RESEARCH ARTICLE

PRELIMINARY FORMULATION STUDY OF Lycopene Self-Assemble Nanoparticles Via Box-Behnken Design Imparting an Efficient Drug Delivery: Statistical and Illustrative Approach

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Abstract

Lycopene, a structurally polyene conjugated antioxidant, is frequently utilized to treat ailments induced by augmented oxidative stress. Its colossal structure and poor water-solubility failed to show the requisite clinical outcomes. To overcome these drawbacks, a formulation study introducing Lycopene as Chitosan-phosphatidylserine self-assembled nanoparticles (LNP) to correlate the dependent and independent variables employing Box-Behnken design has been performed. LNP formulation was achieved by injection technique, using chitosan's intrinsic property of towards Phosphatidylserine for self-assembly. Independent variables, i.e., chitosan concentration (X₁), Phosphatidylserine (X₂), and injecting rate (X₃) were correlated against dependent variables (responses) nanoparticle size, cumulative release, and lycopene encapsulation. Prepared nanocaps were stable, under 300 nm particle size, Zeta potential below +30 mV, close PDI to 0.3, and exhibited invitro 69-76% cumulative release. Overall, the formulation strategy was fruitful to develop a formulation in the future for efficient drug delivery.

Introduction:
Pharmaceutical industries are enforcing well to introduce natural drugs by augmenting their bioavailability. Several studies are going on to overcome poor bioavailability and solubility of these drugs, but only Nano-drg delivery has given hope for natural drugs to be at par with the currently in use chemical drug molecules. Nanoparticles have several advantages i.e., imparting solubility, good bioavailability, high payload and accurate organ targeting of any drug with controlled mechanism, especially to brain [1-3]. Recently, nanoparticles of numerous antioxidants like piperine, curcumin, beta-carotene, and thymoquinone formulated and augmented the bioavailability and solubility of the original compound [4-7]. Lycopene known for its variety of biological activity, mainly acting via Oxidative stress mechanisms[8]. Lycopene’s giant molecular structure, weight, volume, and geometrical isomerism implicates poor pharmacokinetics, these factors often impart poorer water-solubility affecting its bioavailability [9-11].

Formulation of nanoparticles is done using several techniques, including varied raw material concentrations, giving rise to chances of several biohazards posed by the byproducts formed in the design process [12, 13]. Self-assembly approach is the best remedy to deal with this problem, as the technique is devoid of any byproduct or intermediate formation[14, 15]. Natural polysaccharides like Chitosan are safe with higher bio-acceptability and easily processed
inhum gut[16]. Otherwise, phosphatidylserine also approved by FDA as a safer dietary product [17]. Hence, we intended to prepare and optimize LNP from chitosan and phosphatidylserine as no such study reported until now.

**Materials:-**
Lycopene, ethyl-acetate, Chitosan, Phosphatidylserine (PS), hexyl hydride, filter (WF), 33 mm Cellulose dialysis tube-membrane (CD), 0.45μm Whatman, phosphate buffer (PBS), ethanol, HCl, were obtained from Sigma Aldrich.

**Methods:-**

**Lycopene Nanocrops Formulation:**
PS (X₂) and Lycopene (25 mg) dissolved in 90% ethanol (10 ml) to get Solution A. Stock solution of acidified (0.2 M) Chitosan (X₁) was ready and put overnight to get solution B (93cP). Solution A (4 ml) progressively was interspersed in solution-B (46 mL), by a steady injecting rate (X₃) under 1450 rpm homogenization and filtered to obtain Lycopene nanocrops. All nanocrops were lyophilized and stored. Size, charge, and distribution were computed by Zetasizer (Nano-Z90, Malvern, UK) and surface morphology was evaluated by TEM.

**Experimental design:**
Box-Behnken Design optimized all nanocrops via Design-Expert® Version (V.12, Stat-Ease, USA). Independent variables, i.e., chitosan concentration (X₁), Phosphatidylserine (X₂), and injecting rate (X₃) (levels coded −1,0,+1, Table 1) were designated to relate with dependent variables (responses) i.e. nanoparticle size, lycopene Encapsulation, and cumulative release were studied. Design-Expert® performed 17 hits(Table 2)

| Table 1: Experimental design with Independent variables. |
|---|---|---|---|
| Codes | Independent Variables | Levels |
| X₁ | Chitosan (w/v) | -1 | 0 | +1 |
|   |   | 5 | 10 | 15 |
| X₂ | PS (mg/ml) | 2.5 | 5.00 | 7.5 |
| X₃ | Injection rate (ml/min) | 5.0 | 10.0 | 15 |

| Table 2: Box-Behnken Design with level values. |
|---|---|---|---|
| Box-Behnken | Nanocrops | Chitosan (w/v) | PS (mg/ml) | Injection rate (ml/min) |
| NC-1 | -1 | -1 | 0 |
| NC-2 | +1 | -1 | 0 |
| NC-3 | 0 | -1 | -1 |
| NC-4 | 0 | -1 | +1 |
| NC-5 | 0 | 0 | 0 |
| NC-6 | +1 | 0 | -1 |
| NC-7 | 0 | 0 | 0 |
| NC-8 | +1 | 0 | +1 |
| NC-9 | 0 | 0 | 0 |
| NC-10 | -1 | 0 | +1 |
| NC-11 | 0 | 0 | 0 |
| NC-12 | 0 | 0 | 0 |
| NC-13 | -1 | 0 | +1 |
| NC-14 | -1 | +1 | 0 |
| NC-15 | +1 | +1 | 0 |
| NC-16 | 0 | +1 | -1 |
| NC-17 | 0 | +1 | +1 |

**Lycopene% Encapsulation Efficiency (%EE):**
100 μL of Lycopene Nanocrop was extracted in hexyl hydride (3 ml, 3000 rpm, 4 minutes), floating layer of hexyl hydride was parted and diluted to quantify free Lycopene at 471 nm by Ultraviolet spectroscopy equations:

\[
\text{EE}\% = \frac{(\text{Total LYC} - \text{Free LYC})}{\text{Total LC}} \times 100
\]
TEM LNP surface Morphology:
Optimized Nanocrop was visualized via TEM (268D, Philips, USA). Nanocrops kept on 300 size copper grid, with dropping phosphotungstic acid (10 sec), observed under 70-80 KV, magnification 30000x.

Invitro Lycopene cumulative Release (CR) from Lycopene Nanocrop:
Lycopene CR from Nanocrop was detected in PBS (pH 7.4) at 37°C. 1 ml Nanocrop was sandwiched in dialysis bag (Sigma-Aldrich, CA, USA), hanged in PBS (10 ml) kept with constant agitation (150 rpm) at 25°C. At set time intervals, 1 ml was reinstated with fresh volume for 24 hrs and quantified by Ultraviolet spectroscopy (471 nm).

Results and Discussion:-
Statistical assessment of nanocrops:
All the formulation results were examined by Design-ExpertV.12. Numerical optimized method was chosen to access independent variables in relation to the responses. All the responses followed Quadratic polynomial equations (Table 3) and significant findings are reported as per ANOVA analysis.

Table 3:- Polynomial equations as coded factors for Particle size, % EE and Cumulative Release.

| Actual Factors                  | Coded | Particle size | % EE | Cumulative Release |
|--------------------------------|-------|---------------|------|--------------------|
| Intercept                      | 220.14| 30.64         | 72.20|
| Chitosan A                     | +38.58| -0.0500       | -3.50|
| PS B                           | +3.79 | +7.66         | +0.0000|
| Injection rate (ml/min) C      | -2.74 | +0.1375       | +0.0000|
| Chitosan * PS AB               | +1.43 | -0.3000       | +0.0000|
| Chitosan * Injection rate (ml/min) AC | -3.17 | +0.7500       | +0.5000|
| PS * Injection rate (ml/min) BC | +6.45 | -0.1250       | +0.5000|
| Chitosan ² A²                  | +4.30 | -0.8075       | -0.1000|
| PS ² B²                        | +8.58 | +0.6675       | +0.4000|
| Injection rate (ml/min)³ C³    | +7.08 | +1.07         | +0.4000|

Table 4:- Box-Behnken Design with all dependent and independent variables.

| Nanocrops | STD | Run | X₁ | X₂ | X₃ | Chitosan (w/v) | PS (mg/ml) | Injection rate (ml/min) | Dependable Variable |
|-----------|-----|-----|----|----|----|----------------|------------|------------------------|--------------------|
| NC-1      | 1   | 4   | -1 | -1 | 0  | 5              | 2.5        | 10                     | Size               |
| NC-2      | 2   | 8   | +1 | -1 | 0  | 15             | 2.5        | 10                     | % EE               |
| NC-3      | 9   | 1   | 0  | -1 | -1 | 10             | 2.5        | 5                      | Cumulative Release |
| NC-4      | 11  | 17  | 0  | -1 | +1 | 10             | 2.5        | 15                     |                   |
| NC-5      | 17  | 11  | 0  | 0  | 0  | 10             | 5          | 10                     |                   |
| NC-6      | 6   | 14  | +1 | 0  | -1 | 15             | 5          | 5                      |                   |
| NC-7      | 16  | 16  | 0  | 0  | 0  | 10             | 5          | 10                     |                   |
| NC-8      | 8   | 9   | +1 | 0  | +1 | 15             | 5          | 15                     |                   |
| NC-9      | 14  | 2   | 0  | 0  | 0  | 10             | 5          | 10                     |                   |
| NC-10     | 7   | 10  | -1 | 0  | +1 | 5              | 5          | 15                     |                   |
| NC-11     | 13  | 6   | 0  | 0  | 0  | 10             | 5          | 10                     |                   |
| NC-12     | 15  | 3   | 0  | 0  | 0  | 10             | 5          | 10                     |                   |
| NC-13     | 5   | 12  | -1 | 0  | +1 | 5              | 5          | 5                      |                   |
| NC-14     | 3   | 7   | -1 | +1 | 0  | 5              | 7.5        | 10                     |                   |
| NC-15     | 4   | 15  | +1 | +1 | 0  | 15             | 7.5        | 10                     |                   |
| NC-16     | 10  | 13  | 0  | +1 | -1 | 10             | 7.5        | 5                      |                   |
| NC-17     | 12  | 5   | 0  | +1 | +1 | 10             | 7.5        | 15                     |                   |
Particle size reliability on Independent Variables:
Particle size affects bioavailability and physical stability of any drug. Smaller particle size offers high surface area to provide better tissue dispersal and cellular access. Nanoparticles over 230 nm trapped by spleen and decreases the bioavailability of drug[18, 19]. Particle size and responses are shown in Table 4. As per quadratic paradigm, F-value of 311.08 proved a high significance (p=0.0001), whereas P values for chitosan concentration (p=0.0001), Phosphatidylserine (p=0.0016) and injecting rate (p=0.0087) with regression coefficient (R²) of 0.9667 adjusted to 0.9943 representing nearly no variation in experimental model (Figure 6). Adequate precision of 51.351 signifying appropriate navigation for design space. Chitosan concentration increases the particle size, where phosphatidylserine concentration or and injecting rate not affected much. Increase particle size (189-287 nm) may be of high viscosity or chitosan in medium. Relationship between particle size and dependable factors is shown in response surface graphs. (Figure 1)

![Figure 1](image1.png)

Figure 1: Response surface graphs for chitosan concentration, Phosphatidylserine (PS) and injecting rate on particle size

%Encapsulation Efficiency reliability on Independent Variables:
Encapsulation efficacy offers real drug payload to any nanoformulations, it directly affects the drug bioavailability. %EE and responses are illustrated in Table 4. As per quadratic paradigm F-value of 28.81 proves a high significance (p=0.0001) whereas P values for chitosan concentration (p=0.9203), Phosphatidylserine (<0.0001) and injecting rate (p=0.7836) with regression coefficient (R²) of 0.9737 adjusted to 0.9399 representing nearly no variation in experimental model (Figure 6). Adequate precision of 16.70 signifying appropriate navigation for design space. With increase of Phosphatidylserine concentration, this specifies that due to lipophilic nature of lycopene it was solvated in phosphatidylserine core via making electrostatic interaction or conjugated interaction with PS structure, Which has given encapsulation efficiency ranging 21-39%. Relationship between %EE and dependable factors is shown in response surface graphs. (Figure 2)

![Figure 2](image2.png)

Figure 2: Response surface graphs for chitosan concentration, Phosphatidylserine (PS) and injecting rate on %EE

Cumulative release reliability on Independent Variables:
Cumulative release gives prediction of drug deliverance in a similar biological system. The superiority of any formulation directly depends upon the drug amount released and affecting the bioavailability of any drug. Drug-
release is a direct polymer attribute, which affects both stability and therapeutic effectiveness of any drug. Mostly polymer offers a rapid initial release called the burst mode, which also depends upon polymer matrix offering drug encapsulation[20]. Cumulative release and responses are illustrated in Table-4. As per quadratic paradigm, F-value of 8.97 proves a significance (p=0.0043) whereas P values for chitosan concentration (p<0.0001), Phosphatidylserine (p=1), and injecting rate (p=1) with regression coefficient (R²) of 0.9202 adjusted to 0.8175 representing nearly no variation in experimental model (Figure 6). Adequate precision of 9.3 signifying appropriate navigation for design space. Cumulative release was found dependent on all the dependable factors and ranged from 67-76%. Relationship between Cumulative release and dependable factors is shown in response surface graphs[21]. (Figure 3)

![Figure 3: Response surface graphs for chitosan concentration, Phosphatidylserine (PS), and injecting rate on Cumulative release](image)

**Optimization and validation:**
According to desirability, separate optimized Batch of LNP was fabricated (Table 5). The findings are illustrated through digitally generated contour graphs (Figure 4). The relative error (<5%) was calculated between the predicted and actual values showing negotiable variation in the group (Figure 5 and 6).

**Table 5:- Optimized Levels of independent Variables**

| Codes | Independent Variables | Optimized levels |
|-------|-----------------------|------------------|
| X₁    | Chitosan (w/v)        | 12.10            |
| X₂    | PS (mg/ml)            | 3.78             |
| X₃    | Injection rate (ml/min)| 9.31             |

![Fig. 4: Desirability outcomes for chitosan concentration, Phosphatidylserine (PS) and injecting rate](image)
Fig. 5: Desirability outcomes for chitosan concentration, Phosphatidylserine (PS), and injecting rate
Figure 6: Representation of Relative error among chitosan concentration, Phosphatidylserine (PS) and injecting rate.

Figure 7: Predicted and actual responses for optimized formulation.

TEM characterization of optimized Lycopene nanocrop:
Fabricated LNP was evaluated for morphological examination. Actual and Predicted values are shown in Table 6, and Figure 7. TEM picture predicts regular, dispersible, and uniformly distributed formulation (Figure 8). Their size measured around 198.37±2.8 nm within the range PDI of 0.519, Zeta potential of 29.8±3.12 mV, sharing an encapsulation efficiency 39.13±1.25%, thru good cumulative release of 73%.

Table 6: Predicted VS actual results of dependent factors

| Dependent Variables     | Predicted | Actual |
|-------------------------|-----------|--------|
| LNP Size (nm)           | 196.1     | 198.3  |
| EE (%)                  | 39.9      | 39.1   |
| Cumulative release (%)  | 73.8      | 73.0   |
Conclusion:-
Self-assembled nanoparticles of lycopene (LNP) were effectively optimized and prepared from chitosan and Phosphatidylserine through Box-Behnken design to gain desired attributes. In the future LNP can be used for safe and effective delivery of the parent compound to the targeted biological system.

Disclosure:-
There are no conflicts of interest related to present research work.

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