IJBPAS, September, 2024, 13(9): 4378-4387

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

APPLICATION OF EXPERIMENTAL DESIGN IN PREPARATION OF NANOLIPOSOMES CONTAINING ACYCLOVIR

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Received 18th July 2023; Revised 20th Sept. 2023; Accepted 2nd Dec. 2023; Available online 1st Sept. 2024

https://doi.org/10.31032/IJBPAS/2024/13.9.8304

ABSTRACT

In this study DOE based approach is used to prepare and characterized acyclovir loaded liposomes. Different ratios of phospholipid along with cholesterol and different methods of preparation were evaluated by studying various properties of drug loaded liposomes. Acyclovir loaded liposomes are prepared using Phospholipon 90H along with cholesterol. Liposomes are prepared by using three different methods i.e. Ethanol Injection, Film Hydration and Reverse-Phase Evaporation Method. Entrapment efficiency, zeta potential and PDI are three evaluation parameters selected in this study to do detailed statistical analysis and optimization of acyclovir loaded liposomes. Total 24 different batches based on 2 level central composite design were prepared using three different methods of preparation and having different ratios of lipid and cholesterol. Results shows that acyclovir loaded liposomes prepared film hydration method using a ratio of 1:0.15 of HSPC and Cholesterol a maximum entrapment efficiency of 62.3 %, particle size of 91.38 nm and PDI of 0.142.

Keywords: Design of experiment, Liposomes, Acyclovir, Film hydration, Ethanol injection, Reverse phase evaporation

INTRODUCTION:

It is always very important to have a proper understanding of the effect of variables on the overall quality and performance of a product with intended properties. This will also help to prevent batch to batch variation in product quality. To study the effect of process variables one can either use traditional method of checking the effect of

one independent variable on the dependent variable at a given time of can use statistical tools like experimental design. When using experimental design, we can check the effect of all the independent variables, varied together so that the effect of their interactions on the dependent variable can also be analysed. The recent advancement in regulatory requirements advocates that quality by design approach should be practiced to build quality into the product. This can be achieved by defining and analysing the effect of variables on the end result using experimental design. More consistency and robustness has been observed in products developed using these statistical tools. Experimental design using statistical tools like factorial design and response surface methodology permits the researchers to quickly study and optimize the conditions to make products with the finest possible quality and qualities using the inadequate available resources and without dropping any essential information that may affect the outcome **[1]**.

Liposomal drug delivery is one of the most widely studied method for the administration of hydrophobic as well as hydrophilic drugs. It is the versatility of liposomes to accommodate both hydrophobic and hydrophilic drug in their vesicle structure make them a very popular among the researcher throughout the globe. In this study, acyclovir is used as model drug to prepare drug loaded liposome and to evaluate them for size, percentage drug encapsulation and zeta potential. The study has been designed to check the effect of process variables on properties of liposomes. The study has been designed using the concept of DOE and data driven evaluation was done using factorial design and CCD **[2]**.

MATERIAL AND METHODS:

Acyclovir was purchased from Yarrow Chem Products, Mumbai, India.

Phospholipon 90H were given gift samples by Lipoid, GmbH, Germany. Cholesterol (analytical grade), Chloroform (analytical grade) Ethanol (absolute grade), Diethyl ether (analytical grade) and Dichloromethane (analytical grade) were purchased from Loba Chemie Pvt Ltd, India.

Methods:

Preparation of Liposomes:

Design of experiments (DOE):

The effect of variables involved in the preparation of acyclovir loaded liposomes was done by applying factorial design of DOE. The effect of amount of Phospholipon 90H and Cholesterol on responses like particle size, polydispersibility index and % Entrapment efficiency was analysed by using fractional factorial design. Also the liposomes were prepared by three different methods i.e. film hydration, reverse phase evaporation and ethanol injection method. For each methods 4 formulations were

prepared using different ratios of Phospholipon 90H and Cholesterol, and thus total 12 batches were prepared. The effects of interaction of two variables showing the maximum effect on particle size and % Entrapment efficiency was studied using CCD. The level of other variables was fixed based on the results of fractional factorial deign. CCD consisted of 12 runs for the two selected factors. Alpha level was maintained at 1.414.

Film hydration method:

Acyclovir loaded liposomes were prepared using thin film hydration method. In this method Phospholipon 90H and Cholesterol were dissolved in 2ml chloroform and 1 ml Ethanol. Solvents were evaporated using hot air oven at 60°C for 30 min. The resulting solution was allowed to form a thin film and further vacuum dried for 5 in to remove any traces of organic solvents. The dried film was vortexed at 300-320 rpm with the aqueous solution of drug for 1 hour at 60°C. The resulted solution was sonicated for 5 min at room temperature. Further the solution was extruded through a 100 nm pore size polycarbonate membrane (Ministruder-Set, Avanti Polar Lipids, Inc.) to control particle size.

Reverse-Phase Evaporation Method:

Both Phospholipon 90H and cholesterol were mixed and dissolved in a solvent system of chloroform and diethyl ether in the ratio of 1:2. The resulting mixture was further added to aqueous solution of acyclovir. The resulting two phase system was cleaned using ultrasonication for 5 min. further the organic solvent was removed by putting the mixture in over at 50°C for 20 min, rotating at 300-320 rpm. Recover the aqueous suspension containing liposomes and sonicate it for 5 min. Further the solution was extruded through a 100 nm pore size polycarbonate membrane (Ministruder-Set, Avanti Polar Lipids, Inc.) to control particle size.

Ethanol Injection Method:

Phospholipon 90H and Cholesterol were dissolved in 2 ml chloroform and 1 ml of Ethanol solvent system. This organic phase mixture was filled and further used using 2 ml syringe, Fitted with 31-gauge needle. The aqueous phase was prepared by dissolving drug in to water pH-7 at room temperature. The organic phase filled in syringe was further added dropwise to the aqueous phase. The resultant mixture was kept under the agitation and the ethanol was evaporated by maintaining temperature of the mixture at 60°C. The resultant Liposomal solution was then sonicated for 3 minutes at room temperature and further the solution was extruded through a 100 nm pore size polycarbonate membrane (Ministruder-Set, Avanti Polar Lipids, Inc.) to control particle size **[3-6]**.

Particle size determination:

Liposomes formed were sonicated at 60% amplitude control (probe sonicator, Sonics and materials Inc., USA) for total duration of 30 sec consisting of three cycles. Duration of each cycle was 10 sec with 10 sec interval. The formulation was maintained on ice bath during sonication. The liposomes were then transferred into polypropylene tubes and centrifuged at 450 g for 3 min to remove the coarse particles. The supernatant was then centrifuged at 34,600 g for 60 min (Sigma Laborzentrifugen 3k30, Germany) to collect the nanoparticles. The pellet obtained was used for calculating the particle size and percentage drug encapsulation. For size measurement pellet was dispersed in ultrapure water (Milli-Q, Millipore) and analyzed using photon correlation spectroscopy (Malvern Zetasizer). Nanoliposomes were made to release entrapped protein by treating them with 1% triton X-100 and the amount of protein was estimated using Lowry method **[7-9]**.

Particle size distribution analysis:

Vesicle size distribution is also measured along with vesicle diameter in Particle Size Analyzer, Malvern S90 instrument. PDI values display more uniformity and physical stability of the compound. The value of nearby 0 indicates that formulation is homogenous dispersion and PDI greater than 1 indicates heterogeneous nature of dispersion & poor uniformity of mixture **[10]**.

Percentage Entrapment efficiency:

%Entrapment efficiency was measured by using the ultra-centrifugation technique. The sample was centrifuged at 10000 rpm for 10 minutes to separate liposomes from the supernatant. Then the supernatant was collected and analyzed in UV visible spectrophotometer. The %EE was calculated by the following formula;

(The amount of total drug- The amount of drug collected only in the supernatant)/ The amount of total drug * 100 **[11]**.

RESULTS & DISCUSSION:

Determination of Particle size:

The particle size of the acyclovir liposomes prepared by film hydration method was found to be in the range of 147.6 ± 3.5 nm to 210.4 ± 6.8 nm. Liposomes prepared by reverse phase hydration method has particle size in the range of 445.6 ± 5.9 nm to 524.5 ± 5.9 nm. In case of liposomes prepared by ethanol injection method the particle size was found to be in the range of 382.7 ± 4.8 nm to 397.2 ± 6.1 nm.

From the factorial study it can be concluded that there is a significant relationship between Particle size values with the amount of Phospholipon 90H and Cholesterol. The maximum particle size was found in liposomes prepared by reverse phase hydration method and lowest particle size was observed in liposomes prepared by film

hydration method. One-way ANOVA showed that the effect of Phospholipon 90H on particle size was not highly significant with a *P* value less than 0.003. In the present study, the other independent variables did not have any significant effect on the outcome (*P* value > 0.05). Although cholesterol as an independent variable did not have significant effect on the outcome, two-way ANOVA showed that the interaction of Phospholipon 90H with cholesterol has significant influence on the outcome **[12-14]**.

Determination of PDI:

The poly-dispersibility index for prepared acyclovir loaded liposomes was found to be 0.617 ± 0.084 to 0.783 ± 0.068 in case of liposomes prepared by film hydration method, 0.657 ± 0.069 to 0.779 ± 0.058 in case of reverse phase evaporation and 0.639 ± 0.049 to 0.718 ± 0.062 in case of ethanol injection method.

From the factorial study it can be concluded that there is no significant relationship between PDI values with the amount of Phospholipon 90H irrespective of method of preparation. One-way ANOVA showed that the effect of Phospholipon 90H on PDI was not highly significant with a *P* value more than 0.003. In the present study, the other independent variables did not have any significant effect on the outcome (*P* value > 0.05). Although cholesterol as an independent variable did not have significant effect on the outcome, two-way ANOVA showed that the interaction of Phospholipon 90H with cholesterol has significant influence on the outcome **[15-18]**.

Determination of % Entrapment efficiency:

The % entrapment efficiency for prepared acyclovir loaded liposomes was found to be 32.19 ± 3.58 to 41.82 ± 5.01 in case of liposomes prepared by film hydration method, 49.57 ± 3.69 to 58.63 ± 2.58 in case of reverse phase evaporation and 38.69 ± 3.98 to 43.29 ± 4.29 in case of ethanol injection method.

From the factorial study it can be concluded that the % entrapment efficiency was increased with the amount of Phospholipon 90H irrespective of method of preparation. However, the maximum %EE was found in liposomes prepared by film hydration method. One-way ANOVA showed that the effect of Phospholipon 90H on %EE was highly significant with a *P* value less than 0.003. The amount of the Acyclovir that could be entrapped in a liposome depends on the size of the drug molecule and the amount of free aqueous phase within the vesicle. The portion of the aqueous phase interacting with lipid layer will be unavailable for the drug to occupy. In the present study, the other independent variables did not have any

significant effect on the outcome (*P* value > 0.05). Although cholesterol as an independent variable did not have significant effect on the outcome, two-way ANOVA showed that the interaction of Phospholipon 90H with cholesterol has significant influence on the outcome. This is due to the steric stability that cholesterol provides by controlling the lipid layer fluidity. Inclusion of cholesterol is mandatory particularly in case of hydrophilic drugs as cholesterol reduces the leakage of the entrapped drug besides improving the stability of liposomes **[19-21]**.

Table 1

Formulation code	Method of preparation	Phospholipon 90H (mg)	Cholesterol (mg)	acyclovir	Z- Avg (nm)	PDI	%EE
1	Film hydration method	100	15	1%	147.6 ± 3.5	0.617 ± 0.084	32.19 ± 3.58
2		100	30	1%	197.3 ± 4.7	0.783 ± 0.068	39.38 ± 4.21
3		150	15	1%	152.8 ± 5.1	0.740 ± 0.053	41.82 ± 5.01
4		150	30	1%	210.4 ± 6.8	0.607 ± 0.057	39.18 ± 3.11
5	Reverse-phase evaporation	100	15	1%	445.6 ± 5.9	0.674 ± 0.049	58.63 ± 2.58
6		100	30	1%	501.7 ± 7.2	0.779 ± 0.058	49.57 ± 3.69
7		150	15	1%	478.6 ± 6.5	0.683 ± 0.071	55.29 ± 4.03
8		150	30	1%	524.5 ± 5.9	0.657 ± 0.069	56.36 ± 3.87
9	Ethanol Injection Method	100	15	1%	382.7 ± 4.8	0.718 ± 0.062	41.52 ± 3.13
10		100	30	1%	397.2 ± 6.1	0.643 ± 0.055	42.38 ± 4.09
11		150	15	1%	391.9 ± 7.5	0.639 ± 0.049	38.69 ± 3.98
12		150	30	1%	388.4 ± 6.7	0.672 ± 0.039	43.29 ± 4.29
P-values		Phospholipon 90H (A)			0.005	0.905	0.963
		Cholesterol (B)			0.006	0.068	0.02
		AB			0.033	0.59	0.27

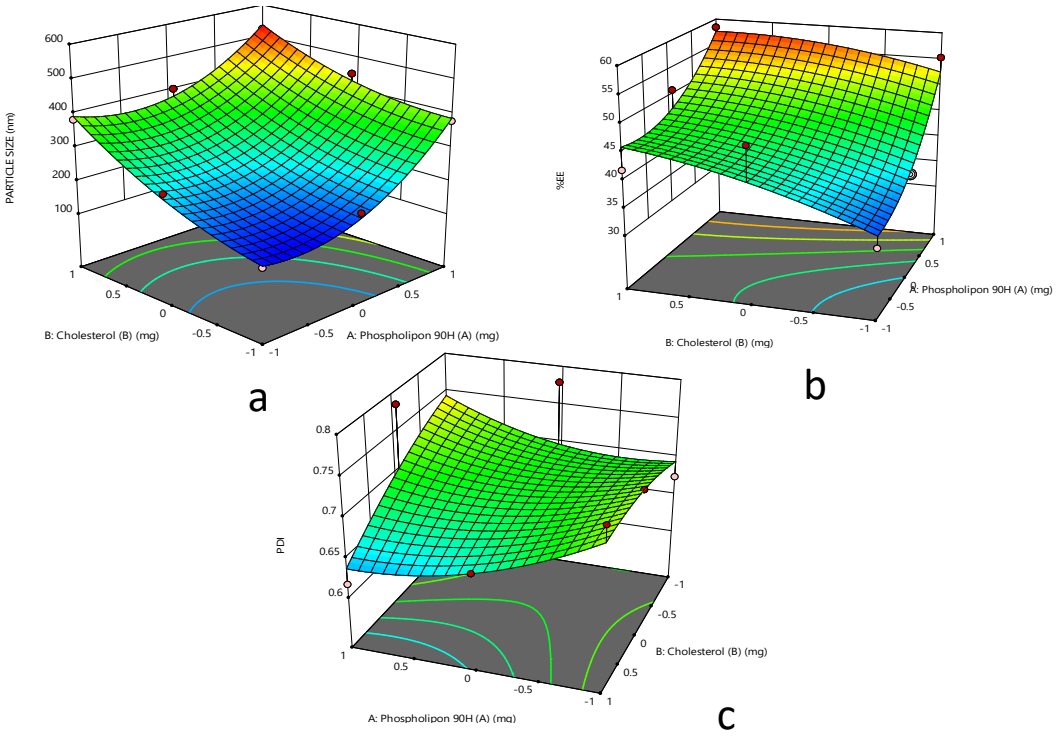

Figure 1: 3D surface plot for factorial studies. (a) The effect of various levels of Phospholipon 90H and cholesterol on particle size. (b) The effect of various levels of Phospholipon 90H and cholesterol on % EE. (c) The effect of various levels of Phospholipon 90H and cholesterol on polydispersibility Index

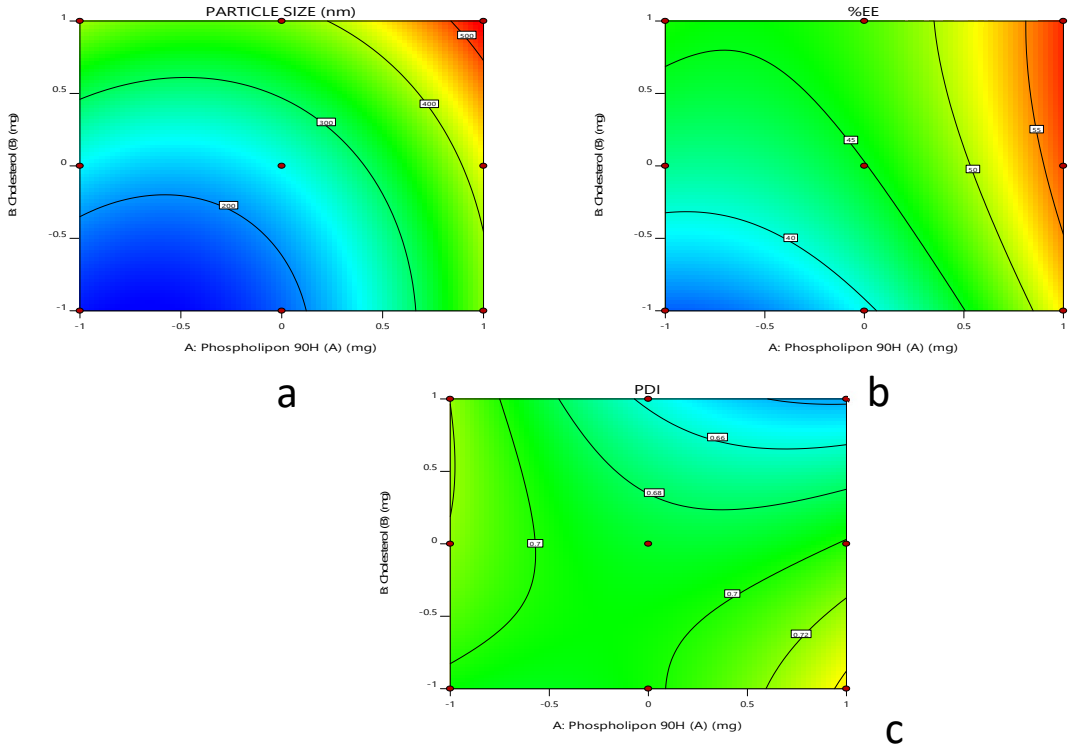

Figure 2: Contour plot for factorial studies. (a) The effect of various levels of Phospholipon 90H and cholesterol on particle size. (b) The effect of various levels of Phospholipon 90H and cholesterol on % EE. (c) The effect of various levels of Phospholipon 90H and cholesterol on polydispersibility Index

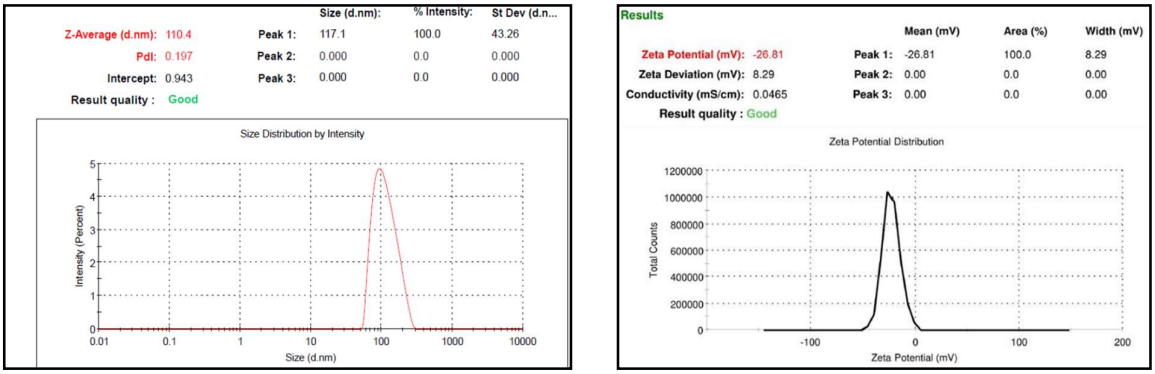

Figure 3: Size (a) and zeta potential (b) of Acyclovir loaded liposomes prepared using optimized conditions

CONCLUSION:

An experimental design was employed in a study to determine the main variables that significantly affect the characteristics of Nanoliposomes containing Acyclovir. Three responses were selected and observed are particle size, Polydispersibility index and % E entrapment efficiency. The application of fractional factorial design indicated that the amount of Phospholipon 90H and cholesterol are very crucial and their levels determines the overall drug loading. The ideal conditions for production of liposomes with least possible particles size and maximum possible Entrapment efficiency with good polydispersibility index were studied and identified using CCD. Under the optimized condition of taking Phospholipon 90H 100 mg and cholesterol ½ of the Phospholipon 90H, temperature during film formation − 50∘ C, and speed of rotation of flask during film formation − 150 rpm) the mean particle size was found to be 117.1 ± 15 nm with percentage entrapment efficiency of 58 ± 2%, PDI 0.197 and zeta potential −26.81 ± 8.29 mV. From the study it can be concluded that in the given experimental conditions reverse phase hydration method appears to be a better option to prepare acyclovir loaded liposomes.

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