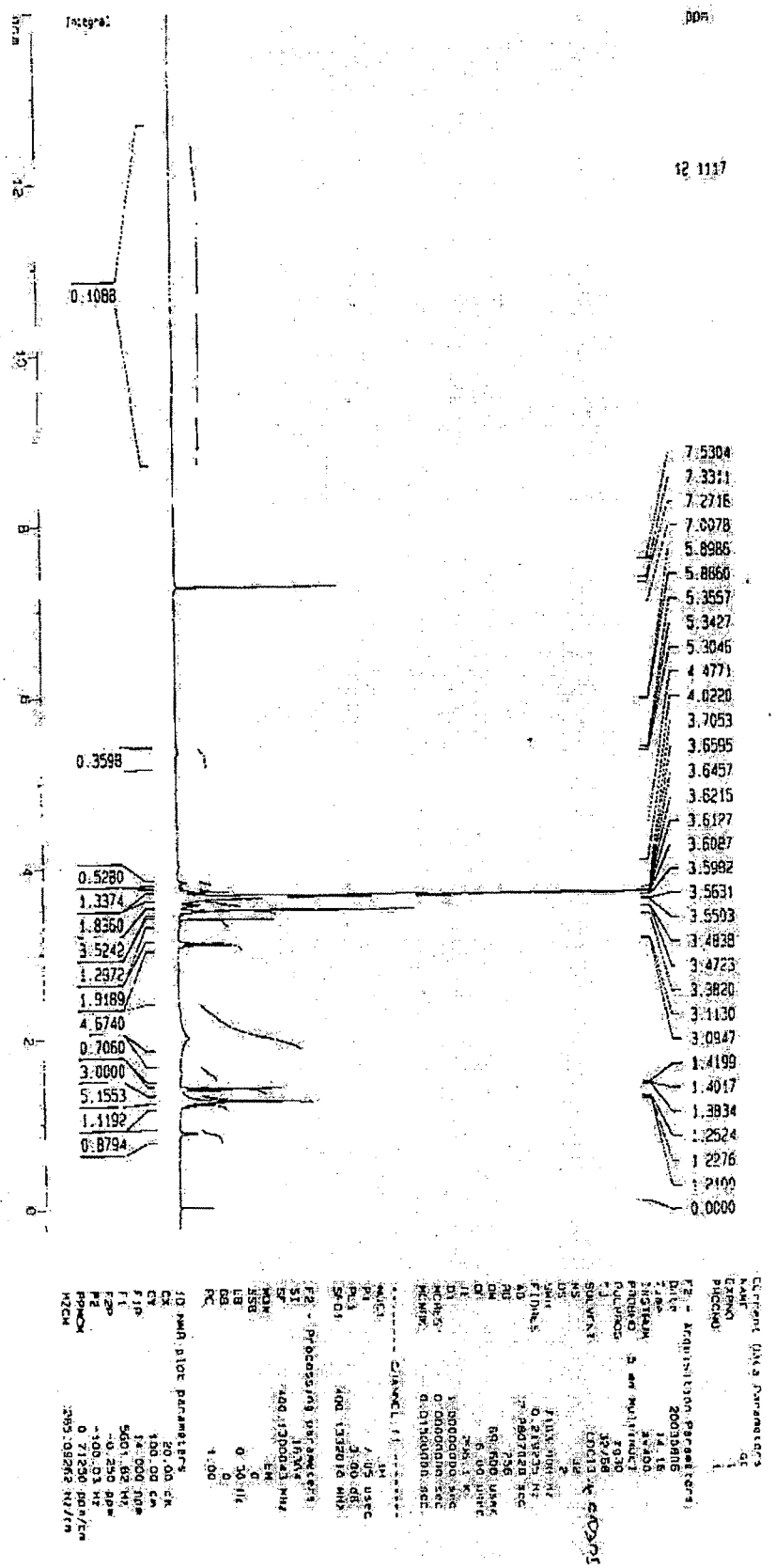


Figure 5.11: ^{13}C-NMR of methoxy polyethylene glycol 2000 activated with cyanuric chloride- phosphatidylethanolamine conjugate ($mPEG_{2000}$-CC-PE)

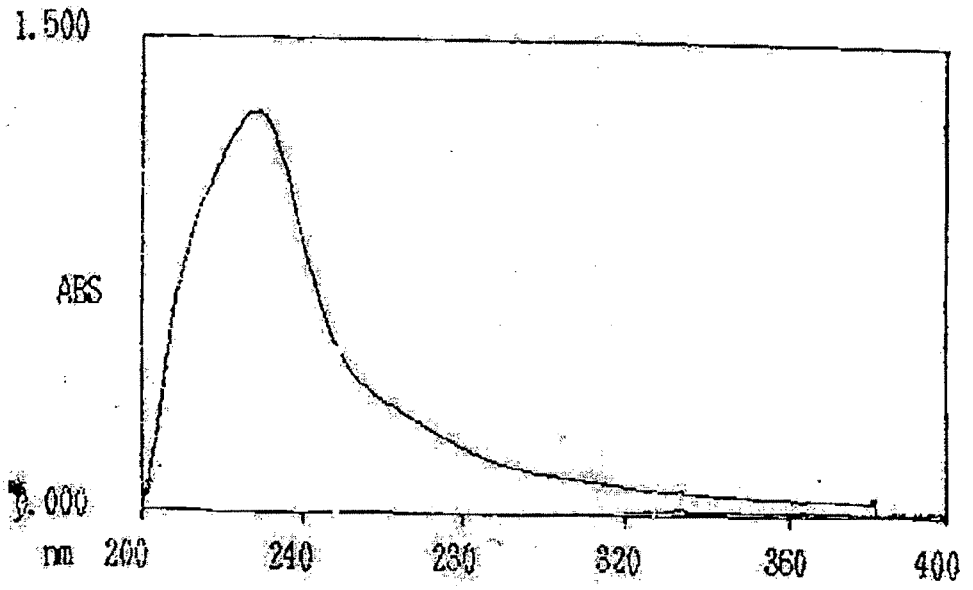


Figure 5.8: Ultraviolet Absorptivity scan of methoxy polyethylene glycol 2000 coupled with cyanuric chloride – phosphatidylethanolamine conjugate ($mPEG_{2000}$-CC-PE) in methanol (1mg/ml)

5.3.2.3 Preparation of flutamide stealth liposomes using $mPEG_{2000}$-CC-PE

The method of preparation of these liposomes is similar to that used to prepare conventional liposomes containing flutamide as described earlier, with the sole modification that an appropriate amount of $mPEG_{2000}$-CC-PE was added to the lipid solution before it was subjected to evaporation for lipid film formation. The amount of $mPEG_{2000}$-CC-PE added was compensated by removal of an equimolar amount of PC from the system. The amount of $mPEG_{2000}$-CC- PE required for steric stabilization was optimized by subjecting triplicate batches of liposomes, formed with different amounts of the $mPEG_{2000}$-CC-PE, to the electrolyte induced flocculation test. Each batch was prepared three times at a three different days and evaluated for % entrapment efficiency. Figure 5.1(b) represents photomicrograph of FLT stealth liposomes.

5.3.2.4 Electrolyte induced flocculation test

Sodium sulphate solutions ranging from 0 M to 2 M were prepared in 16.7 % w/v of sucrose solution. An appropriate volume of conventional and stealth liposome formulation, which gives a final concentration of 1 mg/ml of lipid, was taken and volume was made up to 5 ml using the sodium sulphate solutions of various concentrations. The resulting dispersions were mixed and the absorbance was measured within 5 min at 400 nm on Shimadzu 1601 UV-Visible spectrophotometer against respective blank.

5.3.3 Results and Discussion

5.3.3.1 % Entrapment efficiency

The presence of $mPEG_{2000}$-CC-PE led to reduction in the entrapment of flutamide as compared to conventional liposomes after concentration of steric stabilizing agent increasing above 5-mole%, which may be due to incorporation of steric stabilizing agent in the bilayer where the drug was also incorporated. Stealth liposomes with 3-mole % and 5-mole % showed insignificant difference in % EE from conventional liposomes (Table 5.6).

Table 5.6: % entrapment efficiency of flutamide liposomes at different concentration of steric stabilizing agent

Batch No	Mole % of $mPEG_{2000}$-CC-PE	% EE*
CL	0	99.28±1.94
S1	3	98.81±2.36**
S2	5	98.54 ±2.34**
S3	7	94.62±2.48
S4	9	91.26±2.72

* n=3

** Non-significant difference from % EE of liposomes ($p>0.05$)

5.3.3.2 Electrolyte induced flocculation test

Polyethylene glycol grafting has been widely used as a method for reduction of the rapid clearance of liposomes from circulation by RES. Therefore; PEG-lipid conjugates were incorporated in liposomes for steric stabilization. Table 5.7 showed that the conventional liposomes containing flutamide showed a gradual increase in flocculation as the concentration of the sodium sulphate increases from 0 M to 2 M.

The conventional liposomes are electrostatically stabilized. Addition of electrolyte will compress the electrostatic double layer surrounding the liposomes and results in the aggregation leading to flocculation with corresponding increase in optical turbidity.

Table 5.7: Optimization of $mPEG_{2000}$-CC-PE concentration required for steric stabilization of flutamide containing conventional and stealth liposomes

Concentration of sodium sulphate (M)	Mean absorbance ± SD at 400 nm of Flutamide liposomes containing $mPEG_{2000}$-CC-PE				
	0 mol%	3 mol %	5 mol %	7 mol %	9 mol %
0	0.53 ± 0.014	0.574 ± 0.032	0.532 ± 0.031	0.511± 0.024	0.517± 0.022
0.4	0.585 ± 0.021	0.579 ± 0.018	0.552 ± 0.025	0.51± 0.021	0.52± 0.014
0.8	0.631 ± 0.016	0.608 ± 0.019	0.546 ± 0.011	0.519± 0.014	0.529± 0.018
1.2	0.665 ± 0.031	0.641 ± 0.021	0.561 ± 0.018	0.532± 0.019	0.525± 0.018
1.6	0.728 ± 0.017	0.649 ± 0.025	0.565± 0.015	0.539± 0.018	0.532± 0.011
2	0.776 ± 0.029	0.654 ± 0.021	0.569± 0.027	0.547± 0.023	0.537± 0.035

Figure 5.12: Optimization of $mPEG_{2000}$-CC-PE concentration required for steric stabilization of flutamide containing conventional and stealth liposomes

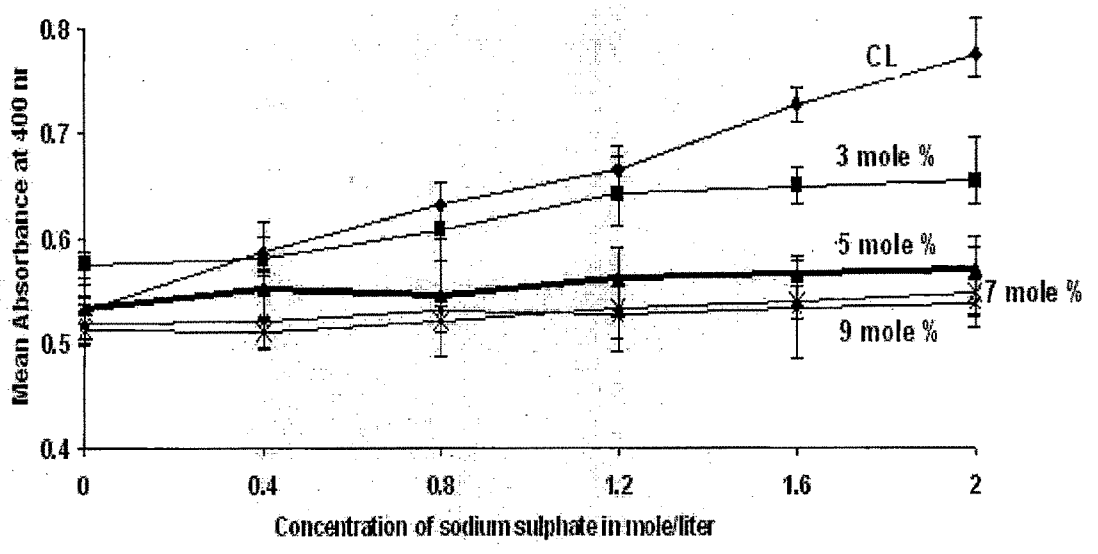


Figure 5.12 also indicated that 3% molar ratio of lipid is insufficient to provide protection against electrolyte induced flocculation probably due to insufficient coverage of polymer on the surface of the prepared liposomes. 5 mole % was found to provide steric stability to the liposomes of flutamide. It may be produced by

surface modification due to the polymer incorporated; the system should be stable even if the electrostatic double layers have been compressed. Further increase in polymer concentration (7% and 9%) does not produce any significant increase in protection. It may be due to dehydration of the hydrated steric stabilized barriers and is measured by finding the change in optical turbidity which can be used to ascertain whether the liposomes are sterically stabilized or not.

5.3.4 Conclusion

The concentration of the steric stabilizing agent, which did not allow any changes in the absorbance when different molar concentrations of the electrolyte were added to the liposomes was selected as the optimum concentration and used for the further studies. 5 mole % of the total lipids of $mPEG_{2000}$-CC-PE was found to be adequate for steric stabilization of the liposomes and had % EE of (98.54 ±2.34).

5.4 PREPARATION OF 6-MERCAPTOPURINE LIPOSOMES

5.4.1 Introduction

6-mercaptopurine (6-MP) is a purine analogue and has been used in cancer chemotherapy, primarily in childhood leukemia (291). It usually used in combination with other anti-cancer drugs and interfered with the synthesis of adenine and guanine ribonucleosides, which are important precursors of DNA and RNA. Because of the low oral bioavailability (16%), the importance of optimizing 6-MP therapy and achieving high and predictable systemic drug exposure has encouraged the use of intravenous administered 6-MP in patients with Acute lymphoblastic leukemia (292). However, parenteral administration of 6-MP has the limitation of short biological half-life and results in an inconveniently high dosing frequency (293). Thus, there is a need for effective delivery systems that not only act as a formulation aid but also alter the biodistribution of drugs in such a way that a greater fraction of the dose reaches the target site. For such delivering system, liposomes are one of the attractive systems for drug delivery due to its composition from natural biological lipids and structural resemblance to cell membranes which suggest compatibility. Liposomes are microparticulate lipoidal vesicles, which are under extensive investigation as drug carriers for improving the delivery of therapeutic agents (294). Due to recent developments in liposome technology, more effective strategies are now available for controlling the stability and reactivity of liposomes after systemic administration (295). The use of experimental designs is the most common method of simultaneously analyzing the influence of different factors on the properties of the drug delivery system being studied. Experimental design techniques and optimization are useful tools in the characterization of pharmaceutical formulation by studying the effect of various factors affecting on it and its possible interactions economically (296). The objective of this study is to determine the effect of the drug: lipid molar ratio, the HSPC: cholesterol molar ratio, the volume of organic phase and the volume of aqueous phase on % entrapment efficiency and to develop 6-MP liposomes for better treatment of leukemia.

5.4.2 Experimental

5.4.2.1 Statistical Design

4^2 factorial design was used to determine the effect of the four independent factors: the drug: lipid molar ratio (X1), the HSPC: cholesterol molar ratio (X2), the volume

of organic phase (X3) and the volume of aqueous phase (X4) on the % entrapment efficiency of liposomes. Each factor was tested at two levels designed as -1 and +1. The normalized factor levels of independent variables are given in Table 5.8.

Table 5.8: Independent variables and their correspondence between real and transformed values as per 4^2 factorial design

	Levels	
Variables	**-1**	**1**
(X1) Drug: lipid molar ratio	1:10	1:15
(X2) HSPC: cholesterol molar ratio	9:1	7:3
(X3) Volume of organic phase (Chloroform: Methanol, 1:2, v/v)	5 ml	7 ml
(X4) Volume of aqueous phase (Distilled Water)	1.5 ml	2 ml

5.4.2.2 Preparation of liposomes

Sixteen batches multi lamellar vesicles of 6-MP liposomes were prepared by conventional thin film hydration technique according to experimental design and order shown in Table 5.9. Briefly HSPC, cholesterol and 6-MP were accurately weighed and dissolved in chloroform and methanol (ratio 2:1 by volume) in 250 ml round bottom flask. To this, 0.5 ml of 0.1 % α-tocopherol solution in chloroform was added. The flask was rotated in the rotary flash evaporator at 120 RPM at 37°C under vacuum (250mmHg) with nitrogen as bleed. The flask was rotated under vacuum for 20 min additionally. The thin dry lipid film formed was hydrated using distilled water and the flask was rotated at the same RPM and 60°C temperature for 20 min. 4-5 pieces of glass-beads were added to improve hydration. The liposomal suspension was sonicated for 20 min using a probe sonicator (model RR-120, Ralsonics, Mumbai) in an ice bath for heat dissipation. The sonicated dispersion was then allowed to stand undisturbed for about 2 hr at room temperature for annealing to be completed. Each batch was prepared three times at a three different days in order mentioned in Table 5.9 and evaluated for % entrapment efficiency.

5.4.2.3 Statistical Analysis

In this study, 4^2 factorial design and multi-linear regression analysis was used to study the dependency of response variable on the four independent variables (X1, X2, X3, X4). The regression model for the four variables can be presented in a general form:

$Y (X1, X2, X3, X4) = b_0 + b_1X1 + b_2X2 + b_3X3 + b_4X4 + b_5X1X2 + b_6X2X3 + b_7X3X4 + b_8X1X4 + b_9X1X2X3 + b_{10}X2X3X4 + b_{11}X3X4X1 + b_{12} X1X2X4 + b_{13}X1X2X3X4$—(Equation1)

Where Y is the predicted response, b_0 is the intercept and b_1-b_{13} is the regression co-efficients of the system. The main effects X1, X2, X3 and X4 denote the drug: lipid molar ratio, the HSPC: cholesterol molar ratio, the volume of organic phase and the volume of aqueous phase, respectively. The X1X2, X2X3, X3X4, X4X1, X1X2X3, X2X3X4, X3X4X1, X1X2X4 and X1X2X3X4 indicate interactions. A full model was established after including all above terms into second order polynomial equation (Equation 2). Neglecting non-significant ($p > 0.05$) terms from the full model equation, the model was re-estimated or reduced (Equation 3). The statistical analysis and the generation of regression model were performed on the basis of data given in (Table 5.10).

$Y (X1, X2, X3, X4) = 72.38 + 13.61 X1 + 5.19 X2 - 1.44 X3 + 3.316 X4 - 1.29 X1X2 - 1.189 X2X3 - 0.244 X3X4 + 0.722 X1X4 + 0.201 X1X2X3 - 0.466 X2X3X4 + 0.0189X3X4X1 + 0.012 X1X2X4 - 0.089 X1X2X3X4$ (Equation 2)

$Y (X1, X2, X3, X4) = 72.29 + 13.07 X1 + 5.74 X2$ (Equation 3)

Table 5.10: Summary of Results of Regression Analysis

Source Effect	Regression Coefficients	p value	t value	Reduced Regression Coefficients
Average Constant	72.83525	0.000206	69.64463	72.29188
X1	13.619*	0.005845	13.02241	13.07563
X2	5.19975*	0.038152	4.97197	5.743125
X3	-1.44225	0.301845	-1.37907	-
X4	3.136	0.095541	2.998624	-
X1X2	-1.299	0.340095	-1.2421	-
X2X3	-1.18275	0.375451	-1.13094	-
X3X4	-0.244	0.837224	-0.23331	-
X4X1	0.72225	0.561191	0.690611	-
X1X2X3	0.201	0.865336	0.192195	-
X2X3X4	-0.466	0.699486	-0.44559	-
X3X4X1	0.18975	0.872747	0.181438	-
X1X2X4	0.01275	0.99138	0.012191	-
X1X2X3X4	-0.08975	0.939429	-0.08582	-
r (correlation coefficient)	0.963	-	-	-

* Statistically significant (p-value<0.05).

Table 5.9: Factorial $4^{2:}$ Matrix of Experiments and results for the measured response

Batch No.	X1	X2	X3	X4	X1*X2	X2*X3	X3*X4	X1*X4	X1*X2*X3	X2*X3*X4	X3*X4*X1	X2*X4*X1	X1*X2*X3*X4	%EE
1	-1	-1	-1	-1	1	1	1	1	-1	-1	-1	-1	1	50.38
2	-1	-1	-1	1	1	1	-1	-1	-1	1	1	1	-1	55.26
3	-1	-1	1	-1	1	-1	-1	1	1	1	1	-1	-1	50.29
4	-1	-1	1	1	1	-1	1	-1	1	-1	-1	1	1	54.94
5	-1	1	-1	-1	-1	-1	1	1	1	1	-1	1	-1	65.33
6	-1	1	-1	1	-1	-1	-1	-1	1	-1	1	-1	1	71.84
7	-1	1	1	-1	-1	1	-1	1	-1	-1	1	1	1	61.21
8	-1	1	1	1	-1	1	1	-1	-1	1	-1	-1	-1	64.48
9	1	-1	-1	-1	-1	1	1	-1	1	-1	1	1	-1	79.67
10	1	-1	-1	1	-1	1	-1	1	1	1	-1	-1	1	86.27
11	1	-1	1	-1	-1	-1	-1	-1	-1	1	-1	1	1	74.34
12	1	-1	1	1	-1	-1	-1	-1	-1	1	-1	1	1	81.24
13	1	1	-1	-1	1	-1	1	-1	-1	1	1	-1	1	88.21
14	1	1	-1	1	1	-1	-1	1	-1	-1	-1	1	-1	97.26
15	1	1	1	-1	1	1	-1	-1	1	-1	-1	-1	-1	84.67
16	1	1	1	1	1	1	1	1	1	1	1	1	1	91.28

5.4.2.4 Characterization of Liposomes

5.4.2.4.1 % Encapsulation Efficiency

From each prepared batch, a definite amount of liposomal dispersion was taken and subjected to centrifugation on laboratory centrifuge (Sigma, 3K30) at 15, 000 RPM for 15 min at 0^{o}C after mixing with 50 µl protamine solution (10 mg/ml) (297) using micropipette. The clear supernatant and sediment were separated. The definite amount of supernatant was diluted to 5 ml with methanol and the absorbance was recorded at 325 nm on Shimadzu 1601 UV-Visible Spectrophotometer. The sediment was resuspended in 1.5 ml distilled water, 0.5 ml of chloroform was added to lyse the liposomes in aliquot and diluted to 10 ml with methanol and the absorbance was recorded at 325 nm. Each time blank containing blank liposome was treated in the same manner to account for any absorbance due to lipid components. Table 5.9 shows the % EE of all batches. % EE was calculated as per Equation 1(Section 5.2.5.1)

5.4.2.4.2Photomicrography

All the batches of the liposomes prepared were observed under Olympus (BX 40F4, Tokyo, Japan) microscope with a polarizing attachment to study their size and lamellarity. A photomicrography under 100X magnification is shown in Figure 5.13(a) and (b).

5.4.3 Results and Discussion

Sixteen batches of 6-MP liposomes were prepared by conventional thin film hydration method using 4^2 factorial design (Table 5.9) by varying four independent variables: the drug: lipid molar ratio (X1), the HSPC: cholesterol molar ratio (X2), the volume of organic phase (X3) and the volume of aqueous phase (X4). The % EE (response variable) of the prepared batches was determined (Table 5.9) and the highest % EE achieved in liposomes was 97.26 % at 1 level of X1 (1:20 molar ratio), 1 level of X2 (7:3 molar ratio), 1 level of X3 (5 ml) and –1 level of X4 (2 ml). The results were subjected to multiple regression analysis. The fitted equation related to % EE and transformed factors shows in Equation 2.

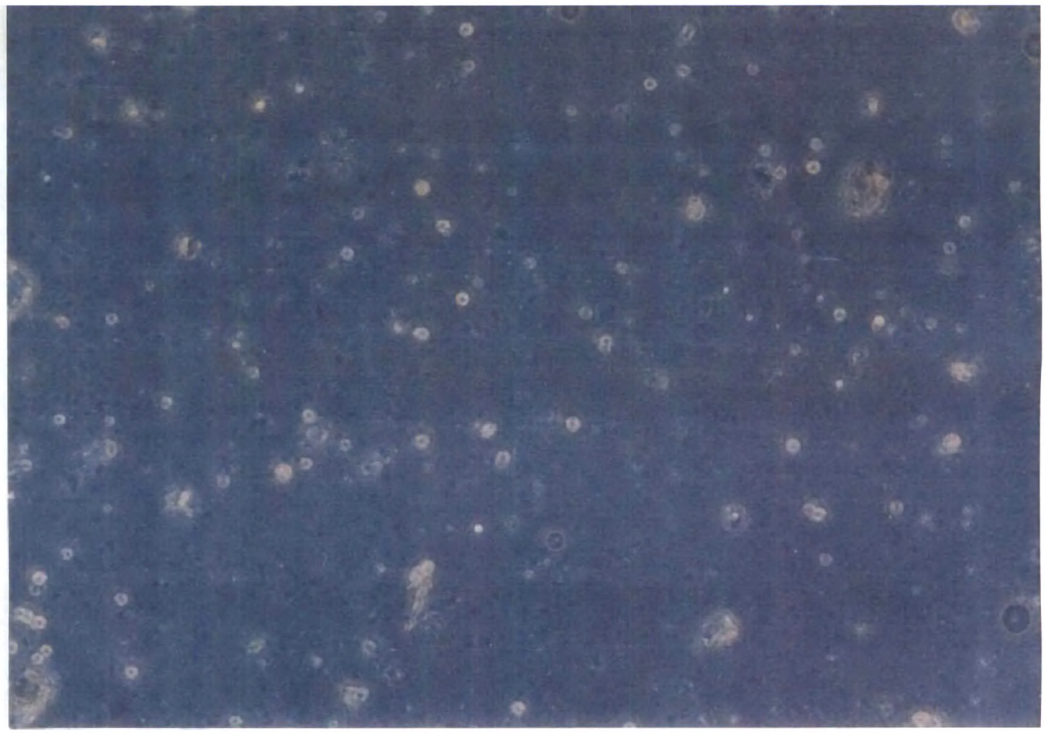


Figure 5.13 (a): 6-Mercaptopurine conventional liposomes

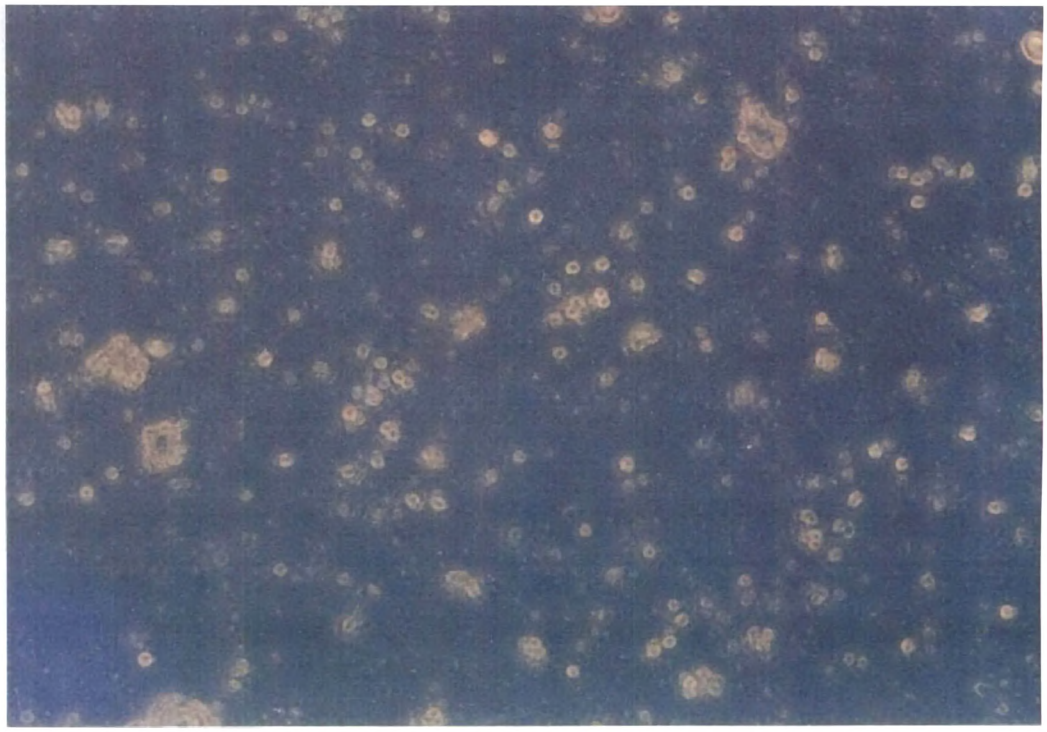


Figure 5.13 (b): 6-Mercaptopurine stealth liposomes

The data clearly indicated that % EE is more dependent on the drug: lipid molar ratio and the HSPC: Chol molar ratio than the volume of organic phase and the volume of aqueous phase. The value of correlation-coefficient (r) was found to be 0.963, indicating a good fit. The small values of coefficients of terms X3, X4, X1X2, X2X3, X3X4, X4X1, X1X2X3, X2X3X4, X3X4X1, X1X2X4 and X1X2X3X4 (Equation 2) were least contributing in preparation of 6-MP liposomes ($p>0.05$). Hence, they were omitted to evolve the reduced model (Equation 3).

Table 5.11: Results of ANOVA of full and reduced models

	df	SS	MS	F value
Regression				
Full Model (FM)	13	3488.677	268.3597	22.49163
Reduced Model (RM)	2	3263.287	1631.644	85.09996
Residuals				
Full Model (FM)	2	23.86308	11.93154	-
Reduced Model (RM)	13	249.2524	19.17326	-

ND= Number of omitted parameters= 11 ($p>0.05$)

$A= (SSE_{RM}-SSE_{FM})/ND= 20.48$

$B=SSE_{FM}/df\ Residual_{FM}=11.93$

$F_{cal}=A/B=1.717$

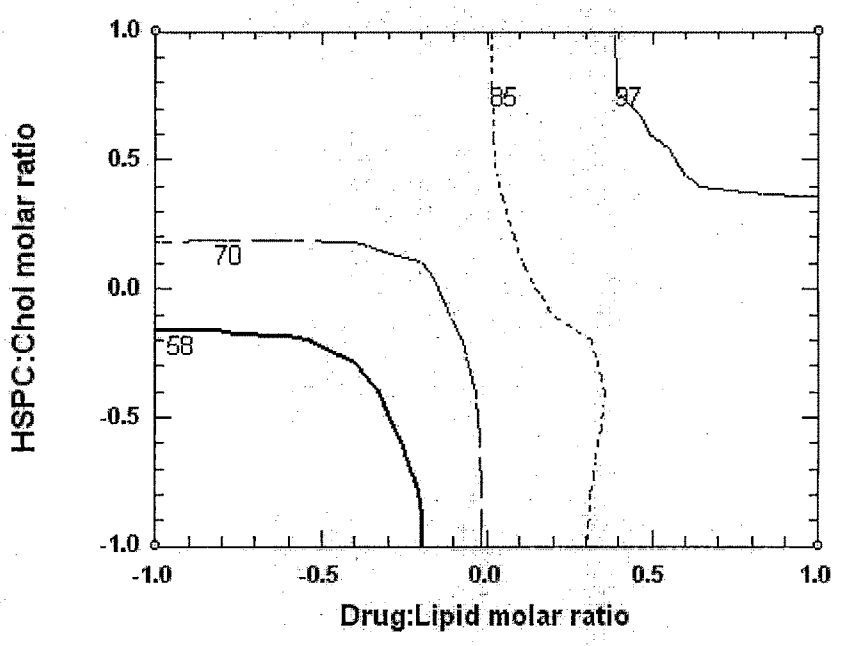
F-statistics of the results of ANOVA of full model and reduced model was applied to confirm omission of non-significant terms of Eq 2). Calculated value of F (1.71) was less than the critical value of F (3.35 at $\alpha= 0.05$, v1=5, v2=10) (298) as mentioned in Table 5.11.

The reduced model (Equation 3) was used to plot two-dimension contour plot (Figures 5.14 using NCSS® Software) and the values of X1 and X2 were computed between -1 and +1 at predetermined value of % EE.

Figure 5.14 represents the contour plot drawn at predetermined % EE values of 58%, 70%, 85% and 97%. Plot was found to be linear which indicated linear relationship between X1 and X2 variables. It was concluded from the linear contour that the % EE (97%) could be obtained with X1 range from 0.4 level (1:17 molar ratio) to 1 level (1:20 molar ratio) and X2 from 0.35 level (7.7:3.3 molar ratio) to 1 level (7:3). It was estimated from this contour plot results that the liposomes with 97% EE could

be prepared with in the range of X1 (1:17 to 1:20 molar ratio) and X2 (7.7:3.3 to 7:3).

Figure 5.14. Contour plot of % Entrapment Efficiency at different levels of X1 and X2



5.4.4 Conclusion

The present study was deal with the study of the optimization of the drug: lipid molar ratio (X1), the HSPC: cholesterol molar ratio (X2), the volume of organic phase (X3) and the volume of aqueous phase (X4) using 4^2 factorial design in the preparation of 6-MP liposomes. A 4^2 factorial design for coded factors X1, X2, X3 and X4 was used to develop a second order response surface regression equation for predicting percent %EE for 6-MP. In turn, the regression equation was used to develop contour plot that shows the %EE was maximized at the level of 1:20 molar ratio of the drug: lipid molar ratio, 7:3 HSPC: Chol molar ratio, the volume of organic phase (Chloroform: Methanol) (1:2) at 5 ml and the volume of distilled water at 2 ml.

5.5 PREPARATION OF 6-MP STEALTH LIPOSOMES USING $MPEG_{2000}$-CC-PE

5.5.1 Experimental

The method of preparation of these liposomes are similar to that used to prepare conventional liposomes containing 6-MP as described earlier, with the sole modification that an appropriate amount of $mPEG_{2000}$-CC-PE was added to the lipid

solution before it was subjected to evaporation for lipid film formation. The amount of $mPEG_{2000}$-CC-PE added was compensated by removal of an equimolar amount of PC from the system. The amount of $mPEG_{2000}$-CC- PE required for steric stabilization was optimized by subjecting triplicate batches of liposomes, formed with different amounts of the $mPEG_{2000}$-CC-PE, to the electrolyte induced flocculation test as discussed earlier. Each batch was prepared three times at a three different days and evaluated for % entrapment efficiency.

5.5.2 Results and Discussion

5.5.2.1 % Entrapment Efficiency

Table 5.12 shows the effect of mole % of mPEG2000-CC-PE on % EE of 6-MP liposomes. It indicated that the significant difference in % EE was found after concentration of 5-mole % of $mPEG_{2000}$-CC-PE. There may be a competition between the drug molecule and the polymer for occupying the lipid bilayer, which led to reduction in the aqueous compartment of liposomes. The inclusion of the steric stabilizing agent did not cause much reduction in the entrapment. Figure 5.13(b) represents photomicrograph of 6-Mercaptopurine stealth liposomes.

Table 5.12: % entrapment efficiency of 6-MP liposomes at different concentration of steric stabilizing agent

Batch No	Mole % of $mPEG_{2000}$-CC-PE	% EE*
CL	0	97.26±2.6
MS1	3	96.02±2.87**
MS2	5	96.38±4.5**
MS3	7	85.37±5.28
MS4	9	77.39±4.67

* n=3

** Non-significant difference from % EE of liposomes ($p>0.05$)

5.5.2.2 Electrolyte induced flocculation test

Table 5.13 showed that the conventional liposomes containing 6-MP showed a gradual increase in flocculation as the concentration of the sodium sulphate increases from 0 M to 2 M. The conventional liposomes are electrostatically stabilized. Addition of electrolyte will compress the electrostatic double layer surrounding the

liposomes and results in the aggregation leading to flocculation with corresponding increase in optical turbidity.

Table 5.13: Optimization of $mPEG_{2000}$-CC-PE concentration required for steric stabilization of 6-MP containing conventional and stealth liposomes

Concentration of sodium sulphate (M)	Mean absorbance ± SD at 400 nm of 6-MP liposomes containing $mPEG_{2000}$-CC-PE				
	0 mol%	3 mol %	5 mol %	7 mol %	9 mol %
0	0.605±0.017	0.551±0.011	0.563±0.025	0.594±0.03	0.531±0.030
0.4	0.636±0.021	0.585±0.028	0.574±0.019	0.587±0.017	0.546±0.013
0.8	0.676±0.023	0.602±0.021	0.588±0.019	0.6120.015	0.544±0.030
1.2	0.747±0.02	0.625±0.12	0.601±0.025	0.627±0.030	0.568±0.028
1.6	0.783±0.023	0.654±0.020	0.592±0.015	0.651±0.021	0.579±0.016
2	0.813±0.024	0.677±0.023	0.611±0.023	0.644±0.015	0.571±0.027

Figure 5.15: Optimization of $mPEG_{2000}$-CC-PE concentration required for steric stabilization of 6-MP containing conventional and stealth liposomes

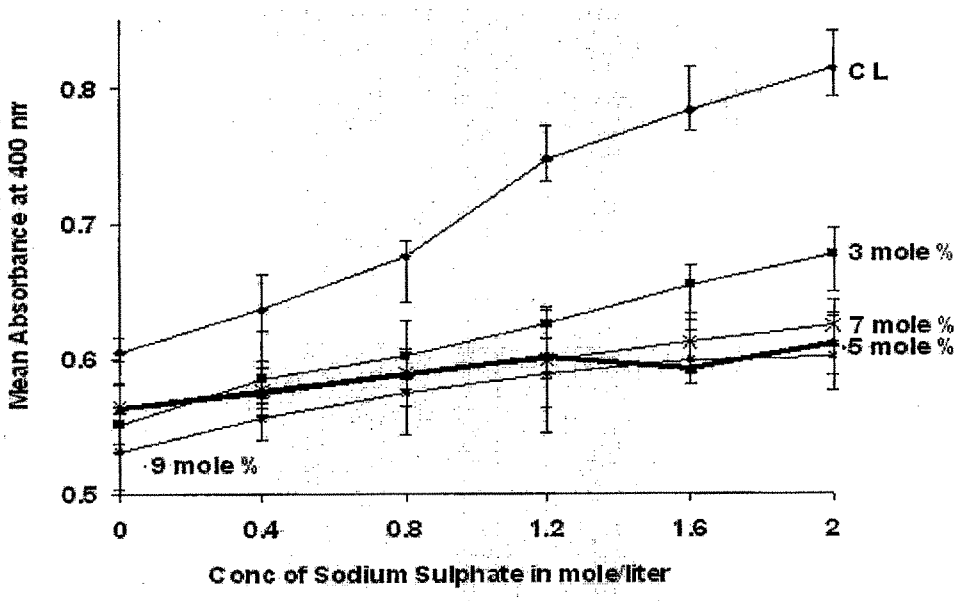


Figure 5.15 also indicated that 3% molar ratio of lipid is insufficient to provide protection against electrolyte induced flocculation probably due to insufficient coverage of polymer on the surface of the prepared liposomes. 5 mole % was found to provide steric stability to the liposomes of 6-MP. It may be produced by surface modification due to the polymer incorporated; the system should be stable even if the electrostatic double layers have been compressed. Further increase in polymer concentration (7% and 9%) does not produce any significant increase in protection. It may be due to dehydration of the hydrated steric stabilized barriers and is measured by finding the change in optical turbidity which can be used to ascertain whether the liposomes are sterically stabilized or not.

5.5.3 Conclusion

The concentration of the steric stabilizing agent, which did not allow any changes in the absorbance when different molar concentrations of the electrolyte were added to the liposomes was selected as the optimum concentration and used for the further studies. 5 mole % of the total lipids of $mPEG_{2000}$-CC-PE was found to be adequate for steric stabilization of the liposomes and had % EE of (96.38±4.5).

5.6 PREPARATION OF MICROBUBBLES

5.6.1 Introduction

Microbubbles have similarities with the naturally occurring and very stable bubble populations found in ocean and other natural waters. The production process for research grade microbubbles is produced simply by mechanical shaking, sonication or by mixing an aqueous suspension of naturally occurring, non-ionic lipids or phospholipids or PEG-lipids. The monolayer forming lipid mixture used to manufacture the microbubbles is also comprised of simple; "off-the-shelf" saturated glycerides and cholesterol esters, in a fixed ratio and of specific chain length (299). Moreover, since formation of the monolayer-stabilized microbubbles is solely molecular self-assembly (300), no organochemical reaction or derivatization is needed to assemble the microbubble structure. Finally, once the microbubbles are self-assembled, purification during manufacturing consists of only a single filtration step (301). While different classes of organic matter coated and thereby stabilized natural microbubbles, it was actually an underlying monolayer of lipids (surrounding the gas bubble), which provides the long-term stability to such coated microbubbles (300). These artificial lipid coated microbubbles were found to be very long-lived, lasting over 6 months in vitro (302). In a view of such longevity, they became a good candidate for potential medical applications. There are various techniques available to produce these microbubbles as describe in Chapter 2, section 2.6.

5.6.2 Experimental

5.6.2.1 Materials

Egg Phosphatidylcholine (PC), Hydrogenated Soya PC (HSPC), methoxy polyethylene glycol (M.Wt-2000), phosphatidylethanolamine (PE). Disteroyl Phosphoglycerol (DSPG) were purchased from Sigma Chemical Co., St.Louis, M.O. Poly (D, L-lactide-co-glycolide) PLGA (50:50) (M_w 40,000-50,000; M_n 38,900; M_w 60,100; PD 1.55), a gift sample from Boehringer Ingelheim Ltd., Germany. All other chemicals and reagents were of analytical grade.

5.6.2.2 Apparatus

Probe sonicator RR-120 (Ralsonics, Mumbai), laboratory centrifuge (Sigma, 3K30), High-pressure homogenizer (Microfluidizer, Avestin, Canada), Vortex Mixer, High speed stirrer, Mechanical Stirrer (Remi Equipments, Mumbai).

5.6.2.3 Methods of Preparation for Flutamide and 6-Mercaptopurine microbubbles

5.6.2.3.1 Method 1: vortexing with freeze and thaw cycles method

Drug, PC or HSPC, DSPG and mPEG2000-PE were accurately weighed and dissolved in 5 ml of organic solvent mixture ($CHCl_3$: Methanol (2:1, v/v)) in a beaker. Organic solvent mixture was evaporated at 40°C. The material was rehydrated with 10 ml of aqueous vehicle (Normal saline (0.9 % w/v), Propylene glycol and glycerol, 8:1:1, v/v/v containing Pluronic 60). Transfer the suspension in 2 ml vials and perfluoropentane was added. The vials were sealed and vortexed for 10 min. The mixture was frozen in liquid nitrogen and kept at room temperature in water bath till it was liquefied. It was frozen again in liquid nitrogen and repeated for 1,4,7 and 9 times. After completion of these cycles, vacuum was applied for 18 hr. The lyophilized material was reconstituted with 5 ml of aqueous media and further vortexed for 10 min. All types of microbubbles were evaluated for particle size and size distribution by Malvern particle size analyzer. Table 5.14 and 5.15 show the effect of the mass ratio of drug/HSPC/ DSPG/ $mPEG_{2000}$-PE, volume of perfluoropentane and number of freeze thaw cycles on particle size of Flutamide and 6-Mercaptopurine microbubbles, respectively.

5.6.2.3.2 Method 2: Modified double emulsion ($W_1/O_1/W_2$) solvent evaporation technique

As described in Chapter 2, Section 2.6.5.3, Literature Review, polymer based gas-filled micro particles were prepared. Briefly, The perfluoropentane was added in sodium chloride solution (W_1 phase). Fixed quantity of PLGA and drug (10 mg) in different drug: polymer mass ratio were dissolved in 10 ml of organic solvent (CH_2Cl_2: Methanol (3:1, v/v) (O_1). Aliquot of O_1 equivalent to 5 mg of drug was emulsified with an aqueous phase using a microtip probe sonicator for 15 sec. The resulting primary emulsion was immediately added to 10 ml of an outer water phase (W_2) (containing 1.5 % of poly vinyl alcohol) and stirred at 7,500 RPM for 45 min. The resulting double emulsion was stirred further using magnetic stirrer for 1 hr to remove CH_2Cl_2 completely. Figure 5.16 represents the steps involved in preparation of gas-filled microparticles. The emulsion was homogenized at 15, 000 psi for 3 cycles and evaluated for particle size. Table 5.16 and 5.17 show the experimental trials for flutamide and 6-MP gas filled microparticles.

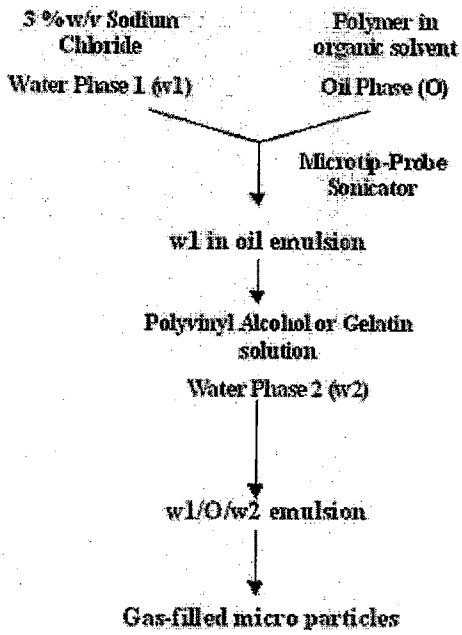
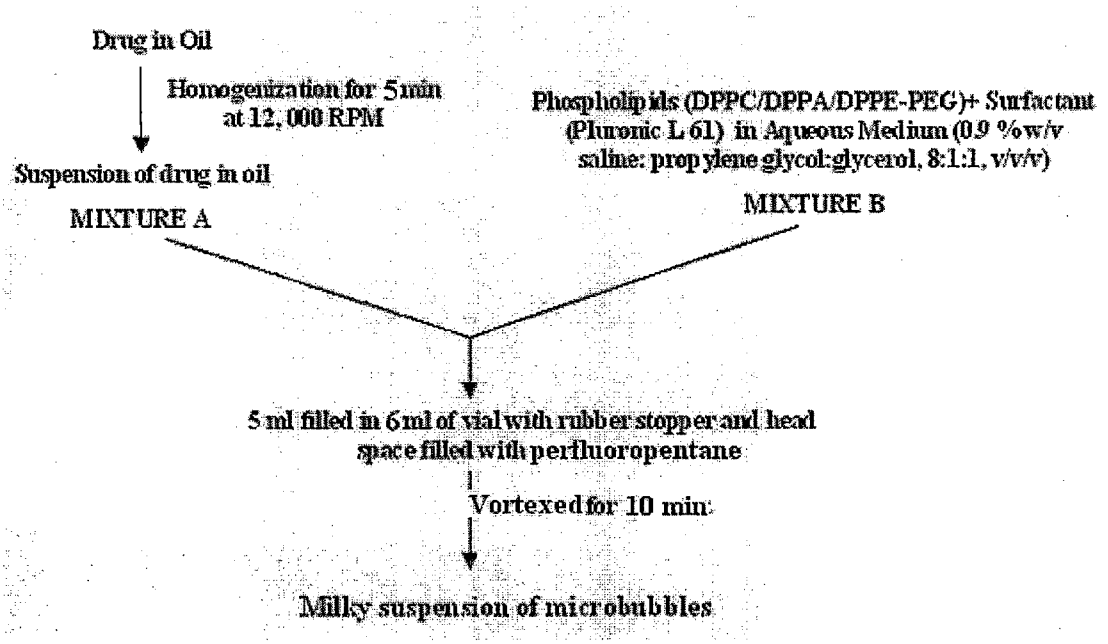


Figure 5.16: Preparation of gas-filled microparticles

5.6.2.3.3 Method 3: Mixing cum sonication technique

As described in Chapter 2, Section 2.6.5.8, Literature Review, acoustically active lipospheres were prepared. Briefly, the drug was suspended in soyabean oil at concentration as mentioned in Table 6.5 and blended at 12, 000 RPM using high speed stirrer for 5 min. Phospholipids (81% w/v HSPC or PC, 5% w/w DSPG and 4% w/w $mPEG_{2000}$-PE) were suspended in aqueous solution containing normal saline (0.9 % w/v), Propylene glycol and glycerol, 8:1:1, v/v/v containing 50 mg Pluronic L 61. The drug-soyabean oil suspension was added to the phospholipid suspension at a 1:1 volume ratio in 6 ml glass vial. The perfluoropentane gas was added, and the vials were sealed. The sealed vials were vortexed for 10 min. The resulting milky suspension contained 1 mg/ml of drug and was studied for particle size and particle size distribution by Malvern particle size analysis. Figure 5.17 represents the steps involved in the preparation of acoustically active lipospheres. Table 5.18 and 5.19 shows the details of batches prepared to formulate FLT and 6-MP acoustically active lipospheres.

Figure 5.17: Preparation of drug loaded acoustically active lipospheres



5.6.3 Results and Discussion

5.6.3.1 Flutamide microbubbles (FLT-MBs)

To design a new formulation in the field of pharmaceutical dosage forms, it is very important to identify the parameters in the preparation since these variables might affect the properties of the final dosage forms. The mean diameters of FLT-MBs determined by Malvern particle size analyzer are shown in Table 6.1. The selection of factors and their concentrations would be based on the results of a preliminary investigation. Depending on the therapeutic application, obtaining a stable suspension with small particle size requires information on the effects that formulation and production variables have on microbubbles properties. Because of the poor solubility of FLT in selected vehicle, it was initially dissolved in organic phase and then rehydrated with aqueous media. The selected composition of aqueous media gave good stability to microbubbles. The percentage of PC (81% w/w), DSPG (10%w/w), $mPEG_{2000}$-PE (9% w/w), volume of organic phase and aqueous phase were fixed depending on a formation of microbubbles of suitable size.

Table 5.14: Effect of various formulation parameters on particle size of flutamide microbubbles

Batch No.	Drug (mg)	Drug : Lipid Ratio	PFP (µl)	PC (mg)	$mPEG_{2000}$-PE (mg)	DSPG (mg)	Org. Phase (ml)	Aq. Phase (ml)	Particle Size* (µm)
M1	5	1:10	100	41	4	5	5	10	28.61±1.4
M2	5	1:10	150	41	4	5	5	10	25.80±1.1
M3	5	1:10	300	41	4	5	5	10	29.17±0.8
M4	5	1:20	100	82	8	10	5	10	31.18±0.7
M5	5	1:20	150	82	8	10	5	10	28.10±1.1
M6	5	1:20	300	82	8	10	5	10	32.28±1.1
M7	5	1:30	100	123	12	15	5	10	56.97±3.4
M8	5	1:30	150	123	12	15	5	10	52.04±1.8
M9	5	1:30	300	123	12	15	5	10	59.69±2.4
Number of freeze and thaw cycles									
M21	1								18.76±0.5
M24	4								15.22±0.3
M27	7								12.8±0.3
M29	9								12.62±0.4

* Particle size in Mean Diameter (n=3)

Organic Phase: Chloroform: Methanol (2:1)

Aq. Phase (Aqueous Phase): Normal saline (0.9 % w/v), Propylene glycol and glycerol, 8:1:1, v/v/v containing 50 mg of Pluoronic L61

PFP: Perfluoropentane

The data indicated that the drug: lipid mass ratio and the volume of perfluoropentane had major influence on the particle size of FLT-MBs. With an increasing the drug: lipid mass ratio, the mean diameter of FLT-MBs increased significantly. Although the volume of PFP showed only slight influence on the particle size, it had a significant influence on the stability of FLT-MBs suspension. As the volume of PFP increasing up to 150 µl, the particle size of microbubbles decreased. Above the volume of 150 µl of PFP, further increased in particle size was observed. The batch M2 (the drug: lipid ratio of 1:10 and the volume of PFP of 150 µl) showed the least particle size of 25.80 µm. The effect of freeze and thaw treatment was given to the selected batch M2. The data of freeze and thaw treatment showed that as the number

of cycle increasing, the mean diameter of microbubbles decreased up to 7 cycles (from 25.80 μm to 12.8 μm). There was no significant difference in particle size reduction was observed after 7 cycles.

5.6.3.2 6-Mercaptopurine microbubbles (6-MP MBs)

The mean diameters of 6-MP-MBs determined by Malvern particle size analyzer are shown in Table 5.15. Because of the poor solubility of 6-MP in selected aqueous vehicle (Normal saline (0.9 % w/v), Propylene glycol and glycerol, 8:1:1, v/v/v containing Pluronic 60), it was initially dissolved in organic phase (5 ml of $CHCl_3$: Methanol, 2:1, v/v) and then rehydrated with 10 ml of aqueous vehicle.

Table 5.15: Effect of various formulation parameters on particle size of 6-MP microbubbles

Batch No.	Drug (mg)	Drug/Lipid Ratio	PFP (μl)	HSPC (mg)	mPEG$_{2000}$-PE (mg)	DSPG (mg)	OP (ml)	AP (ml)	Particle Size* (μm) (Mean±SD)
M1	5	1:10	100	40	5	5	5	10	27.57± 1.28
M2	5	1:10	150	40	5	5	5	10	24.14± 1.1
M3	5	1:10	200	40	5	5	5	10	21.28± 0.9
M4	5	1:20	100	80	10	10	5	10	35.81±1.41
M5	5	1:20	150	80	10	10	5	10	34.28±2.2
M6	5	1:20	200	80	10	10	5	10	31.24±1.82
M7	5	1:30	100	120	15	15	5	10	53.28±1.6
M8	5	1:30	150	120	15	15	5	10	49.2±1.22
M9	5	1:30	200	120	15	15	5	10	45.31±1.8
	Number of freeze and thaw cycles								
M21	1								18.8±1.1
M24	4								14.91±10.8
M27	7								13.24±0.4
M29	9								13.29±0.2

OP: Organic Phase, AP: Aqueous Phase

The selected composition of aqueous media gave good stability to microbubbles. The percentage of PC (80% w/w), DSPG (5 %w/w), mPEG$_{2000}$-PE (5 % w/w), volume of organic phase (5 ml) and aqueous phase (5 ml) were fixed depending on a formation

of microbubbles of suitable size. The data indicated that the drug: lipid mass ratio and the volume of perfluoropentane had major influence on the particle size of 6 MP-MBs. With an increasing the drug: lipid mass ratio, the mean diameter of 6-MP-MBs increased significantly. Although the volume of PFP showed only slight influence on the particle size, it had a significant influence on the stability of 6-MP-MBs suspension. As the volume of PFP increasing, the particle size of microbubbles decreased. The batch M3 (the drug: lipid ratio of 1:10 and the volume of PFP of 200 µl) showed the least particle size of 21.28 µm. The effect of freeze and thaw treatment was given to the selected batch M3. The data of freeze and thaw treatment showed that as the number of cycle increasing, the mean diameter of microbubbles decreased up to 7 cycles (from 21.28 µm to 13.24 µm). There was no significant difference in particle size reduction was observed after 7 cycles.

5.6.3.3 Flutamide Gas filled microparticles (FLT-GFMs)

Table 5.16 shows the particle size distribution and % entrapment efficiency of 9 representative batches (n=3) of FLT encapsulated gas filled microparticles.

Table 5.16: Experimental batches and results of FLT-GFMs

Batch Order	Drug: Polymer ratio	NaCl Concentration (% w/v)	Volume of PFP (µl)	% EE (Mean ±SEM)	Particle Size (µm) (Mean ±SEM)
GFM1	1:5	1	150	40.78±0.51	2.0±0.046
GFM2	1:5	2	150	62.85±0.12	3.1±0.025
GFM3	1:5	3	150	55.23±0.84	3.7±0.059
GFM4	1:10	1	150	74.86±0.68	4.7±0.098
GFM5	1:10	2	150	83.37±0.19	4.9±0.087
GFM6	1:10	3	150	78.37±0.12	5.2±0.015
GFM7	1:15	1	150	79.95±0.23	7.7±0.098
GFM8	1:15	2	150	98.42±0.09	9.0±0.046
GFM9	1:15	3	150	84.76±0.14	9.8±0.099

The microbubbles of spherical in shape were formed when the volume of PFP was 150 µl. The mean diameter of the microparticles of these batches ranged between 2

μm to 9.8 μm. Although the smallest capillary vessels in the blood-vessel system are around 6-9 μm in diameter, the incompatibility of particular formulation intravenously administered increases with particle diameters, especially above 13 μm and here FLT GFMs had mean diameters of 2 μm to 9.8 μm means prepared GFMs are in suitable particle size range. The entrapment efficiency of FLT-GFMs was determined by ultra centrifugation. Batch GFM8 showed entrapment efficiency of 98.42±0.09 %. We found that as the drug: polymer mass ratio increasing, the entrapment efficiency of drug in microparticles was also increased because the higher the viscosity of the polymer (PLGA) solution, the higher the amount of drug entrapped and as the concentration of sodium chloride increasing, the % entrapment efficiency increased up to 2 %w/v concentration and particle size decreased. Finally, the GFM8 was selected for further characterization because of suitable particle mean diameter and high entrapment efficiency.

5.6.3.4 6-Mercaptopurine gas filled microparticles (6-MP-GFMs)

Table 5.17 shows the particle size distribution of 9 representative batches (n=3) of 6-MP encapsulated gas filled microparticles. The microbubbles of spherical in shape were formed when the volume of PFP was 150 μl.

Table 5.17: Experimental batches and results of 6-MP-GFMs

Batch Order	Drug: Polymer ratio	NaCl Concentration (% w/v)	Volume of PFP (μl)	% EE (Mean ±SEM)	Particle Size (μm) (Mean ±SEM)
GFM1	1:5	1	150	64.81± 0.09	2.5±0.015
GFM2	1:5	2	150	69.95± 0.84	2.7±0.084
GFM3	1:5	3	150	66.21±0.41	3.5±0.041
GFM4	1:10	1	150	75.48±0.28	4.2±0.022
GFM5	1:10	2	150	84.68±0.77	4.5±0.091
GFM6	1:10	3	150	82.4±0.41	4.9±0.026
GFM7	1:15	1	150	88.37±0.18	6.2±0.018
GFM8	1:15	2	150	97.61±0.79	6.9±0.055
GFM9	1:15	3	150	93.57±0.23	7.8±0.063

The mean diameter of the microparticles of these batches ranged between 2.5 μm to 7.8 μm means prepared GFMs are in suitable particle size range. The entrapment efficiency of 6-MP-GFMs was determined by ultra centrifugation. Batch GFM8 showed entrapment efficiency of 97.61±0.79 %. We found that as the drug: polymer mass ratio increasing, the entrapment efficiency of drug in microparticles was also increased because the higher the viscosity of the polymer (PLGA) solution, the higher the amount of drug entrapped Finally, the GFM8 was selected for further characterization because of suitable particle mean diameter and high entrapment efficiency.

5.6.3.5 Acoustically active lipospheres (AALs) of flutamide (FLT) and 6-Mercaptopurine (6MP)

Depending on microbubbles-stabilizing materials and the gas selected to fill the microbubbles, contrast agents with sustained circulation in half-lives also can be developed. This procedure resulted in lipospheres with an effective FLT and 6-MP concentration of 1 mg per ml of drug. Spectrophotometric estimation showed that 100% of drug was partitioned in to the oil and into the lipospheres. The change in mean particle diameter depending on formulation parameters changed is shown in Table 5.18 and 5.19 for FLT and 6-MP AALs, respectively.

Table 5.18: Different batches of FLT-AALs and their composition

Batch No.	Drug mg	Drug: Lipid Ratio	PFP μl	AP ml	PC mg	DSPG mg	mPEG$_{2000}$-PE mg	Oil (ml	Particle Size* (μm)
AAL1	5	1:10	300	5	41	4	5	2.5	10.2±0.41
AAL2	5	1:10	450	5	41	4	5	2.5	11.4±0.57
AAL3	5	1:10	300	5	41	4	5	5	13.8±0.78
AAL4	5	1:10	450	5	41	4	5	5	7.5±0.23
AAL5	5	1:20	300	5	82	8	10	2.5	16.0±0.88
AAL6	5	1:20	450	5	82	8	10	2.5	12.4±0.29
AAL7	5	1:20	300	5	82	8	10	5	19.8±0.22
AAL8	5	1:20	450	5	82	8	10	5	17.2±0.46

The volume of aqueous phase, percentage of PC or HSPC, DSPG, mPEG2000-PE, drug: lipid ratio and the volume of oil used were selected on the basis of preliminary

investigations. At all the above-mentioned concentrations of respective formulation parameters, proper size, shaped and number of microbubbles formed.

Table 5.19: Different batches of 6-MP-AALs and their composition

Batch No.	Drug mg	Drug : Lipid Ratio	PFP µl	AP ml	PC mg	DSPG mg	mPEG $_{2000}$-PE mg	Oil ml	Particle Size* (µm)
AAL1	5	1:10	300	5	40	5	5	2.5	9.6±0.15
AAL2	5	1:10	450	5	40	5	5	2.5	11.4±0.24
AAL3	5	1:10	300	5	40	5	5	5	9.8±0.11
AAL4	5	1:10	450	5	40	5	5	5	9.1± 0.38
AAL5	5	1:20	300	5	80	10	10	2.5	15.1±0.71
AAL6	5	1:20	450	5	80	10	10	2.5	14.1±0.63
AAL7	5	1:20	300	5	80	10	10	5	17.5±0.46
AAL8	5	1:20	450	5	80	10	10	5	16.6±0.32

The change in concentrations formed less stable or microbubbles having less particle numbers. The data of Table 5 and 6 indicated that as drug: lipid mass ratio increasing, the mean diameter of microbubbles also increased. The microbubbles with less particle size formed, when the headspace of the vial was filled with 450 µl of PFP and the volume of oil in microbubbles was 1 ml. The selected aqueous vehicle (Normal saline (0.9 % w/v), Propylene glycol and glycerol, 8:1:1, v/v/v containing Pluoronic 60) formed the most stable microbubbles. The Batches FLT AAL4 and 6-MP AAL4 showed the least particle size of 7.5±0.23 µm and 9.1± 0.38 µm.

5.6.4 Conclusion

There were three methods used for preparation of different types of microbubbles: viz. (1) vortexing with freeze and thaw cycles method, (2) modified double emulsion ($W_1/O_1/W_2$) solvent evaporation technique and (3) mixing cum sonication technique. The microbubbles prepared by all three methods were highly stable and had particle size in suitable range. The number of steps involved in the preparation of microbubbles in all these methods is simple and less as compared to liposomes. The selected batches of these methods would be characterized in details in Chapter 6.