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Original Article

Box-behnken study design for optimization of clotrimazole loaded nanostructured lipid carriers

Vipul Sansare*, Govind Pathak, Balisakina Zambharakar, Pradeep Gupta

Department of Pharmaceutics, Indira Institute of Pharmacy, Sadavali, Ratnagiri, Maharashtra, India 415804

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ABSTRACT

Present study deals with development and optimization Clotrimazole loaded nanostructured lipid carriers. From solubility study of Clotrimazole in various lipids, stearic acid and oleic acid were selected as solid lipid and liquid lipid respectively. Nanostructured lipid carriers were formulated by solvent injection-ultrasonication technique. Box Behnken design was used to optimize formulation variables. Different batches were prepared as suggested by software and evaluated for responses, particle size and entrapment efficiency. Response surface plot and perturbation plot were constructed to study effect of independent variables on responses. The optimize formulation containing 5% w/v of total lipid, lipid: drug ratio 20 and 1.5% w/v of surfactant was prepared. The predicted values of responses were found to be close to observed values thus conformed validity of design. CLZ NLCs showed sustained release profile up to 96 hours. Efficacy of CLZ NLCs against Candida albicans was evaluated. The formulated NLCs were found to be more stable at room temperature (25°C ± 2°C/60% RH% ± 5% RH) and in accelerated storage conditions.

Keywords: Clotrimazole, Nanostructured lipid carrier, Box Behnken design

Address for Correspondence: Vipul Sansare, Department of Pharmaceutics, Indira Institute of Pharmacy, Sadavali, Ratnagiri, Maharashtra, India 415804; Email: avipulsansare@gmail.com

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INTRODUCTION

Clotrimazole is a broad spectrum antifungal agent of the imidazole group. It acts by inhibiting ergosterol biosynthesis, which cause lysis of fungal cell membrane because of change in membrane integrity. It is effective in the topical treatment of tinea infections like ringworm.Lipid nanoparticles were developed at the beginning of the 1990s as an alternative nanocarrier system to the existing nanocarriers, such as liposomes, polymeric nanoparticles[1]. The major advantage of solid lipid nanoparticles (SLNs) is the possibility of large scale production, high encapsulation of drug[2]. However some potential problems can occur, such as expulsion of drug, polymorphic change in solid lipid during storage. To overcome limitations associated with SLN, nanostructured lipid carriers (NLCs) have been developed[3]. The addition of liquid lipid in the solid lipid leads to controlled nanostructuring of the lipid particles. Liquid lipid and solid lipid being different molecules create imperfection in the matrix which provides increased space for drug loading[4]. Most of the drugs show high solubility in liquid lipids than that in solid lipids. Thus addition of liquid lipid in solid lipid matrix may increase drug loading.Both SLNs and NLCs possess a number of characteristics features for topical route of application[5]. These nanocarriers are composed of biodegradable, low toxic lipids[6]. The nano size of the nanoparticles ensures close contact to the stratum corneum, which can possibly increase the extent of drug permeation into the skin. Due to encapsulation of drug in lipid matrix, controlled drug release is possible. Thus NLCs becomes an important tool for topical drug delivery[7].

In the formulation development of NLCs, different factors play a critical role in development of optimized formulation. Hence design of experiment concept was used in the current study[8]. In present study, three level-three factor Box Behnken design was applied. In this design lesser runs required as compared to central composite design.

In present study Clotrimazole (CLZ) was selected as lipophilic model drug. The aim of this work was focused on optimization of formulation parameters of CLZ loaded NLCs.

MATERIAL AND METHODS

Materials: Clotrimazole was kindly gifted by Glenmark, India. Soya lecithin S-100 was gifted by lipoid, Germany. Stearic acid was purchased from Loba Chem. ltd., India. Tween 20, Tween 80 and oleic acid were purchased from S.D. Fine-Chem Ltd. India. Kolliphor RH 40, Kolliphor EL were purchased from BASF, India. All other reagents, solvents were purchased locally.

Screening of excipients: In the present study, solid lipids, liquid lipids and surfactants were screened based on their solubilizing capacity of CLZ. In case of solid lipids 50 mg of CLZ was transferred to a glass beaker. Weighed quantity of the solid lipid was taken, added in small increments in the beaker containing CLZ and the mixture was heated using a hot plate at the temperature 10°C above the melting point of respective solid lipid. The addition of solid lipid was continued until a clear melt was obtained. Obtained lipid melt spread on hot glass slide using hot spatula and observed microscopically, once clear lipid mixture obtained the remaining amount of lipid was weighed again and milligram of CLZ soluble in 1 g of solid lipid was determined[9].In case of liquid lipids, 1 ml of liquid lipid was taken in 2 ml eppendorf separately and excess of drug was added and mixture was vortex using cyclomixer. The addition of drug was continued until clear visible drug particles seen. The resulting mixture was shaken for about 72 hours in a water bath shaker at 37 ± 2°C after which it was centrifuged at 5000 rpm for 20 min [10]. The amount of drug dissolved in each liquid lipid at the end of 72 hours was determined by UV Visible spectrophotometer at λmax 243 nm after dilution of supernatant with methanol. In case surfactants an excess of CLZwas added in 1 ml of surfactant. This mixture was vortex for 10 minutes using cyclomixer and shaken for about 72 hours in a water bath shaker at 37 ± 2°C after which it was centrifuged at 5000 rpm for 20 min. The amount of drug dissolved in each surfactant at the end of 72 hours was determined spectroscopically[11]. All experiments were performed in triplicate and results expressed as mean± standard deviation.

Optimization of Ratios of Solid Lipid to Liquid Lipid: In order to optimize the ratio of solid lipid to liquid lipid to provide maximum drug loading, miscibility test between the selected solid lipid and the selected liquid lipid was checked[12]. For the test, the selected solid lipid and liquid lipid were weighed in the different ratios in glass vials. This lipid mixture was heated to a temperature 10°C above the melting point of the solid lipid. After complete melting the liquid mixture vortex and smeared on glass slide. Upon solidification, a dry piece of filter paper was pressed over this lipid blend immediately, at 24 hrs and at 72 hrs and was observed for the presence of any oil droplets on the paper. A binary mixture which did not reveal the presence of any liquid oil droplets on the filter paper was considered as miscible and was selected for use in the development of CLZ loaded NLCs.

Partition coefficient study: After solubility determination the partition behavior of CLZ in selected lipids was determined. Partition coefficient of CLZ in selected solid and liquid lipid was determined as per procedure described in[9]. Partition study was performed using two aqueous media; water and 1.5% Tween 20 in water to determine effect of Tween 20 on partition of CLZ outside the lipid phase. In this study 1 gm of lipid was accurately transferred in clean glass vial and melted above the melting point of lipid. 10mg of CLZwas accurately weighed and added in molten lipid mass. The resulting mixture was mixed using vortexing. 10ml of distilled water was added at last and the mixture was kept in water bath for 1 hr. at 37^0C. Lipid phase and aqueous phase were separated by centrifugation at 2000 rpm for 30 min at 25^0C. The supernatant (aqueous phase) was taken and analyzed for CLZcontent using UV spectroscopy. The partition coefficient was calculated by using following equation.

$$Partition\ coefficient = \frac{Ai - Aw}{Aw}$$

Where,
Ai is initial amount of CLZtaken (10mg)
Aw is the amount of CLZin the aqueous phase
All the experiments were performed in triplicate and results are expressed as mean± SD.

Preparation of CLZloaded Nanostructured Lipid Carrier: CLZ NLCs were prepared by solvent injection ultrasonicationtechnique[13]. In practice calculated quantity of CLZ, stearic acid and oleic acid were dissolved in 10 ml ethanol: acetone (1:1) and maintained at 60^0C. The aqueous surfactant solution containing 10 ml distilled water and Tween 20 was maintained at same temperature. The organic solution injected by using syringe (21 gauge) into aqueous phase and stirred at 4000 rpm for 10 minutes with heating using a hot plate at 60^0C. The resulting hot dispersion was subjected to probe sonication (VCX500, Sonics and materials, U.S.A.) at 20% amplitude for 10 minutes and cooled to room temperature.

Experimental design: For the optimization of the formulation, concept of design expert was used.There were three major factors affecting the formulation, total lipid (%w/v), Lipid: drug ratio, surfactant concentration (% w/v) as well as two responses to be optimized viz., particle size and % entrapment efficiency. A three-level three- factor Box –Behnken design (Design Expert, version 10, Stat-Ease) was used. The design consists of center points in replicate and the set of points lying at the midpoint of each edge of the multidimensional cube that defines the region of interest. The independent variables selected along with their levels are shown in table 1. The seventeen batches of CLZNLCs were prepared as suggested by software and responses were measured.

Particle size and zeta potential measurement: Particle size, polydispersity index and zeta potential of the NLC dispersions were determined using Zetasizer Nano ZS (Malvern Instruments Ltd., UK) equipped with 5-mV He-Ne laser.CLZloaded NLC dispersions were diluted ten times with distilled water and placed in polycarbonate cuvette. The analysis was carried out at an angle of 90° at a temperature of 25°C.

Percent entrapment efficiency: The percentage entrapment efficiency of CLZ in the lipid matrix was measured using the indirect method. The CLZ loaded NLC dispersions were diluted ten times and subjected to ultra-centrifugation at 80,000rpm for 1 hour at 4°C using Optima Max XP ultracentrifuge (Beckman Coulter, U.S.A.) to separate the unentrapped drug. The pellet of lipid was formed at the bottom. The aqueous phase above the pellet (i.e., the supernatant) was carefully separated and analyzed by UV spectrophotometry after suitable dilution with methanol. Percentage entrapment efficiency was calculated by following equation.

$$Percent\ entrapment\ efficiency = (WL - WF) \times 100 \div$$

WL
Where, WL = Theoretical content of CLZ in NLCs dispersion
WF = free CLZ in supernatant as quantified by UV spectrophotometry.

In-vitro CLZ release study: The *in-vitro* release of CLZ from CLZ suspension and CLZ NLCs were performed by a dialysis diffusion technique in phosphate buffer pH 7.4 at 37 ± 0.5^0C. Briefly, 3mg of CLZ and equivalent of CLZ NLCs were separately dispersed in 4 ml of phosphate buffer pH 7.4. The resulting dispersion was put in the dialysis bag (MWCO 13000-14000 Da, Himedia, India) and was dialyzed separately against 150 ml of phosphate buffer pH 7.4. At predetermined intervals 5ml of aliquots were withdrawn, filtered through 0.45 µm membrane filter and CLZ content was determined spectrometrically. The release medium was replenished with an equal volume of fresh phosphate buffer maintained at same temperature. Each experiment was performed in triplicate and the mean value of percent cumulative release and standard deviation at each time point were calculated.

In-vitro antifungal activity: In-vitro antifungal activity study of CLZ NLCs was performed using agar well diffusion technique to investigate whether CLZ activity was maintained in the lipid

particles. Nutrient broth and Candida albicans were used as growth medium and microorganism strain respectively. The wells in agar plate were filled with 100µl of sterile CLZ solution as reference. Each solution was serially two folds diluted to construct the calibration curve by relating inhibition zone diameter to CLZ concentration of standard solution. Other well in agar plate was filled with CLZ NLCs (10 ppm) dissolved in methanol. The plates were incubated at 37 ± 2°C and inhibition zone diameter was measured. The inhibition zone diameter produced by the CLZ NLCs was plotted on the calibration curve to calculate concentration of CLZ in CLZ NLCs.

Stability studies: Stability studies of NLCs dispersion were conducted according to International Conference on Harmonization guideline (Q1AR2). To conduct stability study, 60 ml batch was prepared and it was divided into six different portions each of 10 ml and filled into glass vials, sealed with rubber stopper and metal clips. Of these, three portions were stored at 25^0C/60% RH ± 5% RH and remaining portions stored at 40^0C/75% RH ± 5% RH in stability chamber (Thermolab, India) for a period of three months. After three months the samples were analyzed for drug content and *In-vitro* release profile.

RESULTS AND DISCUSSION

In lipid based colloidal systems such as NLCs, drug is entrapped in lipid matrix**.** Solid lipid provide solid matrix to encapsulate drug and prevent expulsion of drug during storage. Thus, assessment of the drug's solubility in lipid is important preformulation stage.The results of solubility of CLZ in solid lipids are shown in figure 1, where amount of solid lipid required to dissolve 1mg of CLZ was calculated. CLZ showed greater solubility in stearic acid. Solubility of CLZ in liquid lipids was determined (Figure 2). CLZ showed greater solubility in oleic acid (102.11 mg/ml), thus stearic acid and oleic acid were selected as lipid phase for formulation of NLCs. Solubility of CLZ in various surfactants was determined and results are shown in figure 3. It was observed that Tween 20: Kolliphor RH 40 (1:1) had highest solubility for CLZ, it was not selected as surfactant based on the previous studies. Surfactant which shows high solubility for drug may cause the extraction of drug out of the lipid phase leading to reduction of drug loading. Thus surfactant which showed least solubility for CLZ was selected. Hence Tween 20, having solubility (19.86±1.26 mg/ml) for CLZ was selected as surfactant. Structurally Tween 20 is polyoxyethylene sorbitan ester containing multiple polar functional groups (ester, hydroxyl, ether) with high HLB value 15. These polar functional groups confer polarity to the molecule. Whereas CLZ is poor soluble in water, is the reason for reasonable solubility of CLZ in Tween 20.

Optimization of ratios of solid lipid to liquid lipid: Miscibility test between the selected solid lipid and liquid lipid is important to ensure the formation of liquid lipid pockets within the solid lipid matrix, hence maximizing drug loading. The results of miscibility test are shown in table 2. . When stearic acid and oleic acid were mixed in the ratios 5:5, 6:4 and 7:3 by separate heating at 70^0C and smeared on glass slide. After complete solidification filter paper was pressed on it, the presence of oil droplets was observed, denoted separation of oleic acid from the mixture. For the ratios 8:2 and 9:1, the filter paper did not show any oil droplets immediately after solidification and after 24 hours. 8:2 ratio was selected for further study so as to maximize the proportion of liquid lipid in NLCs, so as to maximize drug loading.

Partition coefficient study: Partition coefficient of CLZ in selected solid and liquid lipid was determined as per procedure described in9. Partition study was performed using two aqueous media; water and 1.5% Tween 20 in water to determine effect of Tween 20 on partition of CLZ outside the lipid phase. Observed PC values are shown graphically in figure 4. Partition coefficient of CLZ in oleic acid: water, stearic acid: water and stearic acid: oleic acid (8:2): water was found to be 23.41, 23.22 and 22.74 respectively. No significant change in PC with change in lipid or lipid mixture was observed. Thus high entrapment of CLZ in NLCs can be expected. However generally surfactants are incorporated in NLCs dispersion as stabilizer. When PC of CLZ in lipid and aqueous solution of Tween20 (1.5% w/v) was determined; Low value of PC indicates the extraction of CLZ from lipid phase; this indicates that there could be low entrapment of CLZ in NLCs.

Optimization and validation of formulation parameters of CLZ nanostructured lipid carrier: Seventeen batches of NLCs were prepared as suggested by software DESIGN EXPERT 11 (Statease) and analyzed for particle size and entrapment efficiency. Results are shown in following table 3.The selected independent variables were found to influence the two dependent variables. All batches showed particle size in the range between 435 and 518 nm, entrapment efficiency 43.5-62.35%. The models fitted for each response were linear, cubic, quadratic and two factor interaction. The results found are shown in table 4. The linear model was found to fit best for all two responses. Using the ANOVA, the equation involving main factors were

determined based on the estimation of various statistical parameters.

Influence of independent variables on particle size: Obtained particle size of all the batches, is shown in table 3. The most significant factor contributing to the variation in particle size was C as shown by value of the coefficient. The factor C showed negative effect on the particle size which means an increase in the value of C will show decrease in the value of particle size[14]. This observed decrease in the particle size with increase in surfactant concentration can be explained by greater number of surfactant molecules available to emulsify the lipid particles, leading to more efficient emulsification which results in smaller particle size. The second factor after C contributing to the particle size was A. The factor A showed positive effect on the particle size. The increase in particle size is a logical consequence of the increase in amount of lipid (factor A) since the particles are composed of this lipid[15]. The linear model explaining the effect of various factors on particle size was;

$$Particle\ size = 481.34 + 15.72 * A + 0.7663 * B - 16.40 * C$$

Factor B not showed significant effect on the particle size. Further analysis using ANOVA (table 5) showed significant effects of the independent variable (P>F, 0.0001) on response 1. The model F-value 27.64445 implies that the model is significant. A good correlation between predicted and observed value as indicated by R^2 value of 0.86448. The surface response plot and perturbation plot generated by software are shown in figure 5.

Influence of independent variables on percent entrapment efficiency: The obtained % EE of all the batches is shown in table 3. The most significant factor contributing to the variation in % EE was A as shown by value of the coefficient. Increase in the level of factor A (total lipid % w/v) from -1 to +1 result in increase in the EE. This increase in EE due to increase in factor A is observed because there is a greater amount of lipid available to accommodate the added drug. Similar result reported in[16].The second factor after A contributing in variation of % EE was B. Increase in the level of B (lipid: drug ratio) result in increase in EE. This is because of increase in amount of drug in the formulation with respect to lipid. Factor C showed non-significant negative effect on % EE. The negative effect on % EE was due to extraction of RIF out of the lipid matrix with increase in concentration of surfactant. The linear model explaining the effect of various factors on % EE was;

$$\%EE = 54.26 + 6.91 * A + 5.31 * B - 0.7388 * C$$

Further analysis using ANOVA (table 5) showed significant effects of the independent variable (P>F, 0.0001) on response 2. The model F-value 164.64 implies that the model is significant. A good correlation between predicted and observed value as indicated by R2 value of 0.974356. The surface response plot and perturbation plot generated by software are shown in figure 6.

To get optimized formulation, numerical optimization was performed using Design expert software. The optimization of formulation was based on criteria of minimum particle size, and maximum drug entrapment. The predicted levels of formulation factor obtained by the software were 5% w/v/ of total lipid, lipid: drug ratio of 20, 1.5% w/v of surfactant concentration. The optimized batch of NLCs was prepared and predicted values of responses were compared with observed values and % error was calculated (table 6). The observed values of responses were found to be close to the predicted value evident from less value of % error. By this the validity of the design was proven.

In–vitro CLZ release study: In present study in-vitro release study of CLZ NLCs was performed using dialysis tube diffusion technique by using dissolution apparatus. All studies were performed in triplicate and results are expressed in mean ± SD. Percent cumulative release of CLZ from CLZ suspension and CLZ NLCs is graphically represented in figure 7. CLZ NLCs show sustained release profile up to 96 hours. Whereas CLZ suspension showed 10 hours release profile. This could be due to poor wettability of lipid nanocarrier particles and high lipid solubility of CLZ.

In-vitro antifungal activity: Antifungal activity of CLZ loaded NLCs and CLZ solution against Candida albicans were evaluated using agar well diffusion technique. The zone of inhibition after incubation of plates with 1.25, 2.5, 5 and 10 ppm of CLZ solution were measured and graph of log concentration versus inhibition zone diameter was constructed as shown in figure 8. The inhibition zone diameter produces by the CLZ NLCs containing known CLZ (5 ppm) was plotted on the previously constructed graph as shown below (Figure 8).

By comparing the inhibition zone diameter produce by the dissolved CLZ NLCs with those produce by a serial drug dilutions (1.25 to 10 ppm, correlation coefficient 0.9941), CLZ NLCs showed inhibition zone diameter corresponding to the own CLZ value (5ppm), thus provide evidence of preservation of drug activity during heating and cooling during formation of NLCs. Blank NLCs formulation

showed no zone of inhibition indicated that the material composing NLCs matrix did not interfere with assay. Similar results reported by Marreti et al., 2014[17].

Stability studies

Appearance: Appearance of NLCs stored at both storage conditions was found to be milky white colour which was same as that of before stability study. Thus confirmed no or minimum degradation of drug during storage period.

In-vitro drug release study: CLZ release from NLCs after storage was carried out by using dialysis diffusion technique. The % cumulative release of CLZ graphically represented in figure 9. It was observed that at the end of 96 hours almost equal i.e. 83.02% and 81.77% of CLZ was released from NLCs stored at R.T. and accelerated storage conditions respectively; which was also close to drug release from NLCs before stability study i.e. 84.1% Similarity factor for CLZ NLCs stored at R.T. and accelerated storage conditions was calculated and found to be 65.54 and 56.37. The value of similarity factor above 50 indicates no significant difference in release behavior of formulation after storage.

Percent drug content: Percent CLZ content in NLCs stored at R.T. and accelerated storage conditions was determined by UV spectroscopy. The % CLZ content in CLZ NLCs stored at R.T. and accelerated storage conditions was found to be 99.42± 1.2% and 99.38 ± 0.90% respectively; which was close to that in formulation before storage. The data treated with one way ANOVA to find out significant difference between the %drug content of formulation stored at different storage conditions. The results of ANOVA are shown in table 7. The F value is 0.11927. The p value is 0.88962. Indicated non significance of results, thus there is no significant difference in drug content of formulation stored at R.T., at accelerated storage condition and before stability study. Thus there was no degradation of drug during stability study.

CONCLUSION

The major outcome of this study was the successful entrapment of CLZ within a lipid core and optimization of formulation parameters. Effect of various formulation variables on the formulation can be studied by the Box Behnken design. By considering effect of independent variables on responses, the optimized formulation was selected and evaluated for responses. The CLZ NLCs dispersion showed good quality control parameters. Furthermore, the formulation was found to be stable upto three months of storage at 25^0C/65% RH and at 40^0C/75% RH as per ICH guidelines. However prepared NLCs should convert into proper dosage form. The next step of the study will consider design of dosage form and analysis of its effectiveness in infected cells and animals models.

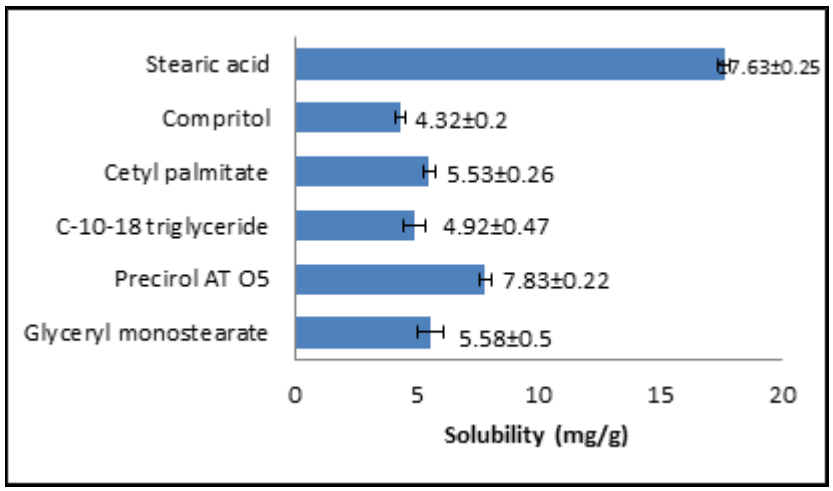

Figure 1: Solubility of CLZ in solid lipids (n=3)

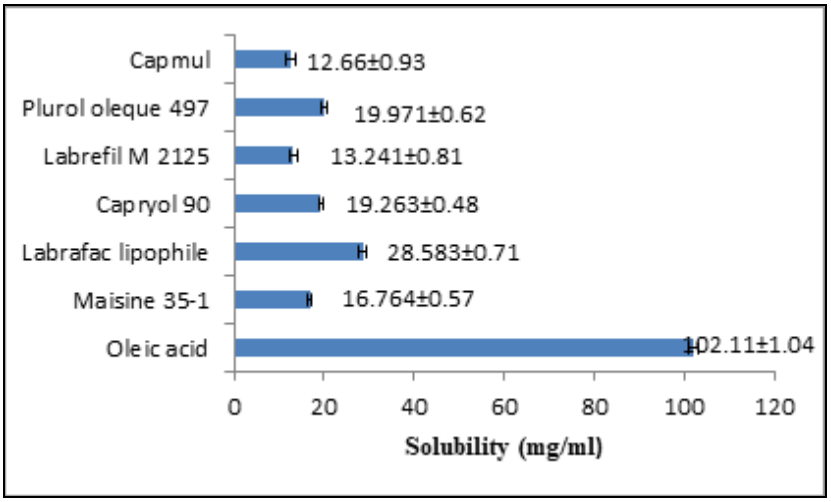

Figure 2: Solubility of CLZ in liquid lipids (n=3)

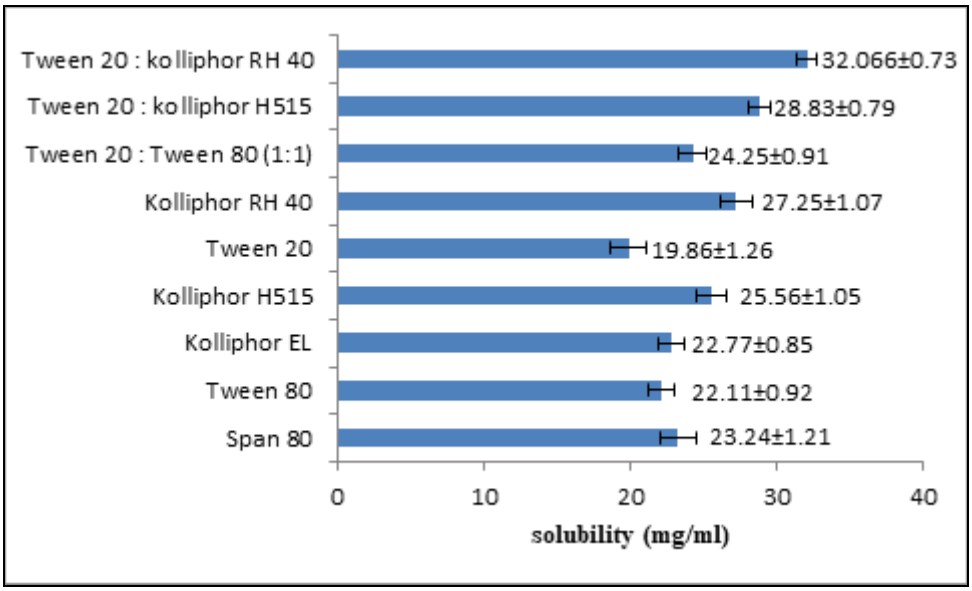

Figure 3: Solubility of CLZ in surfactants (n=3)

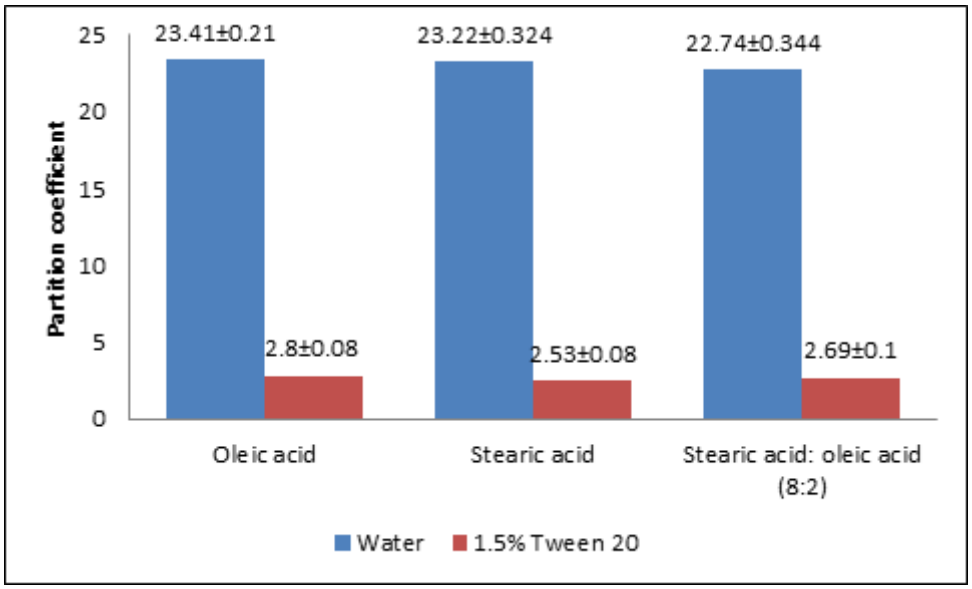

Figure 4: Lipid: water partition coefficient of CLZ in selected lipids (n=3)

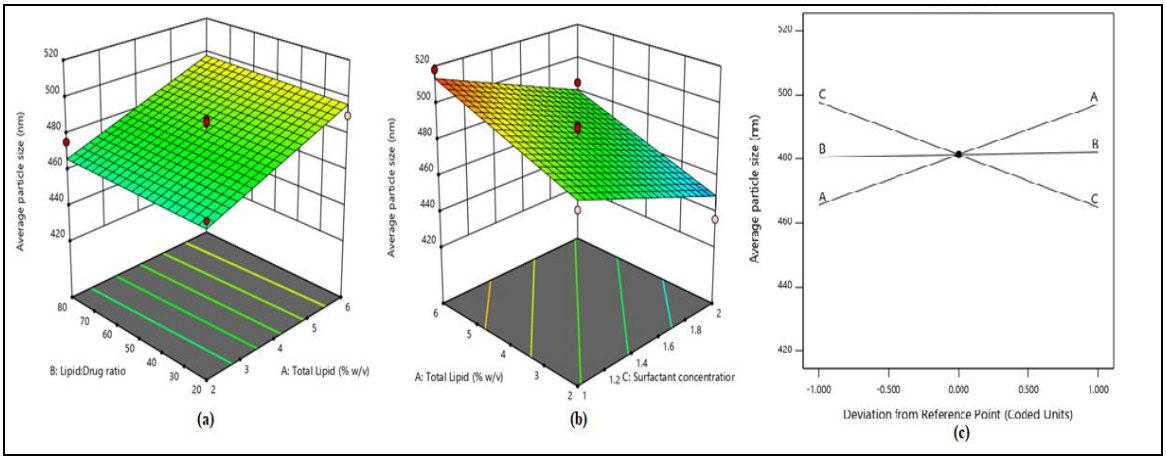

Figure 5: Surface response plot (a, b) and perturbation plot (c) showing effect of formulation variables on particle size

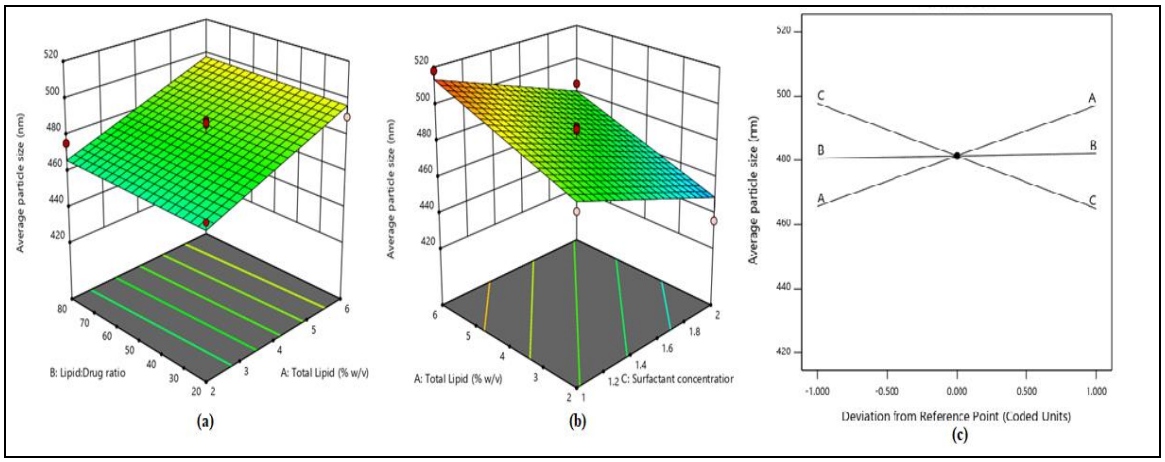

Figure 6: Surface response plot (a, b) and perturbation plot (c) showing effect of formulation variables on entrapment efficiency

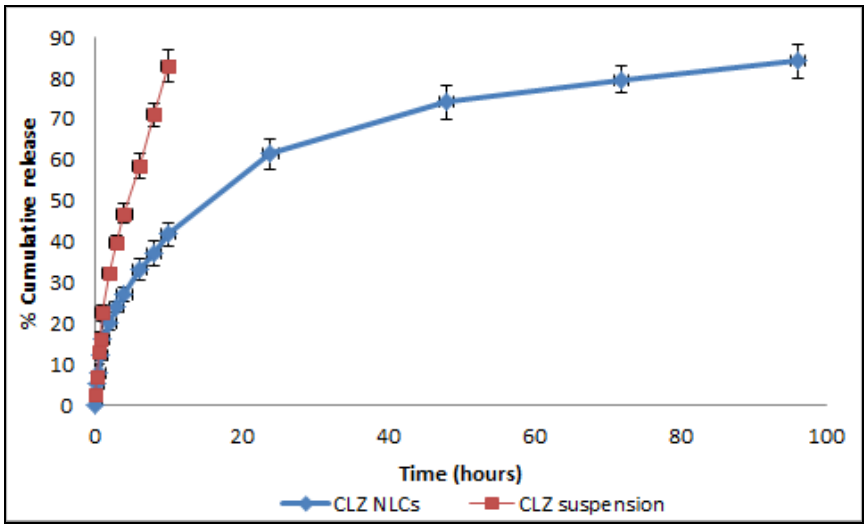

Figure 7: *In-vitro* release behavior of CLZ suspension and CLZ NLCs.

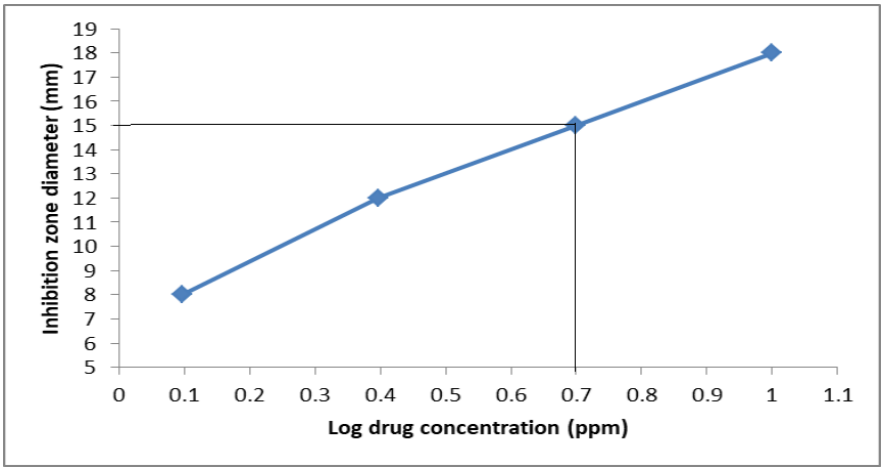

Figure 8: CLZ NLCs antifungal activity in response to CLZ standard solution.

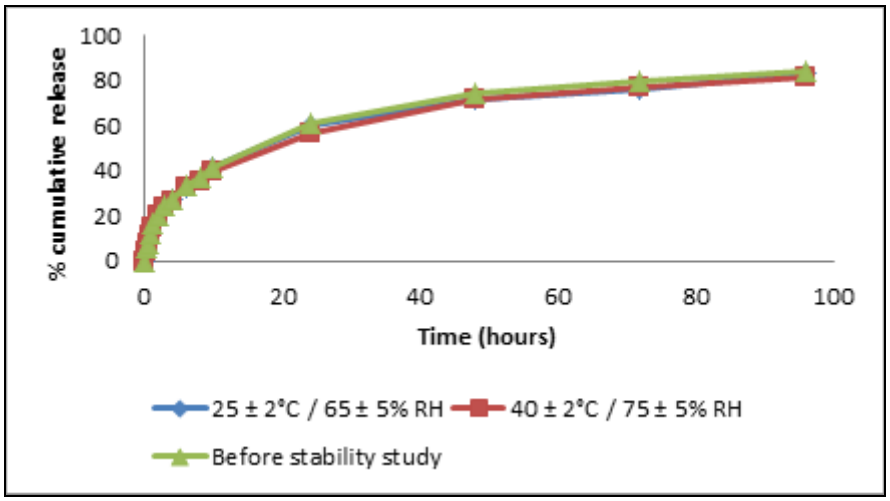

Figure 9: *In-vitro* release behavior of CLZ NLCs stored at R.T. and accelerated storage condition.

Table 1: Variables in Box Behnken design

S.No.	Independent variables	Levels		
		-1	0	+1
1	X_1 = total lipid (% w/v)	2	4	6
2	X_2 = lipid : drug ratio	20	50	80
3	X_3 = surfactant concentration (%w/v)	1	1.5	2

Table 2: Result of the miscibility test between solid lipid and liquid lipid

Ratio of stearic acid: oleic acid	Presence of oil droplets on filter paper	Result
5:5	Yes	Not selected
6:4	Yes	Not selected
7:3	Yes	Not selected
8:2	No	selected
9:1	No	Not selected

Table 3**:** Observed responses in design

Run	A = total lipid (% w/v)	B = lipid : drug ratio	C = surfactant concentration (%w/v)	Response 1 Particle size (nm)	Response 2 Entrapment efficiency (%)
1	2	80	1.5	475.8	52
2	4	80	1	490	60.2
3	4	80	2	462.5	59.6
4	6	50	1	518.1	62.35
5	4	50	1.5	487.8	55
6	2	20	1.5	468.7	43.2
7	4	50	1.5	486	55.7
8	2	50	1	477.2	47.12
9	4	20	2	461.8	48
10	4	50	1.5	486.9	54.6

11	6	50	2	485.2	60.36
12	4	50	1.5	487.2	56.2
13	4	20	1	491.2	49.2
14	6	20	1.5	490.2	54.4
15	6	80	1.5	489.8	54.6
16	4	50	1.5	488.5	54
17	2	50	2	435.8	45

Table 4: Model summery for two responses.

Models	R^2	Adjusted R^2	Predicted R^2	Standard deviation	Remark
Response 1					
Linear	0.864489	0.833218	0.746714	6.295896	Suggested
2FI	0.867643	0.788228	0.44624	7.094412	
Quadratic	0.931415	0.843235	0.08388	6.103891	
Cubic	0.999067	0.996268		0.941807	Aliased
Response 2					
Linear	0.974356	0.968438	0.95641	1.112913	Suggested
2FI	0.976576	0.962521	0.921618	1.212751	
Quadratic	0.98567	0.967245	0.840615	1.133756	
Cubic	0.995158	0.980633		0.87178	Aliased

Table 5: ANOVA for responses.

Source	Response 1		Response 2	
	F	P>F	F	P>F
Model	27.64445	<0.0001	164.64	<0.0001
A	37.46691	<0.0001	308.2952	<0.0001
B	0.093667	0.764412	182.1197	<0.0001
C	45.37278	<0.0001	3.53	0.0831

Table 6: Predicted and observed responses for the optimized formuation.

Response	Predicted value	Observed value	% error
Particle size	488.43	483.23	1.076
% EE	52.4	51.72	1.31

Table 7: One way ANOVA for RIF content in NLCs after storage

Source	Sum of square	Degree of freedom	MS	
Between-treatment	0.7428	2	0.3714	
Within-treatments	18.6825	6	3.1137	F=0.11927
Total	19.4252	8		

REFERENCES

1. Muller R. Arzneistofftrager aus festen lipidteilchen (Feste Lipidnanospharen (SLN)). Eur Patent EP 0605497 B1, 1996.
2. Müller R. Solid-liquid (semi-solid) liquid particles and method of producing highly concentrated lipid particle dispersions. German patent application 45,203.2, 2000.
3. Müller R. Fest-flüssige (halbfeste) Lipidpartikel und Verfahren zur Herstellung hochkonzentrierter Lipidpartikeldispersionen. PCT application EP 00, 04565. 2000.
4. Muchow M et al. Lipid nanoparticles with a solid matrix (SLN, NLC, LDC) for oral drug delivery. Drug Dev Ind Pharm. 2008 Dec;34(12):1394-1405.
5. Mehnert W et al. Solid lipid nanoparticles: production, characterization and applications. Advanced drug delivery reviews. 2012;64:83-101.
6. Müller RH et al. Cytotoxicity of solid lipid nanoparticles as a function of the lipid matrix and the surfactant. Pharmaceutical research. 1997;14(4):458-462.
7. Souto E et al. Development of a controlled release formulation based on SLN and NLC for topical clotrimazole delivery. International Journal of Pharmaceutics. 2004;278(1):71-77.
8. [a]Gohel M et al. Formulation optimization of controlled release diclofenac sodium microspheres using factorial design. Journal of Controlled Release. 1998;51(2-3):115-122;[b]Nazzal Set al. Response surface methodology for the optimization of ubiquinone self-nanoemulsified drug delivery system. AAPS PharmSciTech. 2002;3(1):23-31.
9. Thakkar HP et al. Application of Box-Behnken design for optimization of formulation parameters for nanostructured lipid carriers of candesartan cilexetil. Asian Journal of Pharmaceutics (AJP): Free full text articles from Asian J Pharm. 2014;8(2).
10. Negi LMet al. Development of protocol for screening the formulation components and the assessment of common quality problems of nano-structured lipid carriers. Int J Pharm. 2014 Jan 30;461(1-2):403-410.
11. Gaba Bet al. Nanostructured lipid carrier system for topical delivery of terbinafine hydrochloride. Bulletin of Faculty of Pharmacy, Cairo University. 2015;53(2):147-159.
12. [a]Kasongo KWet al. Selection and characterization of suitable lipid excipients for use in the manufacture of didanosine-loaded solid lipid nanoparticles and nanostructured lipid carriers. J Pharm Sci. 2011 Dec;100(12):5185-5196;[b]Doktorovová St al. Formulating fluticasone propionate in novel PEG-containing nanostructured lipid carriers (PEG-NLC). Colloids and Surfaces B: Biointerfaces. 2010;75(2):538-542.
13. Tran THet al. Preparation and characterization of fenofibrate-loaded nanostructured lipid carriers for oral bioavailability enhancement. AAPS PharmSciTech. 2014 Dec;15(6):1509-1515.
14. Gannu Ret al. Optimization of hydrogels for transdermal delivery of lisinopril by Box–Behnken statistical design. AAPS PharmSciTech. 2009;10(2):505-514.
15. Jia L-Jet al. Preparation and characterization of silybin-loaded nanostructured lipid carriers. Drug Delivery. 2009;17(1):11-18.
16. Ekambaram Pet al. Formulation and evaluation of solid lipid nanoparticles of ramipril. Journal of Young Pharmacists. 2011;3(3):216-220.
17. Maretti Eet al. Inhaled Solid Lipid Microparticles to target alveolar macrophages for tuberculosis. Int J Pharm. 2014 Feb 28;462(1-2):74-82.