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Optimization of Pentoxifylline Liposomes Using 2^4 Factorial Design

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Entrapment efficiency and particle size distribution are important properties affecting pharmacokinetics of liposomes upon intravenous administration. Several factors such as composition of lipids, methods of preparation etc affect these properties of liposomes. A 2^4 complete factorial design with replicated center point was used to quantitate the effect of four factors viz. concentration of cholesterol, phospholipid composition, concentration of stearyl amine and shaking time on the entrapment efficiency of pentoxifylline and particle size distribution of liposomes prepared by thin film hydration method. A mathematical model containing only significant factors affecting each response was predicted using multiple linear regression and ANOVA. Stearyl amine had negative effect on the entrapment efficiency i.e. increase in concentration of stearyl amine did not cause increase in the entrapment efficiency. Particle size distribution was affected by shaking time and phospholipid composition. In addition to main factors, two way and three way interactions also had a significant effect on all the measured responses. A new formulation based on combination of factors between the experimental levels was prepared and evaluated for all responses. Experimental values were found to be in good agreement with the predicted values.

Liposomes are microscopic aggregates of highly ordered lipid molecules, normally dispersed in a hydrophilic solvent like water. Liposomes have been widely used as carriers for drug and vaccine delivery because of their ability to encapsulate drugs of varying solubility in aqueous or lipid phase of liposomes[1]. Their remarkable structural versatility enables design of countless liposome versions to satisfy specific requirements in terms of both technology and optimal function *in vivo*. For achieving optimum *in vivo* performance, attention should be given to a number of properties of liposomal formulation including encapsulation efficiency of drug, particle size distribution, surface charge and stability of entrapped materials. These properties are affected by the choice and composition of ingredients used for bilayer formation, nature of drug to be encapsulated and the method of preparation.

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Optimization of pharmaceutical formulations with regard to one or more attributes has always been important in formulation research[2]. Various optimization techniques have been used for design of dosage forms like tablets[3,4], microspheres[5,6] and liposomes[7]. Factorial designs are widely used in experiments involving determination of effect of several factors on one or more responses. The 2^k design is particularly useful in the early stages of experimental work, when several contributing factors need to be investigated. It provides the smallest number of runs with which k factors can be studied in a complete factorial design.

Factorial designs have been used for evaluating effects of various factors on liposomes. Casals *et al.*[8] have evaluated the effects of four factors viz. pH of hydration medium, cholesterol mole ratio, charge of lipid and time of sonication

on the encapsulation of thioguanine in dehydration-rehydration liposomes. Loukas[6] used response surface methodology for optimization of multicomponent photo protective liposomes. The effects of oil- soluble light absorber, water-soluble light absorber and composition of liposomes on percentage entrapment and photo degradation rate constant of riboflavin was studied. The same multicomponent liposomal system was further studied by applying fractional factorial design to evaluate effect of additional factors like complexation with cyclodextrin and use of different oil soluble light absorbers[8].

Pentoxifylline (PTX), a methyl xanthine derivative, has been recently shown to exhibit antimetastatic activity by inhibiting homing of B16F10 melanoma cells in murine experimental metastasis model[9]. In the present study, PTX was encapsulated in liposomes using lipid film hydration method. An optimum liposomal formulation for intravenous administration should have high entrapment efficiency and a desired narrow particle size distribution. Allen *et al.*[10] have shown an inverse relationship between the liposome size and their uptake by mouse bone marrow macrophages that can be correlated to removal of liposomes from circulation by cells of mononuclear phagocytic system. Also, it is reported that encapsulation efficiency of water-soluble compounds in liposomes is generally low and dependent upon the trapped volume. An optimum balance between entrapment efficiency and liposome size must be achieved for desired *in vivo* performance. In the present study, a 2^4 complete factorial design was used to optimize PTX liposomes with respect to entrapment efficiency and particle size distribution.

MATERIALS AND METHODS

Phospholipon 90 (PL90), soya phosphatidylcholine and Phospholipon 90H (PL90H), hydrogenated soya phosphatidylcholine were obtained as gifts from Nattermann GmBH, Germany. Cholesterol was purchased from Loba Chemie Pvt. Ltd, Mumbai and stearyl amine was purchased from Sigma, USA respectively. Pentoxifylline BP was obtained as a gift sample from Sun Pharmaceutical Industries Ltd., Baroda. Chloroform (Analytical Reagent) was obtained from S. D. Fine-Chem. Ltd, Mumbai. All aqueous solutions and reagents were prepared in double distilled water.

Preparation and characterization of liposomes:

Empty and PTX loaded liposomes were prepared by thin film hydration method as described by Gabizon and Papahadjopoulos[11] with slight modification as reported earlier[12]. Briefly, PL90, PL90H, cholesterol and stearyl amine were taken in a 250 ml round bottom flask (RBF) and dissolved in chloroform. Glass beads were added to increase the surface area available for film formation. Chloroform was evaporated under reduced pressure on a rotary evaporator (Superfit, India) at 40° to form a thin film of phospholipids on the inner surface of flask. The lipid film was hydrated by phosphate buffered saline IP of pH=7.4 (PBS) or PTX solution (20 mg/ml) in PBS above the gel to liquid crystalline phase transition temperature of phospholipids. The RBF was hand shaken vigorously for 5 min followed by heating it again for predetermined time period to anneal liposomes. The RBF was shaken on a horizontal shaker bath (Expo, India) with intermittent sonication (30 sec) in a bath sonicator (Expo, India) after each hour to reduce the vesicle size. For determination of entrapment efficiency (EE), liposomes were diluted with PBS and centrifuged at 21000 g (Remi, India) or ultracentrifuged at 100,000 g (Sorvall Ultra 80) for 30 min. The supernatant was appropriately diluted and absorbance was measured at 274 nm on a Shimadzu 160A UV/Vis Spectrophotometer with respect to similarly treated empty liposomes as blank. Entrapment efficiency was calculated as the difference between the initial amount of PTX added and that present in the supernatant and was expressed as mg of PTX entrapped per mM of total phospholipids (mg/mM). Each value of entrapment efficiency is mean±standard deviation of three batches estimated in duplicate. The particle size distribution of PTX liposomes was evaluated by laser light scattering on a Malvern Mastersizer MS 3 (Malvern Instruments, USA). The data was obtained as diameters (μm) covering range of particle size distribution such as D10% (10% undersize-indicative of 10% particles having diameter less than this value), D50% (50% undersize-indicative of 50% particles having diameter less than this value) and D90% (90% undersize-indicative of 90% particles having diameter less than this value).

Factorial design:

In the present study, molar concentration of cholesterol (CH), composition of phospholipids (PL90:PL90H ratio), molar concentration of stearyl amine (SA) and shaking time (ST) on horizontal shaker were selected as independent variables while entrapment efficiency, specific surface area and particle size distribution were selected as dependent variables. In addition to mean volume diameter (D50%), two other parameters viz. D10% and D90% were selected as dependent variables to have an insight into the effects of independent variables on the entire range of particle size distribution. Potential variables such as concentration of PTX,

TABLE 1: THE LEVELS OF THE FOUR FACTORS INVESTIGATED IN THE 2^4 COMPLETE FACTORIAL DESIGN.

Factor Name	Symbol	Low level (-1)	Medium level (0)	High Level (+1)
Cholesterol (CH)	X_1	4	7.5	11
Phospholipid composition (PL90:PL90H ratio)	X_2	3:8	4.25:6.75	5.5:5.5
Stearyl amine (SA)	X_3	0.22	1.21	2.2
Shaking time (ST)	X_4	2 h	4 h	6 h

Quantities of CH, PL90, PL90H and SA are expressed as mole ratios.

total concentration of phospholipids and volume of hydration medium were kept constant. A complete 2^4 factorial design was used in the present study. Three center points were used to provide protection against curvature and to allow for an independent estimate of error. The experimental conditions chosen for the study are shown in Table 1.

Analysis of the data:

Response parameters (EE, D10%, D50%, D90% and SS) were fitted to first order interactive model ($Y = b_0 + b_1X_1 + b_2X_2....$) by performing multiple linear regression analysis on Unistat 3.0 (Megalon Inc., USA) statistical software package. ANOVA was performed assuming the first order linear model to identify statistically significant effects and a mathematical model that expresses each response was predicted by considering only the significant main and interaction effects.

RESULTS AND DISCUSSION

Several factors and their interactions affect the properties of a stable liposomal formulation. Entrapment efficiency and particle size distribution are amongst the most important properties of liposomes as they play a critical role in determining the pharmacokinetics of liposomes[13]. In the present study, the effect of various formulation factors on entrapment efficiency of PTX and particle size distribution of liposomes was studied by using a 2^4 factorial design. Many variables such as concentration of CH, composition of phospholipids and presence or absence of charged lipids can affect the properties of liposomes, some of which can favourably affect one property while adversely affecting the other. In such cases, traditional experimentation of changing each factor one at a time requires a large number of experiments to be performed. More importantly, these experiments may not yield any information about the possible interactions among the different variables.

Factorial designs at two levels are very useful for preliminary identification of the effect of each variable on a response when a large number of potential variables are present. In preliminary studies, liposomes were prepared using PL90,PL90H:CH in a mole ratio of 11:4. However, this formulation had very low entrapment efficiency (data not shown). Therefore, stearyl amine was incorporated in the formulation in the range of 2 to 20 mol% to impart a positive charge to liposomal bilayers. It is reported that charged lipids increase the entrapment efficiency of water-soluble compounds by increasing the interlamellar distances due to repulsive force[14]. Cholesterol and phospholipids in 1:1 mole ratio are reported to give optimum entrapment efficiency. Therefore, CH:total phospholipid ratio of 1:1 was selected as high level for parameter CH in the factorial design. Phospholipid composition was varied at two different levels of unsaturated (PL90) and saturated (PL90H) phosphatidylcholine because Huang *et al.*[15] have shown that stable hemoglobin containing liposomes could be obtained by selecting appropriate combination of saturated and unsaturated phospholipids. Shaking time and the amount of ultrasonic energy delivered to liposomes can affect entrapment efficiency and particle size distribution of liposomes[16]. In the present design, shaking time was chosen as a variable and varied between 2 to 6 h.

The results of entrapment efficiency, particle size distribution and specific surface area of 19 experiments in the 2^4 complete factorial design with three central points are shown in Table 2. All experiments were carried out in random order to nullify effects of extraneous or nuisance variables.

The data obtained for each response parameter was fitted to the first order interactive model given below:

$$Y=b_0+b_1X_1+b_2X_2+b_3X_3+b_4X_4+b_{12}X_1X_2+b_{13}X_1X_3+b_{14}X_1X_4+b_{23}X_2X_3+b_{24}X_2X_4+b_{34}X_3X_4+b_{123}X_1X_2X_3+b_{234}X_2X_3X_4+b_{124}X_1X_2X_4+b_{134}X_1X_3X_4+b_{1234}X_1X_2X_3X_4. \quad \text{Eqn. 1}$$

TABLE 2: LAYOUT OF 2^4 FACTORIAL DESIGN AND RESULTS OF ENTRAPMENT EFFICIENCY OF PTX AND PARTICLE SIZE DISTRIBUTION.

X_1	X_2	X_3	X_4	Entrapment efficiency*	D10% (μm)	D50% (μm)	D90% (μm)
-1	-1	-1	-1	30.09±3.156	1.51	3.05	5.75
1	-1	-1	-1	30.52±1.553	1.48	3.32	6.32
-1	1	-1	-1	31.04±1.771	1.37	2.9	5.31
1	1	-1	-1	22.46±2.106	0.77	2.65	5.26
-1	-1	1	-1	58.53±4.427	2.14	3.85	6.3
1	-1	1	-1	19.85±2.167	1.38	3.34	6.31
-1	1	1	-1	13.44±5.787	1.41	3.29	6.18
1	1	1	-1	19.16±1.953	1.25	3.03	5.9
-1	-1	-1	1	15.84±2.952	0.36	2.65	7.27
1	-1	-1	1	32.33±1.317	0.42	2.43	5.19
-1	1	-1	1	29.47±0.840	1.33	2.57	4.37
-1	-1	1	1	18.55±3.562	1.8	3.39	5.69
1	1	-1	1	54.35±5.372	1.11	2.33	4.24
1	-1	1	1	17.77±0.689	1.32	3.2	6.01
-1	1	1	1	15.78±0.542	0.23	1.87	3.72
1	1	1	1	36.85±4.627	0.3	1.57	3.46
0	0	0	0	23.68±3.89	1.69	3.08	4.72
0	0	0	0	23.54±4.278	1.7	3.11	4.78
0	0	0	0	18.72±3.719	1.72	3.14	4.82

X_1=cholesterol, X_2=phospholipid composition, X_3=stearyl amine, X_4=Shaking time.*Entrapment efficiency was expressed as mg of PTX entrapped per mM of phospholipids and is mean±standard deviation of three batches.

Where Y is response parameter, b_0 is the arithmetic mean response of 19 experiments and b_i is the estimated coefficient for factor X_i obtained by linear multiple regression. ANOVA was performed to remove nonsignificant terms from the equation and a reduced model containing only statistically significant terms was developed. From these reduced mathematical models, the respective responses can be easily calculated without any further experiments. In addition, the equation provides predictions of the characteristics of different formulations based on various combinations of the above mentioned factors.

The fitted linear model for EE of PTX is given in Eqn. 2.

$$EE=26.808-2.885X_3+4.101X_1X_2-2.866X_1X_3+6.424X_1X_4-3.626X_2X_3+6.553X_2X_4+4.181X_1X_2X_3-2.496X_1X_2X_3X_4 \quad (R^2=0.915) \qquad \text{Eqn. 2}$$

From Eqn. 2 it is seen that the level of stearyl amine is the unique main effect with statistical significance. High levels of SA had a negative effect on the EE of PTX. It is reported that addition of charged moieties like SA in liposome increases the EE of water-soluble compounds[14]. In order to exhibit electrostatic repulsive forces due to charged moieties between the bilayers, liposomes need to be hydrated with media of low ionic strength thereby increasing their trapped volume. However, in the present study, the same effect could not be observed. This can be attributed to the hydration medium, PBS that contains number of ionic moieties like Na^+, K^+, Cl^- and PO_4^- ions. Consequently, SA even with a pKa of 10.6[17] and a net positive charge at pH 7.4 probably had its effect masked by the presence of oppo-

sitely charged Cl^- and PO_4^{--} ions in the hydration medium that nullify the repulsive forces. The results were further supported by particle size measurements where SA did not show any significant increase in the diameter of liposomes. The results are in agreement with previous report of Johnson[18] who has shown that diameter of 4% phosphatidic acid containing liposomes hydrated with 2 M KCl was almost same as that of uncharged liposomes hydrated with 0.16 M KCl.

The two-way interactions viz. CH-phospholipid composition (X_1X_2), CH-SA (X_2X_3), CH-ST (X_1X_4), phospholipid composition-SA (X_2X_3) and phospholipid composition-ST (X_2X_4) showed significant effect on EE of PTX. Although CH on its own did not have any significant effect on EE, its interaction with all other factors was significant. Increasing the concentration of CH along with increase in concentration of unsaturated phosphatidylcholine resulted in more compact packing of liposomal bilayers and a reduction in the leakage of PTX to exterior. Also, a high level of CH along with an increase in shaking time led to increased entrapment. This may be attributed to high levels of CH forming a rigid bilayer that would be completely hydrated by increased shaking time. Phospholipid composition- ST interaction increased entrapment efficiency of PTX probably because the combination of unsaturated phosphatidylcholine and increased shaking time yields a uniformly packed and properly hydrated liposomal bilayer. The interaction terms of SA viz. CH-SA and phospholipid composition-SA had a negative effect due to negating influence of the main effect SA on the entrapment efficacy of PTX. Further, the three-way interaction of CH-phospholipid composition- SA ($X_1X_2X_3$) had a positive effect and four-way interaction viz. CH-phospholipid composition-SA-ST ($X_1X_2X_3X_4$) had a negative effect on the entrapment efficiency. This indicates the complexity of the factors affecting EE of water-soluble compounds. Thus it was observed that interactions of the various factors with each other rather than main effects, affect the properties of the bilayer showing profound effects on the EE of water-soluble compound like PTX. These interaction terms should be therefore systematically evaluated to achieve optimum EE.

The regression coefficients of only significant factors affecting D10%, D50% and D90% along with the regression coefficient for linear model fitted to each response are shown in Table 3. Two main variables, the phospholipid composition and ST had significant effects on the particle size distribution of PTX liposomes. Increase in the concentration of unsaturated (PL90) phosphatidylcholine, which are relatively flexible had no effect on D10% while there was significant decrease in the D50% and D90% values. The results are in agreement with literature[19] where it is reported that relatively flexible phospholipids give rise to smaller vesicles. Increase in ST also resulted in significant decrease in D10%, D50% and D90%. The results were expected because along with increase in shaking time from 2 h (low level) to 6 h (high level), the total sonication time for each sample was also increased from 90 s to 210 s thus aiding reduction in the particle size. Besides these two main effects, two-way interactions of phospholipid composition-SA (X_2X_3) led to decrease in the D10% and D50% due to increase in concentration of unsaturated phospholipids as well as stearyl amine. Also, interaction of phospholipid composition with shaking time (X_2X_4) led to decrease in D90%. A complex three way interaction of phospholipid composition-SA-ST ($X_2X_3X_4$) led to decrease in D10% and D50% values showing that liposome particle size distribution is also affected by main effects as well as their interactions.

The mathematical models representing each response was validated by preparing a formulation with a combination of factors between the experimental design viz. $X_1:X_2:X_3:X_4$=-0.5:1:-0.5:-0.5 and value for each response was determined experimentally and theoretically from the respective mathematical equations. From Table 4, it is evident that

TABLE 3: COEFFICIENTS OF SIGNIFICANT FACTORS AFFECTING THE PARTICLE SIZE DISTRIBUTION OF PTX LIPOSOMES.

Response	Coefficients of significant factors						
	constant	X_2	X_4	X_2X_3	X_2X_4	$X_2X_3X_4$	$(R^2)^a$
D10% (μm)	1.2273	N.S.[b]	-0.2775	-0.2662	N.S.	-0.265	0.623
D50% (μm)	2.8694	-0.3137	-0.3387	-0.1887	N.S.	-0.1825	0.826
D90% (μm)	5.3221	-0.65	-0.4612	N.S.	-0.396	N.S.	0.691

X_2=phospholipid composition, X_3=stearyl amine, X_4=Shaking time. [a]R^2 indicates regression coefficient of the fitted linear model. [b]N.S. indicates nonsignificant factor at $P<0.05$.

TABLE 4: EXPERIMENTAL AND THEORETICAL VALUES OF THE RESPONSES PREDICTED FOR THE FORMULATION (X_1 :X_2 :X_3 :X_4 =-0.5:1:-0.5:-0.5).

Response	Theoretical value	Experimental value	% Deviation
Entrapment efficiency (mg/mM)	26.983	27.307±2.742*	1.2
D10% (μm)	1.432	1.5	4.6
D50% (μm)	2.774	3.06	10
D90% (μm)	5.1	5.56	9

X_1 =cholesterol, X_2 =phospholipid composition, X_3 =stearyl amine, X_4 =Shaking time. *Mean±standard deviation of three batches.

the experimental values of each of the parameter viz. entrapment efficiency, D10%, D50% and D90% compare quite well with the predicted values. The maximum deviation of 10% was observed with the D50% values, which is in agreement with the deviations of 7-15% reported by Loukas[6] for optimization of multicomponent photoprotective liposomes. These findings suggest that entrapment efficiency and particle size distribution of any liposomal formulation of PTX having the composition within the limits of this design can be easily and accurately predicted from the respective mathematical model without any further experimentation.

In conclusion, optimization of liposomal formulation is a complex process that requires consideration of a large number of factors and their interactions with each other. Use of statistical techniques like factorial designs can help in better understanding of these interactions while requiring lesser number of experiments and consequently reducing the cost of formulation development.

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