

DOE-based Development of Liposome Encapsulated Delivery of Azithromycin for Wound Healing

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Abstract- Wound healing is a complex and dynamic biological process that can be compromised by microbial infection, excessive inflammation, and delayed tissue regeneration. Azithromycin, a macrolide antibiotic, possesses broad-spectrum antibacterial activity and has also demonstrated anti-inflammatory and immunomodulatory properties that may be beneficial in wound management. However, conventional delivery of azithromycin may be limited by inadequate localization at the wound site, variable drug availability, and the need for repeated administration. Liposomal drug delivery offers a promising approach for improving the localized and sustained delivery of azithromycin while potentially enhancing its therapeutic effectiveness and reducing systemic exposure. The present study focuses on the development and optimization of azithromycin-loaded liposomes using a Design of Experiments (DOE) approach. Critical formulation variables, such as lipid-to-drug ratio, phospholipid concentration, cholesterol concentration, and process conditions, can be systematically investigated to determine their effects on critical quality attributes, including vesicle size, polydispersity index, zeta potential, encapsulation efficiency, and drug release. A suitable experimental design can be employed to identify significant formulation variables and their interactions and to establish an optimized formulation with desirable physicochemical and drug-release characteristics. The optimized liposomal formulation may subsequently be evaluated for in vitro drug release, stability, antibacterial activity, cytocompatibility, and wound-healing potential. This systematic DOE-based approach is expected to facilitate rational formulation development and provide a potential liposomal platform for localized azithromycin delivery in wound management.

I. INTRODUCTION

Wound healing is a complicated biological process in which several cellular and biochemical events occur in a coordinated manner. Following tissue injury, the

body initiates a sequence of responses involving blood clot formation, inflammation, cell proliferation, new tissue formation, and remodeling. Although these stages normally work together to restore tissue integrity, the healing process can become prolonged when the wound is exposed to microbial contamination, excessive inflammation, or other unfavorable conditions. In particular, bacterial colonization and infection can interfere with tissue regeneration and may increase the duration required for wound closure.

Antimicrobial therapy is therefore an important component of managing wounds in which bacterial infection is present or clinically suspected. Azithromycin is a macrolide antibiotic that acts by interfering with bacterial protein synthesis through interaction with the 50S ribosomal subunit. Apart from its antibacterial effect, azithromycin has been investigated for its ability to influence inflammatory responses. This combination of antimicrobial and potential anti-inflammatory properties makes azithromycin an interesting drug candidate for localized delivery in wound-care applications. Nevertheless, conventional administration does not specifically direct the drug toward the injured tissue and may result in limited drug availability at the desired site.

Liposomal drug delivery represents one possible strategy for addressing some of these limitations. Liposomes are microscopic vesicles generally formed from phospholipids that arrange themselves into one or more bilayer structures in an aqueous environment. Depending on the physicochemical properties of the drug and the composition of the vesicle, liposomes can incorporate therapeutic molecules within their aqueous compartment or lipid bilayer. Their

composition can also be modified to influence drug entrapment, vesicle characteristics, stability, and release behavior. For wound applications, a liposomal system may provide closer contact with the wound surface and facilitate more controlled presentation of the incorporated drug.

II. MATERIAL & METHOD

2.1 Material

Table 1 List of chemical/reagent/drug used

S. No.	Item	Source
1	Azithromycin	Gift Sample from Ind-Swift Pharmaceuticals, Baddi
2	Soy Lecithin	Merck
3	Cholesterol	Merck
4	Ethanolv	Merck
5	Acetonitrile	Rankem
6	Ortho phosphoric acid	Oxford
7	Chloroform	Rankem
8	Methanol	Rankem
9	Water	Merck

2.2 Method

2.2.1 Preformulation Studies

2.2.1.1 Organoleptic properties

A small quantity of pure Azithromycin powder was taken in a butter paper and viewed in well illuminated place to observe its color; the taste and odor were observed using tasting and smelling the drug.

2.2.1.2 Solubility analysis

Solubility of Azithromycin was determined in methanol, ethanol, dimethyl formamide, water and phosphate buffer. Solubility studies were performed by shaking small amount of Azithromycin in test tubes containing the solvent and observing for undissolved particles (if any).

2.2.1.3 Melting point

The melting point of Azithromycin was determined by open capillary method. The pure drug was filled in a capillary tube sealed at one end and placed in the melting point apparatus to observe the temperature at which melting occurs.

2.2.1.4 Loss on drying

It was determined by drying the pure drug in an oven at 100°C to 105°C for 3 h. The percent loss of moisture was calculated by the difference between the initial and final weight of the drug.

2.2.1.5 Partition Coefficient

The partition coefficient of the drugs was performed by using octanol as oil phase (10 mL) and water as aqueous phase (10 mL). Both the phases were mixed by shaking vigorously in a separating funnel and then 5 mg of drug was added. The drug was allowed to dissolve in both the phases by shaking and allowing for equilibration. Both phases were taken in a conical flask and then analyzed against their respective blank solution and the partition coefficient was calculated.

2.2.2 Standard Curve of Azithromycin

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.

2.2.3 Formulation of liposomes

Multilamellar vesicles (MLVs) were generated using a technique based on the established film method [49, 50]. 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.

2.2.4 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:

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

2.2.5 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°.

2.2.6 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.

2.2.7 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.

2.2.8 Stability studies

Liposomal size and drug retention were used as parameters to preliminarily indicate the physical stability of liposomes. 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.

2.2.9 Formulation of liposome loaded cream

A water in oil type of cream formulation loaded with the liposomes was prepared. The oil phase was prepared by melting bees wax along with liquid paraffin by maintaining the temperature to 70°C (Table 5.5). Similarly, aqueous phase was prepared by heating borax, methyl paraben, and propyl paraben with sufficient amount of distilled water by maintaining the temperature to 70°C. When the two phases are attained 70°C temperature, aqueous phase is generally introduced into the oil phase with continuous stirring until the cream consistency is attained. Once the temperature was reduced the prepared liposome suspension was incorporated to the cream base by slow stirring .

Table 2 Formula for cream preparation

S N	Ingredient	Quantity
1	Liposome	500 mg
2	Borax	200 mg

3	Bees Wax	3000 mg
4	Methyl Paraben	50 mg
5	Propyl Paraben	50 mg
6	Distilled Water	Quantity sufficient (to 10g)

2.2.10 Evaluation of the cream

2.2.10.1 Physical appearance and homogeneity

The physical appearance of the cream was observed by its color, and roughness. The formulation was tested for the homogeneity by visual appearance and by touch.

2.2.10.2 pH of the cream

The pH meter was calibrated using standard buffer solution. About 0.5 g of cream was weighed and dissolved in 50 ml of distilled water and its pH was measured using digital pH meter.

2.2.10.3 Spreadability

The spreadability of cream formulation was determined by using parallel plate method. An adequate amount of sample is taken between two glass slides and a weight of 10 g is applied on the slides for 5 minutes. Spreadability can be expressed by the formula:

$$S = m \cdot l / t;$$

where S is spreadability, m is weight applied on the upper slide, l is the length moved by the glass slide and t is the time taken

2.2.10.4 In vitro drug release from the cream

Artificial dialysis membranes were soaked in receptor medium for 12h prior to use and drug release was studied using Franz diffusion cell. Phosphate buffer saline (12 ml) pH

7.4 enriched with 10% v/v methanol was added into the receptor chamber maintained at $34 \pm 1^\circ\text{C}$. Cream equivalent to 10mg of drug was placed into donor compartment and the setup was kept on stirring. Aliquots of 5ml were withdrawn at predetermined time intervals from receptor compartment and replaced with fresh buffer till 12 h. The samples were diluted suitably and analyzed spectrophotometrically

at 285 nm and the amount of drug released was determined using calibration curve.

III. RESULT AND DISCUSSION

3.1 Preformulation studies

The preformulation studies were carried out using the previously reported methods and the results are presented underneath.

Table 3 Physicochemical properties of Azithromycin

Test	Observation
Color	White
Odor	Odorless
Taste	Bitter
Melting Point	117-120°C

Table 4 Solubility of Azithromycin

Solvent	Observation
Water	Insoluble
Ethanol	Soluble
Dimethyl Formamide	Soluble
Methanol	Slightly Soluble
Phosphate Buffer	Slightly Soluble

The partition coefficient of Azithromycin was calculated using octanol and water and the Log P value was found to be 4.05.

The percent loss on drying of Azithromycin was found to be 0.15%.

3.2 Calibration Curve of Azithromycin

Calibration curve of Azithromycin was plotted as absorbance versus concentration ($\mu\text{g/ml}$) at 285 nm.

Table 5 Standard curve absorbance data

Concentration ($\mu\text{g/mL}$)	Absorbance
10	0.089
20	0.179
30	0.241
40	0.311
50	0.368

The linear regression analysis for the calibration curve was $\text{Abs} = 0.006(\text{conc}) + 0.030$ with a regression coefficient of 0.993.

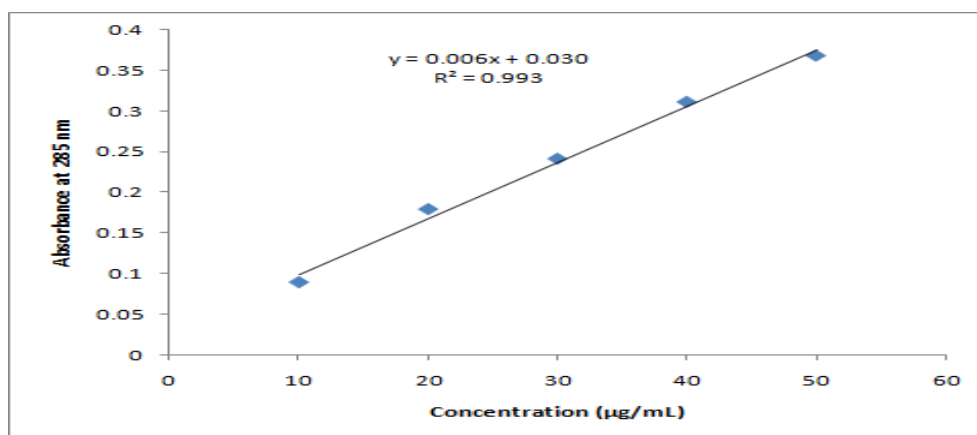


Figure 1 Calibration curve of Azithromycin in phosphate buffer

3.3 Liposomal formulation and optimization

The process and formulation and parameters strongly affect the properties of drug-loaded liposomes. The parameters used to characterize the liposomes in the preliminary experiments included particle size, the encapsulation efficiency and the in vitro drug release profile. Stability studies using particle size as an indicator of stability were also conducted for a 3-month period.

The optimization of liposome formulation was done by design expert software using D-optimal design. The process factors to be optimized included concentration of the phospholipid and the ratio of phospholipid to stearyl alcohol. The response variables selected for optimization were entrapment efficiency (to be maximized) and particle size (to be minimized). The 9 runs were performed and two levels of each process factor and the particle size and entrapment efficiency were determined (Table 6).

Table 6 Effect of process variables on response factors

Experiment Name	PL (mM)	to SA ratio	EE%	Particle size (nm)	Zeta Potential (mV)
AL1	10	10 to 1	64.89	170	-23.7
AL2	10	5 to 1	58.64	162	-21.5
AL3	40	10 to 1	70.07	205	-26.8
AL4	40	5 to 1	68.38	187	-24.3
AL5	40	10 to 1	71.58	211	-25.7
AL6	10	10 to 1	64.11	183	-20.8
AL7	10	5 to 1	61.22	165	-21.5
AL8	40	5 to 1	65.66	192	-24.2
AL9	40	5 to 1	66.48	195	-24.3

The quadratic model was used to create the model that fits perfectly to define the design space for entrapment efficiency and particle size (Table 6).

3.4 Characterization of the optimized formulation

The optimized formulation was prepared and evaluated for particle size, entrapment efficiency, zeta potential and in vitro drug release.

The particle size of the optimized formulation was found to be 180.6 nm with a poly dispersity index of

0.213 (Figure 6.5) whereas the zeta potential was -21.7 mV (Figure 6.6). The entrapment efficiency was found to be 65.80 ± 0.361 %. The drug release was determined for 24 h and the formulation was able to release 98.10 ± 0.200 % drug by the end of 24h of the study (Table 7).

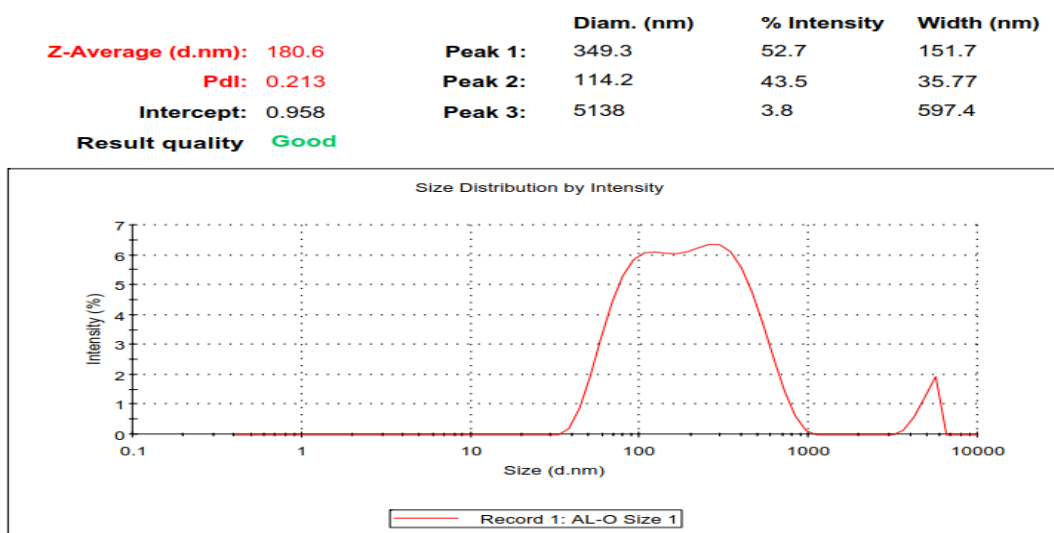


Figure 2 Particle size of optimized formulat

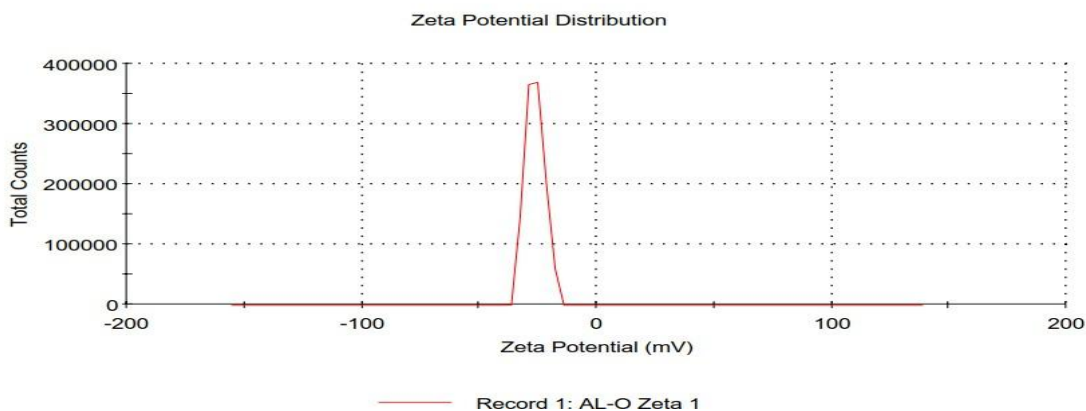
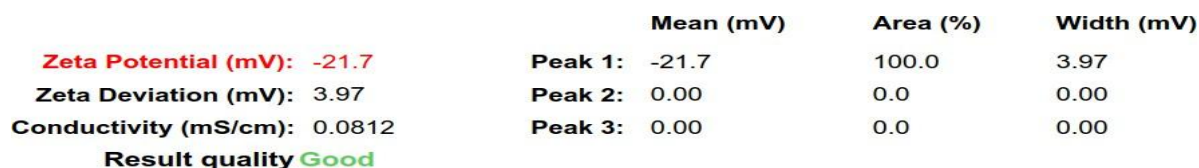


Figure 3 Zeta Potential of optimized formulation

Table 7 In vitro release of azithromycin from optimized formulation

Time (h)	Cumulative % release
0	0.00 ± 0.00
2	6.73 ± 0.451
4	14.27 ± 0.451
6	27.93 ± 0.929
8	48.73 ± 0.551
12	65.57 ± 0.473
24	98.10 ± 0.200

Mean ± SD, n = 3

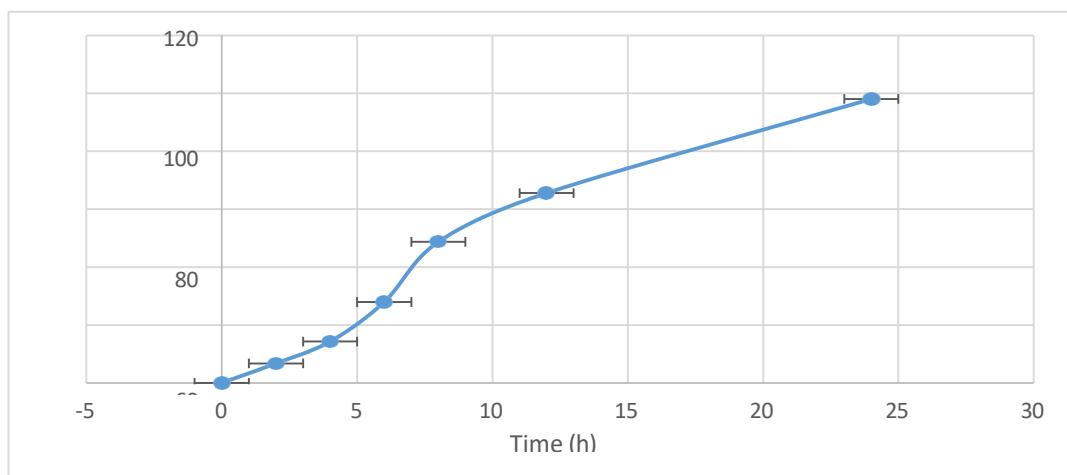


Figure 4 Release graph of azithromycin from liposome

3.5 Stability of liposomes

The change in particle size over a period of three months was considered to ascertain the stability of the liposomal formulation.

No significant change in particle size was observed suggesting that the formulations were stable at the storage conditions.

3.6 Evaluation of liposomal cream of azithromycin

The oil in water cream containing azithromycin optimized liposome was prepared and evaluated for various features (Table 8).

Table 8 Physical parameters of cream

Parameter	Observation
Homogeneity	Non-gritty and homogenous
Color	White

Odor	Pleasant
Texture	Smooth
pH	6.10 ± 0.021
Spreadability (g.cm/5min)	56.67 ± 3.055

The in vitro release of azithromycin from the cream was compared with that of liposome (Table 9).

Table 9 Release data of azithromycin from cream

Time	Cumulative % release
0	0.00 ± 0.00
2	5.77 ± 0.153
4	11.40 ± 0.265
6	23.80 ± 0.361
8	46.57 ± 0.503
12	62.83 ± 0.451
24	93.53 ± 0.404

Mean ± SD, n = 3

The release profile data from liposome and cream was plotted and revealed complete release of the drug from

the cream by the end of 24 h as similar to the liposome (Figure 5).

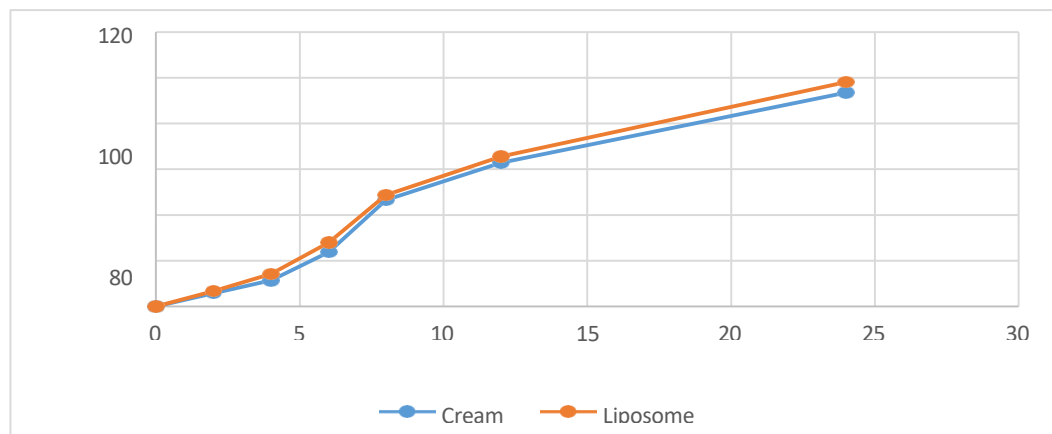


Figure 5 Comparative release plot of azithromycin from liposome and cream

IV. SUMMARY AND CONCLUSION

The hypothesis of this project was that Azithromycin could be successfully encapsulated in liposomes that would preserve the stability of the drug and increase the total duration of the release of the drug followed by incorporation of the liposome into cream formulation suitable for topical application of azithromycin with higher bioavailability (due to controlled release).

4.1 Summary

Drug loaded liposomes were prepared by a film hydration method using phosphatidylcholine and stearyl alcohol as the lipids require to prepared the liposomes and maintain its stability. The optimization of process parameters (PL concentration and ratio of PL:SA) as done by D-optimal design using design expert software for maximum entrapment efficiency and minimum particle size. The characterization of the liposomes was carried out by estimation of Azithromycin, estimation of encapsulation efficiency, in vitro drug release, particles size, and stability of the liposomes. The optimized liposome was incorporated in to oil in water cream formulation and evaluated. The particle size of the formulations ranged from 162 to 211 nm and the entrapment efficiency ranged from 58.64 to 71.58 %.

The optimized formulation was predicted to have entrapment efficiency of 65.262% and particle size of 181.592 nm for the liposomes when the phospholipid concentration was 14.1611 mM and the PL:SA ratio was 10:1.

The optimized formulation was prepared and evaluated and was found to have 65.80 ± 0.361 % entrapment of azithromycin and a particle size of 180.6 nm with a poly dispersity index of 0.213. The zeta potential of the optimized formulation was found to be -21.7 mV. The drug release was determined for 24 h and the formulation was able to release 98.10 ± 0.200 % drug by the end of 24h of the study.

No significant change in particle size was observed over the three-month storage duration suggesting that the formulations were stable at the storage conditions. The oil in water cream prepared by incorporating the liposome was found to be homogenous, non-gritty, smooth, white in color with a pH of 6.10 ± 0.021 and spreadability of 56.67 ± 3.055 g.cm/5min. The amount of azithromycin released from the cream by the end of 24 h (93.53 ± 0.404 %) almost equivalent to the liposome.

4.2 Conclusion

The present study successfully demonstrated the feasibility of encapsulating Azithromycin in liposomes prepared by the film hydration method

using phosphatidylcholine and stearyl alcohol. Optimization through D-optimal design yielded a formulation with desirable physicochemical properties, including high entrapment efficiency ($65.80 \pm 0.361\%$), small particle size (180.6 nm, PDI 0.213), and adequate stability (zeta potential -21.7 mV). The liposomal system provided sustained drug release, achieving $98.10 \pm 0.200\%$ release over 24 hours, thereby confirming its potential for controlled delivery. Incorporation of the optimized liposomes into an oil-in-water cream base resulted in a formulation that was homogenous, smooth, stable, and cosmetically acceptable, with a near-neutral pH and good spreadability. Importantly, the cream maintained the controlled release profile of the liposomes, with $93.53 \pm 0.404\%$ drug release at 24 hours, closely matching the performance of the liposomal suspension.

Overall, the findings validate the initial hypothesis: liposomal encapsulation of Azithromycin enhances drug stability, prolongs release duration, and enables effective incorporation into a topical cream formulation with improved bioavailability. This approach offers a promising strategy for the development of topical Azithromycin formulations aimed at achieving sustained therapeutic action and patient compliance.

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