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Research Article

Design, Development and Characterization of Nanostructure Lipid Carriers (NLCs) by HPH Method Loaded with Anticancer Drug

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Abstract

The Nanostructured Lipid Carriers (NLCs) was formulated with the aim to improve the aqueous solubility and thus ultimately oral bioavailability of Axitinib. Axitinib loaded NLCs were formulated using Compritol ATO 888 as solid-lipid, Oleic acid as liquid-lipid and Tween 80 as surfactant by the High-Pressure Homogenization technique. A full 2^3 factorial design was utilized to study the effect of the independent parameters, solid-lipid to the liquid-lipid ratio (6:4) and surfactant, on the dependent variables such as Mean Particle Size (MPS) and Entrapment Efficiency (%EE). Optimized formulation showed 202.2 nm MPS, -21.5 mV zeta-potential and 0.44 PDI, and 88% EE, which imparts good stability of developed NLCs. The physicochemical characterization of AXT loaded NLCs was done by examining the results of Differential Scanning Calorimetry, Fourier transform infrared spectroscopy study. The stability study of optimized formulation was found to be stable with no significant change in particle size, and drug content. In vitro release profiles of NLCs indicated a burst release for the first 2 hrs followed by a prolonged-release profile for AXT until about more than 10 hrs. The Axitinib NLCs may provide a better bioavailability, reduction in dose, dosing frequency, dose-related side effects and better control of the disease. A foresaid results showed the potential of NLCs for significant improvement in oral bioavailability of poorly soluble Axitinib in cancer treatment.

Keywords: NLCs, full factorial design, high-pressure homogenization, in-vitro release

INTRODUCTION

Renal cell carcinoma (RCC) is a diverse category of malignancies that emerges from renal tubular epithelial cells. Kidney cancer is responsible for 2% of all cancer diagnosis and deaths globally[1]. More than females, males are more prone to get kidney cancer, and the incidence has been increasing in many regions of the world since several decades. RCC is responsible for 80-85% of kidney malignancies[2]. Hypertension, cigarette smoking and obesity are well-known risk factors contributing for RCC. Epidemiological research suggests that kidney transplantation, chronic kidney illness, haemodialysis, previous RCC history, and perhaps diabetes mellitus has all been linked to RCC. RCC is also related with variety of lifestyle, nutritional, and environmental factors, with varied degrees of evidence[1]. The Von Hippel-Lindau gene (VHL) is a tumour suppressor (TS) gene that is important in RCC development. The VHL gene produces a protein, pVHL. It acts as a TS protein. The amounts of various intracellular proteins, including hypoxia-inducible factor 1 and 2 alpha are regulates by pVHL. When these intracellular proteins connect to the DNA, they operate as transcription factors, resulting in the overexpression of mRNA, which codes for various growth factors, including vascular endothelial growth factor (VEGF) and platelet-derived growth factor-β (PDGFB). These growth factors are important for developing highly vascular malignancies (such as RCC) linked to VHL gene mutations[3]. Different angiogenesis-promoting signaling systems are triggered in the tumor region to promote cancer cell survival. The angiogenesis process is mediated by VEGF once it interacts with VEGF receptors 1, 2, and 3 [4].

Axitinib (AXT) is an anticancer agent that inhibits the tyrosine kinase (TK) portion of all VEGF receptors at picomolar levels and PDGFR and KIT at nanomolar levels [5]. When compared to earlier VEGFR inhibitors that shown multi kinase activity with off-target effects, Axitinib, as a second-generation inhibitor, provides enhanced potency and the VEGFR specificity [6]. Axitinib outperforms multi-targeted TK inhibitors such as pazopanib, sorafenib, and sunitinib in terms of potency and selectivity for VEGFRs. Endothelial nitric oxide synthase suppresses VEGF-mediated endothelial cell survival, tube formation, and signalling pathways downstream. Axitinib inhibits VEGFR2 phosphorylation in a dose-dependent way, which results in reduced vascular permeability and angiogenesis, as well as tumour cell death induction, indicating therapeutic potential [7].

A variety of lipid-based nano-delivery systems, including solid-lipid nanoparticles (SLNs), nanoemulsions, nanostructured lipid carriers (NLCs), nanoparticles, and liposomes, have recently been described for targeted tumour delivery of anticancer medicines. These nano-formulations were employed to deliver drugs through various routes [8]. Axitinib is classified as BCS class II agent, with a mean absolute bioavailability of 58%. NLCs higher drug loading capacity can improve the oral bioavailability of weakly water-soluble drugs, like Axitinib. These are lipidic nanoparticles

with an unstructured solid-lipid core that allows encapsulating highly lipophilic drugs, thus, preserving them from degrading and increasing their stability. They have several benefits over conventional nanoparticulated drug delivery technologies. They are composed of FDA and/or EMA approved lipids and surfactants and are commercially available in marketed pharmaceutical products [9]. Furthermore, NLCs have various advantages, including improved targeting, controlled release, biodegradability, less harmful effects, preparation without organic solvents, and high %EE of both hydrophilic and lipophilic compounds [8]. Henceforth, NLCs development increase the lipophilicity of drug and therefore increases bioavailability of formulation. Nanostructured Lipid Carrier will be one of the most promising drug delivery systems for carrying highly lipophilic material.

MATERIALS AND METHOD

Axitinib was supplied by Glenmark Pharmaceuticals, Nashik. Compritol 888 ATO was supplied by Gattefosse, France. Oleic Acid and Tween 80 was procured by Loba Chemie Pvt. Ltd., Mumbai. Methanol was procured by Merck Specialities Pvt. Ltd., Mumbai. All the analytical reagents graded were used chemicals used for the work.

Partitioning behavior of AXT between the lipids

To check the partition behaviour, the solubility of drug was determined in different solid-lipids and liquid-lipids. Lipid screening is important to determine the most suitable lipid for incorporation into NLCs. For this, increasing amount of active ingredient was dissolved in various melted solid-lipids and liquid-lipids and maximum amount of active ingredient that could be dissolved was determined. The lipid/active mixtures are cooled to room temperature after dissolution. It led to solidification. The presence or absence of crystalline active is visually determined in the solid mixture. The miscibility of the melted lipid and liquid-lipid is observed if the active ingredient is oil. After the solidification to room temperature, the lipid will solidify again and the incorporation of the oil in the solid-lipid matrix is investigated. This can be performed by smearing used as cooling system to control the rate of cooling of the obtained product. [10]

Solubility of AXT in liquid-lipids

For the evaluation of the solubility of AXT in liquid-lipids, 15 mg AXT was weighed and mixed with 2 mL tested oil in a test tube. Methanol was gradually added until the mixture became homogenous. The aqueous phase of the mixture was separated from the lipid by centrifuging it at 25000 rpm for 20 minutes. The resultant clear supernatant was appropriately diluted and examined using UV-spectroscopy at 260 nm to investigate its partitioning behaviour with different lipids. The liquid oil that dissolved the highest amount of AXT was selected for use. [11]

Solubility of AXT in solid-lipids

For the evaluation of solubility in solid-lipids, accurately weighed 10 mg of AXT was weighed and taken in a test tube; the solid-lipid was added in 0.5 g increments. The temperature of test tube was maintained at 80°C on water bath till the clear melt formation. The lipid sufficient to solubilize 10 mg AXT was established by this process. [12]

Formulation and optimization of AXT loaded NLCs

Hot high-pressure homogenization was used to formulate AXT-loaded NLCs. Following a detailed literature review, formulations were developed using a hit-and-trial technique. Compritol 888 ATO (solid-lipid) and Oleic acid (liquid-lipid) concentration 60:40 was kept constant. The solid-lipid was melted in the lipidic phase. After dissolving 10 mg of AXT in the melted solid-lipid phase, the liquid-lipid was added to the melted lipidic phase and further heated to 80°C to obtain a clear lipid phase. [13] Meanwhile, an aqueous surfactant solution was prepared with hydrophilic surfactant (Tween 80) and heated at the abovesaid temperature. The heated surfactant solution (aqueous phase) was then added dropwise in the hot lipid phase to form a milky white pre-emulsion. It was continuously stirred on mechanical stirrer (Remi Instruments Ltd., Mumbai, India) for 15 min at 1500 rpm. [14] The heated pre-emulsion was subjected to high-pressure homogenization (PANDA 2K, Niro Soavi, Italy) for 7 cycles at 800 bar. The resulted NLCs were then cooled down to room temperature, and further used for characterization. [15]

Table 1: The coded and actual values of the variables used in Full Factorial designs of AXT-NLCs

Independent Variables	Levels	
	Low (-1)	High (+1)
A: Concentration of Solid-lipid	60	80
B: Concentration of Liquid-lipid	20	40
C: Concentration of Surfactant	2	4
Dependent Variables	**Constraints**	
Y1: MPS (nm)	Minimum	
Y2: %EE	Maximum	

Optimization of the ingredients using a full factorial design

The formulation batches were optimized using 2^3 simple full factorial designs, as shown in Table 2. Formulation was optimized by considering Concentration of Solid-lipid (X_1), Liquid-lipid (X_2) and Surfactant (X_3) as independent variables and MPS and %EE as responses. The results of experimental design were analysed using Design Expert software. Each response coefficient was studied for its statistical significance at 95% confidence level. The *P* value less than 0.05 indicates that model terms are significant, and greater than 0.05 indicates that model terms as insignificant and should be removed from analysis to generate the reduced model. All independent variables, their levels along with actual and coded values are shown in Table 5. Mean Particle Size of NLCs (Y1) and %EE (Y2) were selected as response parameter and the dependent variables. The design is used to choose the best model among the two-factor interaction, linear, and quadratic model by analysis of variance (ANOVA) *F*-value. Statistical analysis and graph plotting was done using Design-Expert software. The ANOVA was used to calculate the effect of independent variables on the responses were through Fisher's test. Statistical significance was defined as a *P*-value less than 0.05. [16] Evaluation of multiple correlation coefficients (R^2) and adjusted R^2 were employed for best suitability of model. The relationship and interaction between the coded variables and the responses were revealed using contour and three-dimensional surface plots. For optimization, selection of the MPS was based at its minimum, and %EE at maximum levels. [17]

Table 2 Formulation of AXT-NLC by 2^3 simple Full Factorial design

Run	X1	X2	X3
1	60	20	4
2	80	20	4
3	60	40	4
4	60	40	2
5	80	20	2
6	60	20	2
7	80	40	4
8	80	40	2

Note: X_1, Concentration of solid-lipid (High level: 80gm, Low level: 60gm); X_2, Concentration of liquid-lipid (High level: 40, Low level: 20); X_3. Concentration of surfactant (High level: 4, Low level: 2)

Screening of optimized batches

MPS and PDI

The MPS analysis of NLC was performed by photon correlation spectroscopy (PCS) using Zeta-sizer (Nano ZS 90, Malvern Instruments, UK) at fixed angle of 90°, at 25°C temperature. To determine MPS and PDI, the NLCs dispersion was diluted with distilled water before analysis to obtain optimum 50-200 kcps. [18]

Zeta potential

Zeta potential measurements were run at 25°C with electric field strength of 23 V/m, using Zeta-sizer (Nano ZS 90, Malvern Instruments, UK). To determine the zeta-potential, samples of AXT-NLCs were diluted and placed in electrophoretic cell. The zeta-potential was calculated as described by Helmholtz–Smoluchowski equation. [19]

Determination of % entrapment efficiency

%EE was determined by calculating the amount of free AXT in aqueous surfactant solution. Quantity sufficient volume of AXT-NLCs dispersion was placed in thin wall polyallomer tubes and centrifuged using Ultracentrifuge (Optima max XP, Beckman coulter, USA) at 45,000 rpm for 30 min at 4°C. The free AXT concentration was determined using UV–visible spectrophotometer (UV 1700, Shimadzu, Japan) at λmax 260 nm. Values of %EE were calculated using following equation. [10]

$$(\%)\ \text{Entrapment Efficiency:} = \frac{\text{Total amount of drug-Amount of drug in supernatant}}{\text{Total amount of drug}} \times 100$$

Selection of optimized batch

The design matrix created by Design-Expert® (version 7.0.0; Stat-Ease, Inc., Minneapolis, Minnesota, USA) was utilised for optimization data analysis. [20]

Lyophilization of NLCs

The AXT-NLCs aqueous dispersion with 3% cryoprotectant (Mannitol) was frozen in a refrigerator at −75°C for 24 hours. Then samples were lyophilized using a lab freeze-dryer (VirTis Benchtop K, SP scientific, Warminster USA). The vials were freeze-dried for two weeks before being sealed with rubber caps. [21]

In-vitro release study

It is usually known that drug release from nanocarriers is influenced by various factors such as, structure and composition of the NLC. Hence, in-vitro release of AXT from NLCs was studied. *In-vitro* drug release was studied in phosphate buffer pH 6.8 using dialysis bag diffusion method. The Dialysis bag membrane with molecular weight cut off 12-14 kDa was soaked in phosphate buffer saline (PBS) solution 12 hours prior usage. [24] The dialysis bag was magnetically stirred at 100 rpm and 37 ± 2 °C in beaker containing 250 mL of dissolution medium (PBS pH 6.8). Dialysis bag was sealed from one end; 2 mL of NLCs dispersion was poured into it and tightly sealed from another end. Then it was subjected again to magnetic stirring in a beaker containing 200 mL PBS at 50 rpm and 37 ± 2 °C. mL of dissolution medium was withdrawn and replaced with the fresh buffer at regular intervals of 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 24 hours to maintain sink condition. The filtrate was suitably diluted, if necessary, and analyzed using UV-spectrophotometer at 260 nm. [8]

Solid state characterization

DSC studies

DSC analysis was carried out using a differential scanning calorimeter (DSC 1 STAR^e system, Mettler-toledo, Switzerland). For DSC measurement; a scan rate of 10°C/min was employed over the heating rate range of 40-400°C under a nitrogen purge. DSC studies were conducted for AXT, and freeze dried AXT-NLCs of the optimized batch. [22]

FTIR studies

FTIR spectrum of drug was measured in the solid state as potassium bromide (KBr) mixture. Freeze dried drug loaded AXT-NLCs were mixed thoroughly with KBr in 1:100 (AXT-NLCs:KBr) ratio and IR-transparent matrix pellet was prepared. The pellet was then scanned across a wave range of 4000-400 cm^{-1} using a FTIR spectrometer-430 (Shimadzu 8400S, Japan) and spectra was obtained. [23]

Stability studies

In general, a shelf life of at least one year is an important prerequisite criterion for a commercial product, to be pharmaceutically acceptable with high drug retention capacity. During storage, the particle size should be maintained. Hence the drug leakage, zeta-potential, and particle size growth should be studied. Stability studies for prepared AXT-NLCs were carried out for 1 month and were accessed by, particle size, PDI, and %EE. [20]

RESULT AND DISCUSSION

Partitioning behavior of AXT between the lipids

Based on the screening of liquid-lipids for the maximum solubility of the drug, the solubility data are shown in Fig. 1 and Fig. 2. Oleic acid and Compritol 888 ATO was screened out as most suitable liquid-lipid and solid-lipid, respectively, to be used in the AXT-NLCs. Moreover, both the lipids are non-toxic in nature, approved by regulatory status (GRAS), inexpensive etc., and hence were used in the formulation.

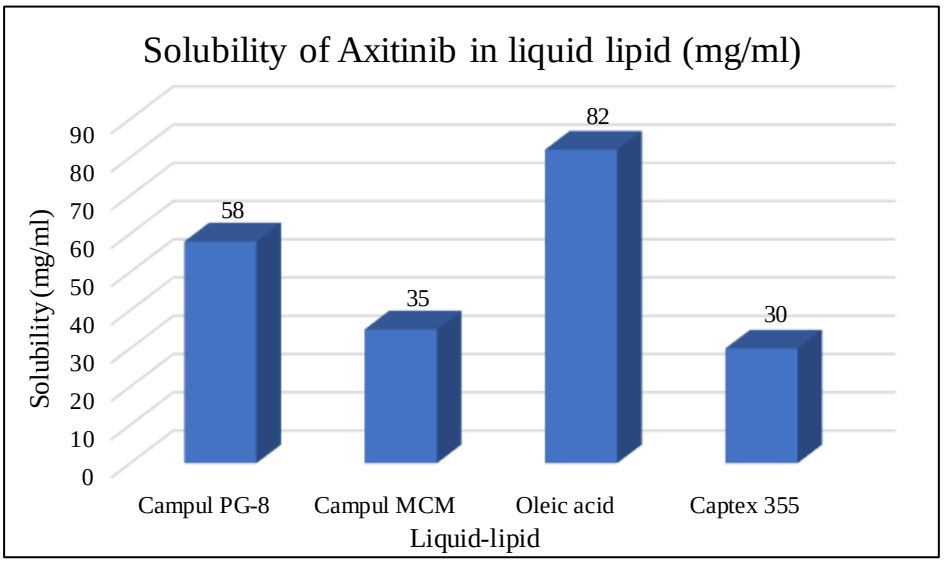

Figure 1: Solubility of AXT in different Liquid-lipids

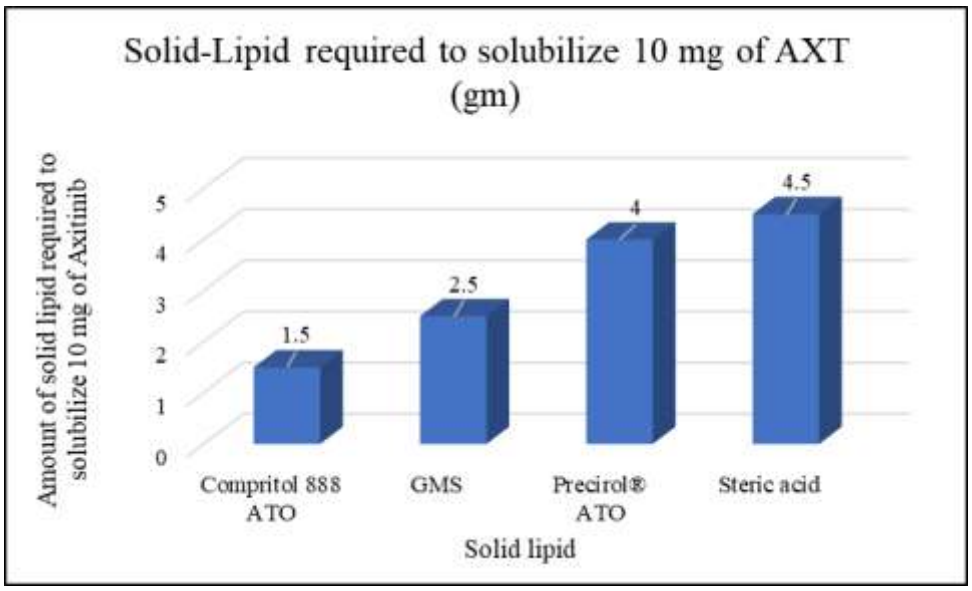

Figure 2: Amount of different Solid-lipids required to Solubilize 10 mg of Axitinib.

Preliminary trials

For designing AXT-NLCs, total lipid i.e., solid-lipid: liquid-lipid (Compritol 888 ATO: Oleic Acid) taken in ratio of 6:4 which was optimized by preliminary experiments indicating that total concentration of solid-lipid, liquid-lipid, and surfactant during preparation were the main factor that affected the MPS and %EE of the AXT-NLCs. Solid-lipid (X_1), Liquid-lipid(X_2), Surfactant(X_3) as an independent variable and MPS (Y_1), %EE (Y_2), are the dependent variables. MPS and %EE increases as the surfactant concentration increases, the MPS and %EE also increases and vice versa. MPS decreases as the lipid concentration increases. Thus, based on the obtained results, optimal range of independent variables was chosen as concentration of surfactant 2-4 %, concentration of solid-lipid 6-8 mg, concentration of liquid-lipid 2-4 ml. Total eight tests were conducted for the purpose of optimization.

Formulation and optimization of AXT loaded NLCs.

The AXT-NLC dispersions were prepared by hot high-pressure homogenization (HPH) as described in literature with suitable modification. The lipid phase containing the solid and liquid-lipids obtain a homogeneous solution. Drug was dissolved in the melted lipid (60-70°C above the melting point of lipid), and the drug loaded lipid dispersed in a hot aqueous surfactant solution to form a pre-emulsion in a water bath at 80 °C which was consequently introduced in to hot HPH (PANDA 2K, Niro Soavi, Italy) at optimized pressure and cycles to form the NLC dispersion. Then NLCs so formulated were allowed to cool at room temperature which was further used for characterization. To prepare the NLCs, hot HPH was selected as it is the best, reliable and powerful technique used to scale up production. Preliminary studies shown that concentration of solid-lipid and liquid-lipid should be constant up to 6:4 ratios.

Considering the low solubility and absorption of AXT from the gastrointestinal tract, the actual amount of drug bioavailable to the body is only about 58%, due to its extensive first-pass metabolism. For this reason, the NLCs were selected to enhance the oral bioavailability of AXT, which is supposed to bypass the hepatic metabolism through the lymphatic absorption pathway. NLCs may be formulated by involving many parameters in the formulation process. To minimize the number of tests and in attempt to optimize the NLCs, Simple full factorial design was used for designing the least number of runs. The AXT-loaded NLCs were prepared using HPH technique.

For the preparation of AXT-NLCs, Compritol 888 ATO was selected as a solid matrix and Oleic acid as liquid-lipid with constant concentration 60:40, respectively, as optimized in partition behavior study. Thus, depending upon the result obtained the optimal value range of the variables was taken as solid-lipid concentration (X1) of 60–80 gm, liquid-lipid concentration (X2) of 20-40 ml and Concentration of surfactant 2-4 ml (X3).

Formulation optimization based on the factorial design software 7.0.0.

Table 3 expresses the 2^3 full factorial design with concentration of solid-lipid (Factor A) ,Concentration of liquid-lipid (Factor B) and Concentration of Surfactant (Factor C) as independent variables while MPS and %EE as Response 1 and Response 2, resp. Table 2 also includes the PDI and DL for the prepared batches .The results of experimental design were observed using Design-Expert software. Each response coefficient was studied for its statistical significance at 90% confidence level. The p value of 'Prob > F' less than 0.05 indicates that model terms are significant and values greater than 0.05 indicates that model terms are insignificant and should be removed from analysis to generate the reduced mode**.**

Table 3: Design of formulations AXT- NLCs and its results

Run	Factor A	Factor B	Factor C	Response 1	Response 2
	X1: Conc. of Solid-lipid (mg)	X2: Conc. of Liquid-lipid (ml)	X3: Conc. of Surfactant (%)	Y1: Particle Size (nm)	Y2: %EE
1	60	20	4	224.7	65.6
2	80	20	4	235.4	75.8
3	60	40	4	230.9	77
4	60	40	2	228.9	75.6
5	**80**	**20**	**4**	**202.2**	**88**
6	60	20	2	248.2	58.2
7	80	40	4	209.6	82.6
8	80	20	4	227.2	80.3

Optimization data analysis and model validation

Fitting of data to the model

The ranges of responses Y_1, Y_2 and were 202 to 248.2 nm and 58.2 to 88 % respectively. All the responses observed for 8 formulations prepared were fitted to various models using Design- Expert® software. It was observed that the best fitted models showed effects on particle size, entrapment efficiency (EE%). The values of R^2, adjusted R^2, predicted R^2, SD, % CV and Mean are given in Table 4, along with the regression equation generated for each response. The results of ANOVA in Table 7 for the dependent variables demonstrates that the model was significant for all three the response variables.

Table 4: Values of R^2, Adjusted R^2, Predicted R^2, SD, % CV and Mean

Response	R^2	Adjusted R^2	Predicted R^2	SD (±)	% CV	Mean
Particle Size	0.9619	0.9111	0.7291	4.29	1.90	225.89
% Entrapment Efficiency	0.9403	0.8607	0.5755	3.54	4.69	75.39

Abbreviations: SD, Standard Deviation; CV, Coefficient of Variance

Table 5: Results of analysis of variance for MPS (Y_1) and % EE (Y_2)

Parameters	SS	DF	MS	*F*-Value	*P*-Value	Model Significance
MPS						
Model	1396.73	4	349.18	18.94	0.0182	Significant
Residual	55.31	3	18.44	-	-	
Total	1452.05	7	-	-	-	
EE (%)						
Model	591.68	4	147.92	11.81	0.0352	Significant
Residual	37.56	3	12.52	-	-	
Total	629.5	7	-	-	-	

(Note: *P* Values less than 0.05 are significant while greater than 0.1 are not significant)

Abbreviations: SS, Sum of Square; DF, Degrees of Freedom; MS, Mean Sum of Square; F, Fischer's ratio

Effects of independent variables on mean particle size (Y1)

The polynomial equation for MPS is as follows (Figure 8, 9, 10)

Y1= MPS (nm) = 225.89-7.29A-9.04C-3.66AC+5.14BC

From the ANOVA data all the three independent factors and their interactions were found to significantly affect the MPS (p-value < 0.0001). The R^2 value (0.9619) near to one indicates linearity. Decrease in concentration of Tween 80, decreases the MPS as it helps in formation of stable NLCs suspension by reducing the interfacial tension. Decrease in the concentration of lipid led to a very significant decrease in particle size of NLC formulation. Pre-emulsion with large globules & NLCs with large particles were obtained with 0.1% concentration which may be due to the inadequate amount of surfactant to reduce the interfacial tension. Pre-emulsion with small globules & NLCs with reduction in MPS was observed with 0.4% concentration which may be due to effective reduction in the interfacial tension between the aqueous and the lipid phase. In addition, the surfactant helps to stabilize the newly generated surface and prevent particle aggregation. A remarkable increase in PDI was also observed which supports the aggregation phenomenon. Increase in viscosity of aqueous phase causes decrease in size reduction with sonication.

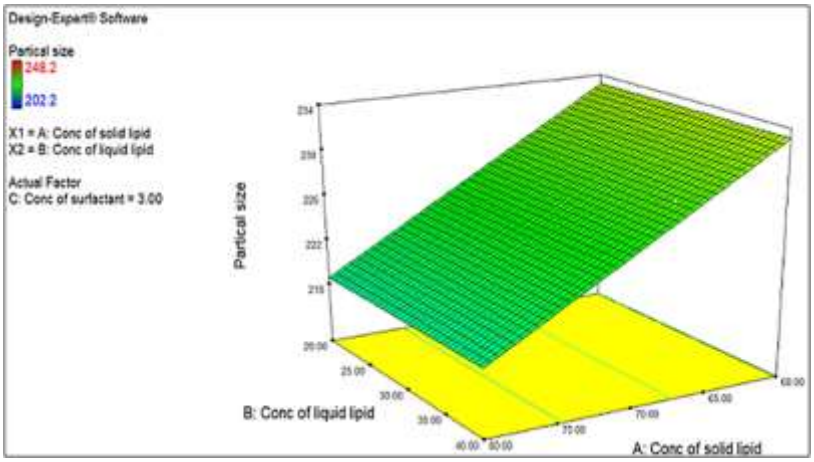

Figure 3: Response surface plot for Y1 MPS (AB).

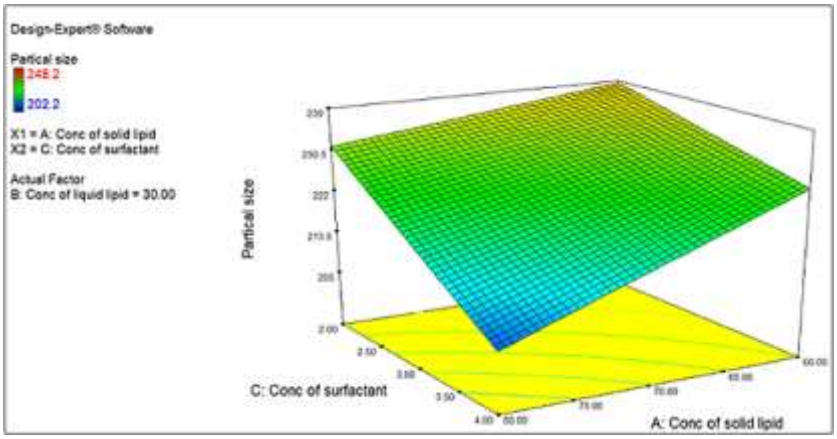

Figure 4: Response surface plot for Y1 MPS (AC).

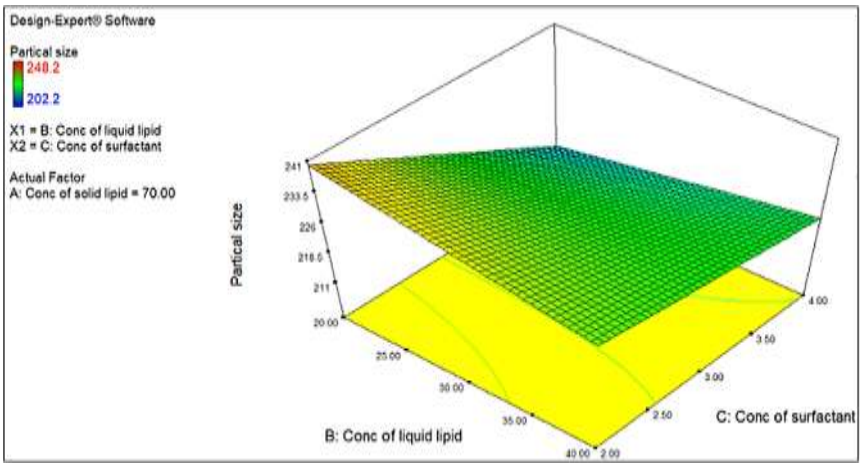

Figure 5: Response surface plot for Y1 MPS (BC).

Effect of independent variables on drug % entrapment efficiency (Y2)

The polynomial equation for %EE is as follows (Figure 11, 12, 13)

Y2 = EE (%) = +75.39+6.29A+3.49B+2.91C-3.71AB

Values of 'Prob > F' less than 0.0500 indicate model terms are significant. In this case A, B, C, are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The R^2 value (0.9403) near to 1 indicates linearity. The non-significant factors and interactions were removed from above polynomial equation. AXT shows good solubility in Oleic acid and thus increases the amount of drug dissolved in total lipid. Increase in liquid-lipid amount may increase the drug entrapment in lipid matrix. This may be due to increase in the lattice imperfection. The solution recommended from design expert software indicated that the optimized AXT-NLCs with high %EE and least MPS obtained when the formulation contains 0.5% of Tween 80, concentration of solid-lipid and liquid-lipid. These observed responses of MPS (202.2 nm) and %EE (88%), presented by the optimized AXT-NLCs formulation, and the predicted value of MPS (225.89 nm) and %EE (75.39%) generated by the Design Experts software, were found to be in good agreement. This finding demonstrates that the optimized formulation was reliable in predicting the response of the AXT-NLCs system.

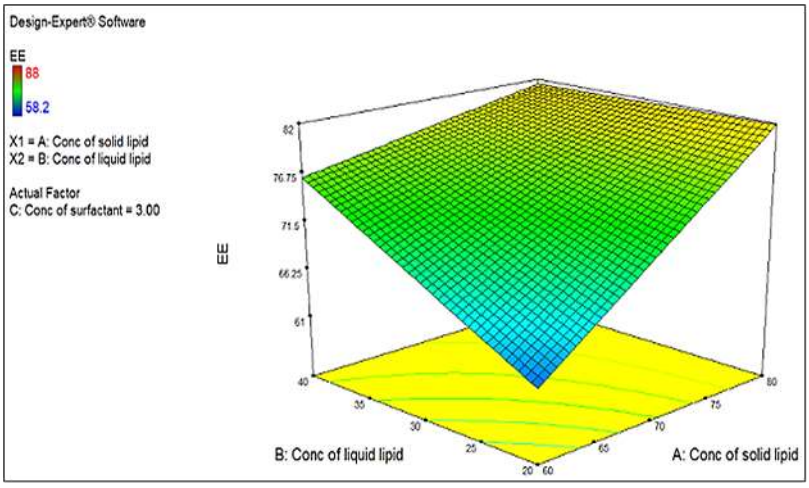

Figure 6: Response surface plot for Y2 %EE (AB).

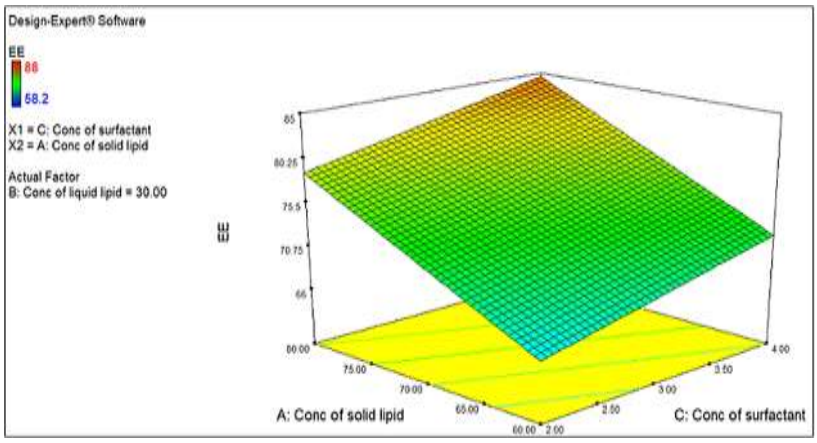

Figure 7: Response surface plot for Y2 %EE (AC).

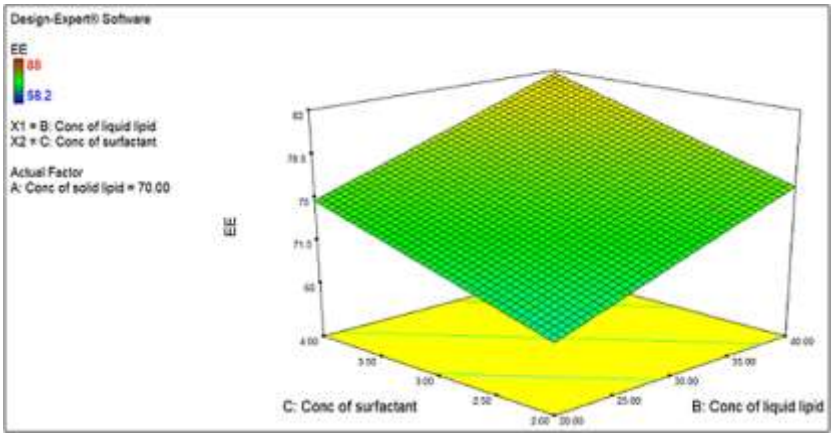

Figure 8: Response surface plot for Y2 %EE (BC).

Screening of optimized batch of NLCs

The result of optimized batch (F5) showed the NLC dispersion with average particle size of 202.2 nm and Zeta-Potential -21.5 mV immediately after preparation. The lipid nanoparticles with optimum stabilizer composition are usually stable for prolonged period .The ξ value of -30 mV to -60 mV was considered optimized batch.

Table 6: Characterization of Mean Particle Size & PDI

Batch	Mean Particle Size	PDI
F1	224.7	0.56
F2	235.4	0.44
F3	230.9	0.24
F4	228.9	0.47
F5	**202.2**	**0.44**
F6	248.2	0.17
F7	209.6	0.31
F8	227.2	0.52

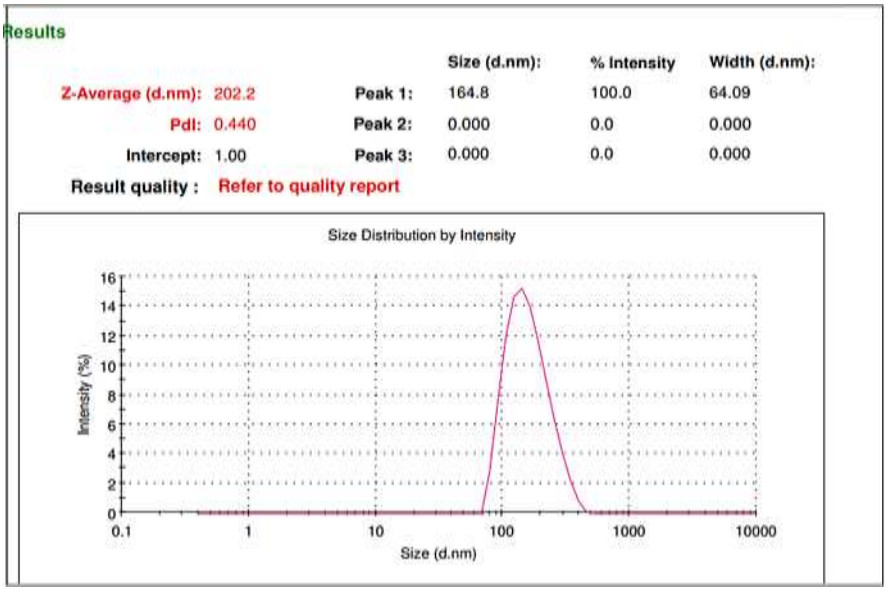

Figure 9: Particle size of AXT-Loaded NLCs (F5).

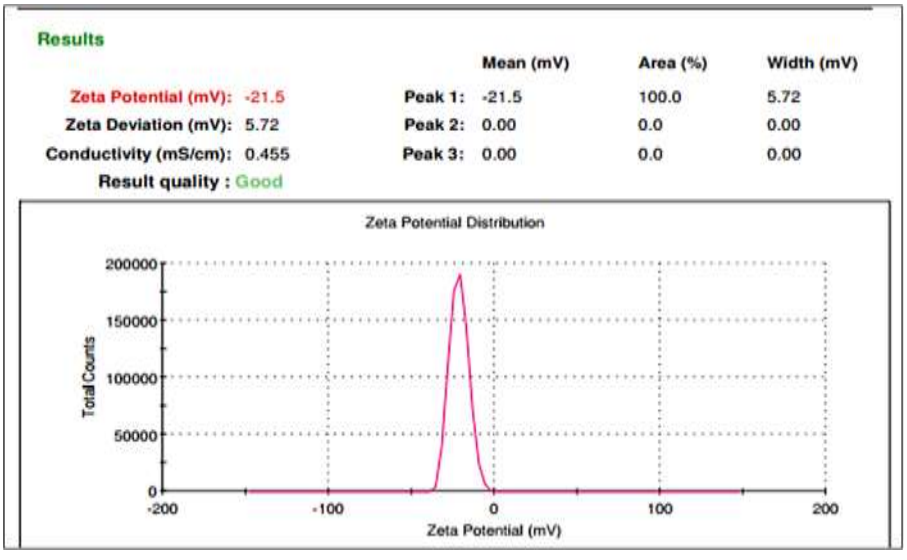

Figure 10: Zeta potential AXT-Loaded NLCs (F5).

Lyophilization of AXT– NLCs

The lipid dispersion was successfully freeze dried using the benchtop freeze dryer. Lyophilization was carried out for optimized batch at -75 °C with 2-3% mannitol as a cryoprotectant. The role of cryoprotectant is to decrease nanoparticle aggregation during the process of freeze-drying. The obtained lyophilized powder was found to be dry, porous and friable after 72 h. The vacuum was maintained at 76 mTorr. To improve the storage stability of the AXT-NLCs, they were lyophilized. To protect nanoparticles from stresses and subsequent aggregation, a cryoprotectant is generally used. Here, when dried powders are reconstituted in an aqueous system, the ability of the nanoparticles to go back to their original particle size is defined as 're-dispersibility'. As the concentration of a cryoprotectant increases, the aggregation of nanoparticles may either decrease or increase depending on the system of nanoparticles and the freeze-drying conditions. The 2-3% mannitol showed to be the most appropriate properties including free flowing nature of lyophilized powder. Thus, the AXT-NLCs batch containing 2% mannitol was selected and further taken for DSC, FTIR and in-vitro drug release studies.

In-vitro drug release study

The cumulative percentage drug release of Axitinib from Axitinib suspension and optimized AXT-NLCs were observed *in-vitro* by dialysis bag over a time of 1 h to 24 h (Table 9). F5 batch which was optimized based on low particle size and higher %EE, was selected for in vitro study. Each sample was analyzed in triplicate and release profiles are shown in Fig. 16. The release profile of Axitinib from NLCs was biphasic. It was observed that, from AXT-NLCs, drug was released slowly up to 71.83% at the end of 24 h. This may be due to penetration of aqueous diffusion medium slowly taking place into the lipid layer as they are lipophilic in nature. Then dissolution and diffusion of the drug would occur and Axitinib was released.

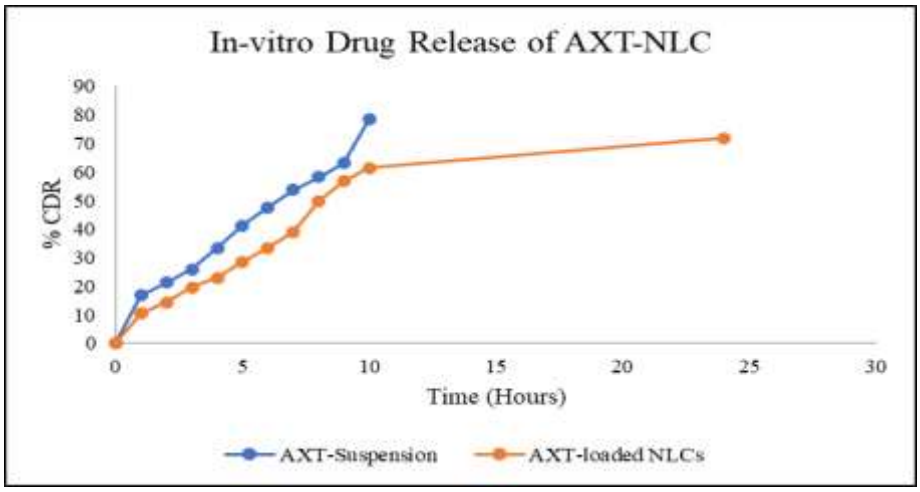

Figure 11: Drug release profile of Axitinib-suspension and optimized batch-NLC (F5).

Table 7: In-vitro diffusion study for pure drug suspension and optimized batch (F5)

Time (hrs)	AXT-Suspension	AXT-loaded NLCs
0	0	0
1	16.75±0.71	10.41±0.71
2	21.23±0.71	14.45±1.08
3	25.89±0.71	19.53±1.47
4	33.50±0.41	22.92±0.71
5	41.34±0.71	28.64±1.47
6	47.69±0.71	33.51±0.71
7	53.83±1.08	39.01±1.00
8	58.28±0.71	49.80±1.08
9	63.15±0.71	56.80±0.71
10	78.38±1.08	61.46±1.08
24	-----	71.83±1.47

Solid state characterization of optimized NLCs

Figure 11 shows DSC curve of Compritol 888 ATO and lyophilized powder of AXT–NLCs. The thermogram of AXT (Figure 17) shows a melting peak of AXT at around 220°C. The melting process of Compritol 888 ATO took place with maximum peak at 71°. However, the melting peak for the AXT around 220°C was not detected in the thermograms of the lyophilized AXT–NLCs. The disappearance of the endothermic peak of AXT in the AXT–NLCs powder suggests that the drug is completely encapsulated in the lipid matrix and converted to amorphous state from crystalline state.

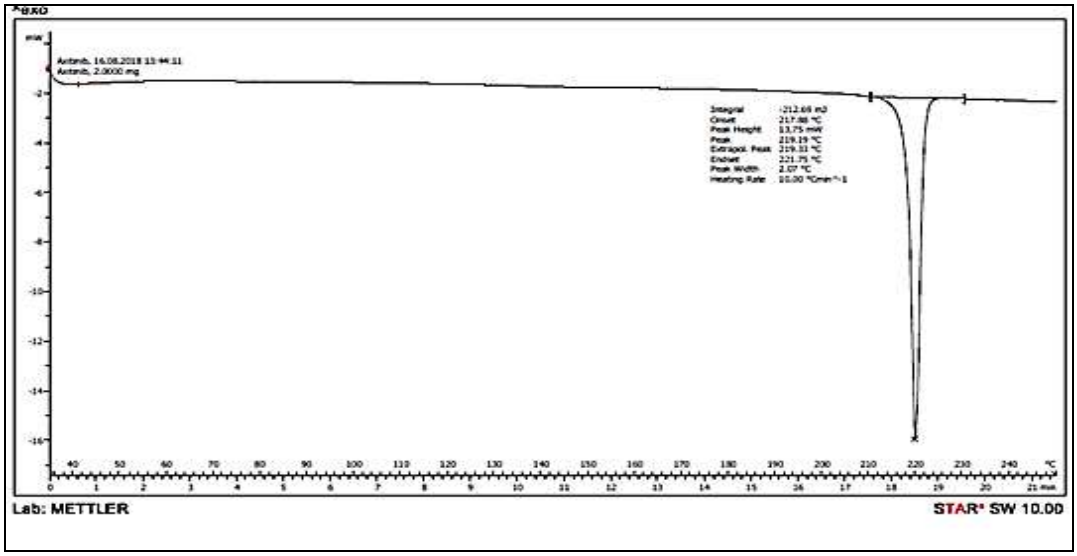

Figure 12: DSC study of pure drug AXT

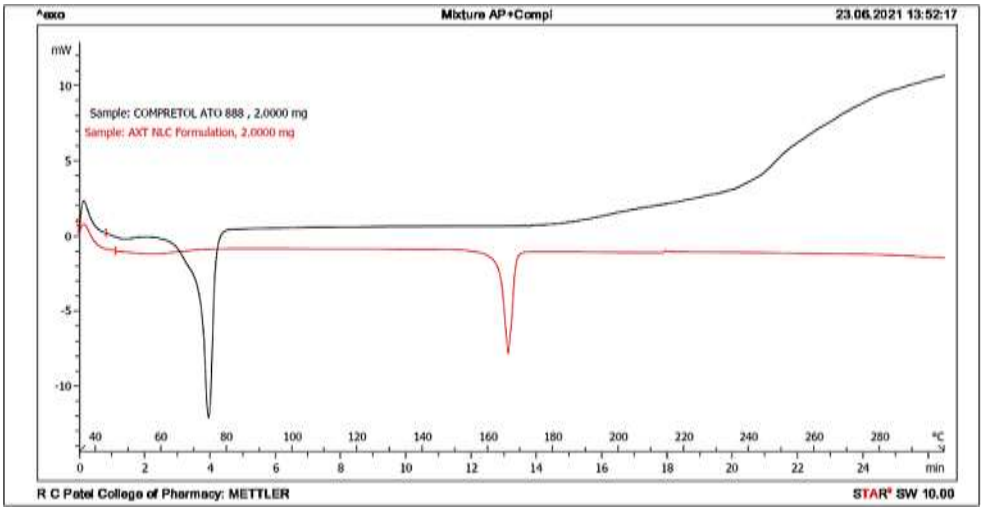

Figure 13: DSC Overlay of Compritol 888 ATO, AXT-NLCs (F5).

FTIR

The FTIR spectra of ACT, Compritol 888 ATO and AXT NLC has been showed in Figure 19. No detectable peaks were observed in the FTIR spectra, implying that there were no chemical interactions occurred between the drug and the lipid.

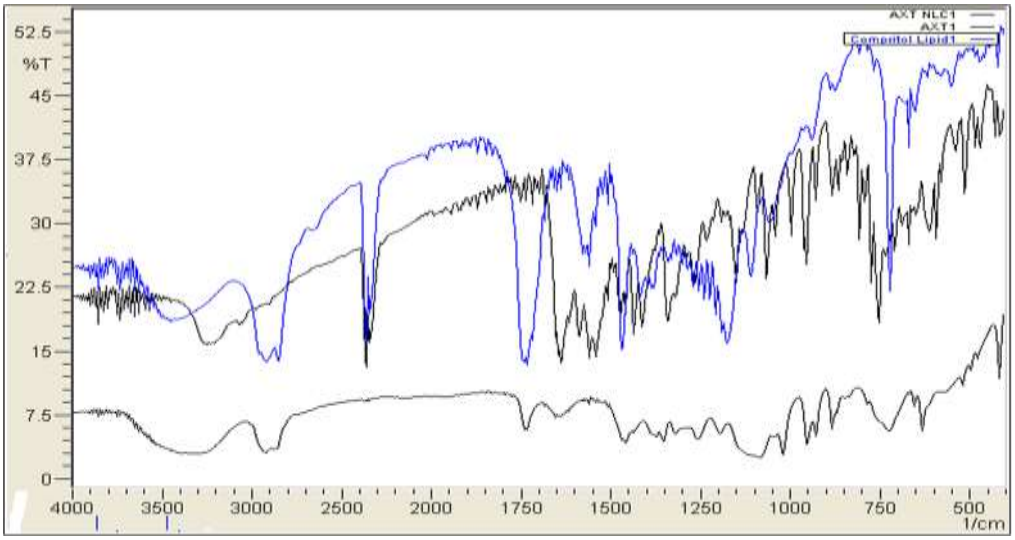

Figure 14: FTIR spectra of AXT-NLC formulation (F5).

Accelerated stability study of optimized AXT-NLCs

Temperature was found to have an impact on NLCs. The initial MPS, PDI, and % EE were calculated. The batch was divided into two equal portions at the time of preparation, stored at various temperatures in a refrigerated (2-8^{o}C) and at room temperature (28-30 ^{o}C). Particle size analysis, PDI, and % EE of the samples were determined up to one month. The formulation was stable at room temperature for one week, after which aggregation was noticed. As a result, Axitinib-NLCs were less stable at room temperature than in cold conditions. According to the findings of the stability investigation, the improved batch was very stable at refrigerator temperature. The results are tabulated in Table 12.a, 12.b, 12.c.

Table 8. Stability study **a.** at the time of preparation, **b.** storage at room temperature (28-30 ^{o}C), **c.** Storage Condition in refrigerator (2-8^{o}C).

a.

Storage Condition	MPS (nm)	PDI	% EE
At the time of preparation	202.2	0.4	88

b.

Sampling Time (Days)	MPS (nm)	PDI	% EE
15	207.2	0.215	83.60%
30	235.7	0.439	80.50%

c.

Sampling Time (Days)	MPS (nm)	PDI	% EE
15	204.7	0.413	84.30%
30	207.7	0.434	82.30%

Abbreviations: MPS, Mean Particle Size; PDI, Polydispersity Index; EE, Entrapment Efficiency

CONCLUSION

In the present investigation Axitinib (AXT) was successfully loaded into NLCs by using Compritol 888 ATO as solid-lipid, Oleic acid as liquid-lipid and Tween 80 as surfactant. The general 2^3 factorial design based on particle size and entrapment efficiency, was successful in predicting the optimized formulation of NLCs.

The optimized formulation produced nanoparticles with a particle size of 202.2 nm, PDI of 0.440, zeta-potential of 21.5 mV, and %EE of 88%. The physicochemical characterization of AXT-loaded NLCs was done by examining the results of DSC, FTIR spectroscopy study. The stability study of optimized formulation was found to be stable with no significant change in particle size, PDI and %EE. In-vitro release profiles of NLCs indicated a burst release for first 2 hrs followed by prolonged-release profile for AXT until about more than 10 hrs. Nanostructured Lipid Carrier are considered to be one of the most promising drug delivery systems for carrying highly lipophilic materials. The Axitinib-NLCs can provide a better bioavailability, reduction in dose, dosing frequency, dose related side effects and better control of the disease.

The field of nanomedicine has a bright future with the emergence of several promising approaches for delivery of therapeutic agents and imaging using the advantages of the nanoscale carriers. However, it is also recognized that as research moves toward developing smaller and smaller devices and agents, larger multidisciplinary teams are needed for success.

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