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APPLICATION OF QUALITY BY DESIGN (QBD) APPROACH IN FORMULATION OF AZITHROMYCIN LIPOSOMES FOR LOCALIZED TREATMENT OF BACTERIAL INFECTIONS

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ABSTRACT

The objective of the present investigation was to formulate liposomes of azithromycin using quality by design (QbD) approach and evaluate the formulation for various parameters. A D-optimal experiment design with two independent and two dependent variables was used to optimize the formulation with the best QTPP. Phospholipid (Lecithin) concentration, lecithin to stearyl alcohol molar ratio were selected as the critical parameters affecting the desired CQAs. Multilamellar vesicles (MLVs) were generated using a technique based on the established film method using lecithin and stearyl alcohol as the lipid components. The effect of lecithin concentration and the ratio of lecithin to stearyl alcohol was statistically validated using ANOVA and the 2 factorial model depicted a significant F-value of 38.35 for entrapment efficiency. The model presented a regression coefficient value of 0.9901 and adequate precision value of 17.302. The particles of the optimized liposome were found to be having an average particle size of 163.2 nm with a poly dispersity index of 0.391 and a zeta potential of -20.4 mV. The entrapment efficiency was found to be 72.79 ± 0.793 % (n=3). The *in vitro* release showed that the optimal liposomal formulation released only 82.77 ± 0.8098 % azithromycin after 48 h. The formulation was subjected to stability analysis for 28 days and the amount of azithormycin retained in the formulation was considered as the stability indicator. It was seen that around 0.8 % drug was lost in the 28 days of keeping the formulation.

KEYWORDS: Quality by Design, Azithromycin, stability, liposome, anti-bacterial.

INTRODUCTION

Until recently, treatment of topical bacterial infections has lagged far behind in part, because the most common bacterial infections in humans have been relatively superficial infections of the skin and mucosal membranes.

Azithromycin (AZI) is a potent, broad-spectrum macrolide antibiotic that is available in dosage forms for oral, ophthalmic and parenteral administration. It is recommended for the treatment of respiratory, skin and soft tissue infections, including sexually transmitted bacterial diseases caused by C. trachomatis and Neisseria gonorrhoeae. AZI is usually available as a dehydrate (Mw 785) and is characterized by its limited solubility in water (log P=3.98).[1-3] Incorporation of AZI into liposomes could increase its solubility, favor its compatibility in the urinogenital area, enable prolonged release and help in attaining better localization of the drug.[4] Use of QbD approach will be helpful in obtaining liposomal formulation with the required set of properties that would be helpful in high localization at the site of infection and in turn improve the antibacterial efficacy on topical application.

MATERIAL AND METHODS

Azithromycin was obtained as gift sample from Ind-Swift Pharmaceutical, Baddi. Phosphatidylcholine was obtained from Himedia whereas stearyl alcohol was purchased from Suvidhinath Laboratories. All other solvent, reagents and chemicals were purchased from Loba, CDH and Rankem. UV-Visible spectrophotometer (Labtronics, LT-2201) was used for measuring the absorbance of the samples.

Drug-Excipient Compatibility analysis

The FTIR spectra of the pure drug and a physical mixture of the drug and the polymers under study were obtained and observed for deletion of the characteristic peaks of the drug.[5]

Preparation of calibration curve

The maximum absorption of Azithromycin was observed at 285 nm using phosphate buffer pH6.8 as the solvent. The calibration curve was obtained using different concentrations of the drug at the above wave length. The stock solution was freshly prepared by dissolving 10 mg of Azithromycin in 1 ml of ethanol in a 10 ml volumetric flask and then made up the solution upto the mark using

phosphate buffer pH6.8 for obtaining the solution of strength 1000 µg/mL (stock I). 1 ml of this solution is diluted to 10 ml with phosphate buffer pH6.8 to obtain a solution of strength 100 µg/mL (stock II). From this solution with draw 1, 2, 3, 4 & 5 ml of solution in to the 10 ml volumetric flask and volume made up to 10 ml by using phosphate buffer pH6.8 to get the solutions of 10, 20, 30, 40 & 50µg/ml.

Design of Experiments

A D-optimal experimental design was used to optimize the identified process variables that affect the CQAs. The matrix of independent variables included the two formulation factors identified as critical for liposomal quality. These factors include phospholipid concentration (X1), lecithin to stearyl alcohol molar ratio (X2). On the other hand the matrix of dependent variables included EE% (%) (Y1) and liposomal size (nm) (Y2). The design matrix is presented in Table 1.

Table 1: Design matrix for optimization of liposome by QbD.

Run Code	X1 (mM)	X2
A1	70	10:1
A2	10	10:1
A3	40	7.5:1
A4	70	10:1
A5	10	5:1
A6	10	5:1
A7	10	7.5:1
A8	70	5:1
A9	40	5:1
A10	40	10:1
A11	70	7.5:1
A12	40	7.5:1

Formulation of liposomes

Multilamellar vesicles (MLVs) were generated using a technique based on the established film method.[6,7] Briefly, the lipid entities (lecithin and stearyl alcohol) were dissolved in chloroform:methanol (9 : 1) and the solvent evaporated on a rotary evaporator to yield a dry film as per the standard lipid film hydration method. To entrap azithromycin within the bilayer, the required amount of drug (1.00 mg) was added to the solvent mixture and subsequently hydrated as per the normal hydration method. In all cases, the film was hydrated with 2 ml double distilled water to give a final lipid concentration of 16–24 mmol/ml dependent on formulation.

Entrapment Efficiency evaluation

The drug loading of liposomes was determined by measuring the non-incorporated drug present in the hydration and wash media after separation of liposomes by centrifugation (Remi) at 27200g for 30 min. All samples were diluted enough (with respect to solubility values) to avoid drug precipitation. The drug content of the supernatant was analysed by UV spectroscopy at 285 nm.[8] The amount of entrapped drug was calculated by subtracting the un-entrapped drug from total amount of drug used. The entrapped drug was expressed as encapsulation efficiency (EE%), using the formula.

$$EE\% = \frac{\text{Entrapped drug concentration}}{\text{Total drug concentration}} \times 100$$

Determination of vesicle size

Liposomal size was determined by dynamic light scattering method, using a Zetasizer Nano ZS analyser (Malvern Instruments Co., Malvern, UK) after the dilution of the liposomes in distilled water (1:200 v/v). All the measurements were performed in triplicate at 25 °C, with a scattering angle of 90°.

Zeta Potential

Zeta potential was measured by laser Doppler electrophoresis, using a Zetasizer Nano ZS90 analyser (Malvern Instruments Co., Malvern, UK). The measurements were performed in distilled water at 25 °C, three times for each sample.

***In Vitro* Drug Release**

The release rate of drug was determined by incubating drug-loaded vesicles (after separation of non-incorporated drug) in 30 ml PBS at 37°C in a shaking (constant; 150 oscillations/min) water bath. At time intervals of 0, 2, 4, 8, 24, 48 and 72 h, the medium was centrifuged at 27200g for 30 min. The supernatant was analyzed spectrophotometrically at the 285 nm and the amount of drug released was assayed by comparison with a calibration curve for drug.

Stability studies

Liposomal size and drug retention were used as parameters to preliminarily indicate the physical stability of liposomes.[9,10] The stability of formulations, with respect to retention of the entrapped drug and changes in the size distribution, was determined by incubating vesicles (after separation of the free drug) in 10 ml PBS at 4 and 25°C. At time intervals of 0 (immediately after preparation), 7, 14 and 28 day samples were centrifuged to separate loaded from 'free' drug, and supernatants analyzed spectrophotometrically at 285 nm. The amount of drug released was assayed by comparison with a calibration curve for drug.

RESULTS AND DISCUSSION

Calibration Curve of Azithromycin

Calibration curve of Azithromycin was plotted as absorbance versus concentration (µg/ml) at 285 nm (Figure 1).

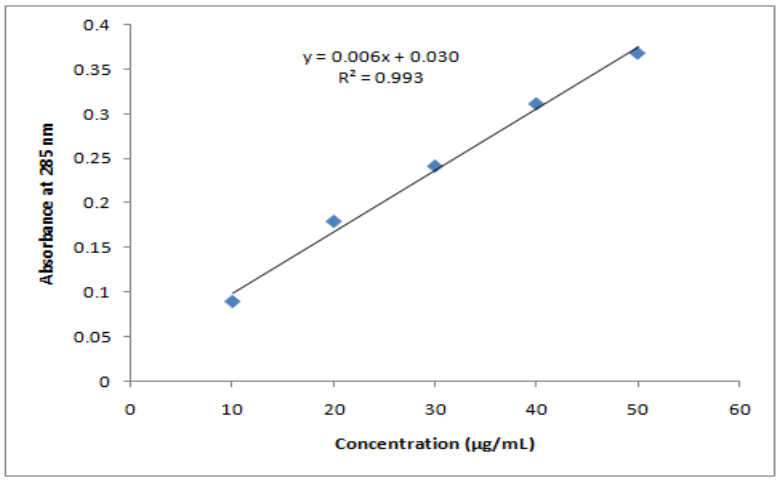


Figure 1: Calibration curve of Azithromycin in phosphate buffer.

Compatibility study by FTIR

The FTIR spectrum of azithromycin (figure 2a) exhibited significant peaks of C-N stretch, C=O stretch, C-O-C stretch, and O-H stretch and the peaks were compared to the standard spectra available at NIST. No deletion of the characteristic peaks of azithromcyin was found in the FTIR spectrum of the physical mixture of drug and polymer (figure 2b).

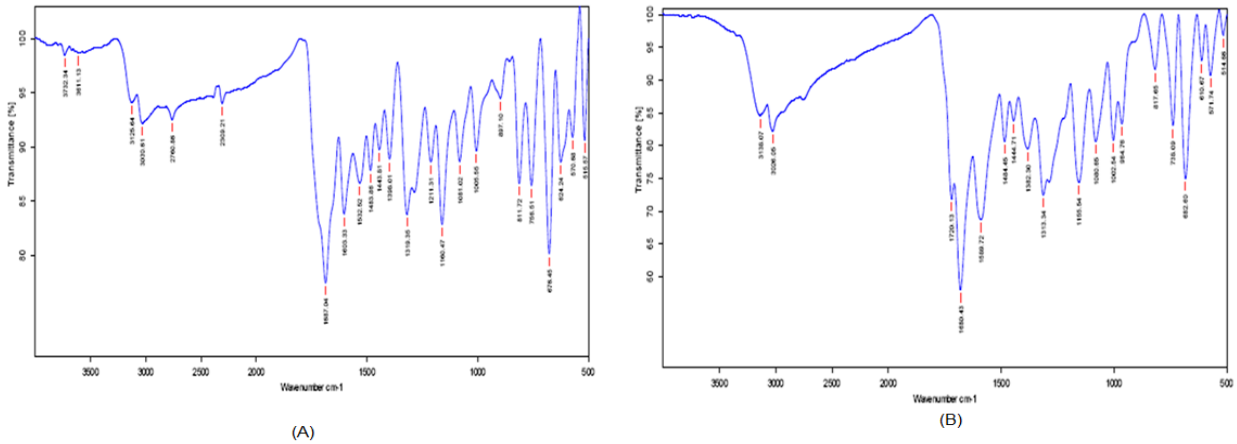


Figure 2: FTIR spectra of (A) Azithromycin (B) physical mixture.

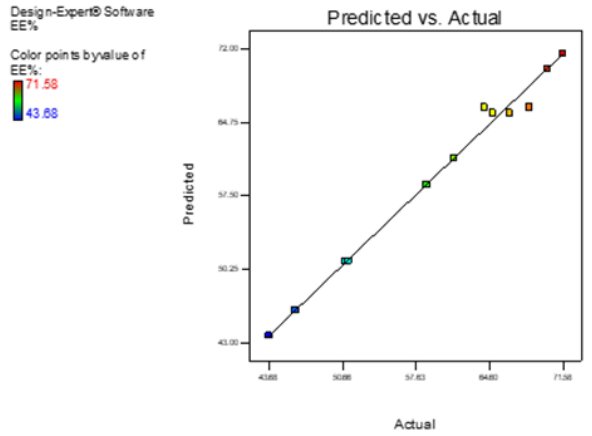
Optimization of Formulation

The DOE was done using Design Expert 7.0.0 trial version using D-optimal design with two independent variables and two dependent variables. The result of EE% and particle size were statistically analyzed in order to study the influence of the independent variables of them (Table 2).

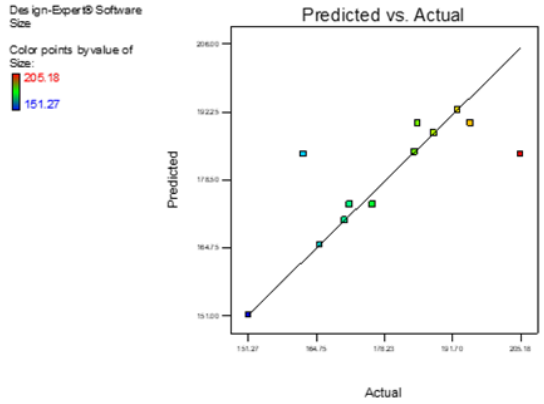
Table 2: D-optimal design results.

Experiment Name	Lecithin (mM)	Lecithin to Stearyl Alcohol ratio	EE (%)	Particle diameter (nm)
A1	70	10:1	47.69	212
A2	10	10:1	68.43	155
A3	40	7.5:1	61.69	189
A4	70	10:1	48.02	166
A5	10	5:1	66.21	179
A6	10	5:1	61.06	175
A7	10	7.5:1	58.13	196
A8	70	5:1	40.58	191
A9	40	5:1	55.41	169
A10	40	10:1	67.01	175
A11	70	7.5:1	43.07	188
A12	40	7.5:1	13.25	199

The effect of lecithin concentration and the ratio of phospholipid to stearyl alcohol was statistically validated using ANOVA and the 2 factorial model depicted a significant F-value of 38.35 for entrapment efficiency. The model presented a regression coefficient value of 0.9901 and adequate precision value of 17.302. The adequate precision measures the signal to noise ratio and a ratio greater than 4 is desirable. The predicted vs. actual entrapment efficiency was studied and two runs were found to exceed the residual limits.



(A)



(B)

The model was not significant for the particle size suggesting that there is a very high probability that the predicted size may be due to noise. From the above figure 2, it is evident that the predicted particle size was unable to match the actual size and the outliers were very high.

The optimization was done with respect obtaining with highest entrapment efficiency percent and lowest particle size of the liposomes. A total of 8 solutions were obtained of which the solution with 10mM lecithin and 10:1 ratio of lecithin to stearyl alcohol was having the highest desirability (0.523). It was selected as the optimized formulation in the design space (Figure 3).

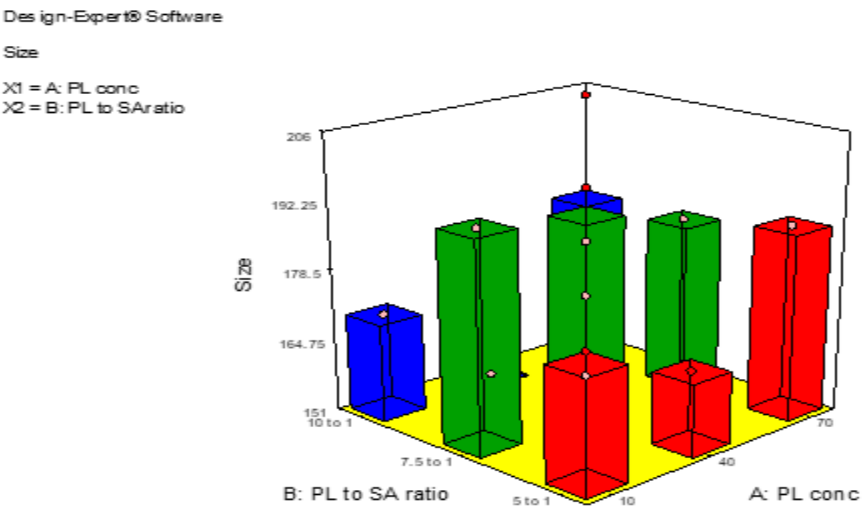


Figure 3: 3D surface plot of particle size.

Formulation of liposome using optimized parameters

The optimization experiments suggested a phospholipid concentration of 10 mM and lecithin to stearyl alcohol ratio of 10:1 as the optimized conditions to obtain lowest particle size and highest entrapment efficiency. Formulation of liposome was done by replicating these conditions and the liposome was evaluated for various characteristics.

Particle size and zeta potential

The particle size and zeta potential were studied using Malvern zeta sizer and the particles were found to be having an average particle size of 163.2 nm with a poly dispersity index of 0.391. The zeta potential of the formulation was found to be -20.4 mV. The zeta potential values around 20 mV are considered to provide sufficient repulsion among the particles for preventing aggregation. The higher poly dispersity index of the

particles could be attributed to the low zeta potential of the formulation.

Entrapment efficiency

The percentage of azithromycin encapsulated or entrapped in the core of the liposomal formulation was calculated by studying the amount of non-incorporated azithromycin. The entrapment efficiency was found to be 72.79 ± 0.793 % (n=3). The high incorporation of drug was beneficial as a higher EE% is associated with a reduced drug loss during the manufacturing process and with a low-cost production.

***In vitro* drug release**

The release of azithromycin was studied using diffusion method by calculating the amount of drug in the solution at predetermined time intervals. As the attribute required in the formulation was a prolonged duration of action of the drug, the drug release was studied up to 48 h. The cumulative amount of azithromycin released from the liposome was calculated and plotted as a function of time (Figure 4).

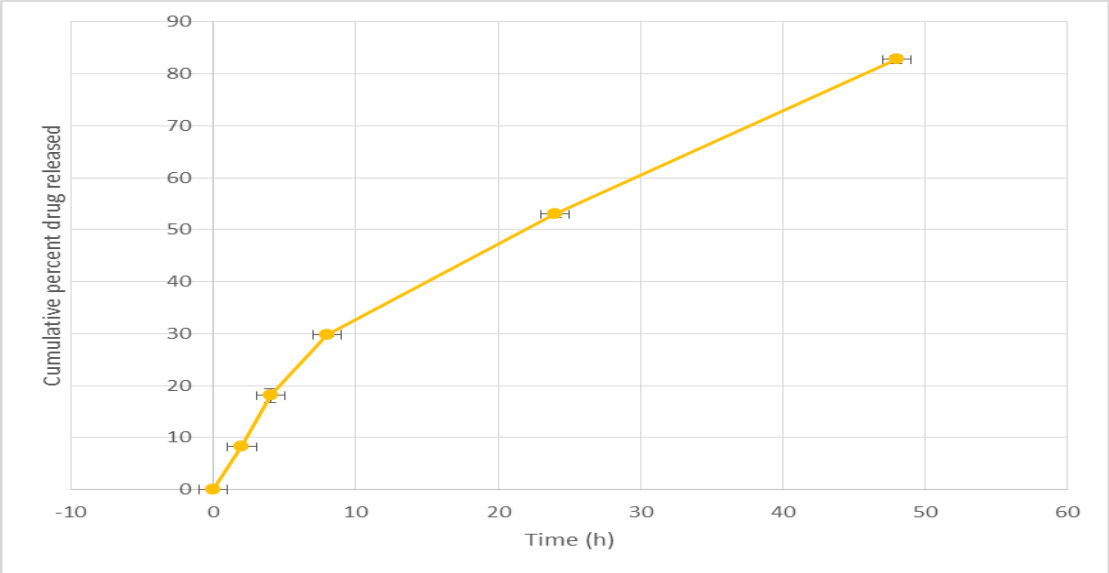


Figure 4: Release of azithromycin from the liposome.

The *in vitro* release showed that the optimal liposomal formulation released 82.77 ± 0.8098 % azithromycin after 48 h. The amount of drug that released in the initial hours of the study increased rapidly whereas it steadied thereafter. The *in vitro* release study proved a continuous and sustained release of azithromycin from the optimal formulation, for at least 48 h, fulfilling the desired attribute in the formulation as per our quality requirements (EE>40%, size<200nm).

Stability study

The formulation was subjected to stability analysis for 28 days and the amount of azithromycin retained in the formulation was considered as the stability indicator. The entrapment efficiency on day of preparation (day 0), day 7, 14 and 28 was determined from the formulation as per the reported procedure (Table 3).

Table 3: Entrapment efficiency in stability sample.

Day	Entrapment Efficiency (%)
0	82.77 ± 0.8098
7	82.33 ± 0.7528
14	82.18 ± 0.6676
28	82.07 ± 0.4954

It was seen that around 0.8 % drug was lost in the 28 days of storing the formulation warranting the lyophilization of the formulation for long term storage.

CONCLUSION

The QbD approach proved to be a key element in azithromycin liposome development, by providing information regarding the impact of the formulation factors and process parameters on the CQAs of the liposomes. The developed liposomal formulation presented a release of azithromycin for at least 48 hours, suggesting an improved half-life, and bioavailability of the drug. The diffusion method used for assessing the drug release simulates drug release by oral administration and hence the liposome could be believed to be administered orally and fulfil all the QTPPs. The study also establishes the use of stearyl alcohol as a prominent replacement of traditionally used cholesterol in formulation of liposomes.

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