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A Quality by Design Concept on Lipid Based Nanoformulation Containing Antipsychotic Drug: Screening Design and Optimization using Response Surface Methodology

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Abstract

The purpose of this study was to implement Quality by Design (QbD) concept to Solid Lipid Nanoparticles (SLN) containing Asenapine maleate (AM) in order to identify critical process and formulation variables which can affect product quality such as particle size (PS) and entrapment efficiency (EE). Initially, risk assessment using ishikawa diagram and preliminary investigation of critical variables was carried out. Two statistical designs were used to optimize critical variables which can affect product quality attributes i.e. PS and EE. Plackett Burman Design (PBD) was used to screen 8 variables and results showed that lipid concentration, surfactant concentration and sonication time had significant effect on PS and EE. These critical factors were further optimized using Central Composite Design (CCD), a type of response surface methodology, to assess its effect on PS and EE. Design space was identified and implementation of control strategy for responses generated quality of the desired product. Design space was generated for SLN for reducing intra-batch and inter-batch variability in formulation development process. Analysis of robustness of design space predicted that the formulation must be prepared in established design space to reduce batch variations. The results conclusively demonstrated the potential of QbD concept to build quality in SLN formulation.

Keywords: Asenapine maleate; Solid lipid nanoparticles; Quality by design; Ishikawa diagram; Plackett Burman design; Central composite design; Design space

Introduction

Solid Lipid Nanoparticles (SLNs) have attracted increasing scientific and commercial attention as colloidal drug carriers during the last few years. They have emerged as a potential alternative compared to other colloidal systems like polymeric nanoparticles, liposomes and fat emulsions, as they have been claimed to combine their advantages but successfully overcome their drawbacks [1]. SLNs are colloidal systems composed of physiological and biocompatible lipids that are solid at room and body temperatures and stabilized with non-toxic emulsifiers. The three main features of SLNs-solid nature, lipid matrix and nano-sized particles-have been theorized to impart biocompatibility, controlled drug release and improved drug dissolution [2]. It was also reported that SLN can be efficiently taken up by intestinal lymphatics which aids in improving oral bioavailability [3].

Asenapine maleate (AM) is an atypical antipsychotic drug which is slightly soluble in water. Its oral bioavailability is <2% due to its extensive first pass metabolism. It has been reported that delivery of drugs with extensive first pass first metabolism could be improved by formulating nano-encapsulated lipid based drug delivery system [4]. Hence, we proposed to formulate SLNs of AM with the aim of improving its oral bioavailability.

Pharmaceutical formulation development involves complex procedure which includes various process and formulation variables that can affect quality of final product. The effect of these individual parameters and their interaction can affect the critical quality attribute (CQA). Quality by Design (QbD) was first introduced to the pharmaceutical industry in 2006 by the International Conference on Harmonization (ICH) Q8 guidance [5]. QbD is "a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management". QbD has been promoted by pharmaceutical agencies as a way to enhance pharmaceutical development through design efforts from product conceptualization to commercialization [6].

According to ICH Q8 R2, "A critical quality attribute (CQA) is a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality" [7]. Some methods of risk assessment mentioned in ICH guideline Q9 are Failure Mode Effects Analysis (FMEA), Failure Mode, Effects and Criticality Analysis (FMECA) etc. These principles help to identify Critical Process Parameters (CPPs) that can affect the quality of final product. The relationship between the process inputs (material attributes and process parameters) and the critical quality attributes can be described in the design space. Design space is proposed by the applicant and is subject to regulatory assessment and approval of ICH Q8 (R2) [8].

Thus, QbD aids in understanding the effect of critical processing parameters (CPPs) by identifying risk identification (Ishikawa diagram), risk analysis (Screening design) and optimization using Design of Experiment (Box Behnken Design, Central Composite Design, Factorial design etc.) on CQA of final product [9]. Therefore, we aimed to implement Quality by Design (QbD) concept to aid formulation and process design of solid lipid nanoparticles and to understand effects of variables in order to improve product quality in terms of particle size (PS) and entrapment efficiency (EE), which are critical parameters affecting the performance of the SLNs.

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Materials and Methods

Materials

Asenapine maleate was received as a gift sample from Alembic pharmaceuticals Ltd., Vadodara, India. Glyceryl Monostearate was purchased from Loba Chemie Pvt Ltd., Mumbai, India. Poloxamer 188 was purchased from Sigma Aldrich, Germany. D-α-Tocopheryl Polyethylene Glycol 1000 Succinate (TPGS) was obtained from Antares Health Products, Inc, USA. All other chemicals and reagents used were of analytical grade.

Screening of solid lipid

Briefly, 500 mg of lipid was melted at a temperature 5°C above the melting point of lipid. Accurately weighed quantity of drug was added in increments and dissolved in molten lipid under stirring till drug was completely dissolved. This solution was diluted with methanol:chloroform (1:1) and the amount of dissolved drug was determined by measuring absorbance using UV-spectroscopy (UV 1700, Shimadzu AS, Japan) at 270 nm [10].

Preparation of solid lipid nanoparticles

SLNs were prepared by high speed homogenization followed by ultrasonication method as reported previously [11]. Briefly, lipid was melted 5°C above its melting point; drug was added to molten lipid and this lipid phase was dispersed in the aqueous surfactant solution at the same temperature of lipid using a homogenizer (T-25 digital Ultra-Turrax, IKA® India Private Limited, India). The obtained emulsion was ultrasonicated using a probe sonicator (Labsonic, Sartorius, Germany). The resulting dispersion was cooled in an ice bath to produce SLN.

Experimental design

Initial risk assessment: Ishikawa diagram: Firstly, Ishikawa (fishbone) diagram was used to identify critical processing/formulation variables which can have an impact on the CQAs of asenapine maleate SLN. Particle size and entrapment efficiency were selected as product QAs [12].

Preliminary investigation of critical variables: After identifying the variables that might affect the product QAs from Ishikawa diagram, preliminary investigation of variables was carried out on the basis of risk priority. Preliminary optimization for various process and formulation variables was carried out by changing one variable at a time while keeping the other constant. The effects of selected variables on the particle size and entrapment efficiency were studied to determine the optimal lower and upper values for a screening design study.

Risk analysis: Plackett Burman design (PBD): Initial screening of significant variables was carried out using PBD for their relative influence on the particle size and entrapment efficiency of the SLNs. The high and low values for each factor were selected on the basis of the results obtained from preliminary investigation. The PBD was constructed with 12 runs using Minitab version 16 (Minitab Inc., State College, PA, USA).

Key factors were X_1: Homogenization speed, X_2: Homogenization time, X_3: Sonication time, X_4: Sonication amplitude, X_5: Concentration of surfactant, X_6: Concentration of lipid, X_7: Concentration of drug and X_8: Concentration of TPGS. The responses selected were particle size and entrapment efficiency.

Optimization using central composite design (CCD): A response surface methodology

A central composite design, a type of response surface methodology, was used to statistically optimize critical factors and to estimate main, interaction, and quadratic effects of the factors on properties CQA of AM loaded SLN. Based on the results obtained from PBD, three critical variables i.e. X_1: Lipid concentration, X_2: Surfactant concentration and X_3: Sonication times were taken as independent variables and particle size and entrapment efficiency were taken as dependent variables.

The regression equation for the response was calculated using the following equation:

$$Y=B_o+B_1X_1+B_2X_2+B_3X_3+B_{11}X_1^2+B_{22}X_2^2+B_{33}X_3^2+B_{12}X_1X_2+B_{13}X_1X_3+B_{23}X_2X_3. \quad (1)$$

In this mathematical approach, each experimental response (Y) can be represented by a quadratic equation of the response surface. Y is the measured response and b is the estimated co-efficient for the factor X. The co-efficient corresponding to linear effects (X_1 and X_2), determination (X_1X_2) and the quadratic effects (X_1^2 and X_2^2) were determined from the results of experiments [13]. Coded and actual values are shown in Table 1.

Response surface plots are helpful to understand effect of two variables on response while keeping the third variable constant. Response surface plot aids in understanding of main and interaction effects of independent variables on responses [14]. Response surface plots were generated using Design expert software version 7.0.0.

Establishment of design space

The ICH Q8 defines design space as "the multidimensional combination and interaction of input variables and process parameters that have been demonstrated to provide assurance of quality". Design space was generated using JMP software (SAS, SAS institute, Cary, NC, USA) and constraints for the desired response were selected. The batch suggested by software was prepared using same procedure as described above and predicted value was compared with observed value.

Analysis of design space robustness

Analysis of design space robustness was performed using Minitab 16 by plotting overlay plot with response higher and lower to the established design space. The software suggested values for variables in and around established design space along with value of the desired responses.

Statistical analysis

The results were presented as means ± standard error of the mean. The results were analyzed using the statistical software Minitab 16, Design expert 7 and JMP (SAS, SAS institute, Cary, NC, USA). The experimental data were validated by ANOVA, regression coefficient, and p value less than 0.05 was considered as significant.

Factors	Levels		
	-1	0	+1
X_1: Lipid Concentration (%)	2.5	5	7.5
X_2: Surfactant Concentration (%)	2	2.5	3
X_3: Sonication Time (min)	5	10	15

Table 1: The coded and actual values of independent variables.

Characterization of SLN

Determination of particle size

The particle size (PS) of the SLN was determined by photon correlation spectroscopy using a Zetasizer Nano instrument (Malvern Instruments, Malvern, UK) [14].

Determination of entrapment efficiency

The percentage entrapment efficiency was estimated by measuring amount of unentrapped drug in SLN dispersion. SLN dispersion was centrifuged at 12000 rpm for 30 min so as to settle the SLN pellet. 1 ml of supernatant was dissolved in 10 ml of methanol, the solution was filtered and amount of free drug in the supernatant was determined by measuring absorbance at 270 nm in UV spectrophotometer (UV 1700, Shimadzu AS, Japan) [15]. %EE was calculated from the following equation:

$$\%EE = \frac{\text{Total amount of drug added} - \text{free drug}}{\text{Total amount of drug added}} \times 100 \cdot \quad (2)$$

Results and Discussion

Screening of solid lipid

The maximum solubility of ASM was obtained in Glyceryl monostearate (GMS). So GMS was selected as a lipid phase for formulation development.

Risk identification: Ishikawa diagram: Ishikawa diagram was generated for factors affecting QAs of SLN and these factors were divided in three category viz., Process, formulation and environment. Critical factors were identified amongst all and were studied to evaluate their effect on QAs. Ishikawa diagram for particle size and entrapment efficiency are shown in Figure 1a and 1b respectively.

Preliminary investigation of critical variables: Influence of various process and formulation variables are shown in Table 2.

Influence of homogenization speed: It was observed that homogenization speed had major effect on particle size as compared to entrapment efficiency (Table 2). As the homogenization speed was increased from 8000 to 10000 rpm, the particle size was decreased. However, increase in homogenization speed from 10000 to 16000 increased the particle size. This might be because at higher homogenization speed higher shear rate was generated lead to increase in viscosity of lipid melt which affects emulsification process. Frothing was also observed at 16000 rpm, which increased particle size [16]. Entrapment efficiency was found to be slightly lower at 8000 rpm as compared to other speeds.

Influence of homogenization time: The high speed accompanied by time is very important factor for the SLN dispersion. It was observed that homogenization time had significant effect on particle size. As homogenization time was increased from 1 min to 10 min, the particle size was gradually decreased from 607.2 nm to 120.3 nm. With further increase to 15 min, particle size increased from 120.3 nm to 379.0 nm as longer time may cause instability for colloidal particles due to high input of energy that leads to aggregation of colloidal particles into larger particles [16,17]. Entrapment efficiency was slightly lower in case of 1 min homogenization.

Influence of sonication time: Sonication time had major effect on particle size and was the critical step in reduction in particle size. It was observed that as sonication time was increased from 1 min to 12.5 min, the particle size was gradually decreased which might be attributed to energy provided by sonication which reduced the size of coarse dispersion into nano-droplets [18]. Entrapment efficiency was not significantly affected by sonication time upto 10 min. But at 12.5 min sonication time, entrapment efficiency was found to be decreased from 77.91% to 62.34% which was attributed to breakdown of particles causing loss of drug and reduced entrapment efficiency with higher sonication time.

Influence of sonication amplitude: Sonication amplitude has direct correlation with sonication intensity. At low amplitude setting, low intensity of sonication will be delivered. Sonication works by producing cavitation transit in the dispersion and its effectiveness depends on viscosity and density of dispersion which in turn is affected by formulation temperature. It is essential to maintain temperature above melting point of lipid to facilitate breakdown of dispersion [18]. The results showed that at 20 to 40% sonication amplitude, particle size

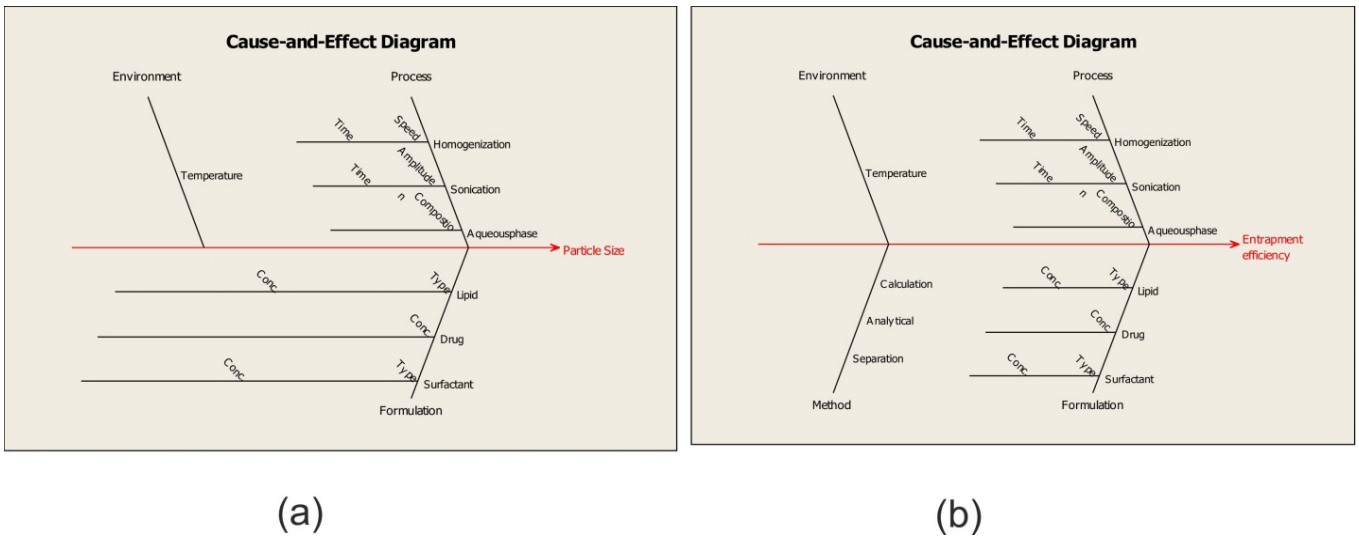

Figure 1: Ishikawa diagram for (a) Particle size and (b) Entrapment efficiency.

Process variables		Particle size (nm)	Entrapment efficiency (%)
Homogenization speed (rpm)	8000	251.2 ± 3.1	67.34 ± 3.58
	10000	149.7 ± 2.7	77.40 ± 4.40
	12000	187.9 ± 4.8	73.00 ± 2.06
	14000	213.8 ± 3.2	78.91 ± 2.86
	16000	277.5 ± 2.5	71.00 ± 3.18
Homogenization time (min)	1	607.2 ± 6.7	68.38 ± 3.58
	2.5	320.3 ± 7.5	72.56 ± 4.12
	5	218.4 ± 5.2	75.55 ± 2.51
	10	120.3 ± 4.1	76.96 ± 3.40
	15	379.0 ± 6.5	71.38 ± 2.82
Sonication time (min)	1	341.0 ± 6.9	77.92 ± 3.80
	2.5	261.3 ± 3.2	79.23 ± 4.45
	5	236.7 ± 2.6	78.34 ± 2.36
	7.5	213.0 ± 4.7	74.34 ± 3.51
	10	147.4 ± 6.8	77.91 ± 5.20
	12.5	108.3 ± 3.5	62.34 ± 1.89
Sonication amplitude (%)	20	261.2 ± 5.5	78.70 ± 2.87
	40	208.7 ± 8.4	72.14 ± 1.58
	60	127.3± 4.6	75.63 ± 3.45
	80	125.4 ± 3.9	73.04 ± 4.78
Lipid concentration (%)	1	69.6 ± 3.5	18.83 ± 5.77
	2.5	122.3 ± 5.1	46.52 ± 7.03
	5	150.7 ± 6.7	84.96 ± 6.34
	7.5	207.6 ± 8.4	89.47 ± 4.55
	10	242.3 ± 4.8	95.45 ± 3.19
Surfactant concentration (%)	0.5	347.5 ± 3.7	46.35 ± 3.92
	1	251.3 ± 2.3	56.54 ± 4.11
	2	217.9 ± 4.9	66.19 ± 5.34
	2.5	145.1 ± 4.1	77.41 ± 4.32
	5	133.5 ± 2.8	62.49 ± 6.73
TPGS concentration (%)	0.01	121.2 ± 4.3	73.21 ± 1.93
	0.02	117.4 ± 2.7	80.91 ± 4.27
	0.03	110.2 ± 2.4	68.93 ± 3.51
	0.04	105.3 ± 3.9	57.98 ± 3.92
	0.05	104.3 ± 4.0	51.23 ± 4.90
Type of lipid	GMS	133.9 ± 3.6	75.46 ± 5.31
	Precirol	224.9 ± 3.1	52.23 ± 4.29
Type of surfactant	Poloxamer 188	307.9 ± 6.9	68.03 ± 3.72
	Cremophor EL	498.1 ± 5.5	67.97 ± 3.29
	Tween 80	394.2 ± 3.6	70.78 ± 5.62
	Poloxamer 188: TPGS	145.5 ± 4.9	80.32 ± 2.76
	Tween 80: TPGS	237.5 ± 4.5	73.23 ± 3.89
	Tween 80: Poloxamer 188	292.3 ± 3.7	62.34 ± 4.33

Table 2: Influence of process and formulation variables on particle size and entrapment efficiency.

was more than 200 nm indicating that low intensity of sonication was unable to reduce particle size efficiently. When sonication amplitude was increased from 40 to 60% particle size was reduced to 140 nm. Further increase in sonication amplitude didn't affect particle size. There was not a significant difference in entrapment efficiency with change in sonication amplitude.

Influence of lipid concentration: It was observed that lipid concentration was the major influencing factor on both particle size and entrapment efficiency. As the lipid concentration was increased from 1 to 10%, particle size increased from 69.6 nm to 242.3 nm. As the lipid amount is increased, the viscosity of lipid melts increases, ultimately affecting the shearing efficiency of homogenizer during the initial phase of emulsification. This also increased collision of particles and lead to aggregation and particle size increased [19]. Another reason might be that the sonication energy is less efficiently distributed in viscous dispersion as compared to viscous dispersion. As lipid concentration was increased, the entrapment efficiency was found to be increased from 18 to 95%. This was expected because at higher lipid concentration, viscosity of lipid phase will be increased which will result into faster solidification which in turn will prevent drug diffusion from inner phase to aqueous phase [20].

Influence of surfactant concentration: The amount of surfactant plays important role in nanoparticle formation. It was observed that as surfactant concentration was increased from 0.5% to 2.5%, particle size was gradually decreased from 340 nm to 140 nm. Upon further increase in surfactant concentration from 2.5% to 5%, particle size didn't change significantly. At lower surfactant concentration, the amount of surfactant available is not able to cover the nano-droplets which lead to their coalescence which in turn increases the particle size. At higher surfactant concentration, more amount of surfactant

will be available to reduce interfacial tension between two phases, enable the lipid to become efficiently emulsified in the aqueous phase, and stabilize the nano-droplets and prevent their coalescence [21]. As surfactant concentration increased from 0.5% to 2.5%, entrapment efficiency was found to be increased. Further increase in surfactant concentration from 2.5% to 5% reduced entrapment efficiency. With increase in the concentration of surfactant in external phase, drug may diffuse out from lipid nanodroplets and solubilize in the micelles in aqueous phase, leading to reduced entrapment efficiency [19].

Type of lipid: Two different lipids GMS and Precirol were studied. GMS produced smaller particle size as compared to Precirol. The possible explanation might be the longer chain lehgth of Precirol (C_{37}) as compared to GMS (C_{21}) and its higher molecular weight which makes it bulkier and less susceptible to packaging into small particle size and produces larger particles [21]. Entrapment efficiency was found to be higher with GMS because solubility of drug in Precirol was less as compared to GMS.

Type of surfactant: It was observed that combination of surfactants efficiently reduced particle size as compared to single surfactant. Among 6 types of surfactants, it was observed that Poloxamer: TPGS produced smallest particle size. Particle size increased as follows: Cremophor EL>Tween 80>Poloxamer 188>Tween 80: Poloxamer 188>Tween 80: TPGS>Poloxamer 188: TPGS. This might be because combination of surfactants helps in reducing interfacial tension more efficienlty and helps in stabilization of nano-droplets [22,23].

There were insignificant difference in entrapment efficiency among batches prepared using different surfactants. Entrapment efficiency increased as follows: Tween 80: Poloxamer 188>Cremophor EL>Tween 80>Poloxamer 188>Tween 80>Tween 80:TPGS>Poloxamer 188: TPGS.

It is also reported that combination of ionic and non-ionic stabilizers are preferred for emulsification technology; therefore, combination of ionic and non-ionic surfactants may provide SLNs with special properties due to their steric and electostatic effects [23]. Hence, combination of Poloxamer and TPGS was selected as surfactant.

Concentration of TPGS: It was observed that as the TPGS concentration increased, both particle size and entrapment efficiency was decreased. It was reported that critical micelle concentration (CMC) of TPGS is 0.025% w/v [22]. Therefore, increase in concentration of TPGS from 0.02% to 0.04%, drug gets diffuse out from nanodroplets and solubilizes drug in micelles so entrapment efficiency was reduced.

Analysis of critical variables using screening design: PBD

PBD helps in the beginning of formulation development to segregate various factors for their influence on major characteristics of SLN. PBD allows identifying critical factors with small number of runs (12 runs) but it doesn't include interaction effects of factors.

Influence on particle size and entrapment efficiency: PBD helps in the beginning of formulation development to segregate various factors for their influence on major characteristics of SLN. PBD allows identifying critical factors with small number of runs (12 runs) but it doesn't include interaction effects of factors.

Pareto chart helps to priotirize main affecting variables amongst all selected variables. It indicates that any effects that extend beyond the reference line are considered as significant. Here, the pareto chart of Figures 2a and 3a show that sonication time, lipid concentration and surfactant concentration are beyond the reference line and were considered as critical variables for particle size and entrapment efficiency. Normal plot indicated that lipid concentration and surfactant concentration had positive whereas sonication time had negative effect on particle size (Figure 2b). It was observed from normal plot that lipid concentration has positive effect on particle size whereas sonication time and surfactant concentration had negative effect on entrapment efficiency (Figure 3b).

It was observed from actual vs. predicted plot that R^2 value for particle size and entrapment efficiency is 0.95 and 0.94 respectively which indicate a good correlation. From effect test parameters, it was confirmed that sonication time, surfactant concentration and lipid concentration had significant effect with p value less than 0.005 for particle size (0.0184, 0.0427 and 0.0204) and entrapment efficiency (0.0440, 0.0423 and 0.0297) which was in accordance with result obtained from pareto chart and normal plots.

Optimization using CCD

Influence of independent variables on particle size: From the statistical analysis of particle size data (Table 3), it can be observed that X_1 had significant effect on particle size as compared to X_2 and X_3

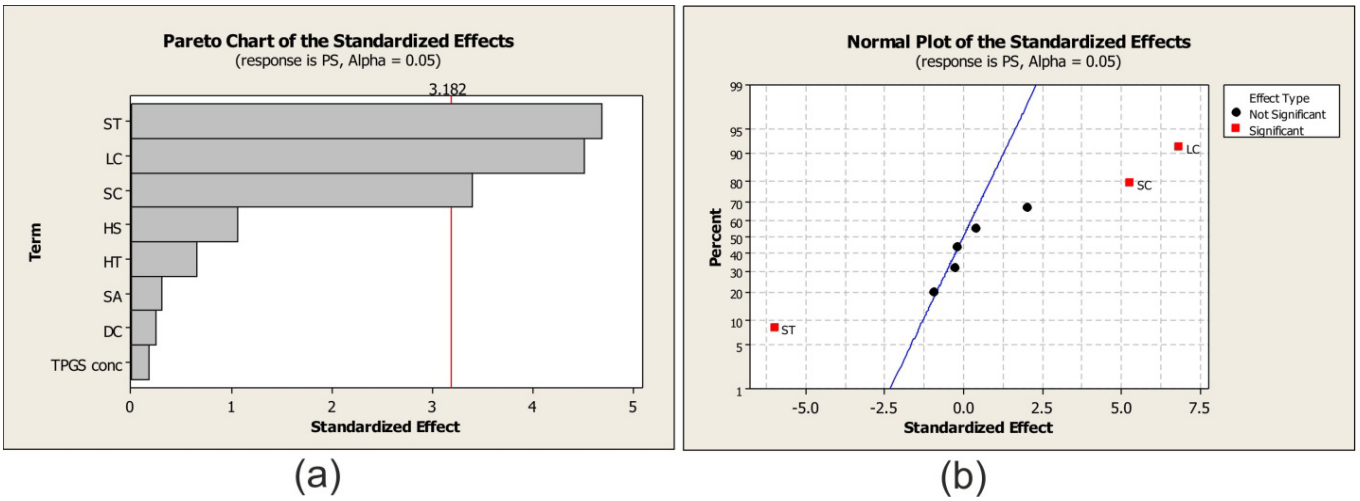

Figure 2: (a) Pareto chart and (b) Normal plot for particle size of SLN.

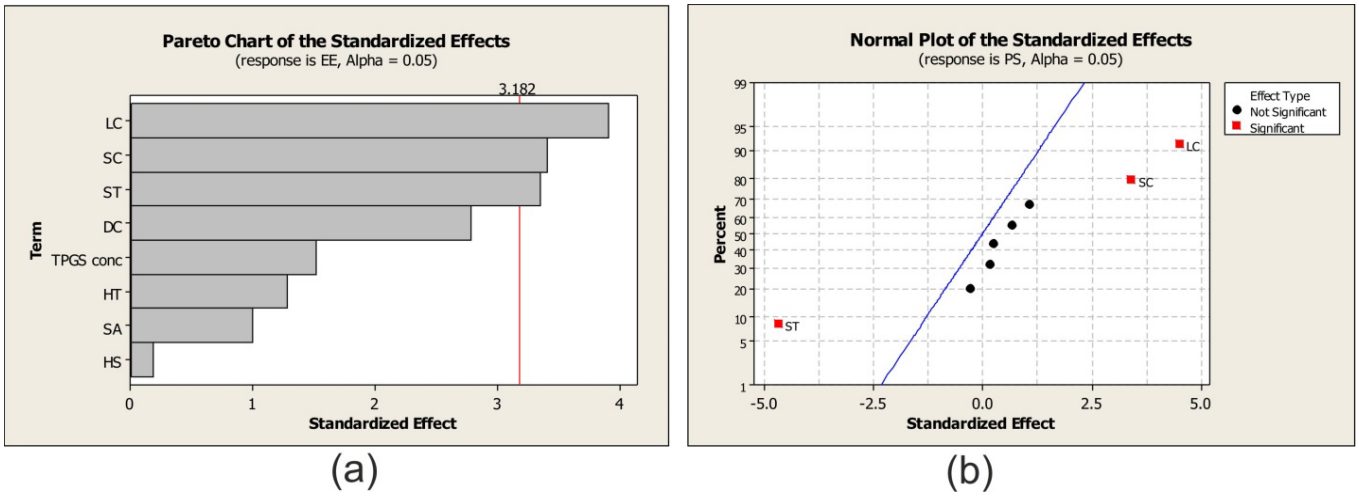

Figure 3: (a) Pareto chart and (b) Normal plot for entrapment efficiency of SLN.

Variable term (levels)	Nparm	DF	Sum of squares	F Ratio	Prob>F
Lipid conc. (X_1) (2.5,7.5)	1	1	41266.720	18.2298	0.0037
Surfactant conc. (X_2) (2,3)	1	1	993.659	0.4390	0.5288
Sonication time (X_3) (5,15)	1	1	3198.973	1.4132	0.2733
Lipid conc. (X_1)*Surfactant conc. (X_2)	1	1	3003.125	1.3266	0.2872
Lipid conc. (X_1)*Sonication time (X_3)	1	1	3264.320	1.4420	0.2689
Surfactant conc. (X_2)*Sonication time (X_3)	1	1	59.405	0.0262	0.8759
Lipid conc. (X_1)*Lipid conc. (X_1)	1	1	24569.177	10.8536	0.0132
Surfactant conc. (X_2)*Surfactant conc. (X_2)	1	1	9617.400	4.2485	0.0782
Sonication time (X_3)*Sonication time (X_3)	1	1	59797.263	26.4158	0.0013

Table 3: Effect tests analysis of independent variables on particle size of SLN.

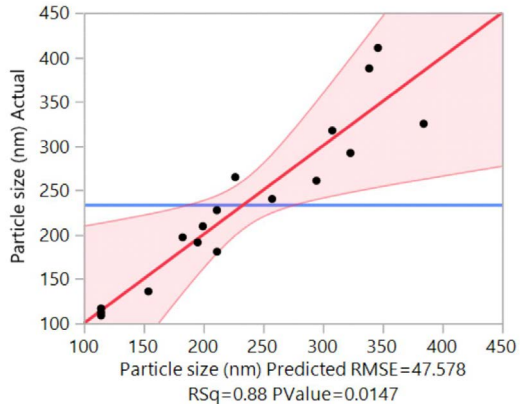

Figure 4: Actual vs. predicted plot for particle size of SLN.

Source	DF	Sum of squares	Mean square	F ratio
Model	9	119483.27	13275.9	5.8647
Error	7	15845.83	2263.7	**Prob>F**
C. Total	16	135329.10		0.0147

Table 4: ANOVA for particle size of SLN.

($p<0.005$). Interaction term X_1^2 and X_3^2 also had significant effect on particle size. Actual vs. predicated plot showed R^2 value of 0.88 which indicates a good correlation (Figure 4).

The full mode regression equation from effect analysis was obtained for particle size by software:

$$PS=+114.40+54.99X_1-8.53X_2-15.30X_3-19.38X_1X_2-20.20X_1X_3+2.72X_2X_3+46.75X_1^2+29.25X_2^2+72.76X_3^2. \quad (3)$$

The regression coefficients having p value <0.05 were considered as significant factors. The terms having coefficients with p value>0.05 were least contributing in the prediction of response. Thus, neglecting non-significant ($p>0.05$) terms from the full model and applying regression between significant terms gave equation of reduced model (equation 4) [13].

$$PS=+151.73+54.99X_1-15.30X_3+38.07X_1^2+64.22X_3^2. \quad (4)$$

As can be seen from the multiple regression equation 4, X_1 had positive effect i.e. increase in X_1 increased the value of particle size. X_3 had negative effect on the particle size i.e. increase in X_3 reduced the particle size. In ANOVA, the Fisher F test with probability ($P>F=0.0147$) indicated that the model was significant indicating significant effect of selected variables on particle size (Table 4).

The effect of individual independent variables on particle size can be visualized using bubble plot as shown in Figure 5. Bubble plot indicates an insight about the variation caused in response with changing independent variable. Dense area in the graph represents more probability of the desired response in that area. It was observed that as the lipid concentration increased, particle size also increased (Figure 5a). But more dense area was observed at 5% lipid concentration. As the surfactant concentration increased, particle size was decreased and at 2.5% surfactant concentration, minimum particle size with higher density was observed (Figure 5b). As the sonication time increased,

particle size was decreased upto 10 min. Increase in sonication time from 10 min to 15 min, increased particle size. It was reported that sonication efficiency increases when the medium temperature is low. When the temperature of the medium increases due to cavitation, the medium expands, leading to the production of less energetic shock waves from bubble implosion [18]. So, at higher sonication time, efficiency of sonication decreased which increased particle size (Figure 5c).

The relationship between independent variables and dependent variables were elucidated using response surface plots. It was observed that increase in X_1 increased particle size but no significant change in particle size was observed with change in X_2 (Figure 6a) which was in accordance with results obtained from effect analysis. It can also be seen from that increase in X_3 decreased particle size (Figure 6b) which is also in accordance with effect analysis. No prominent effect was observed on particle size while changing X_2 (Figure 6c).

Influence of independent variables on entrapment efficiency

From the statistical analysis of entrapment efficiency data (Table 5), it can be observed that X_1 had significant effect on particle size as compared to X_2 and X_3 ($p<0.005$). Interaction term X_1^2 and X_2^2 also had significant effect on entrapment efficiency. Actual vs. predicated plot showed R^2 value of 0.87 which indicated a good correlation (Figure 7).

The full mode regression equation from effect analysis was obtained for particle size by software:

$$EE=+85.35+7.21X_1-2.14X_2-1.34X_3-3.07X_1X_2-2.73X_1X_3+2.23X_2X_3-8.77X_1^2-10.75X_2^2-5.23X_3^2. \quad (5)$$

Reduced model equation:

$$EE=+78.67+7.21X_1-2.14X_2-7.22X_1^2-9.20X_2^2 \quad (6)$$

As seen in equation, X_1 had positive effect i.e. increase in X_1 increased the value of entrapment efficiency. X_2 had negative effect on the entrapment efficiency i.e. increase in X_2 reduced the entrapment efficiency. The Fisher F test with probability ($P>F=0.0213$) indicated the model was significant (Table 6).

It was observed from bubble plot that as the lipid concentration increased, entrapment efficiency was increased (Figure 8a). As the

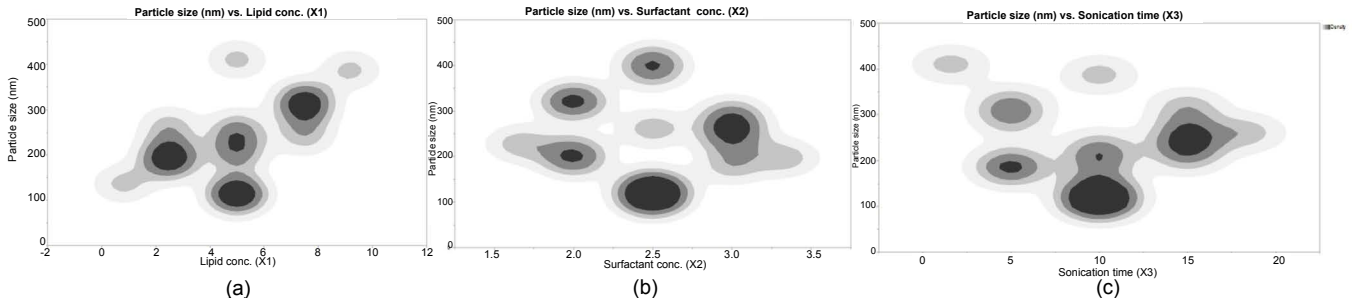

Figure 5: Bubble plots of (a) Lipid concentration (b) Surfactant concentration and (c) Sonication time on particle size of SLN.

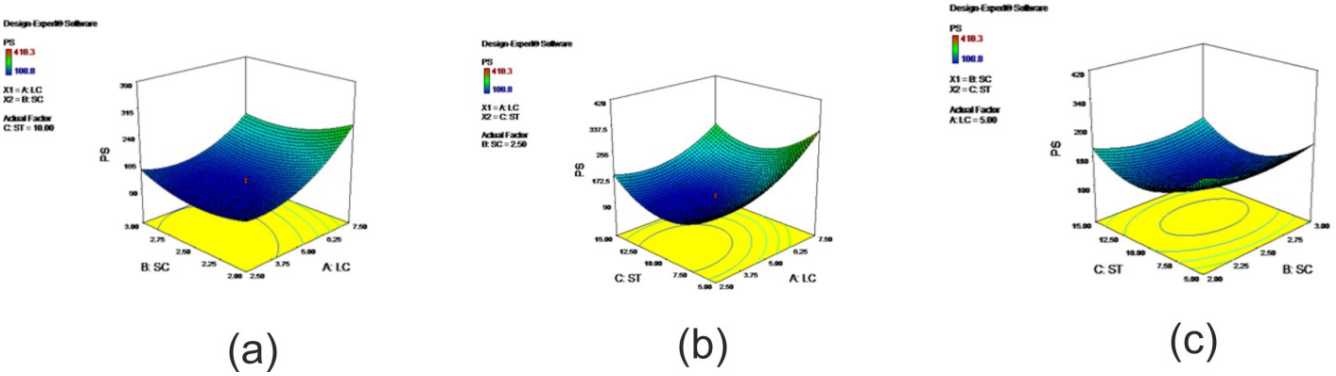

Figure 6: Response surface plot showing effect of (a) X1 and X2 (b) X1 and X3 and (c) X2 and X3 on particle size of SLN.

Variable term (levels)	Nparm	DF	Sum of squares	F Ratio	Prob>F
Lipid conc. (X_1) (2.5,7.5)	1	1	709.2520	12.2639	0.0100*
Surfactant conc. (X_2) (2,3)	1	1	62.8088	1.0860	0.3320
Sonication time (X_3) (5,15)	1	1	24.5570	0.4246	0.5354
Lipid conc. (X_1) Surfactant conc. (X_2)	1	1	75.2151	1.3006	0.2916
Lipid conc. (X_1) Sonication time (X_3)	1	1	59.7871	1.0338	0.3431
Surfactant conc. (X_2) Sonication time (X_3)	1	1	39.7386	0.6871	0.4345
Lipid conc. (X_1) Lipid conc. (X_1)	1	1	865.6587	14.9683	0.0061
Surfactant conc. (X_2) Surfactant conc. (X_2)	1	1	1300.0302	22.4792	0.0021
Sonication time (X_3) Sonication time (X_3)	1	1	308.0936	5.3273	0.0543

Table 5: Effect Tests analysis of independent variables for entrapment efficiency of SLN.

Source	DF	Sum of squares	Mean square	F Ratio
Model	9	2666.5116	296.279	5.1230
Error	7	404.8285	57.833	**Prob>F**
C. Total	16	3071.3400		0.0213

Table 6: ANOVA for entrapment efficiency of SLN.

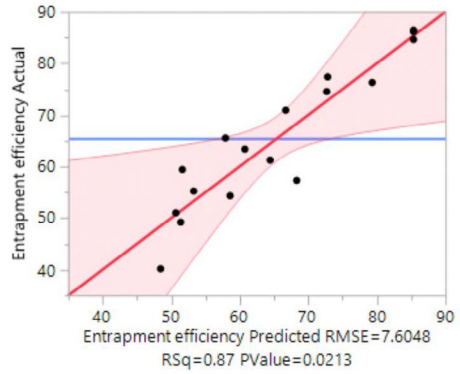

Figure 7: Actual vs predicted plot for entrapment efficiency of SLN.

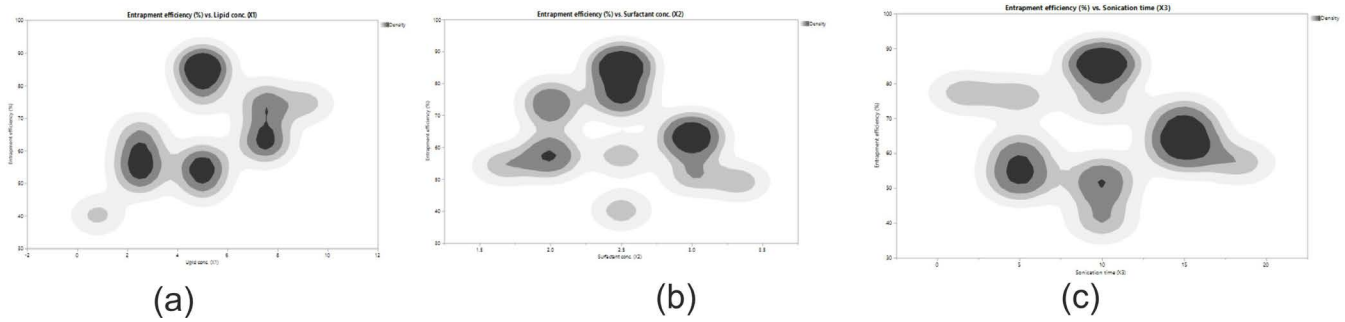

Figure 8: Bubble plots of (a) Lipid concentration (b) Surfactant concentration and (c) Sonication time on entrapment efficiency of SLN.

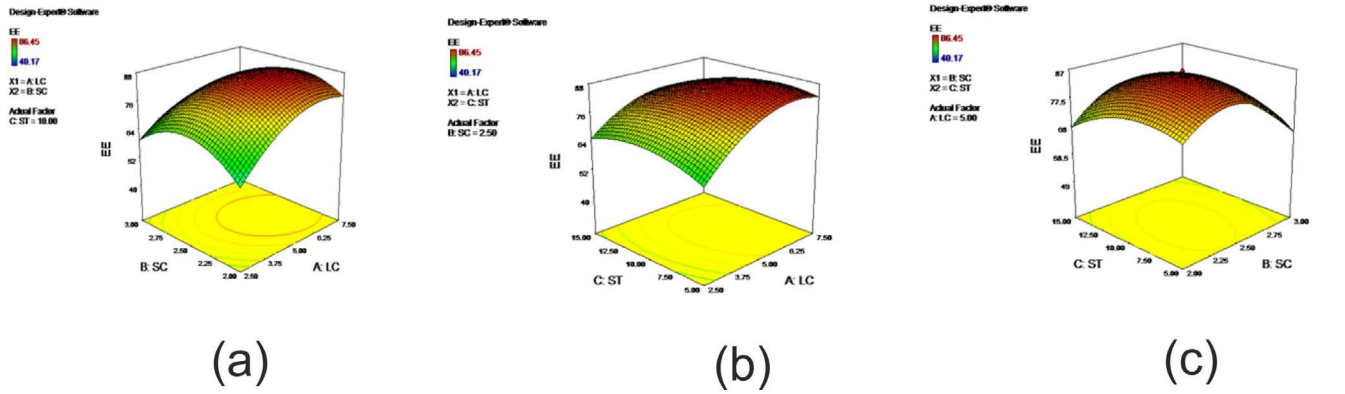

Figure 9: Response surface plot showing effect of (a) X1 and X2 (b) X1 and X3 and (c) X2 and X3 on entrapment efficiency of SLN.

surfactant concentration increased, entrapment efficiency increased upto 2.5%. Further increase in surfactant concentration to 3% decreased entrapment efficiency (Figure 8b). As the sonication time increased, entrapment efficiency increased upto 10 min thereafter entrapment efficiency was decreased at 15 min (Figure 8c).

The effect of independent factors on response variable entrapment efficiency was elucidated using response surface plots. It was observed that increase in X_1 increased entrapment efficiency while increase in X_2 reduced entrapment efficiency which is in accordance with results obtained from effect analysis. At lowest X_1 and highest X_2 entrapment efficiency was decreased so it proved that interaction between these factors affected entrapment efficiency (Figure 9a). Amongst factors X_1 and X_3, it was observed that slight change in entrapment efficiency was observed with X_3 (Figure 9b). Response surface plot also indicated that X_2 had profound effect on entrapment efficiency (Figure 9c).

All the above considerations (effect test analysis, ANOVA and Lack of fit) for particle size and entrapment efficiency indicated adequacy of the developed regression model.

Establishment of design space

Design space is the space within which the quality of the product can be built. The constraints for the resposes were selected and predicted value was compared with experimental value. There was no significant difference between observed and predicted value of particle size and entrapment efficiency (Table 7).

Desirability plot was genertd using JMP software which was shown in Figure 10. The desirabilty obtained was 0.9210, which is near to 1 which indicates suitability of predicted desirability for responses.

Analysis of design space robustness

Analysis of design space is necessary for scale up point of view. The upper and lower ranges of the desired response were selected.

Independent variable	**Value**	
Lipid concentration	4.92	
Surfactant concentration	2.49	
Sonication time	10.13	
Response	**Predicted**	**Experimental**
Particle size (nm)	112.8	115.3 ± 5.45
Entrapment efficiency (%)	85.10	83.10 ± 3.72

Table 7: Predicted and experimental value of optimized batch of SLN suggested by software.

Contour plots show how response variables (particle size and entrapment efficiency) relate to two continuous design variables (lipid concentration and surfactant concentration) while holding the third variable (sonication time) at 10 min. The white area inside each plot shows the range of lipid concentration and surfactant concentration where the criteria for both response variables are satisfied (Figure 11).

It was observed (Table 8) that value of independent variables outside the design space showed variation in response so it proved that the design space was sensitive to variation in independent variables. The area selected inside the circle showed desired response proving the robustness of design space.

Conclusion

A QbD concept was used to understand the effect of various formulation and process variables on CQAs of SLN such as particle size and entrapment efficiency. Ishikawa diagram aided in the initial risk assessment for formulation development process. Preliminary investigation helped to define range for each factor for further study. PBD and CCD were useful to fully understand influence (main, interactive and quadratic effect) of various variables of high speed homogenization followed by ultrasonication method and their relative influence on product quality attributes. The optimized formulation prepared using the predicted level suggested by software showed the desired response of particle size and entrapment efficiency with desirability near to 1 confirming the suitability of the developed model. Therefore, statistical investigation of AS SLN formulation confirmed

Lipid conc. (%)	**Surfactant conc. (%)**	**Sonication time (min)**	**Particle size (nm)**		**Entrapment efficiency (%)**	
			Predicted	**Observed**	**Predicted**	**Observed**
3.88	2.51	10	99.42	106.4 ± 3.2	80.36	76.62 ± 3.54
4.72	2.49	10	109.06	112.7 ± 5.8	84.48	82.03 ± 4.21
5.18	2.16	10	138.68	143.3 ± 2.9	82.52	80.93 ± 2.91

Table 8: Evaluation of sensitivity of obtained design space for Asenapine maleate SLN.

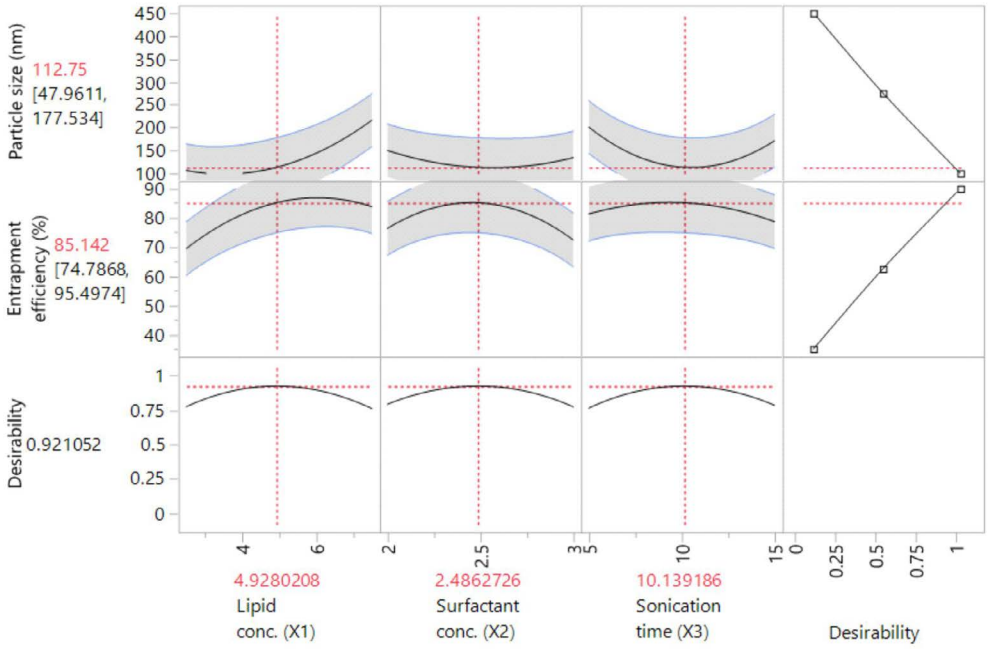

Figure 10: Desirability plot for optimized batch of AM SLN.

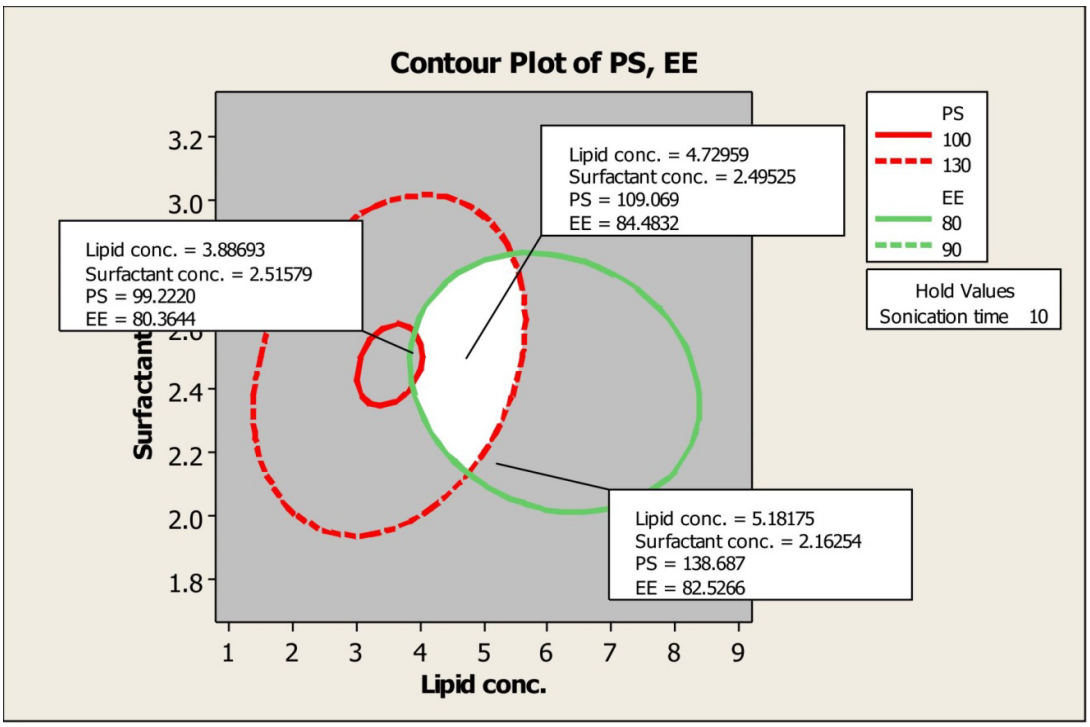

Figure 11: Overlay plot showing robustness of design space for AM SLN.

the potential of QbD concept in optimization of independent variables for SLN preparation.

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Conflict of Interest

The authors report no conflict of interest.

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