RESEARCH PAPER

DESIGN OF EXPERIMENTS ASSISTED CHARACTERIZATION AND DEVELOPMENT OF ISOQUINOLINE ALKALOID LOADED NANOSTRUCTURED LIPID CARRIERS

Abhishek Verma[1*], Shubhini A. Saraf[1] and Vijayakumar Mahalingam Rajamanickam[1]
[1] Department of Pharmaceutical Sciences, Babasaheb Bhimrao Ambedkar University, Vidya Vihar, Raebareli Road, Lucknow-226025, Uttar Pradesh, India
*** Corresponding author:**
Abhishek Verma
Department of Pharmaceutical Sciences, Babasaheb Bhimrao Ambedkar University, Lucknow - 226029, Uttar Pradesh, India, Email: bbau.abhishek@gmail.com

Abstract:
Lipid-based nanocarriers are a promising strategy to improve the pharmaceutical performance of poorly water-soluble compounds. The present study aimed to develop and optimize nanostructured lipid carriers (NLCs) loaded with the isoquinoline alkaloid M1 using a design of experiments (DOE) approach. M1-loaded NLCs were prepared using Precirol ATO 5 as solid lipid, olive oil as liquid lipid and Tween 80 and Span 80 as surfactants. The effects of total lipid concentration, total surfactant concentration, and sonication time on particle size and entrapment efficiency were evaluated using a Box–Behnken design. The experimental data were analyzed using response surface methodology and analysis of variance. The optimized formulation (F-7) was prepared using 5% (w/v) total lipid and 4% (w/v) total surfactant, then sonicated for 20 min. The particle size (PS) was 151.53 ± 5.79 nm, and the entrapment efficiency was 82.3 ± 2.53%. The zeta potential (ZP) was measured at -15.20 ± 1.34 mV, and the DLS analysis indicated PS of 151.53 ±5.79 nm with a polydispersity index of 0.297 ± 0.061. To summarize, the DOE based approach facilitated rational formulation optimization and the successful preparation of M1-loaded NLCs with nanosized particles and satisfactory entrapment efficiency. Further investigation is required on their storage stability and pharmaceutical performance.

Keywords:
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Abbreviations:
SLN: Solid lipid nanoparticles; NLCs: Nanostructured lipid carriers; M1: 6,7-dimethoxy-1,2,3,4-tetrahydro-isoquinoline-3-carboxylic acid; DMSO: Dimethyl sulfoxide; DOE: Design of Experiments; QbD: Quality by Design; BBD: Box–Behnken design; PS: mean particle size; PDI: Polydispersity index; ZP: Zeta potential; DLS: Dynamic Light Scattering; EE: Entrapment efficiency; M1-NLCs: M1-loaded nanostructured lipid carriers.

1. Introduction

The development of efficient and reliable drug delivery systems is an important aspect of modern pharmaceutical research. The therapeutic performance of a drug is dependent not only on its intrinsic pharmacological activity but also on its physicochemical properties, the characteristics of the formulation and its ability to reach the site of action in an adequate concentration. Several drug candidates are limited by poor aqueous solubility, inadequate dissolution and low permeability which may limit their pharmaceutical applications [1]. These challenges may not be adequately addressed by conventional dosage forms, especially when the drug has unfavourable physicochemical characteristics. Hence, increasing attention has been paid to the development of new carrier systems that can improve drug incorporation, dispersion, protection and release. An example of this is the use of nanoformulation delivery systems for drugs, where the properties of the formulation can be designed by controlling particle composition, size and surface characteristics [2, 3]. Lipid-based nanoparticles have received extensive attention as a nanocarrier platform due to their promising biocompatibility, formulation flexibility and capacity to encapsulate a wide variety of therapeutic compounds. Lipid-based nanocarriers include liposomes, nanoemulsion, solid lipid nanoparticles (SLNs), and nanostructured lipid carriers (NLCs). SLNs were developed as alternatives to conventional polymeric nanoparticles and liposomal systems. The matrix is mainly composed of lipids, solid at room and body temperatures. Although SLNs provide many formulation benefits, the highly organized crystalline lipid matrix of SLNs may limit drug loading and, in some cases, result in drug expulsion during storage due to lipid crystallization or polymorphic transitions. These limitations led to the development of NLCs as a second generation lipid nanoparticle system [4, 5]. NLCs contain a mixture of solid and liquid lipids within the lipid

matrix, thereby altering its internal organization and possibly improving drug accommodation [6]. NLCs generally consist of a solid lipid, a liquid lipid, one or more surfactants and an aqueous phase. The solid lipid constitutes the structural framework of the particle, while the liquid lipid is incorporated into the matrix to form a less ordered arrangement. This lipid structure may be imperfect or have areas of low crystallinity, which may allow more space to load the drug. Depending on the composition and arrangement of the lipid components, NLCs may exhibit different internal structures, such as imperfect crystalline, amorphous, and multiple-type matrices. Such structural variations may affect drug loading, drug distribution in the particle and release behaviour. The presence of liquid lipid may also reduce the tendency of the matrix to form a highly ordered crystalline structure and thus may minimize drug expulsion during storage. However, these effects depend on the physicochemical compatibility of drug and lipid components, the ratio of solid to liquid lipid, and the manufacturing conditions. Thus, the advantages of NLCs should be evaluated for each drug and formulation rather than assumed to be universal. The selection of appropriate lipid and surfactant components is a crucial step in the development of NLCs [7]. The solid lipid is expected to provide a suitable matrix, while the liquid lipid may influence the drug solubilization in the lipid phase and the extent of structural disorder. Therefore, drug-lipid compatibility and the active compound's solubility in the selected lipid mixture may affect entrapment efficiency and drug loading. Surfactants are added to lower the interfacial tension during particle formation and to stabilize the resultant dispersion. The type of nanoparticles and their concentration may affect the formation of nanoformulations, particle size, surface characteristics and the tendency of particles to aggregate. Additionally, processing parameters such as homogenization or sonication conditions can influence energy input during formulation and the final particle size distribution. Imbalance in formulation composition or processing conditions might cause larger particles, lower entrapment efficiency, or insufficiently stable dispersion. Thus, a systematic study of formulation and process variables is necessary to establish a suitable relationship between composition, manufacturing conditions and the physicochemical characteristics of the developed NLCs [8].

Isoquinoline alkaloids are a large group of compounds defined by the presence of an isoquinoline-derived chemical structure, and are found in a wide range of natural and synthetic sources [9, 10]. The alkaloids belonging to this class have been studied for different biological activities, however, the pharmaceutical development of each alkaloid depends on its molecular and physicochemical properties [11]. In particular, limited aqueous solubility can be a challenge for formulation due to its impact on drug dispersion and dissolution in aqueous environments.

Previously, our research group reported the isolation of an isoquinoline alkaloid, 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (M1), from MP seeds. Their study demonstrated that M1 exhibited potent antiproliferative activity against the Huh-7 cell line [12].

In preliminary assessment, M1 was found to be insoluble in water but soluble in methanol and dimethyl sulfoxide (DMSO). These observations warranted exploring an appropriate formulation strategy for its incorporation into a pharmaceutical carrier system. Hence, a lipid-based nanoparticulate approach was considered, as the lipid matrix may provide a suitable environment for drug incorporation, depending on M1's compatibility and solubility within the selected lipid components. The preparation of M1-loaded NLCs would allow the study of their incorporation into a lipid matrix and the characterization of the physicochemical properties of the resulting formulation. But the choice of NLC system alone is not sufficient to demonstrate improvement in solubility, bioavailability or therapeutic activity and requires appropriate experimental evaluation. The development of NLC formulations is inherently complex because multiple formulation and process variables influence the critical quality attributes of the final product. Traditional formulation approaches, such as one-factor-at-a-time (OFAT) methods, examine the effect of a single variable while holding other variables constant. Such approaches may provide some initial information but may not be sufficient to identify interactions among variables and may require a large number of experiments to explore a formulation space. The Design of Experiments (DOE) is a systematic, statistical approach to studying the effects of multiple independent variables on defined responses [13]. Experimental designs are used to determine the relationship between formulation variables and responses by mathematical models. The effect of individual factors and interactions can be evaluated. Thus, DOE is seen as a vital tool in the Quality by Design (QbD) framework, which involves formulation development driven by a systematic understanding of material attributes, process parameters, and product quality [14]. Response surface methodology is commonly employed in pharmaceutical formulation development to explore and optimize the influence of multiple variables. One of its approaches is the Box–Behnken design (BBD), a response surface design that enables the estimation of quadratic effects and interactions through a structured set of experimental runs [15]. BBD can be especially helpful when the goal is to study the effect of multiple formulation factors with fewer experiments than some full factorial designs.

In the development of lipid nanoparticles, the effects of variables such as lipid concentration, surfactant concentration, and processing time on particle size and entrapment efficiency have been investigated. Particle size is an important physicochemical property as it indicates the size of the dispersed particles and can influence dispersion behaviour and subsequent formulation performance[16, 17]. Entrapment efficiency is a measure of the amount of drug associated with the nanoparticle formulation compared to the total amount of drug used. Together, these responses provide a more complete basis for selecting a formulation than simply evaluating a single parameter [18]. In the present study, M1-NLCs were developed using a DOE-assisted formulation strategy. The effects of total lipid concentration, surfactant concentration, and sonication time on particle size and entrapment efficiency were studied using a Box–Behnken design. The experimental data were analyzed to find the relationship between the chosen independent variables and the measured responses. The prepared formulations were further characterized for particle size, polydispersity index, zeta potential, and entrapment efficiency.

2. Material and Methods

2.1. Materials

M1 (6,7-dimethoxy-1,2,3,4-tetrahydro-isoquinoline-3-carboxylic acid. Olive oil was obtained from m/s Deve Herbs. Precirol ATO-5 (glycerol distearate) was obtained as a gift sample from Gattefossé. Methanol, Tween 80 (Polyoxyethylene (80) sorbitan monooleate), and Span 80 (Sorbitan monooleate) and other analytical grade chemicals were purchased from SRL (Sisco Research Laboratories Pvt. Ltd.).

2.2. Methods

2.2.1. Preformulation studies of M1

2.2.1.1. Physical appearance

The physical characteristics of M1 were evaluated by macroscopic visual and organoleptic examination under ambient laboratory conditions. The sample was assessed for its physical state, colour, and odour, and the observations were systematically recorded.

2.2.1.2. Melting point determination

The melting point of M1 was determined by the capillary tube method using a melting point apparatus. A clean glass capillary tube was sealed at one end, and the powdered M1 sample was introduced through the open end and compacted at the bottom of the tube. The loaded capillary was placed in the sample chamber of the melting point apparatus, and the temperature was slowly increased [19]. The temperature at which the sample fully transitioned from solid to liquid was recorded as the melting point. The determination was performed in triplicate.

2.2.1.3. UV Spectroscopy analysis (determination of absorption maxima/ λmax)

To determine the wavelength of maximum absorption (λmax), a standard stock solution of M1 (1 mg/mL) was prepared by dissolving the active alkaloid in analytical-grade methanol. A suitable aliquot of this solution was subsequently scanned across the ultraviolet-visible spectrophotometric spectrum ranging from 200 to 800 nm using a double-beam UV-Vis spectrophotometer (Labtronics, Model LT-2910, India) [20]. Pure methanol was utilized as a baseline blank to calibrate the system and subtract solvent background noise. The peak corresponding to the maximum absorbance was identified and recorded as the characteristic λ_{max} of the M1.

2.2.2. Development of HPLC method

2.2.2.1. Preparation of Mobile phase

All the chemicals used were of HPLC grade. Methanol and water in a 95:5 (v/v) ratio were used to prepare the mobile phase and flow rate was set to 1 mL/min. A reverse phase C18 column (250*4.6mm, 5µm) was used as the stationary phase [20].

2.2.2.2. Preparation of calibration curve

A standard curve for M1 was prepared in methanol to evaluate the drug concentration. A stock solution of M1 (1mg/mL) was prepared by dissolving 10 mg of the drug in 10 mL of methanol. Different dilutions ranging from 1 to 100 µg/ml were prepared in triplicate to plot the calibration curve. A least-squares linear regression was applied to the average area versus concentration to obtain the slope, intercept, correlation, etc.

2.2.3. Experimental Design (DOE)

To systematically evaluate the individual and combined effects of critical processing variables (independent variables) on the physicochemical properties of the nanoformulation, a 3-factor, 3-level Box-Behnken Design (BBD) was implemented using Design-Expert® software (trial version 8.0) for the development of M1-NLCs. The impacts of independent factors like total lipid content (A), total surfactant content (B), and sonication time (C) on response variables like PS (R1) and EE (R2) were explored while the importance of the outcome was kept in mind. To obtain M1-NLCs with a particle size range of 140 to 180 nm, the impact of independent factors on the dependent factors of M1-NLCs was investigated [21].

2.2.4. Preparation of M1-NLCs

M1-NLCs were prepared using a modified emulsification-solvent evaporation method combined with high-energy probe sonication [21]. The lipid phase was briefly prepared by mixing a solid lipid (Precirol® ATO-5) and a liquid lipid (Olive oil) at a fixed mass ratio of 70:30 with a lipophilic surfactant (Span 80, 200 mg). The lipidic mixture was heated at 75 ± 2 °C under constant magnetic stirring until a transparent, homogeneous molten mass was obtained. Simultaneously, the isoquinoline alkaloid (6,7-dimethoxy-1,2,3,4-

tetrahydroisoquinoline-3-carboxylic acid; M1, 10 mg) was completely pre-dissolved in 800 µL of methanol and was added directly into the hot molten lipid blend. The mixture was stirred continuously and kept at 75 °C for 10 min to ensure full thermal evaporation of the organic solvent. Simultaneously, a second aqueous phase (10 mL) containing a dispersed hydrophilic surfactant stabilizer (Tween 80, 200 mg) was prepared and equilibrated to the same temperature (75 ± 2 °C). The ration of surfactant used was 1:1 (Span 80: Tween 80). The heated aqueous phase was then added dropwise to the solvent-free lipid melt under magnetic stirring at high speed to form a coarse hot pre-emulsion. The amounts of total lipid content, total aqueous surfactant and final processing duration were exactly adjusted in different batches according to the constraints defined by the Design of Experiments (DOE) parameters. Finally, the warm emulsion was cooled with ice-cooled water to induce lipid recrystallization, and the structured NLCs were stored at 4 °C for further examination.

2.2.5. Physicochemical analysis of M1-NLCs

2.2.5.1. Determination of surface charge properties and particle size distribution of NLCs

The PS, PDI and ZP of M1-NLCs were determined by DLS using a Zetasizer (Malvern Instruments, Nano ZS, UK). To minimize particle-particle interactions and to avoid multiple-scattering effects, a 100 µL portion of the fresh nanoparticle dispersion was diluted 100-fold with filtered deionized water before measurement. All analytical runs were performed at a fixed temperature (25 °C) and a fixed light scattering angle (90°). All determinations were performed in triplicate (n=3) to ensure reproducibility [22].

2.2.5.2. Analysis of Entrapment efficiency (EE)

To evaluate the EE, the synthesized M1-NLCs formulations were subjected to ultrafiltration-centrifugation. Briefly, a 2 mL aliquot of the fresh NLCs dispersion was transferred into a centrifugal filter unit (Briscale™ Ultrafiltration 4 mL, MWCO 10 kDa, Cobetter) and processed in a temperature-regulated centrifuge at 10,000 rpm and 4 °C for approximately 15 minutes. This configuration effectively separated the unencapsulated free drug (supernatant) from the lipid nanoparticles retained by the membrane. The concentration of the unbound free drug present in the separated supernatant fraction was quantified at a wavelength of 229 nm using a double-beam UV spectrophotometer (Labtronics, Model LT-2910, India) [23]. The EE was subsequently calculated using the following equation:

EE (%) = [DTq – DFq / DTq] * 100

Here, 'DTq' represents the total quantity of drug (mg), whereas 'DFq' denotes the free drug remaining in the supernatant.

3. Results

3.1. Preformulation studies

3.1.1. Physical appearance of M1

Macroscopic visual and organoleptic examination showed that M1 was a white, odourless solid (presented in Table 1).

Table 1: Organoleptic Properties of M1

S. No.	Characteristic	Observation
1.	Physical state	Solid powder
2.	Colour	White
3.	Odour	Odourless

3.1.2. Melting point determination of M1

The experimentally determined melting point of M1 was found to be 280.5 ± 0.2 °C, compared with the reference value of 281.0 ± 0.1 to 281.3 ± 0.1 °C (Table 2).

Table 2: Melting point determination of M1

S. No.	Observed melting point (°C)	Reference value (°C)
1.	280.5 ± 0.2	281 ± 0.1 - 281.3 ± 0.1 °C

3.1.3. Solubility Profile of M1

M1 was soluble in methanol and DMSO at the tested concentration of 1 mg/mL, whereas it was insoluble in distilled water at the same concentration (Table 3).

Table 3: Preliminary solubility profile of M1

S. No.	Solvent	Concentration tested	Solubility
1.	Methanol	1mg/mL	Soluble
2.	DMSO	1mg/mL	Soluble
3.	Distilled water	1mg/mL	Insoluble

3.1.4. UV Spectroscopy analysis (determination of absorption maxima/ λmax)

The optical absorption profile of the isolated alkaloid was characterized by recording its spectrum in analytical-grade methanol across the ultraviolet-visible range of 200–800 nm. The resulting UV-visible absorption spectrum of M1 displayed a prominent, sharp absorption maximum (λmax) at 229 nm **(Fig. 1)**.

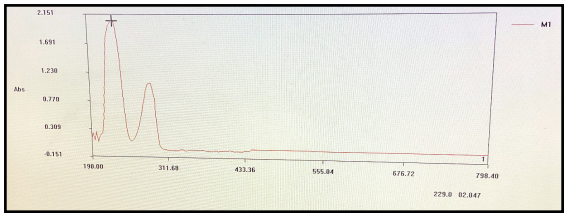


Fig. 1: UV–visible absorption spectrum of M1.

3.2. Development of HPLC method

The chromatographic separation of the active isoquinoline alkaloid was successfully established using the developed reversed-phase HPLC (RP-HPLC) protocol. Under the optimized mobile phase conditions consisting of methanol and water (95:5 v/v) passing through the reversed-phase C18 stationary column at 1 mL/min flow rate at 40 ºC, M1 exhibited a sharp, distinct, and symmetric chromatographic peak at a highly stable retention time of 2.988 minutes, within a total chromatographic run profile of 6 minutes **(Fig. 2 (A))**. No interfering matrix peaks from the formulation excipients were observed within this window, confirming the method's high selectivity. The standard calibration curve generated from the working dilutions demonstrated an excellent proportional increase in chromatographic response across the wide investigated concentration range of 1 to 100 µg/mL. The least-squares linear regression analysis mapping the mean peak area responses (y, arbitrary units) against the corresponding analyte concentrations (x) µg/mL yielded the robust linear equation:

$y = 118.09x-180.07$

The calculated coefficient of determination (R^2) was 0.9936 **(Fig. 2 (B))**. This high R^2 value indicates a strong, reliable linear relationship between the chromatographic signal and the sample mass concentration.

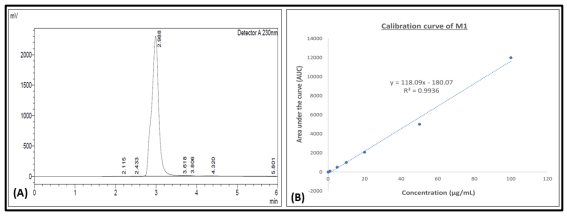


Fig. 2: (A) High-performance liquid chromatography (HPLC) chromatogram of the optimized M1-loaded NLCs matrix. **(B)** Linear standard calibration curve of M1

3.3. Experimental Design (DOE)

The effect of formulation variables on the physicochemical properties of NLCs was investigated using a Box–Behnken design (BBD). The relationship between independent variables (A), (B), (C) and responses, PS (R1) and EE (R2), was established using a quadratic polynomial model. The model equations were expressed in terms of coded independent variables, where A, B, and C represent total lipid concentration, total surfactant concentration, and sonication time, respectively.

3.3.1. Effect of formulation parameters on PS (R1)

The quadratic model developed for PS was significant, having an F-value of 69.69 and a p-value of 0.0001. The model showed a high coefficient of determination ($R^2 = 0.9921$) with an adjusted $R^2 = 0.9779$ and a predicted $R^2 = 0.8773$, which indicated a satisfactory agreement between experimental and predicted responses. The model was adequate to navigate the experimental design space, as the adequate precision value was 24.03, which is well above the recommended threshold of 4. Among the linear factors (A), (B) and (C) had a significant effect on particle size ($p < 0.0001$). Interaction terms AB, AC, and BC were also significant, with p-values of 0.0296, 0.0007, and 0.0015, respectively. After excluding non-significant terms, the final quadratic model for response variable R1 is given as Eq. (1).

R1 (PS) = +202.27+26.80*A-22.25*B-22.23*C+7.88*AB+19.57*AC-16.48*BC+9.28*A^2+2.13*B^2+9.38*C^2 …. **Eq. (1)**

Where (A), (B) and (C) stands for total lipid content, aggregate surfactant concentration and sonication time respectively.

The positive coefficient associated with the total lipid concentration indicated an increasing contribution to particle size, whereas the negative coefficients for the total surfactant concentration and the sonication time denoted a decreasing contribution in the investigated design space. The significant interaction terms indicated that the effect of one individual formulation variable on particle size depended on the level of another interacting variable. Overall, the statistical parameters indicated that the developed model had a high predictive ability for particle size in the range of the studied formulation.

3.3.2. Influence of formulation variables on EE (R2)

The quadratic model developed for entrapment efficiency was significant, with an F value of 40.25 and a p-value of 0.0004. The model gave a high R^2 value of 0.9864, an adjusted R^2 value of 0.9619, and a predicted R^2 value of 0.9132. The difference between adjusted and predicted R^2 was less than 0.2, indicating good agreement between the model fit and predictive performance. The experimental data were consistent, as shown by the relatively low coefficient of variation (0.84%). The linear terms (A), (B) and (C) showed significant effects on entrapment efficiency, with p-values of 0.0001, <0.0001, and 0.0056, respectively. The final quadratic model for response variables R2 is given below as Eq. (2).

R2 (EE) = +83.73+2.65*A+2.85*B-1.13*C+1.05*AB-2.10*AC-0.8500*BC-1.14*A^2-1.59*B^2-0.6917* C^2 …….. **Eq. (2)**

Where (A), (B) and (C) stands for total lipid content, aggregate surfactant concentration and sonication time respectively.

The linear terms A and B were positive, indicating that increases in total lipid and surfactant concentrations within the studied range increased entrapment efficiency. In contrast, the negative coefficient for the sonication time indicated a decreasing contribution to the entrapment efficiency. The significant quadratic terms indicated curvature in the response and suggested that the relationship between the formulation variables and entrapment efficiency was not only linear.

3.3.3. Optimization of M1-NLCs

Based on preliminary testing, 15 M1-NLCs formulations were created by varying three parameters: total lipid content, total surfactant content and sonication time at three levels: low (-1), medium (0), and high (+1), as indicated by preliminary assessment in **Table 4**. The 3-D response surface plots **(Fig. 3 (a-f))** represent the effect of variations in formulation factors on the responses measured for M1-loaded NLCs. The optimized NLCs formulation was selected as formulation F-7, which was based on the overall evaluation of low particle size and high encapsulation. The chosen formulation consisted of 5% (w/v) total lipid and 4% (w/v) total surfactant, and was sonicated for 20 min. F-7 had a PS of 151.53 ± 5.79 nm and EE (%) of 82.3 ± 2.53 %. F-12 showed a similar PS of 152.6 ± 12.64 nm but the EE and ZP was lower (80.1 ± 2.64%) than that of F-7. Hence, F-7 was found to be a good formulation, with a comparatively small PS and satisfactory drug entrapment. The optimized formulation was chosen for further physicochemical characterization.

Table 4. M1-NLCs formulation and optimization: Box-Behnken experimental design matrix with independent variables and corresponding response values.

	Low level (-1)	Mid-level (0)	High level (+1)
Independent variables			
A: Total lipid (%)	4	5	6
B: Total surfactant (%)	2	3	4
C: Sonication time (min)	10	15	20
Response variables **R1:** Particle size (nm) **R2:** Entrapment efficiency (%)			

Run	**A:**	**B:**	**C:**	**R1:**	**R2:**
	Total lipid (%)	**Total surfactant (%)**	**Sonication time (min)**	**Particle size (PS) (nm)**	**Entrapment efficiency (EE) (%)**
F-1	6	4	15	231.2 ± 4.13	87.2 ± 2.21
F-2	5	4	10	225.4 ± 2.61	86.5 ± 1.79
F-3	4	4	15	158.2 ± 7.36	80.3 ± 2.47
F-4	5	3	15	200.8 ± 4.77	82.9 ± 1.33
F-5	4	3	10	239.3 ± 2.93	77.9 ± 2.80
F-6	6	3	10	250.1 ± 6.74	87.9 ± 3.74
F-7	**5**	**4**	**20**	**151.53 ± 5.79**	**82.3 ± 2.53**
F-8	5	3	15	202.1 ± 11.52	84.7 ± 1.32
F-9	6	3	20	241.7 ± 4.85	81.7 ± 3.41
F-10	5	2	10	243.5 ± 8.62	78.9 ± 1.72
F-11	5	3	15	203.9 ± 9.48	83.6 ± 2.49
F-12	4	3	20	152.6 ± 12.64	80.1 ± 2.64
F-13	4	2	15	211.9 ± 3.82	76.9 ± 2.04
F-14	6	2	15	253.4 ± 7.88	79.6 ± 1.86
F-15	5	2	20	235.1 ± 15.39	78.1 ± 3.58

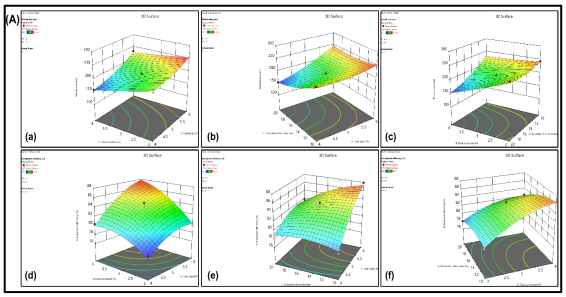


Fig. 3 (A) Three-dimensional response surface plots showing the effect of formulation variables on particle size (PS) and entrapment efficiency (EE): (a-c) Effect of Total lipid concentration vs Total surfactant concentration, Total lipid concentration vs sonication time, and sonication time vs Total surfactant concentration on PS, respectively; (d-f) Effect of Total lipid concentration vs Total surfactant concentration, Total lipid concentration vs sonication time and Total surfactant concentration vs sonication time on EE, respectively.

3.4. Physicochemical analysis of NLCs

3.4.1. Determination of surface charge properties and particle size distribution of NLCs

The formulation exhibited a PS of 151.53 ± 5.79 nm with a PDI of 0.297 ± 0.061, which suggests a relatively narrow size distribution of particles, as shown in **Fig. 4 (A)**. The ZP analysis showed a negative surface charge of -15.20 ± 1.34 mV, as shown in **Fig. 4 (B)**. The negative charge observed can be explained by the properties of the lipid matrix surface. The optimized formulation showed good nanoscale characteristics with a relatively uniform particle size distribution overall.

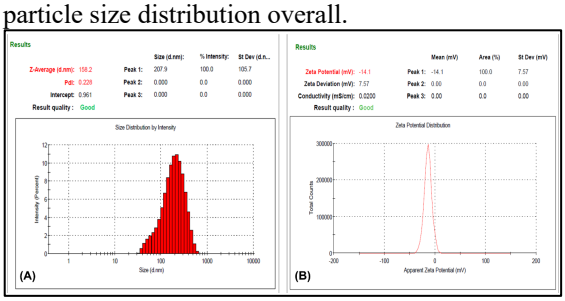


Fig. 4 (A) Representatives profile of **(A)** PS of M1-NLCs and **(B)** ZP profiles of M1-NLCs

3.4.2. Assessment of drug entrapment efficiency (EE)

To determine the amount of drug successfully incorporated within the lipid matrix, the drug entrapment efficiency (EE) of the optimized M1-loaded NLCs formulation was evaluated. The optimized formulation (F-7) showed an EE of 82.3 ± 2.53%. The observed entrapment efficiency may be attributed to the lipid matrix providing an appropriate environment for drug incorporation. These results showed the potential of the developed NLCs formulation to achieve good drug entrapment, thus making it suitable for further characterization and evaluation.

4. Discussion

The present study aimed to develop and optimize M1-loaded nanostructured lipid carriers (NLCs) by using Box–Behnken design and to evaluate their physicochemical characteristics. The results of the preformulation studies, analytical method development, and formulation optimization together provided a rationale for selecting an NLCs formulation with a nanometric particle size and adequate drug entrapment efficiency. Preformulation studies showed that M1 was a white, odourless solid with a melting point experimentally determined to be near the reported value. This agreement provides initial evidence of the material's consistency. Melting point alone cannot determine chemical purity. The solubility study showed that M1 was soluble in methanol and DMSO, and insoluble in distilled water at the tested concentration (1 mg/mL). Its low aqueous solubility is a formulation challenge and requires the development of a lipid-based carrier system. NLCs are characterized by a mixture of solid and liquid lipids resulting in a less ordered lipid matrix and potentially better incorporation of drug molecules than highly crystalline solid lipid systems. The UV spectrum of M1 showed a maximum absorption wavelength of 229 nm. This wavelength was then employed for spectrophotometric quantification. The developed RP-HPLC method showed a well-defined M1 peak with a retention time of 2.988 min, and the calibration equation was $y = 118.09x - 180.07$, with a coefficient of determination (R^2) of 0.9936 over the specified concentration range. The results show a strong linear relationship between concentration and chromatographic response under the experimental conditions. Linearity and a specific retention time do not constitute a complete validation of an analytical method. The effects of total lipid concentration (A), total surfactant concentration (B), and sonication time (C) on particle size and entrapment efficiency were studied using a Box-Behnken design. The quadratic model for particle size was statistically significant ($F = 69.69$, $p = 0.0001$) and explained a high proportion of the variance ($R^2 = 0.9921$). The model fit and predictive performance were reasonably consistent, as shown by the adjusted R^2 (0.9779) and predicted R^2 (0.8773). The positive coefficient for total lipid concentration indicated that increasing lipid levels tended to increase particle size within the range studied. This could relate to the formation of a larger, more dispersed lipid phase when the amount of lipid exceeds the capacity of the applied emulsification and sonication conditions to reduce droplet size [21]. However, the negative coefficients for surfactant concentration and sonication time indicated that the tendency for smaller particle formation increased with an increase in these variables. The increased concentration of surfactant may help stabilize the newly formed interfaces and reduce coalescence, while sonication can provide energy to break larger

droplets into smaller particles. These effects are consistent with the known effects of lipid composition, surfactant selection, and processing conditions on the properties of lipid nanoparticles [21]. The significance of quadratic terms of A and C suggested that the response was not purely linear in the range investigated. Studies on similar formulations have described the effects of sonication and lipid composition on NLCs particle size, with the result depending on the formulation and processing conditions. The developed model for entrapment efficiency was significant ($F=40.25$, $p=0.0004$) with $R^2=0.9864$, adjusted $R^2=0.9619$ and predicted $R^2=0.9132$. The adjusted R^2 and predicted R^2 values were close, and the coefficient of variation (0.84%) was low, indicating that the model fit the experimental data well. The non-significant lack of fit ($p = 0.8359$) further confirmed the adequacy of the model in the design space studied. Positive coefficients for total lipid and surfactant concentrations indicated that these factors were generally responsible for increased entrapment efficiency [18]. A higher lipid content may provide a greater matrix volume for drug incorporation, while an optimal level of surfactant may improve dispersion and stabilization during formulation. The negative coefficient of sonication time, however, indicates that an increase in sonication time in the tested range may decrease the measured entrapment efficiency. Perhaps processing may improve the distribution of the drug within the lipid matrix or enhance the transfer of the drug to the surrounding aqueous phase. This mechanism was not directly addressed in the present study. Thus, the observed effects should be interpreted as statistical associations rather than definitive mechanistic proof. The choice of formulation F-7 was guided by the overall particle size and entrapment efficiency. It contained 5% (w/v) total lipid and 4% (w/v) total surfactant and was sonicated for 20 min. F-7 had a particle size of 151.53 ± 5.79 nm and entrapment efficiency of 82.3 ± 2.53%. The particle size of F-12 was similar (152.6 ± 12.64 nm), whereas its entrapment efficiency was lower (80.1 ± 2.64%). Therefore, F-7 was a suitable formulation within the studied design space with respect to particle size and drug incorporation. The particle size of the optimized formulation was further confirmed by dynamic light scattering, which showed a Z-average diameter of 151.53 ± 5.79 nm. The polydispersity index (PDI) was 0.297 ± 0.061, indicating a fairly narrow distribution. The particle size and PDI are important characteristics of lipid-based nanocarriers, as they provide information on the colloidal size and width of the particle population. The observed nanoscale diameter is consistent with the targeted formulation range of 140-180 nm. The ZP of the optimized NLCs was -15.20 ± 1.34 mV. The negative charge of the surface can be explained by the nature of the lipid components and the interface created between the lipid phase and the aqueous medium. The magnitude of the measured potential is, however, moderate and does not, by itself, constitute evidence of strong electrostatic stabilization. Steric stabilization by surfactants, particle composition and the surrounding medium can also affect the colloidal stability of lipid nanoparticle dispersions. As such, the ZP result has to be considered alongside the particle size and PDI. The entrapment efficiency of 82.3 ± 2.53% indicates that a significant amount of the initially added M1 was associated with the optimized NLCs formulation under the conditions of the study. The presence of both solid and liquid lipids can result in a less ordered matrix that accommodates drug molecules and mitigates the limitations of highly crystalline lipid structures. However, the internal arrangement of the lipid matrix or molecular state of M1 was not characterized directly in the present study. This means that the entrapment efficiency observed can be discussed in terms of the overall formulation system, but a specific mechanism of drug localization cannot be confirmed from these data alone. The results indicate that it is possible to prepare M1-loaded NLCs with a particle size in the desired nanoscale and an entrapment efficiency of more than 80%. Systematic evaluation of formulation and processing variables using a Box–Behnken design identified F-7 as a potential candidate for further characterization. These results provide a basis for future investigations at the formulation level. Further studies on drug release, structural characterization, biological activity and in vivo performance would be required to ascertain the wider pharmaceutical potential of the developed formulation.

5. Conclusion

M1-loaded NLCs were successfully developed using a lipid-based formulation approach and optimized using a three-factor, three-level Box-Behnken design. The DOE assisted approach allowed the systematic development and optimization of the M1 loaded nanostructured lipid carriers (M1-NLCs). The optimized formulation exhibited PS of 151.53 ± 5.79 nm, an entrapment efficiency of 82.3 ± 2.53 %, a ZP of -14.1 mV and a PDI value of 0.297 ± 0.061. The results show that M1 can be incorporated into a nanosized lipid based carrier system. Further studies on storage stability, drug release, structural characteristics and biological performance are required to confirm its pharmaceutical potential.

6. Conflict of Interest – Nil

7. References

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