Formulation optimisation and characterisation of azithromycin proniosome

Ramkanth S*, Aravind M, Gayathri R, Benedict Jose C, Swetha V, Mohan S
Department of Pharmaceutics, Karpagam College of Pharmacy, Coimbatore – 641032, Tamilnadu, India

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ABSTRACT
The research was featured to formulate, characterise and optimise the Azithromycin Proniosome using the three-factor three-level Box Behnken scheme. The independent variables chosen were span 20 (X1), span 80 (X2) and phospholipids (X3) to assess their individual and shared response on entrapment efficiency (Y1) and % drug released at six h (Y2). Based on Box Behnken design, 15 formulations were prepared and optimised using Design Expert Version 12.0.10.0. The entrapment efficiency and \textit{in-vitro} drug release were exposed to different regressions to setup a polynomial equation. The counterplots understood the connections between the independent and dependent factors. The congruity of the polynomial equations was tested by fixing three checkpoint groups. The optimised formulation was determined by point prediction method using the various constraints. The optimised batch was subjected to multiple parameters such as vesicle shape, viscosity, spread ability, drug content, entrapment efficiency, \textit{in-vitro} drug diffusion and stability studies. The optimised formulation shows better physical and chemical stability which is confirmed by the results of various parameters. The \textit{in-vitro} drug diffusion has observed to be 98.53% at 24th h obeying zero-order drug release with diffusion mediated non-fickian type of drug release kinetics. The formulation preserved at both refrigerated and room temperature shows better stability. This approach might be an additional finding in enhancing the adherence of patient and improves compliance.

*Corresponding Author
Name: Ramkanth S
Phone: +91-9618312122
Email: ramkanthsg@gmail.com

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INTRODUCTION
Vesicular systems are turning out to be driving aspects in the arena of drug delivery. Numerous investigators are progressively working on these areas for refining their physical, chemical and biological properties. Liposomes, niosomes, pharmacosomes, proniosomes, proliposomes and transferosomes are few instances of the vesicular delivery that are appealing much consideration because of their adaptability to be customised benefits for varied attractive purposes (Talegaonkar \textit{et al.}, 2006). Proniosomes are formulation having water-soluble transporter particles that are layered with surfactant and can be allotted varying and dried out to shape niosomal dispersion preceding use on transitory agitation within a few minutes in hot aqueous media. The resultant niosomes are fundamentally the same as conventional niosomes and progressively uniform in size.
for aerobes and anaerobes found in the periodontal pocket. Azithromycin is commonly used for a wide variety of less to average bacterial contaminations brought about by vulnerable strains of the assigned microorganisms in the particular conditions: *Streptococcus pyogenes*, *Mycoplasma pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *Staphylococcus agal*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Chlamydia pneumoniae*, etc. Azithromycin (AZI) extends a spectrum of antibiotic action and expands tissue pharmacokinetic features compared with erythromycin (Newman and Takei, 2006; Dunn et al., 1996). AZI absorbs quickly and has a two-compartment model with extravascular administration, while its treatment frequently has a contrary effect on the gastrointestinal tract. The bio availability of azithromycin is 38 %, which is low bio availability for the oral dosage form. Hence, Proniosome based azithromycin is planned to formulate and optimise by three factors three levels Box-Behnken Design to improve bio availability through better penetration, overcome the first-pass metabolism, minimise the GI side effects and improve patient compliance through enhancing the dosage adherence.

**MATERIALS AND METHODS**

Azithromycin was a gift sample obtained from Medopharm Pvt Ltd., Chennai. Carbolop 940 was purchased from Himedia Laboratories, Mumbai. Phospholipid was a gift sample received from Lipoid, Switzerland. Cholesterol, Disodium hydrogen phosphate, Sodium chloride, Potassium dihydrogen orthophosphate and triethanolamine were purchased from Nice chemicals Pvt Ltd., Coimbatore. All the reagents and chemicals used were of analytical grade.

**Methodology**

**Drug-excipient compatibility study**

The drug-excipient compatibility studies were accomplished to find the interaction among drug and excipients. A liquid mixture of medicine, span 20, span 80, phospholipid and cholesterol was scanned from 4000-400cm⁻¹ in an FTIR-8400 Shimadzu, Japan. The individual spectra of drug, phospholipid, and cholesterol also were performed to identify the interactions and compatibility (Imam et al., 2015).

**Box-Behnken Experimental Design**

The utilisation of experimental design takes into account for testing countless factors concurrently. It excludes the utilisation of a massive number of independent runs when the conventional process-wise approach is utilised. Logical optimisation procedures are performed by choosing an objective, discovering the most significant factors and exploring the connections between responses and factors is named as response surface methodology. Hence, a Box-Behnken statistical design (Design-Expert version 12.1.10.0) with three variables, three levels, and 15 runs was chosen to statistically optimise the dosage form preparation parameters and evaluate the vital effects, interaction effects and quadratic effects of the components used for formulation on the % encapsulation efficiency of proniosomes and % drug released (Motwani et al., 2007; Box and Behnken, 1960). A 3-variable, 3-level design was utilised to investigate the quadratic response surfaces and for developing second-order polynomial models thus serving in optimising a procedure using a few trial runs (Cansee et al., 2008). The Box-Behnken design was precisely chosen since it requires fewer runs compared with a central composite design, in instances of three or four factors. The experimental design comprises of a lot of points lying at the midpoint of each edge and the replicated centre point of the multidimensional cube.

The independent variables designated were span 20 (X1), span 80 (X2) and phospholipids (X3) to assess their distinct and collective effects on entrapment efficiency (Y1) and % drug released at six h (Y2). The independent and dependent variables are listed in Table 1.

**Formulation of Azithromycin loaded proniosomes**

The gel formulation of azithromycin was set up by utilising the vesicular method (proniosome). Initially, the proniosome was formed and then blended with carbopol 934 gel which is used as a penetration enhancer. Proniosome was formulated by coacervation phase separation method. A precisely weighed measure of surfactant (span 20), (span 80), phospholipid (carrier), cholesterol, and the drug was mixed in a fresh and wide-mouthed dry glass vial and add 1.3 ml of alcohol. After warming, by using a glass rod, all the ingredients were mixed well, later the glass bottle was enclosed with a closure to avoid the waste of solvent from it and warmed-over water bath at 60-70°C for a time duration of 5 min until the mixture of surfactant was dissolved absolutely. At this point, the aqueous phase (7.4 phosphate buffer solution) 1.6 ml was added and warmed on a water bath for about 2 min. Finally, the preparation is mixed with 2% w/v of carbopol neutralised with triethanolamine to form a proniosomal gel (Vashist et al., 2015).
Table 1: Variables and their Levels in Box-Behnken Design

| Independent variables                                      | Levels       |
|------------------------------------------------------------|--------------|
| X1 = Span 20 concentration (mg)                            | Low: 300     |
|                                                             | Medium: 600  |
|                                                             | High: 900    |
| X2 = Span 80 concentration (mg)                            | Low: 300     |
|                                                             | Medium: 600  |
|                                                             | High: 900    |
| X3 = Phospholipid concentration (mg)                       | Low: 700     |
|                                                             | Medium: 900  |
|                                                             | High: 1100   |

Independent variables

Y1 = Encapsulation efficiency

Constraints

Maximize

Y2 = % drug released at 6 hours

Minimize

Table 2: Interpretation of the FTIR spectrum

| S.No | Functional Group | Wavenumber | Azithromycin | Phospholipids | Cholesterol | Proniosome |
|------|------------------|------------|--------------|---------------|-------------|------------|
| 1    | C6H6             | 1600 & 1500-1430 | -            | -             | 1465.90     | 1458.18    |
| 2    | C-O              | 1780-1650   | 1651.07      | 1735.93       | 1705.07     | 1651.07    |
| 3    | C-O              | 1250-1050   | 1080.14      | 1087.85       | 1134.14     | 1049.28    |
| 4    | C-O-C            | 1300-1000   | 1010.70      | 1242.16       | -           | 1226.73    |
| 5    | P-O-C            | 1145-970    | -            | 1064.71       | -           | 1087.85    |
| 6    | O-H              | 3700-3500   | 3549.02      | 3741.90       | 3441.01     | 3734.19    |
| 9    | C-H              | 3300-2700   | 2877.79      | 2924.09       | 2893.22     | 2924.09    |
| 10   | -CH3             | 3200-2800   | 2978.09      | 2854.65       | 2931.80     | 2978.09    |
| 11   | C-N              | 1350-1000   | 1303.88      | 1242.16       | -           | 1381.03    |
| 12   | PO2              | 1200-1145   | -            | 1165          | -           | 1165       |
| 13   | P-O-C + PO2      | 1200-970    | -            | 964.71        | -           | 956.69     |
| 14   | C=C              | 1722-1665   | -            | -             | 1705.07     | 1735.93    |

Figure 1: FTIR spectrum of azithromycin
Table 3: Observed responses in Box–Behnken design

| Formulation Code | Factor 1  | Factor 2  | Factor 3  | Response 1 Encapsulation efficiency | Response 2 % drug diffusion at 6th h |
|------------------|-----------|-----------|-----------|-------------------------------------|-------------------------------------|
| AZI 1            | 900       | 600       | 700       | 92.48                               | 22                                  |
| AZI 2            | 300       | 600       | 700       | 93.58                               | 23.8                                |
| AZI 3            | 900       | 300       | 900       | 97.16                               | 27.2                                |
| AZI 4            | 900       | 600       | 1100      | 94.76                               | 24.7                                |
| AZI 5            | 600       | 600       | 900       | 93.94                               | 24.9                                |
| AZI 6            | 600       | 300       | 700       | 94.75                               | 24.6                                |
| AZI 7            | 900       | 900       | 900       | 93.51                               | 22.4                                |
| AZI 8            | 600       | 900       | 700       | 93.16                               | 22.9                                |
| AZI 9            | 300       | 900       | 900       | 92.81                               | 22.5                                |
| AZI 10           | 600       | 900       | 1100      | 95.17                               | 24.5                                |
| AZI 11           | 600       | 300       | 1100      | 96.61                               | 27.3                                |
| AZI 12           | 300       | 300       | 900       | 95.73                               | 23.7                                |
| AZI 13           | 600       | 600       | 900       | 95.49                               | 24.3                                |
| AZI 14           | 300       | 600       | 1100      | 95.41                               | 25.6                                |
| AZI 15           | 600       | 600       | 900       | 95.51                               | 25.1                                |

Figure 2: FTIR spectrum of Phospholipids

Table 4: Statistical parameters obtained for various responses

| Responses                          | Sequential p-value | Lack of fit p-value | Predicted R² | Adjusted R² |
|------------------------------------|--------------------|---------------------|--------------|-------------|
| Encapsulation efficiency           | 0.5942             | 0.4387              | 0.4710       | 0.9844      |
| % drug diffusion at 6 h             | 0.5462             | 0.0971              | 0.5818       | 0.8332      |
Figure 3: FTIR spectrum of Cholesterol

Figure 4: FTIR spectrum of proniosome formulation

Table 5: Stability studies data of the optimized formulation

| Time of storage in days | 4 °C-2 °C (Refrigerator temperature) | 37 °C ± 2 °C (Room temperature) |
|------------------------|--------------------------------------|----------------------------------|
|                        | Drug content (%)                     | Encapsulation efficiency (%)     | Drug content (%) | Encapsulation efficiency (%) |
| 0                      | 95.55                                | 95.08                            | 95.37            | 95.16                        |
| 15                     | 95.31                                | 94.72                            | 94.64            | 94.35                        |
| 30                     | 95.05                                | 94.25                            | 93.56            | 93.22                        |
Evaluation of Independent Variables

Determination of encapsulation efficiency

Proniosome gel (0.2 g) was weighed in a beaker containing 10 ml of phosphate buffer (pH 7.4 to convert to niosomal dispersion), and it was sonicated for 10 mins. The azithromycin containing niosomes was separated from the untrapped drug by centrifugation at 6,000 rpm at 37°C for 30 min. The supernatant was recovered and assayed spectrophotometrically at 275 nm (Vashist et al., 2015).

The following equation calculated the percentage of encapsulation efficiency (EE (%)):

\[
EE(\%) = \left( \frac{C_t - C_r}{C_t} \right) \times 100\%
\]

Where,

- \( C_t \) is the concentration of total azithromycin.
- \( C_r \) is the concentration of free azithromycin.

In-vitro percentage drug release for six h

In vitro diffusion rate were carried out by Franz-diffusion cell. The active permeation area of the chamber was 1.41 cm² and accommodated 15 ml in the receptor compartment. The dialysis film of cel-lophane was placed partitioning the superior donor and lower receptor compartment. The medium of the receptor chamber holds phosphate saline buffer pH 7.4. The proniosomal gel was positioned on either side of the dialysis membrane. The receptor was circulated with a controlled temperature of...
water jacket at 37 ± 1°C. The fluid in the receptor was agitated by a magnetic bead (Teflon-coated) controlled by a magnetic stirrer. Samples in the receptor chamber were removed periodical time interval and substituted instantly with an identical volume of receptor media. The concentration of drug was analysed by UV spectrophotometrically (Shimadzu-1800) at 275 nm (Ramkanth et al., 2018; Anitha et al., 2011).

Characterisation of Optimised Azithromycin Loaded Proniosomal Gel

Vesicle Size

The confirmation of niosomal hydration of the Proniosomal formulations was viewed under optical microscopy. Momentarily, a tinny layer of the proniosome gel was flattened over the glass slide, covered with a coverslip and focused under projection microscope at 100 x magnification (Gupta et al., 2007).

Shape & surface morphological

0.2 g proniosome gel was diluted in a glass tube with 10 ml of (pH 7.4) phosphate buffer. The diluted dispersion was scattered and secured on a scanning electron microscopy (SEM) frame with dual - side adhesion with a gold coat for 3 min, implying a sputter coater. Niosome prepared after hydration of proniosomes were evaluated for their surface morphology, shape, size. The samples were examined using a SEM (Tescan – Mira3 XMU) at 15 kV accelerating voltage (Azarbayjani et al., 2009).

Drug Content

100 mg equivalent Proniosomes was transferred in a standard volumetric flask. 50 ml methanol for 15
min shaking is incorporated to induce lysis. The resulting solution was diluted with methanol to 100 ml. Withdraw 10 ml from the solution and makeup to 100 ml with (7.4) phosphate buffer. The final diluted solution was measured at 275 nm, and drug content was estimated (Tomar and Singhal, 2015).

\[ \text{% Drug content} = \frac{\text{Amount of the drug in gel}}{\text{Amount of total gel}} \times 100 \]

**Measurement of pH**

The pH of the formulations was estimated by a digital pH meter. One gram of gel was allowed to get into solution in 100 ml of distilled water and preserved for two hours. A triplicate measure was estimated for respective formulations.

**Measurement of viscosity**

The prepared gels were assessed for viscosity with Brookfield viscometer (Brookfield viscometer RVT) with spindle NO.62 (Tomar and Singhal, 2015).

**Measurement of spreadability**

Spreadability was performed by applying an excess of the sample in between 2 glass slide & pressure was applied in such a manner to form a thickness in a similar pattern by employing 1000 gm weight for 5 minutes. In the opposite side, 50 gm weight has been added. The time duration to detached both the slides is measured.

\[ S = \frac{(m \times l)}{t} \]

\[ S = \text{Spreadability} \quad m = \text{Weight tied to upper slide} \quad L = \text{Length moved on upper glass slide} \quad t = \text{Time taken.} \]

**In-vitro drug diffusion study**

In vitro diffusion studies on were performed as mentioned earlier whereas the samples were withdrawn for 24 h and examined by UV spectrophotometry (Ramkanth et al., 2018; Anitha et al., 2011).

**Release kinetic studies**

The precise mechanism of drug diffusion from the gels, drug diffusion data were predicted according to zero, first-order, Higuchi’s and Korsmeyer-Peppas equations. Most fitting models were chosen based on the best of fit test (Rani and Singh, 2018).

**Stability Study**

The prepared gel formulation was packed in a tight container keeping away from the air at two different temperature environments, i.e. cooling temperature (4–8 °C) and room temperature (37±2°C). At various time interval, the samples were withdrawn at intervals upon one month and evaluated for drug content and encapsulation efficiency.

**RESULTS AND DISCUSSION**

**Drug-excipient compatibility study**

The FTIR spectrum of the drug and carrier blends shown in spectrum indicates no modification in distinctive peaks of the drug. Hence no interaction occurred amongst the drug and carrier. The FTIR spectra and its interpretation were shown in Figures 1, 2, 3 and 4 and Table 2.

**Formulation of azithromycin loaded proniosomes**

By using box – Behnken experimental design, 15 different azithromycins proniosome formulation was prepared, which is converted into proniosome gel using carpololl and triethanolamine. Gel base was formulated in the 1 % ratio of 0.2 g in 10 ml purified water. The prepared formulations were stable and consistence and stored in refrigerator temperature until used for further studies.

**Encapsulation efficiency**

All the formulation were observed for encapsulation efficiency by using UV spectrophotometer. EE % for all the formulation range from 92.48 % to 97.16%.

**In-vitro drug diffusion studies for six h**

In-vitro drug diffusion for all 15 formulations to be performed and observed in UV spectrophotometer.
at 275 nm range. **In-vitro** drug release range for all the formulations was found to be in the range from 22 to 27.3 %. The drug diffusion graph for all was shown in Figure 5.

**Data Analysis**

Three independent variables at three different levels Box-Behnken design was applied to know the effects on dependent variables. All 15 formulations of proniosomes executed among the experimental design yielded niosomes on hydration, and these were assessed for the entrapment efficiency (EE %) and % drug diffusion at six h. The factors and its response were shown in Table 3. The Predicted Vs Actual, Response Surface and Counter plot of encapsulation efficiency & % drug diffusion at six h were shown in Figures 6, 7 and 8 respectively.

The quadratic model resulted in the following polynomial equation for the independent variables in terms of coded factors (Table 4), as are follows:

Encapsulation efficiency = 94.98 + 0.0474 A - 1.2 B + 0.9975 C - 0.1824 AB + 0.1125 AC + 0.0375 BC - 0.5212A² + 0.3437B² - 0.4012C²

% Drug Release at 6th h = 24.76 + 0.087A - 1.3175B + 1.105 C - 0.9 AB + 0.225 AC - 0.813 B² - 0.0033 A² + 0.0716 C²

**Optimum formulation**

The optimum proniosomal formulation was chosen based on a high % of EE and a controlled drug diffusion with maximum quantity. It is apparent from the polynomial equation and plots that increasing the amount of phospholipid increases the EE% and the % drug released after six h.

High level was selected as optimum for the phospholipid concentration % (X3). It is clear that the total lipid concentration increases the EE% within niosomes and decreases the % drug released after six h from niosomes. So, the high level was selected as optimum for the total lipid concentration % (X3). Based on the optimisation and contour plots for X3, we chose the medium level of 100 mg of the drug, which gives the theoretical value of 95.51%, 24.9 % for EE% and % drug released after six h, respectively. Hence, 600 mg of span 20 concentration (X1), 600 mg of span 80 concentration (X2), and 900 mg of phospholipid concentration (X3) were selected as optimal. To confirm, a new formulation was formulated at the optimised quantity of the independent variables, and the resulting proniosomes were converted to niosomes and estimated for the responses. The observed values of EE% and % drug released at six h were found to be 95.8 %, 25.4%, respectively, which were in close agreement with the theoretical values. So, that AZI 15 was the optimised formulation which is further evaluated.

**Characterisation of optimised formulation**

Particles are round spherical shape. The particles were of good morphological characteristics, having a rough surface with a particle size range of 1-500 μm is shown in Figure 9. Drug content for the optimised azithromycin proniosomal transdermal gel was found to be 99.58%. Encapsulation efficiency for the optimised azithromycin proniosomal transdermal gel was found to be 95.8%. High viscosity gels do not easily squeeze out from container whereas, low viscous may immediately flow away, and hence appropriate viscosity is required to extrude a gel. AZI 15 have the excellent viscosity of 71280 cps. The values of spreadability were 23.25 gm.cm/sec, which shows the gel with minimal shear is easily spreadable.

**In-vitro drug diffusion study**

The **in-vitro** drug diffusion study states that the drug release was 98.53% at 24th h for the final formulation. The drug diffusion graph was shown in Figure 10. The r² value of the in-vitro graph shows 0.998, which denotes the drug release obeys zero kinetics drug release ensuring the controlled release pattern. The r² value of Higuchi’s plot was found to be 0.9734, and the n value of peppa’s plot was found to be 0.646 which denoted that it undergoes diffusion mediate the non-fickian type of drug diffusion process.

**Stability studies**

The optimised final formulation AZI 15 was found to be stable for one month; it was observed that the gel shows better drug content and encapsulation efficiency without significant deviations at both the temperature. However, at the end of the 30th-day study, the formulation stored at 4° C shows better results and physical stability, drug content and encapsulation efficiency. Hence it is suggested to store the formulation at refrigerator temperature. The stability studies were represented in Table 5.

**CONCLUSIONS**

The present study demonstrates the prepared proniosomes by coacervation phase separation method using Box Behnken design was stable and effective, which shows better release with good characteristics features. The prepared formulation shows better stability and meets out the objective of the study. Thus this approach might be an additional finding in enhancing the adherence of patient and improves compliance.
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Conflict of Interest

The authors have declared no conflict of interest for this study.

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