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RESEARCH ARTICLE
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FORMULATION AND CHARACTERIZATION OF GRISEOFULVIN LOADED LIPOSOMES USING QUALITY BY DESIGN (QBD) APPROACH

Indra Kumar[1*], Kapil Malviya[1], Pushpendra Soni[1], Pushpendra Kumar[1], Lavakesh Kumar Omray[1]

[1]Radharaman Institute of Pharmaceutical Sciences, Bhopal

***Corresponding Author:** Indra Kumar
Email – indrakumarsgrl@gmail.com

Abstract
The objective of the present investigation was to formulate liposomes of griseofulvin 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 concentration, phospholipid to stearyl alcohol molar ratio were selected as the critical parameters affecting the desired CQAs. The effect of phospholipid 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 35.83 for entrapment efficiency. The model presented a regression coefficient value of 0.9896 and adequate precision value of 17.274. The particles of the optimized liposome were found to be having an average particle size of 149.1 nm with a poly dispersity index of 0.428 and a zeta potential of -17.1 mV. The entrapment efficiency was found to be 72.71 ± 1.047 % (n=3). The *in vitro* release showed that the optimal liposomal formulation released only 74.78 ± 1.8680 % griseofulvin after 72 h. The formulation was subjected to stability analysis for 28 days and the amount of griseofulvin retained in the formulation was considered as the stability indicator. It was seen that around 3.08% drug was lost in the 28 days of keeping the formulation.

Keywords: QbD, optimization liposome, antifungal, griseofulvin

Introduction

Quality by design (QbD) is a systematic approach of development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management[1]. It helps to establish a control strategy for the entire process that may include input material controls, process controls and monitors, design spaces around individual or multiple unit operations, and/or final product tests. The control strategy should encompass expected changes in scale and can be guided by a risk assessment.

Until recently, chemotherapy of fungal infections has lagged far behind hemotherapy of bacterial infections. This lack of progress has resulted, in part, because the most common fungal infections in humans have been relatively superficial infections of the skin and mucosal membranes and potentially lethal deep-seated infections have been quite rare. Because most humans with a normally functioning immune system are able to ward off invading fungal pathogens with little difficulty, the demand for improvements in antifungal therapy has been small. Oral antifungal drugs are reserved for extensive or severe infection for which topical antifungal agents are inappropriate or ineffective, because of

high cost, potential side effects and drug interactions.
Griseofulvin is a widely acknowledged antifungal drug used used orally to treat superficial fungal infections, primarily fingernail and toenail infections, but it does not penetrate skin or nails if used topically. The lower aqueous solubility and absorption causes the dose of griseofulvin to be very high (500 mg/kg, twice a day in adults). On the other hand only 25-50% of the drug is absorbed and with around 42% drug excreted unchanged in urine after 4 hours thereby decreasing its half-life.
Liposomes have been widely studied for improving bioavailability of formulations[2-6]. As the absorption of drug is low, formulating it as liposome using QbD approach would be helpful to obtain a formulation with improved efficacy and in turn its oral absorption and bioavailability.

Material and Methods

Griseofulvin was purchased from Yarrow Pharmaceuticals, Mumbai. 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.

Compatibility analysis of drug and excipients

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.

Preparation of Calibration Curve in methanol

Accurately weighed 10 mg of Griseofulvin was taken in 10 mL volumetric flask and dissolved in methanol to the mark resulting in a stock solution of 1000 µg/mL. 1 mL of the above stock solution was taken in another 10 mL volumetric and volume was made up with methanol to mark resulting in a solution of 100 µg/mL. Aliquots of 1-6 mL of stock solution were taken into a series of 10 mL volumetric flask and volume was made up to the mark using methanol and were analyzed at 295 nm using UV spectrophotometer. A standard curve was constructed against absorbance and concentration.

Quality by design implementation

The quality target product profile (QTPP) and critical quality attributes (CQAs) are presented in Table 1.

Table 1 QTPP and CQAs for griseofulvin liposomes

QTPP	
Formulation type	Oral liposomes
Route of administration	Buccal cavity
Required properties	Prolonged release duration
CQAs	
CQA	**Target to be achieved**
Encapsulation Efficiency, EE (%)	> 30%
Size	< 200 nm

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), phospholipid 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 2.

Table 2 Design matrix for optimization of liposome by QbD

Experiment Name	X1 (mM)	X2
L1	70	10 to 1
L2	10	10 to 1
L3	40	7.5 to 1
L4	70	10 to 1
L5	10	5 to 1
L6	10	5 to 1
L7	10	7.5 to 1
L8	70	5 to 1
L9	40	5 to 1
L10	40	10 to 1
L11	70	7.5 to 1
L12	40	7.5 to 1

Formulation of liposomes

Multilamellar vesicles (MLVs) were generated using a technique based on the established film method[7,8]. Briefly, the lipid entities (phosphatidylcholine 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 drugs 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 295 nm. 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 appropriate wavelength 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. The protocol was adapted from du Plessis et al[9] and Vangala et a[l10]. 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 295 nm. The amount of drug released was assayed by comparison with a calibration curve for drug.

Results and Discussion

The objective of the present investigation was to formulate liposomes of griseofulvin 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.

Compatibility study by FTIR

The FTIR spectrum of griseofulvin (figure 1a) exhibited significant peaks of C-N stretch, C=O stretch, C-O-C stretch, N-H and O-H stretch and the peaks were compared to the standard spectra available at NIST. No deletion of the characteristic peaks of griseofulvin was found in the FTIR spectrum of the physical mixture of drug and polymer (figure 1b).

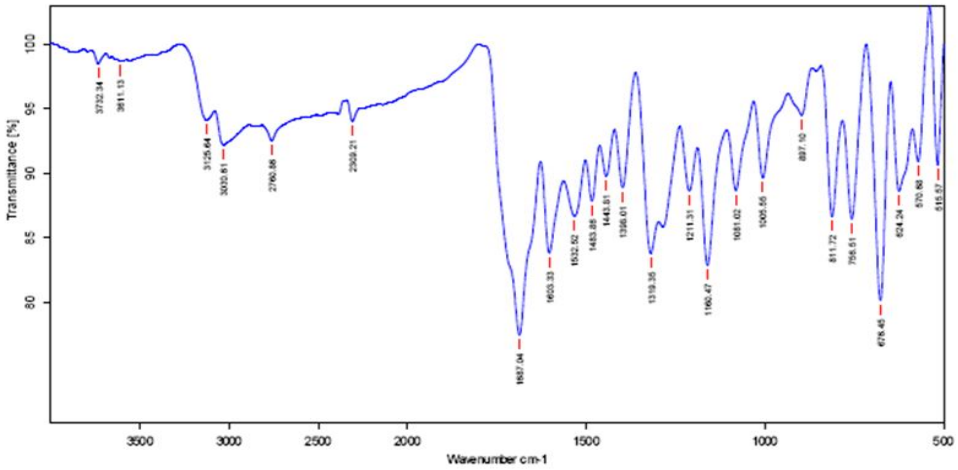


Figure 1a FTIR spectra of griseofulvin

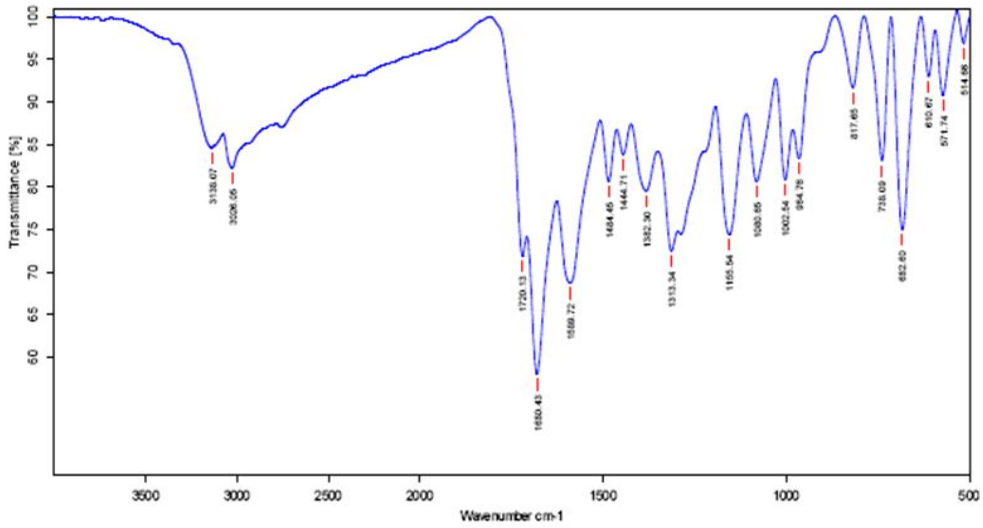
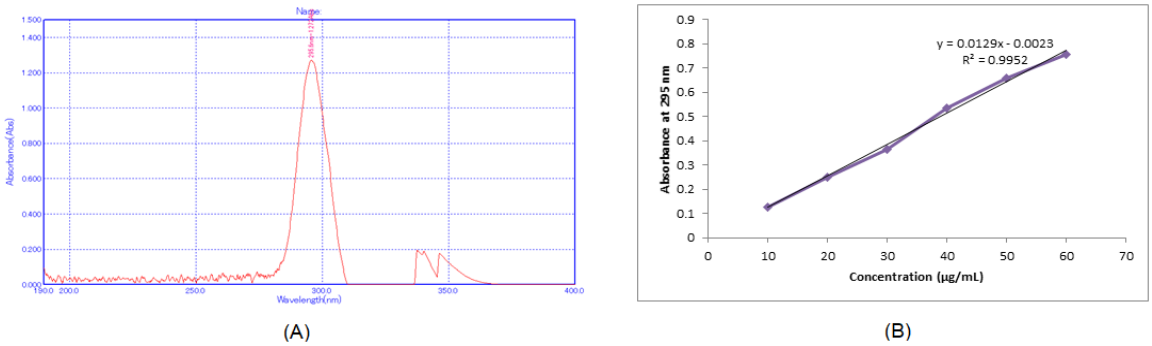


Figure 1b FTIR spectrum of physical mixture

5.3 Construction of calibration curve

The calibration curve of griseofulvin was constructed in methanol at concentration range of 10-60 µg/mL. The λmax was found to be 295 nm and was used for all the analysis of drug (Figure 2).



(A) (B)

Figure 2 UV spectrum and calibration curve of griseofulvin

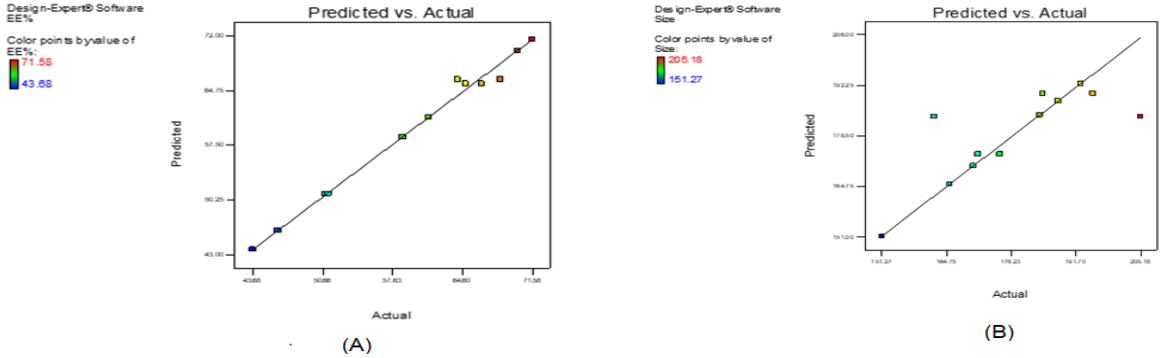
5.4 Optimization of formulation

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 3).

Table 3 D-optimal design results

Experiment Name	PL (mM)	PL to SA ratio	EE%	Particle size
L1	70	10 to 1	50.89	205
L2	10	10 to 1	70.07	170
L3	40	7.5 to 1	64.89	184
L4	70	10 to 1	51.22	162
L5	10	5 to 1	68.38	175
L6	10	5 to 1	64.11	171
L7	10	7.5 to 1	61.22	192
L8	70	5 to 1	43.68	187
L9	40	5 to 1	58.64	165
L10	40	10 to 1	71.58	151
L11	70	7.5 to 1	46.22	184
L12	40	7.5 to 1	66.48	195

The effect of phospholipid 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 35.83 for entrapment efficiency. The model presented a regression coefficient value of 0.9896 and adequate precision value of 17.274. 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 (Figure 3).



(A) (B)

Figure 3 Predicted vs. actual (A) entrapment efficiency (B) particle size using the model

The model was not significant for the particle size suggesting that there is a very high probability than the predicted size may be due to noise. From the above figure 3 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 40mM phospholipid and 10 to 1 ratio of phospholipid to stearyl alcohol was having the highest desirability (0.517). It was selected as the optimized formulation in the design space.

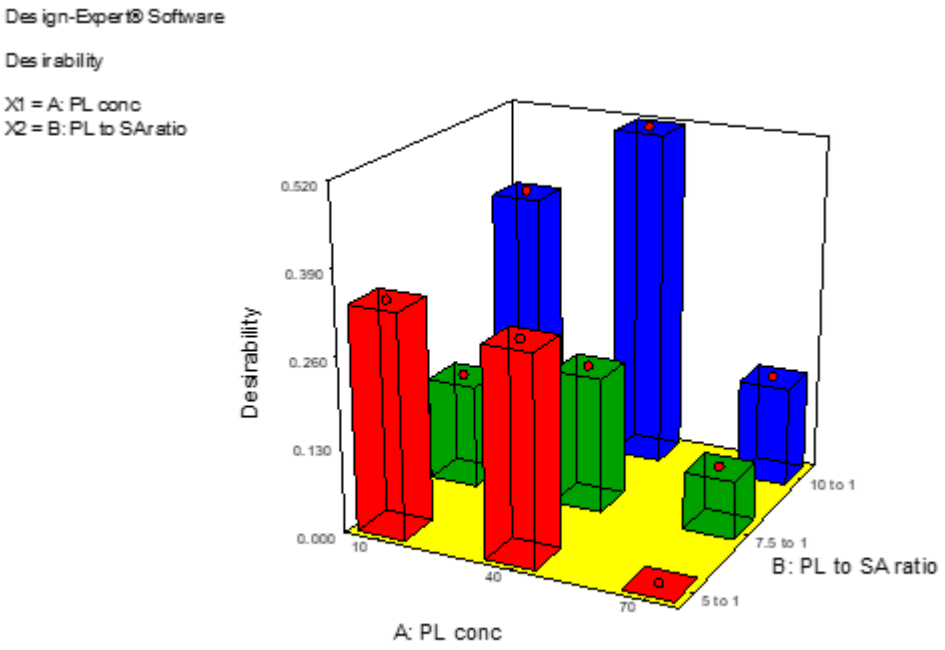


Figure 4 3D surface plot of desirability of the solutions

Formulation of liposome using optimized parameters

The optimization experiments suggested a phospholipid concentration of 40 mM and phospholipid to stearyl alcohol ratio of 10 to 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.

Evaluation of the optimized formulation

The formulation using the optimized conditions was evaluated for entrapment efficiency, particle size, zeta potential and *in vitro* release of griseofulvin.

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 149.1 nm with a poly dispersity index of 0.428 (Figure 5). The zeta potential of the formulation was found to be -17.1 mV (Figure 6). Though the value was not very optimum, yet values around 20 mV are considered to provide sufficient repulsion among the particles for preventing aggregation. The high poly dispersity index of the particles could be attributed to the low zeta potential of the formulation.

Size Distribution Report by Intensity

Sample Details

Sample Name: PL- 3 Size 1
SOP Name: mansettings.dat
General Notes:

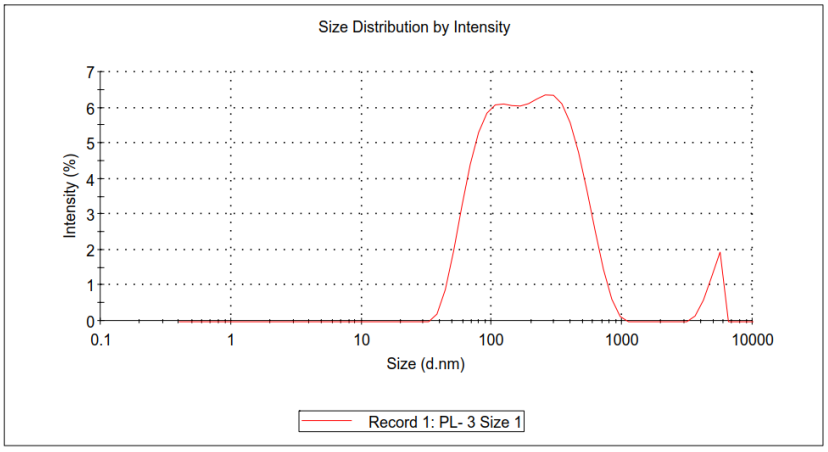
File Name:	PL- 3 Size.dts	**Dispersant Name:**	Water
Record Number:	1	**Dispersant RI:**	1.330
Material RI:	1.59	**Viscosity (cP):**	0.8872
Material Absorbtion:	0.01	**Measurement Date and Time:**	Saturday, July 22, 2023 12...

System

Temperature (°C):	24.9	**Duration Used (s):**	70
Count Rate (kcps):	189.8	**Measurement Position (mm):**	4.65
Cell Description:	Disposable sizing cuvette	**Attenuator:**	6

Results

			Diam. (nm)	% Intensity	Width (nm)
Z-Average (d.nm):	149.1	**Peak 1:**	334.3	52.7	151.7
PdI:	0.428	**Peak 2:**	104.6	43.5	35.77
Intercept:	0.958	**Peak 3:**	5024	3.8	597.4
Result quality	Good				



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DTS Ver. 5.03
Serial Number : MAL1023461

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Figure 5 Particle size by intensity of optimized formulation

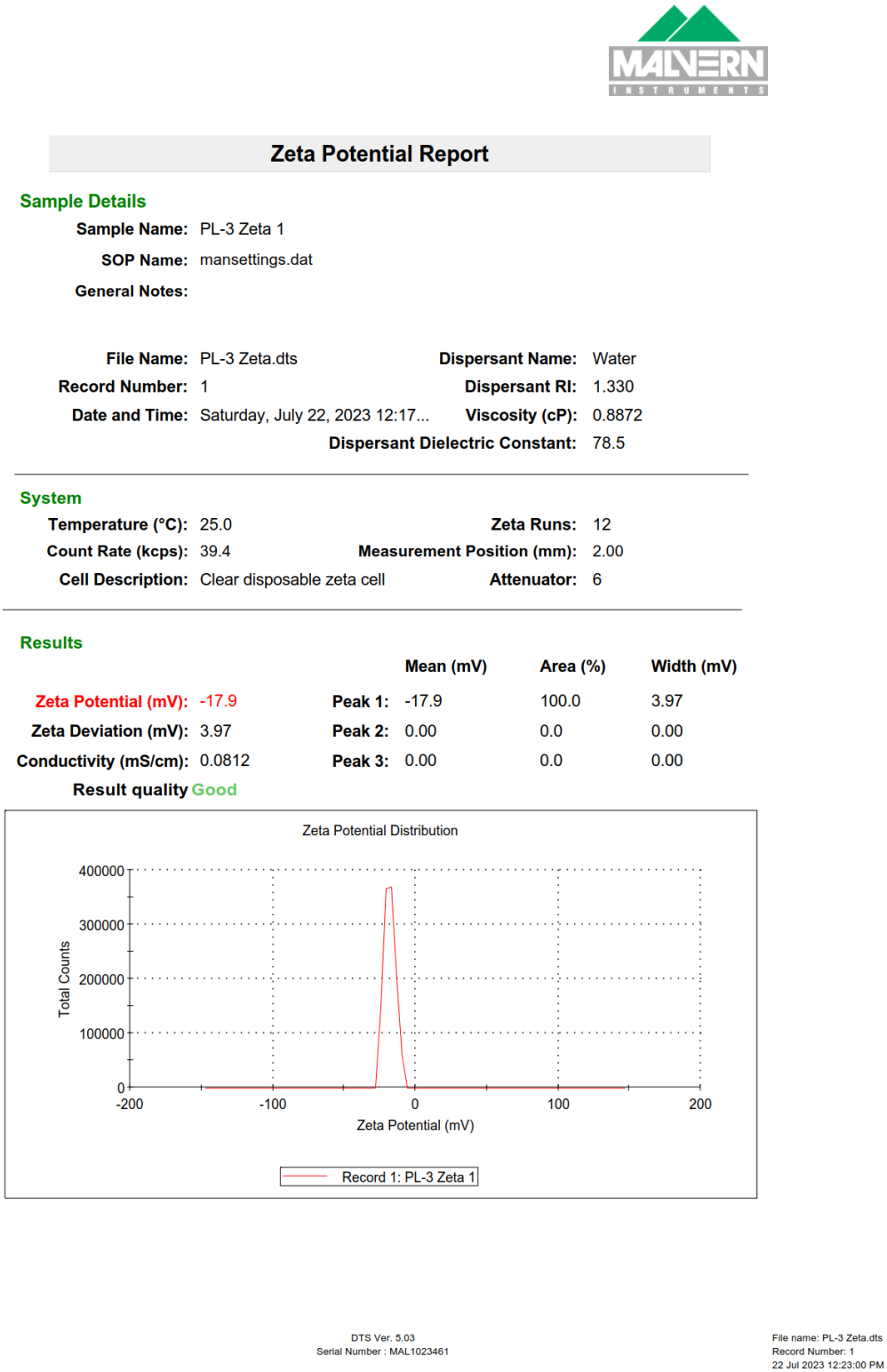

Figure 6 Zeta potential of the optimized formulation

Entrapment efficiency

The percentage of griseofulvin encapsulated or entrapped in the core of the liposomal formulation was calculated by studying the amount of non-incorporated griseofulvin. The entrapment efficiency was found to be 72.71 ± 1.047 % (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 griseofulvin 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 72 h. The cumulative amount of griseofulvin released from the liposome was calculated and plotted as a function of time (Figure 7).

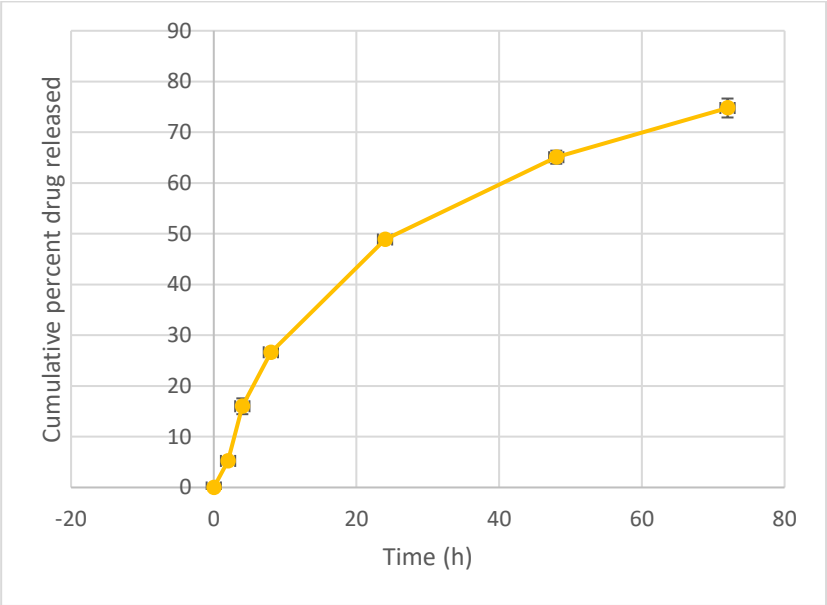

Figure 7 Plot depicting a steady release of griseofulvin from the liposome

The *in vitro* release showed that the optimal liposomal formulation released only 74.78 ± 1.8680 % griseofulvin after 72 h. The amount of drug that released in the initial hours of the study increased rapidly whereas an almost plateau was attained post 48 hours of the study. The in vitro release study proved a continuous and sustained release of griseofulvin from the optimal formulation, for at least 72 h, fulfilling the desired attribute in the formulation as mentioned in Table 1.

Stability study

The formulation was subjected to stability analysis for 28 days and the amount of griseofulvin 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 4).

Table 4 Entrapment efficiency in stability sample

Day	Entrapment Efficiency (%)
0	71.89 ± 0.8901
7	70.74 ± 0.6326
14	70.70 ± 0.5896
28	69.67 ± 0.9707

It was seen that around 3.08% drug was lost in the 28 days of keeping the formulation. This warrants the use of lyophilization of the formulation for long term storage of the liposomal formulation.

Conclusion

The QbD approach proved to be a key element in griseofulvin 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 griseofulvin for at least

72 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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