ORIGINAL ARTICLE

Application of Box-Behnken design for optimization of formulation parameters for nanostructured lipid carriers of candesartan cilexetil

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This study deals with development and optimization of nanostructured lipid carriers (NLCs) of candesartan cilexetil (CC) for improving its oral bioavailability. From solubility and lipid-water partition studies of CC in various lipids, glyceryl monostearate (GMS) and glyceryl monocaprylate were selected as solid lipid and liquid lipid, respectively. NLCs were formulated by hot melt-emulsification-ultrasonication method. A three-factor, three-level Box–Behnken design was used to optimize the independent variables, lipid: drug ratio (X1), solid lipid: liquid lipid ratio (X2) and surfactant concentration (X3). Different batches were prepared and evaluated for responses, particle size (Y1), zeta potential (Y2) and % entrapment efficiency (Y3). Response surface plots and perturbation plots were constructed to study the effect of factors on responses. The optimized formulation containing X1 - 22.47:1, X2 - 7.23:1 and X3 - 1.97% was prepared and evaluated. Observed values for Y1, Y2, andY3 were found to be closer to the predicted values thus validating the optimization method. Differential scanning calorimetry thermograms of pure drug, GMS and lyophilized drug loaded NLCs indicated complete miscibility of drug into lipids. The release of CC from the NLCs conducted in artificial gastric fluid (pH 1.2) was much higher than in phosphate buffer solution (pH 6.8). The formulated NLCs were found to be more stable at refrigerated condition (5°C ± 3°C) as compared with room temperature (25°C ± 2°C/60% RH% ± 5% RH). The use of design approach helped to identify critical formulation parameters in CC loaded NLCs preparation.

Key words: *Candesartan, hypertension, nanostructured lipid carriers, oral bioavailability partition study*

INTRODUCTION

In the year 2000, 26.4% of the adults in the world had high blood pressure (hypertension) and it is estimated to increase up to 29.2% by 2025.[1] Moreover, hypertension and high cholesterol levels in the blood are considered the two main risk factors for various cardiovascular diseases.[2] Treating hypertension in elderly patients can decrease the risks of heart failure and stroke thus prolonging the life.[3] Hence, treatment of hypertension is very essential for prevention of other disorders. Various medications are available for its treatment, which includes thiazide diuretics, β-adrenergic receptor antagonists, α1-adrenergic antagonist, angiotensin II type-1 receptor antagonists, angiotensin-converting enzyme inhibitors as well as calcium channel blockers.[4]

Candesartan cilexetil (CC) is approved for use in mild to moderate hypertension. It is an inactive prodrug, getting completely bio-activated (in the gastrointestinal tract) by hydrolysis to form active candesartan, which gets absorbed majorly from the small intestine.[5] Candesartan and other AT1 receptor antagonists shows vasculoprotective, cardioprotective, renoprotective and other organ protective effects, while showing minimal effects on renal function and lipid metabolism and thus

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proved to be safer antihypertensive agents.[6] Unfortunately, CC shows poor aqueous solubility ($<5 \times 10^{-5}$ g/L) leading to low oral bioavailability of about 15%.[7]

Various strategies for improvement in oral bioavailability of such lipophilic drugs have been tried such as liquisolid compact,[8] solid lipid nanoparticles (SLNs)[9] and nanoemulsion.[10] Lipid based formulation have emerged as excellent carriers for oral formulations due to their diverse physicochemical properties of lipids, their bioacompatibility and lymphatic uptake.[11] Among many such delivery options including incorporation of drugs in oils,[12] emulsions,[13] self-emulsions[7] and SLNs,[14] one of the popular approaches is nanostructured lipid carriers (NLCs) composed of mixture of solid lipid and liquid lipid in appropriate proportions. However, SLNs and NLCs contain a matrix of lipids similar to that of polymeric nanoparticles thus allowing slow release of incorporated drug.[15] Unlike NLCs, SLNs may result to conversion in to lower energy state forms of some part of solid lipid leading to expulsion of drug incorporated.[16] Furthermore, NLCs have high loading capacity in comparison to SLNs because of the reason that they contain liquid lipid that results in uneven lipid matrix having imperfections that leads to higher drug entrapment.[17,18] Thus, NLCs preparation of CC has been tried in the present work to increase its oral bioavailability.

In the design of NLCs, different factors play a critical role in developing an optimized batch. Hence, design of experiments (DOEs) concept was used in the present work, which gives optimized parameters using lesser number of experimental runs.[19,20] For this study, three levels-three factor design was required and hence Box-Behnken, a widely used response surface method was applied. In this design, for three factors, the design is a 22 that holds all other factors at their 0 level thus lesser runs required. Moreover, this design holds the advantage that it avoids all the corner points and star points, which are extreme points in terms of region experiment.[21]

This study describes the development of suitable NLCs of CC in order to improve its oral bioavailability. Box-Behnken design was used to optimize independent variables-lipid: Drug ratio, solid lipid: liquid lipid ratio and surfactant concentration. Effects of these variables on particle size, zeta potential, and % entrapment efficiency were estimated.

MATERIALS AND METHODS

Materials

Candesartan cilexetil was kindly gifted by Torrent Research Center (Gandhinagar, India). Cutina® GMS VPH (glyceryl monostearate (GMS) 40-55 type-II) was obtained as a gift sample from BASF (Germany). Geleol® mono and diglycerides (GMS 40-55 type-I), Precirol® ATO 5 (glyceryl distearate), Gelucire® 50/13 pellets (stearoyl macrogol-32 glycerides), Compritol® 888 ATO (glyceryl dibehenate) and arlamol were purchased from Gattefosse (Germany), Lutrol® F 68 (poloxamer 188) was purchased from BASF (Germany); Dynasan® 112, Dynasan® 114, Miglyol® 829 (caprylic/capric/succinic triglyceride), Miglyol® 840 (propylene glycol dicaprylate/dicaprate), Imwitor® 491 (GMS >90%), Imwitor® 948 (glyceryl monooleate) from Sasol (Germany); stearic acid was purchased from Titan Pharma (India) Pvt. Limited; chloroform from RFCL Limited (India); protamine sulfate from Merck (India); Capmul® MCM (medium chain mono- and diglycerides and Acconon® CC-6 (Polyoxyethylene[6] caprylic/capric glycerides) from Abitec Corporation; methanol from Spectrochem pvt. Limited (Mumbai, India). All other materials and solvents were of analytical reagent grade.

Selection of solid lipid and liquid lipid

Solubility of CC in various solid lipids and liquid lipids was determined. In case of solid lipids, accurately weighed 50 mg of CC was transferred to a 5 ml beaker. Weighed quantity of the solid lipid was taken, added in small increments in the beaker containing CC and the mixture was heated using a hot plate cum magnetic stirrer at the temperature 5°C above the melting point of respective solid lipid. The addition of solid lipid was continued until a clear melt was obtained after which its remaining amount was weighed again and quantity required to dissolve 50 mg of CC was determined.[22] For determining the solubility in liquid lipids, 200 mg of CC was taken in a vial, 5 ml of liquid lipid was added and vial was closed. The mixture was shaken using a vortex mixer for 30 min and kept for 24 h after which it was centrifuged at 5000 rpm for 20 min. The supernatant was taken and analyzed by ultraviolet (UV) spectrophotometer after suitable dilution with chloroform: methanol (3:7) at 303 nm.[22]

After solubility determination of CC in various lipids, the partition study was carried out to select the lipids. In this study, 1 g lipid, 50 mg CC and 10 ml distilled water were taken in a test tube. The mixture was heated using a water bath until the lipid melted and mixed properly using a vortex mixer. The solidification of the melted lipid was prevented by frequent exposure to heated water bath and vortex mixer. After shaking for 30 min, the mixture was centrifuged at 2000 rpm for 30 min at 25°C to separate lipid. The supernatant (aqueous phase) was taken, diluted appropriately and analyzed by UV spectrophotometer to determine the amount of CC in the aqueous phase. The partition coefficient (PC) was calculated by the Equation 1 given below:

$$PC = (A_l - A_w)/A_w \quad (1)$$

Where,
A_l is initial amount of CC taken (50 mg)
A_w is the amount of CC in the aqueous phase.

A compatibility screening between selected liquid and solid lipid in the ratio of 1:9 was performed.[23] Briefly, accurately

weighed bulk lipids were heated up to 80°C in glass vials. Mixture was checked for homogeneity immediately after solidification, after 1 h and later at 24 h.

Formulation of candesartan cilexetil loaded nanostructured lipid nanocarriers

Candesartan cilexetil-NLCs (CC-NLCs) were prepared by melt emulsification ultrasonication method[24] with slight modification. Briefly, the solid lipid, liquid lipid and CC were taken in a beaker and heated up to 60°C. In another beaker, poloxamer 188 (1-3%) was dissolved in distilled water and heated up to the same temperature. The melted lipid phase was added into the aqueous phase while stirring using Ultra-turrax® T 25 Basic (IKA® India Pvt. Limited, India) at 19,000 rpm for 5 min with heating using a hot plate at 60°C in order to obtain an o/w emulsion. The prepared emulsion was ultrasonicated using probe sonicator (Ultrasonic processer [UP] 100H, Labsonic® M, Sartorius, Germany) for 5 min. The emulsion was allowed to cool at room temperature naturally. For lyophilization, sucrose was added to NLCs dispersion in the ratio of 10:1 (sucrose to lipid ratio by weight). The dispersion was exposed to lyophilization cycle on Virtis® lyophilizer (Spinco Biotech pvt. Limited, India). The lyophilized sample was reconstituted with deionized water and particle size, polydispersibility index, zeta potential, and entrapment efficiency were measured.

Experimental design

For the optimization of the formulation, concept of DOE was used.[25] As there were three major factors affecting the formulation, lipid: drug ratio (X1), solid lipid: Liquid lipid ratio (X2) and surfactant concentration (X3) as well as three major responses to be optimized viz., particle size (Y1), zeta potential (Y2), % entrapment efficiency (Y3), a three-level three-factorial Box-Behnken experimental design (Design Expert, Version 8.0.3, Stat-Ease Inc., Minneapolis, MN) was used. This design is suitable for exploring quadratic response surfaces and constructing second order polynomial models. The design consists of replicated center points and the set of points lying at the midpoint of each edge of the multidimensional cube that defines the region of interest. The nonlinear quadratic model generated by design is as follows:

$$Y_i = b_0 + b_1 X_1 + b_2 X_2 + b_3 X_3 + b_{12} X_1 X_2 + b_{23} X_2 X_3 + b_{13} X_1 X_3 + b_{11} X_1^2 + b_{22} X_2^2 + b_{33} X_3^2$$

in which Yi represents the response associated with each factor level combination, b0 is an intercept and b1 - b33 are the regression coefficients of the factors. X1, X2 and X3 are the coded levels of independent factors.[26] The independent and dependent variables selected are shown in Table 1 along with their high, medium and low levels. Seventeen batches of CC-NLCs were formulated (including five center points) as suggested by the software [Table 2]. Responses Y1, Y2 and Y3 were measured.

Table 1: Variables in Box-Behnken design

Factor	Level		
	−1	0	1
X_1: Lipid: Drug	18	21.50	25
X_2: Solid lipid: Liquid lipid	7	8	9
X_3: % surfactant concentration (% w/v)	1	1.5	2
Responses	**Constraint**		**Importance**
Y_1: Particle size (nm)	Minimize		4
Y_2: Zeta potential (mV)	In range (−30 to −49.12)		1
Y_3: % entrapment efficiency	Maximize		5

Table 2: Variables in Box-Behnken design

Batch number	Independent variables			Dependent variables		
	X_1	X_2	X_3	Y_1 (nm)	Y_2 (mV)	Y_3 (%)
1	1	−1	0	277	−35.62	79.72±1.89
2	1	0	1	259	−45.12	77.37±1.22
3	0	1	−1	275	−31.25	65.54±0.97
4	1	1	0	307	−34.56	74.37±1.51
5	−1	0	1	280	−49.12	59.22±3.46
6	−1	1	0	290	−40.32	56.46±1.51
7	0	0	0	246	−41.10	75.48±1.80
8	1	0	−1	289	−28.76	69.48±0.79
9	0	0	0	241	−40.55	77.44±0.82
10	0	−1	1	234	−45.31	78.68±1.06
11	0	1	1	240	−48.12	66.48±1.61
12	0	0	0	244	−41.21	73.88±0.82
13	0	0	0	251	−38.32	77.60±0.67
14	−1	0	−1	288	−27.12	58.37±3.49
15	−1	−1	0	270	−23.45	52.50±2.36
16	0	−1	−1	268	−25.14	66.48±2.11
17	0	0	0	245	−41.60	74.42±1.56

Particle size analysis

The dispersions of CC-NLCs were diluted 10 times with distilled water. The diluted sample were filled in the disposable transparent sizing cuvette and the particle size of samples were measured using Malvern Zetasizer (Nano ZS, Zen 3600, Malvern Instruments ltd., UK) equipped with 5-mV He-Ne laser (633 nm) at 25°C. The size was measured in triplicate and the average value was calculated.[27]

Zeta potential analysis

The zeta potential was measured using Malvern Zetasizer, Zeta-nano particle electrophoresis analyzer setup (Nano ZS, Zen 3600, Malvern Instruments Limited, UK). The dispersion was diluted 10 times with distilled water. The pH was between 6.0 and 7.0. The average of the zeta potential is given from 30 runs.[27]

Percentage entrapment efficiency

For determination of % entrapment efficiency, protamine sulfate conjugation method was used. The CC-NLCs in

dispersion were aggregated by adding 0.1 ml of 10 mg/ml protamine sulfate solution and centrifuged at 8000 rpm for 10 min to obtain a pellet. The supernatant was suitably diluted with chloroform: methanol (3:7) solution and the free drug content was determined spectrophotometrically. The pellet obtained was dissolved in a mixture of chloroform: methanol (3:7) and analyzed spectrophotometrically for the entrapped drug against a solvent blank.

Compatibility of candesartan cilexetil with excipients

Differential scanning calorimetry (DSC) was used to ascertain physical state of drug and lipid in formulated NLCs using Schimadzu thermal analyzer (Schimadzu DSC-60, TA-60 WS, Japan) at a heating rate of 20°C/min in the range of 30-300°C under inert nitrogen atmosphere at a flow rate of 40 ml/min. DSC thermo grams were recorded for pure drug, GMS and lyophilized CC-NLCs.[28]

In vitro drug release study

The *in vitro* release of CC from CC suspension and CC-NLC was performed by a dialysis method in simulated gastric fluid and simulated intestinal fluid (both without enzymes). The cellulose membrane dialysis bags (MWCO-12 000, Sigma, USA) were soaked in the boiling water for 30 min before use. A volume of 1 ml of freshly prepared CC-NLC and CC suspension (1 mg/ml) were put into the dialysis bags and tightly sealed. The bags were placed in conical flasks with 100 ml of release media. The temperature was maintained at 37°C. Gentle stirring was done using a magnetic stirrer at 100 rpm. Two ml of release media was withdrawn at a predetermined time intervals until 8 h and the same volume of fresh media was added to the conical flask to maintain sink condition. Meanwhile, the release of free CC from CC suspension (1 mg/ml) was performed in the similar manner. The aliquots were analyzed by UV spectrophotometery.

Stability studies

Accelerated stability studies for NLC dispersion were conducted according to International Conference on Harmonization 2003.[29] To conduct the stability study, 30 ml batch was prepared and it was divided in six different portions each of 5 ml and lyophilized. The lyophilized samples were filled into 20 ml glass vials, sealed with rubber stopper and metal clips. Of these, three portions were stored in a stability chamber maintained at 25°C ± 2°C/60% RH ± 5% RH and three portions were stored in a refrigerator (5°C ± 3°C) for the period of 3 months.[30] The samples were analyzed for particle size and % drug retained at a definite time intervals.

RESULTS AND DISCUSSION

The primary step in formulating NLC dispersions is always prediction of drug solubility in lipids. Hence, solubility of CC in various solid and liquid lipids was determined. The results of solubility studies in solid lipids are shown in Figure 1, where the amount of solid lipid required to dissolve 50 mg of CC was calculated. CC showed greater solubility in both grades of GMS-Geleol® Mono and diglycerides and Cutina® GMS VPH. Also, properties of Cutina® like good flowability, nontoxicity, approved regulatory status and low cost favors its choice as a solid lipid. Solubility of CC was checked in different liquid lipids [Figure 2] to find liquid lipid that shows better solubility than others. Furthermore, solubility of most lipophilic drugs is usually more in liquid lipid than solid lipid.[31] Capmul® MCM showed higher drug solubility than other liquid lipids. Besides solubility study in lipids, partition study of drug between lipid and aqueous media is necessary since the drug although having high solubility in lipid may precipitate out into aqueous media *in vivo* if its PC is low.[13] Hence, partition study was performed in various solid and liquid lipids. The results showed higher PCs in Cutina® GMS VPH and Capmul® MCM in comparison to other lipids [Figure 3]. Higher entrapment efficiency can be

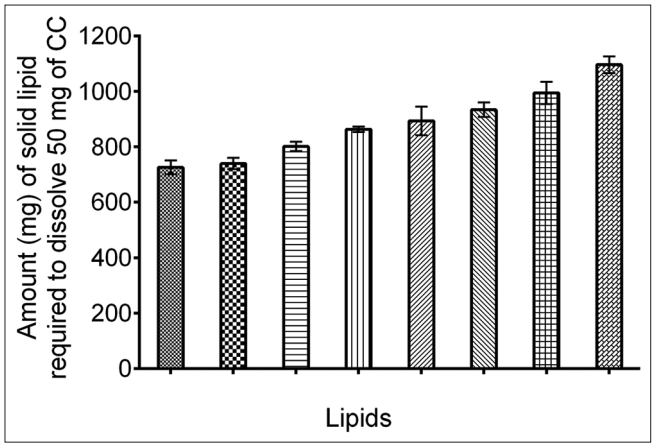

Figure 1: Amount (mg) of solid lipid required to dissolve 50 mg of candesartan cilexetil (a) Cutina® glyceryl monostearate verapamil hydrochloride (b) Geleol® mono and diglycerides (c) Gelucire® 50/13 pellets (d) Compritol® 888 ATO (e) Stearic acid (f) Dynasan® 114 (g) Dynasan® 112 (h) Precirol® ATO 5

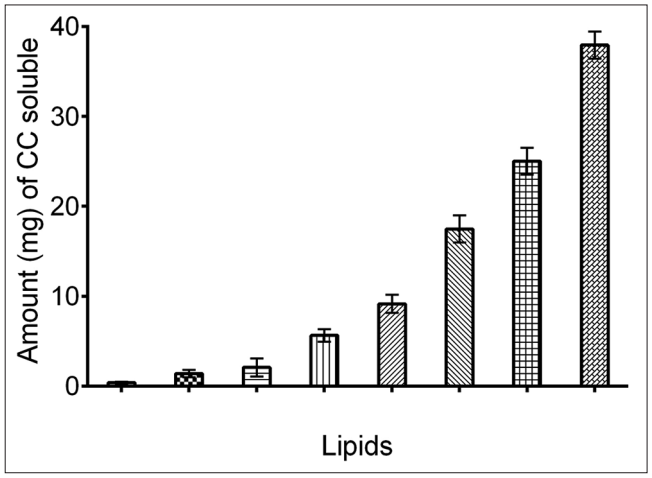

Figure 2: Solubility of candesartan cilexetil in liquid lipid (mg/ml) (a) Miglyol® 840 (b) Miglyol® 829 (c) Imwitor® 948 (d) Olive oil (e) Imwitor® 491 (f) Arlamol (g) Acconon® CC-6 (h) Capmul® medium chain mono

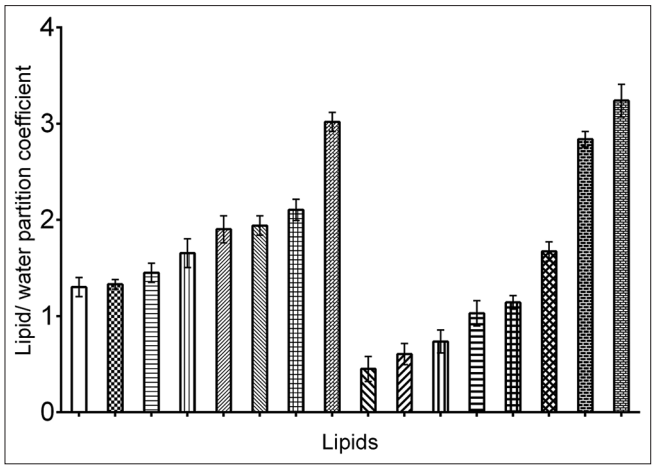

Figure 3: Partition coefficients of candesartan cilexetil in various solid and liquid lipids (a) Precirol® ATO 5 (b) Dynasan® 112 (c) Dynasan® 114 (d) stearic acid (e) compritol® 888 (f) Gelucire® 50/13 pellets (g) Geleol® (h) Cutina® (i) Miglyol® 840 (j) Miglyol® 829 (k) Imwitor® 948 (l) Olive oil (m) Imwitor® 491 (n) Arlamol (o) Acconon® (p) Capmul® medium chain mono

expected from lipids, which show higher PCs as the transfer of the drug from internal lipid phase to the external aqueous phase would be minimum. Moreover, miscibility of solid lipid with liquid lipid was checked by compatibility study. It was found that Cutina® GMS VPH formed homogenous mixture with Capmul® MCM and no phase separation was observed for 24 h. This is the primary step in the development of stable NLCs formulations and allows considering that the liquid lipid is fully entrapped in solid lipid. Hence, these two lipids, Cutina® GMS VPH and Capmul® MCM were selected as solid and liquid lipid, respectively.

Seventeen NLC formulations according to the Box–Behnken design were prepared by emulsification ultrasonication method. The observed values of the three responses viz. particle size, zeta potential and % entrapment efficiency for all batches are shown in Table 2. The selected independent variables were found to influence the three responses measured. All batches showed particle size in the range between 234 and 307 nm, zeta potential -49.12 to -23.45 and % EE 52.50-79.72%. The various models fitted for each response were linear, cubic, and quadratic and two factor interaction models. The results obtained are shown in Table 3. Quadratic model was found to fit best for all three responses as indicated by greater R2 values [Table 3] in comparison to other models. Using the ANOVA provision available in the software, the polynomial equations involving the main effects and interaction factors were determined based on estimation of various statistical parameters. The results of ANOVA study are shown Table 4. Accordingly, model F-value for response Y1, Y2 and Y3 were found to be 9.29, 35.49, and 13.30, respectively which implied that the quadratic model selected was significant for all three responses. Moreover, value of "prob >F" <0.05 indicate that the model terms are significant.

Table 3: Model summary statistics

Models	R^2	Adjusted R^2	Predicted R^2	SD	Remarks
Response (Y_1)					
Linear	0.2541	0.0820	−0.3121	20.87	
2FI	0.2744	−0.1626	−1.6972	23.49	
Quadratic	0.9228	0.8235	−0.1346	9.15	Suggested
Cubic	0.9930	0.9720	-	3.65	Aliased
Response (Y_2)					
Linear	0.7814	0.7310	0.5937	4.14	
2FI	0.8696	0.7913	0.5433	3.65	
Quadratic	0.9786	0.9510	0.7533	1.77	Suggested
Cubic	0.9933	0.9733	-	1.31	Aliased
Response (Y_3)					
Linear	0.6488	0.5677	0.4270	5.73	
2FI	0.6985	0.5176	0.1255	6.05	
Quadratic	0.9447	0.8737	0.2540	3.10	Suggested
Cubic	0.9904	0.9617	-	1.70	Aliased

SD: Standard deviation, 2FI: Two factor interaction

Table 4: ANOVA for response surface quadratic model

Source	Y_1		Y_2		Y_3	
	F	*P>F*	*F*	*P>F*	*F*	*P>F*
Model	9.29	0.0038	35.49	<0.0001	13.30	0.0013
X_1	0.024	0.8816	0.79	0.4030	72.20	<0.0001
X_2	5.92	0.0452	24.46	0.0017	3.15	0.1194
X_3	17.08	0.0044	229.83	<0.0001	6.83	0.0347
X_1X_2	0.30	0.6019	25.72	0.0014	2.26	0.1763
X_1X_3	1.44	0.2685	2.20	0.1819	1.29	0.2929
X_2X_3	2.984E-003	0.9580	0.87	0.3817	2.75	0.1414
X_{12}	56.66	0.0002	16.01	0.0052	18.39	0.0036
X_{22}	3.16	0.1189	17.67	0.0040	5.49	0.0517
X_{32}	0.043	0.8416	0.37	0.5646	4.46	0.0726

ANOVA: Analysys of variance

Hence, for response Y1-particle size, X2, X3 and X12 were found to be significant model terms. Values >0.1000 indicated the model terms were not significant. The adequate precision of 9.277 indicated an adequate signal. The response surface analysis plots in three-dimensional model graphs were constructed using the software. These plots were used to study the interaction effects of two independent variables on the responses while holding the third factor at a constant level. The graphs obtained for responses Y1, Y2 and Y3 are shown in Figures 4-6a-c. Moreover, the perturbation plots for each responses were plotted that helped to compare the effects of all three factors at any particular point in the design space. The responses were plotted by changing only one factor in its constrained range while keeping other two factors constant [Figures 4-6d].[32] The effect of independent variables on particle size could be quantified by the following quadratic equation.

$$Y_1 = 245.40 + 0.5X_1 + 7.88X_2 - 7.88X_2 - 13.38X_3 + 2.50X_1X_2 - 5.50X_1X_3 - 0.25X_2X_3 + 32.67X_1^2 + 7.92X_2^2 + 0.93X_3^2$$

The positive values before a factor in the above regression equation indicate that the response increases with the factor and vice versa.[33] It was observed that on increasing the solid lipid: liquid lipid ratio, the size of the NLCs increased. This effect could be attributed to the fact that during NLCs formulation, by increasing the solid content, the dispersion viscosity also increases that result into higher surface tension and thus higher particle size.[34] This effect was also promoted by increasing the concentration of surfactant in the formulation, which is required to stabilize the higher % of solid lipid [Figure 4c]. This result could be attributed to the accumulation of excess surfactant molecules on the NLCs surface probably due to a hydrophobic interaction, in which nonpolar groups such as alkyl chains of the surfactant and solid lipid molecules could interact with each other.[35] The effects of factors on particle size could be further justified by the perturbation plot shown.

For response Y2-zeta potential, the significant model terms obtained were X2, X3, X1X2, X12 and B2. The Adequate precision of 18.54 indicated an adequate signal. Quadratic equation generated for effect on zeta potential is as follows:

$$Y_2 = -40.56 - 0.56X_1 - 3.09X_2 - 9.48X_3 + 4.48X_1X_2 + 1.31X_1X_3 + 0.83X_2X_3 + 3.45X_1^2 + 3.62X_2^2 - 0.52X_3^2$$

Surfactant concentration alone (X3) showed greater influence on zeta potential as indicated by its exponent - 9.48 in the equation. Zeta potential is an important parameter indicating the stability of NLCs. Theoretically, higher zeta potential values on either side stabilizes the particle suspension.[30] The results of Box-Behnken design suggested that surfactant concentration greatly affected zeta potential while the other two factors had negligible effect on zeta potential. As the surfactant concentration increased, the zeta potential also increased in the negative side [Figure 5]. This could be further justified that the use of nonionic type surfactant (as in this case) imparts stearic stabilization avoiding aggregation of fine particles in the colloidal system.[36]

Similarly, for response Y3-% entrapment efficiency, it was found that X1, X3 and X12 were significant model terms. The "adequate precision" of 10.244 indicates an adequate signal. The quadratic equation generated is:

$$Y_3 = 75.76 + 9.30X_1 - 1.94X_2 + 2.86X_3 - 2.33X_1X_2 + 1.76X_1X_3 - 2.57X_2X_3 - 6.47X_1^2 - 3.53X_2^2 - 3.19X_3^2$$

The higher value before X1 indicate the lipid: drug ratio greatly influenced % entrapment efficiency. Drug: lipid ratio was found to be a major factor affecting entrapment efficiency. The response surface plots [Figure 6] showed the effects of all three factors on entrapment efficiency. As the lipid portion was increased relative to drug, more amount of drug could be entrapped into the lipid matrix and hence entrapment efficiency also increased. Moreover, increasing amount of liquid lipids lead to increased solubility of drugs and hence, entrapment efficiency increased.

To get optimized formulation, numerical optimization was performed using Design expert software (Design Expert, Version 8.0.3, Stat-Ease Inc., Minneapolis, MN). The various desirabilities were fed into the software as constraints for responses. The optimum formulation was based on set criteria of minimum particle size, zeta potential in range of -30 to -49.12 and maximum drug entrapment [Table 2]. The predicted levels of formulation factors obtained by the software were 22.47:1 ratio of lipid: drug, 7.23:1 ratio solid lipid: liquid lipid and 1.97% surfactant concentrations.

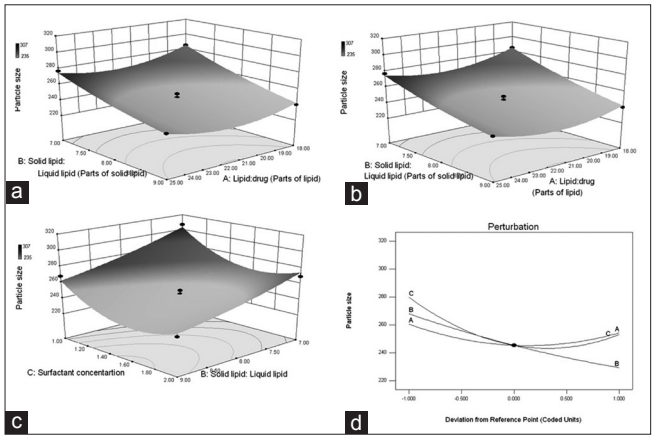

Figure 4: Response surface plot three-dimension showing effect of, (a) Lipid: Drug ratio (X1) and solid lipid: Liquid lipid ratio (X2), (b) Lipid: Drug ratio (X1) and surfactant concentration (X3), (c) Solid lipid: Liquid lipid ratio (X2) and surfactant concentration (X3) on particle size, (d) Perturbation plot showing effect of lipid: Drug ratio (X1), solid lipid: Liquid lipid ratio (X2) and surfactant concentration (X3) on particle size

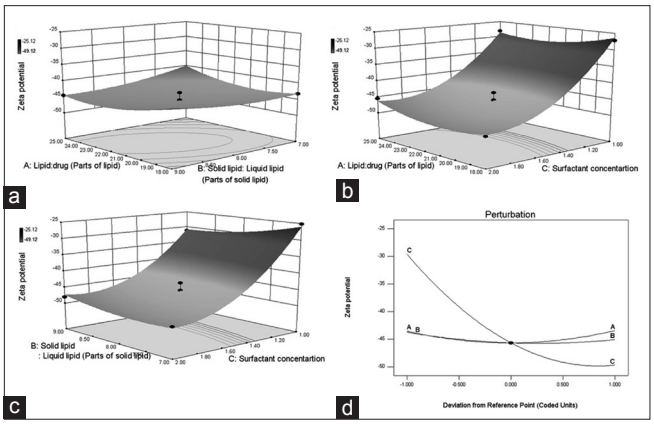

Figure 5: Response surface plot three-dimension showing effect of, (a) Lipid: Drug ratio (X1) and solid lipid: Liquid lipid ratio (X2), (b) Lipid: Drug ratio (X1) and surfactant concentration (X3), (c) Solid lipid: Liquid lipid ratio (X2) and surfactant concentration (X3) on zeta potential, (d) Perturbation plot showing effect of lipid: Drug ratio (X1), solid lipid: Liquid lipid ratio (X2) and surfactant concentration (X3) on zeta potential

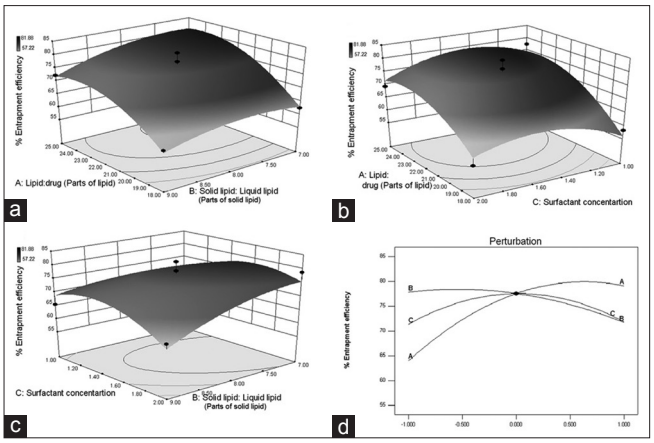

Figure 6: Response surface plot three-dimension showing effect of; (a) Lipid: Drug ratio (X1) and solid lipid: Liquid lipid ratio (X2), (b) Lipid: Drug ratio (X1) and surfactant concentration (X3), (c) Solid lipid: Liquid lipid ratio (X2) and surfactant concentration (X3) on percentage entrapment efficiency, (d) Perturbation plot showing effect of lipid: Drug ratio (X1), solid lipid: Liquid lipid ratio (X2) and surfactant concentration (X3) on % entrapment efficiency

This new batch of NLCs was formulated and responses were measured. The observed value of responses were compared to the predicted values and % error was calculated [Table 5] to validate the method. The observed value of Y1, Y2 and Y3 were in a very close agreement to the predicted ones. By this the validity of the optimization procedure was proven.

The optimized batch was lyophilized using sucrose as cryoprotectant. After lyophilization, particle size increased insignificantly from 238 to 244 nm. Moreover, entrapment efficiency was found to be 76.23%, which also did not change significantly. Thus, NLCs were effectively lyophilized using sucrose.

Differential scanning calorimetry measures the heat loss or gain resulting from physical or chemical changes within a sample as a function of the temperature. DSC experiments are useful to understand solid dispersions such as solid solutions, simple eutectic mixtures or, as in the case of SLN and NLC, drug and lipid interactions and the mixing behavior of solid lipids with liquid lipids, such as oils.[37] DSC thermograms of CC bulk powder, GMS and of freeze dried NLCs were measured and graphs obtained are shown in Figure 7. Bulk CC powder showed endothermic peak at 180°C while lipid showed peak at 85°C. The NLC dispersion shows no endothermic peak around 180°C that indicates complete entrapment of drug into lipids.

The drug release profile of free CC and CC-NLCs at pH 1.2 and 6.8 measured are shown in Figure 8. There was 24.13% and 34.99% CC release found at the end of 2 h in simulated gastric fluid from free CC and CC-NLCs respectively. In simulated intestinal fluid (pH 6.8), CC release was found to be higher from CC-NLCs (73.12%) as compared to free CC (39.74%). Thus in both medias, the release from CC-NLCs was found to be prolonged as compared to free drug which could be attributed to the solubilizing effect of

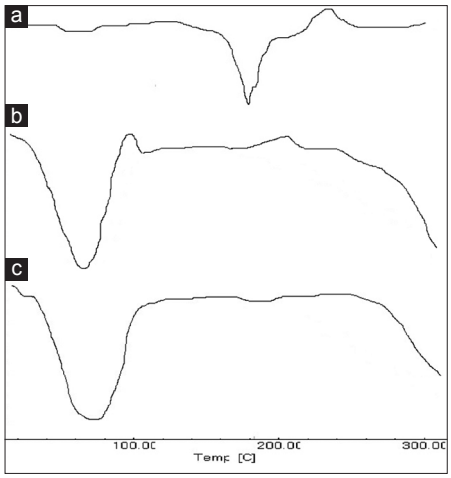

Figure 7: Differential scanning calorimetry thermograms of (a) candesartan cilexetilc bulk powder (b) Glyceryl monostearate and (c) freeze dried nanostructured lipid carriers

Table 5: Comparative levels of predicted and observed responses for the optimized formulation

Response	Predicted value	Observed value	% error*
Y_1	233.19 nm	238 nm	2.06
Y_2	−46.47 mV	−45.33 mV	2.45
Y_3	79.94%	77.15%	3.49

*: % error = (observed value – predicted value)/predicted value × 100

nanoparticles. The release of CC from CC-NLC at stomach pH was much higher than that at intestinal pH, which could result from the higher solubility of CC at low pH values.[38] The lipids gets digested in presence of enzymes of gastric and intestinal media resulting in formation of emulsion and micellar solution.[39,40] Furthermore, the presence of surfactant in NLCs allows the formation of drug micelles.[41] Thus, CC remains in the dissolved form in emulsion and micellar forms, which in absence of lipids and surfactant has limited solubility *in vivo*. This result in increased solubility and hence absorption of CC *in vivo*.

Results of stability data are shown in Figures 9 and 10. At room temperature, there was a significant increase of about 30 nm in particle size, while at refrigerated condition; there was an increase of about only 10 nm at the end of 90 days. Thus, NLCs maintained their particle size better when kept under refrigerated condition as compared to room temperature. Results of % drug retained showed that the formulation was found to be stable at both condition with a slight higher stability in refrigerator and hence formulated NLCs need to be stored in this condition.

CONCLUSION

There is real need to develop novel type of drug delivery for the effective treatment of hypertension with CC. Incorporation of CC in NLCs can increase the absorption of CC through oral route. Effect of various factors on the formulation can be studied by the Box-Behnken design. With

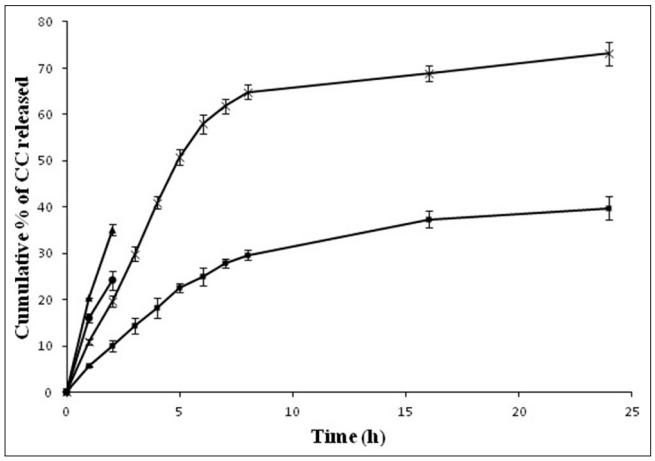

Figure 8: Cumulative percentage of drug release in simulated gastric fluid and simulated intestinal fluid. (a) candesartan cilexetil (CC) suspension in artificial gastric fluid pH 1.2, (b) CC suspension in phosphate buffer pH 6.8, (c) CC-nanostructured lipid carrier in artificial gastric fluid pH 1.2, (d) CC-NLCs in phosphate buffer pH 6.8

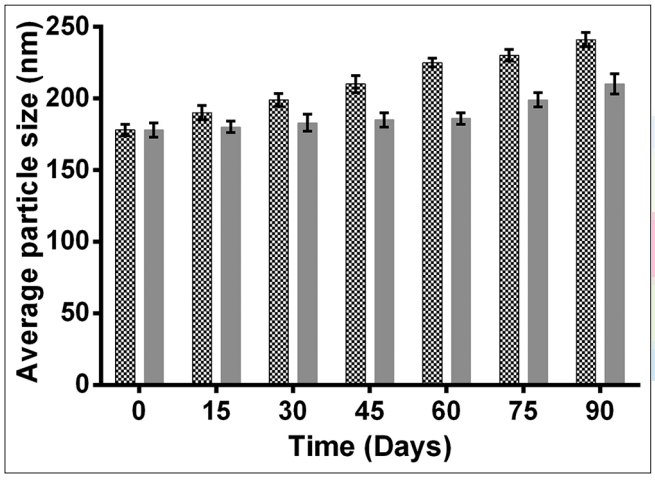

Figure 9: Effect of storage condition on particle size of candesartan cilexetil-nanostructured lipid carriers as a function of time

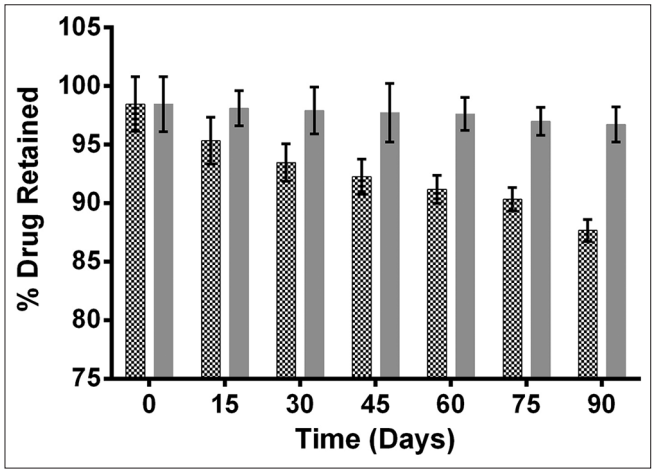

Figure 10: Effect of storage condition on percentage drug retained of candesartan cilexetil-nanostructured lipid carriers as a function of time

the use of desirability plots minor change in the formulation is possible for the required response. However, *in vivo* pharmacokinetic as well as pharmacodynamic studies are required for confirmation of improvement in bioavailability.

REFERENCES

1. Kearney PM, Whelton M, Reynolds K, Muntner P, Whelton PK, He J. Global burden of hypertension: Analysis of worldwide data. Lancet 2005;365:217-23.
2. High blood pressure and cholesterol: out of control in US. Atlanta. Available from: http://www.cdc.gov/Features/Vitalsigns/CardiovascularDisease. [Last cited on 2013 Apr 25].
3. Tadevosyan A, Maclaughlin EJ, Karamyan VT. Angiotensin II type 1 receptor antagonists in the treatment of hypertension in elderly patients: Focus on patient outcomes. Patient Relat Outcome Meas 2011;2:27-39.
4. David PW. Antihypertensive drugs. In: Charles R, Craig RE, editors. Modern Pharmacology with Clinical Applications. Beltimore: Lippincott Williams and Wilkins; 1997. p. 225-38.
5. Ferreiros N, Dresen S, Alonso RM, Weinmann W. Hydrolysis and transesterification reactions of candesartan cilexetil observed during the solid phase extraction procedure. J Chromatogr B Analyt Technol Biomed Life Sci 2007;855:134-8.
6. Nishida Y, Takahashi Y, Nakayama T, Soma M, Asai S. Comparative effect of olmesartan and candesartan on lipid metabolism and renal function in patients with hypertension: A retrospective observational study. Cardiovasc Diabetol 2011;10:74.
7. Nekkanti V, Karatgi P, Prabhu R, Pillai R. Solid self-microemulsifying formulation for candesartan cilexetil. AAPS PharmSciTech 2010;11:9-17.
8. Al Omari AA, Al Omari MM, Badwan AA, Al-Sou'od KA. Effect of cyclodextrins on the solubility and stability of candesartan cilexetil in solution and solid state. J Pharm Biomed Anal 2011;54:503-9.
9. Sayyad FJ, Tulsankar SL, Kolap UB. Design and development of liquisolid compact of candesartan cilexetil to enhance dissolution. J Pharm Res 2013;7:381-8.
10. Zhang Z, Gao F, Bu H, Xiao J, Li Y. Solid lipid nanoparticles loading candesartan cilexetil enhance oral bioavailability: *In vitro* characteristics and absorption mechanism in rats. Nanomedicine 2012;8:740-7.
11. Gao F, Zhang Z, Bu H, Huang Y, Gao Z, Shen J, *et al*. Nanoemulsion improves the oral absorption of candesartan cilexetil in rats: Performance and mechanism. J Control Release 2011;149:168-74.
12. Burcham DL, Maurin MB, Hausner EA, Huang SM. Improved oral bioavailability of the hypocholesterolemic DMP 565 in dogs following oral dosing in oil and glycol solutions. Biopharm Drug Dispos 1997;18:737-42.
13. Shafiq S, Shakeel F, Talegaonkar S, Ahmad FJ, Khar RK, Ali M. Development and bioavailability assessment of ramipril nanoemulsion formulation. Eur J Pharm Biopharm 2007;66:227-43.
14. Venishetty VK, Chede R, Komuravelli R, Adepu L, Sistla R, Diwan PV. Design and evaluation of polymer coated carvedilol loaded solid lipid nanoparticles to improve the oral bioavailability: A novel strategy to avoid intraduodenal administration. Colloids Surf B Biointerfaces 2012;95:1-9.
15. Severino P, Andreani T, Macedo AS, Fangueiro JF, Santana MH, Silva AM, *et al*. Current state-of-art and new trends on lipid nanoparticles (SLN and NLC) for oral drug delivery. J Drug Deliv 2012;2012:750891.
16. Müller RH, Mäder K, Gohla S. Solid lipid nanoparticles (SLN) for controlled drug delivery-A review of the state of the art. Eur J Pharm Biopharm 2000;50:161-77.
17. Muchow M, Maincent P, Muller RH. Lipid nanoparticles with a solid matrix (SLN, NLC, LDC) for oral drug delivery. Drug Dev Ind Pharm 2008;34:1394-405.
18. Kasongo KW, Shegokar R, Müller RH, Walker RB. Formulation

development and *in vitro* evaluation of didanosine-loaded nanostructured lipid carriers for the potential treatment of AIDS dementia complex. Drug Dev Ind Pharm 2011;37:396-407.
19. Gohel MC, Amin AF. Formulation optimization of controlled release diclofenac sodium microspheres using factorial design. J Control Release 1998;51:115-22.
20. Nazzal S, Khan MA. Response surface methodology for the optimization of ubiquinone self-nanoemulsified drug delivery system. AAPS Pharm Sci Tech 2002;3:E3.
21. Gareth A. Lewis DM, Phan-Tan-Luu R. Pharmaceutical Experimental Design. New York, Basel: Marcel Deckker Inc.; 2005.
22. Joshi M, Patravale V. Formulation and evaluation of nanostructured lipid carrier (NLC)-based gel of valdecoxib. Drug Dev Ind Pharm 2006;32:911-8.
23. Doktorovová S, Araújo J, Garcia ML, Rakovský E, Souto EB. Formulating fluticasone propionate in novel PEG-containing nanostructured lipid carriers (PEG-NLC). Colloids Surf B Biointerfaces 2010;75:538-42.
24. Aji Alex MR, Chacko AJ, Jose S, Souto EB. Lopinavir loaded solid lipid nanoparticles (SLN) for intestinal lymphatic targeting. Eur J Pharm Sci 2011;42:11-8.
25. Box GE, Behnken DW. Some new three-level designs for the study of quantitative variables. Technometrics 1960;2:455-75.
26. Dong CH, Xie XQ, Wang XL, Zhan Y, Yao YJ. Application of Box-Behnken design in optimisation for polysaccharides extraction from cultured mycelium of Cordyceps sinensis. Food Bioprod Process 2009;87:139-44.
27. Gulbake A, Jain A, Khare P, Jain SK. Solid lipid nanoparticles bearing oxybenzone: In-vitro and in-vivo evaluation. J Microencapsul 2010;27:226-33.
28. Unruh T, Bunjes H, Westsen K. Observation of size-dependent melting in lipid nanoparticles. J Phys Chem 1999;103:10373-7.
29. ICH Q 1 A (R2). Stability Testing of New Drug Substances and Products (CPMP/ICH/2736/99); 2003.
30. Gonzalez-Mira E, Egea MA, Garcia ML, Souto EB. Design and ocular tolerance of flurbiprofen loaded ultrasound-engineered NLC. Colloids Surf B Biointerfaces 2010;81:412-21.
31. Uner M, Yener G. Importance of solid lipid nanoparticles (SLN) in various administration routes and future perspectives. Int J Nanomedicine 2007;2:289-300.
32. Woitiski CB, Veiga F, Ribeiro A, Neufeld R. Design for optimization of nanoparticles integrating biomaterials for orally dosed insulin. Eur J Pharm Biopharm 2009;73:25-33.
33. Gannu R, Yamsani VV, Yamsani SK, Palem CR, Yamsani MR. Optimization of hydrogels for transdermal delivery of lisinopril by Box-Behnken statistical design. AAPS Pharm Sci Tech 2009;10:505-14.
34. Jia LJ, Zhang DR, Li ZY, Feng FF, Wang YC, Dai WT, *et al*. Preparation and characterization of silybin-loaded nanostructured lipid carriers. Drug Deliv 2010;17:11-8.
35. Finney J. The structural basis of the hydrohobic interaction. In: Bellissent-Funel MC, editor. Hydration Processes in Biology: Theoretical and Experimental Approaches. France: IOS Press; 1998. p. 115-26.
36. Han F, Sanming L, Yin R, Liu H, Xu L. Effect of surfactants on the formation and characterization of a new type of colloidal drug delivery system: Nanostructured lipid carriers. Colloids Surf A Physicochem Eng Asp 2008;315:210-6.
37. Unruh T, Bunjes H, Westesen K, Koch MH, Investigations on the melting behaviour of triglyceride nanoparticles. Colloid Polym Sci 2001;279:398-403.
38. Nekkanti V, Pillai R, Venkateshwarlu V, Harisudhan T. Development and characterization of solid oral dosage form incorporating candesartan nanoparticles. Pharm Dev Technol 2009;14:290-8.
39. Chakraborty S, Shukla D, Mishra B, Singh S. Lipid – An emerging platform for oral delivery of drugs with poor bioavailability. Eur J Pharm Biopharm 2009;73:1-15.
40. Beloqui A, Solinís MÁ, Delgado A, Evora C, Isla A, Rodríguez-Gascón A. Fate of nanostructured lipid carriers (NLCs) following the oral route: Design, pharmacokinetics and biodistribution. J Microencapsul 2014;31:1-8.
41. Joshi M, Patravale V. Nanostructured lipid carrier (NLC) based gel of celecoxib. Int J Pharm 2008;346:124-32.

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