Available online on 15.06.2019 at http://jddtonline.info

Journal of Drug Delivery and Therapeutics

Open Access to Pharmaceutical and Medical Research

Open Access Research Article

Application of Design Of Expert for the Development and Systematic Optimisation of L-Asparaginase loaded Nanoparticulate Carrier Drug Delivery Systems

Sharma Gazal[1]*, Dr. Goyal Amit Kumar*[2], Dr. Singh A.P.[3]

[1] Research Scholar, IKG Punjab Technical University, Kapurthala, Punjab India

[2] Scientist E, National Animal Institute of Biotechnology, Hyderabad, India

[3] Dean, IKG Punjab Technical University, Kapurthala, Punjab India 146001

ABSTRACT

L-Asparaginase (L-ASN) is a clinically approved chemotherapeutic agent for the treatment of acute lymphoblastic leukaemia and lymphosarcoma. The aim of this research study was to develop and to optimize solid lipid nanoparticle formulation loaded with enzyme L-Asparaginase using response surface methodology (RSM) [1]. The formulation was prepared by a modified double emulsion method followed by solvent evaporation technique using a combination of high-speed homogeniser (10000 rpm) and an automatic hotplate for a temperature 40°C. Box-Behnken Design (BBD) was involved in the study to establish and to understand the relationship between selected design factors and the experimental data thus obtained. A set of 29 formulations were prepared in triplicate based on the recommendations of BBD.[2] The desired results obtained were found to be in close agreement with the experimental results. The responses were fitted to a quadratic; polynomial model. The statistical validation using Analysis of Variance (ANOVA) was done for the respective fitted models.[3] Response Surface Graphs and 3D contour plots were constructed to understand the effect of independent variables in different combinations on the desired responses. SLN prepared were found to be spherical in shape and the mean particle size <198 nm.[4] The polydispersity index (PDI) and the zeta potential recorded for the prepared formulation corresponding to the particle size was 0.096 ± 0.043 and −10.39 mV respectively. The enzyme drug loading was 10.11% ± 2.02 and the enzyme entrapment efficiency was found to be 76.19% ± 1.23. BBD found to be very effective in considering the effects of independent formulation variables to develop an optimised enzyme loaded SLN formulation with sufficient activity of the L-ASN enzyme.

Keywords: Solid Lipid Nanoparticle, Response Surface Methodology, Box-Behnken Design

Article Info: Received 20 April 2019; Review Completed 30 May 2019; Accepted 02 June 2019; Available online 15 June 2019

Cite this article as:

Sharma G, Goyal AK, Singh AP. Application of Design Of Expert for the Development and Systematic Optimisation of L-Asparaginase loaded Nanoparticulate Carrier Drug Delivery Systems, Journal of Drug Delivery and Therapeutics. 2019; 9(3-s):303-308 http://dx.doi.org/10.22270/jddt.v9i3-s.3017

***Address for Correspondence:**

Gazal Sharma, Research Scholar, IKG Punjab Technical University, Kapurthala, Punjab India

1.0 INTRODUCTION

Chemically, L-ASN is a tetrameric protein known to produce selective cytotoxic effect on the cancer cells. The cytotoxicity is produced through a hydrolytic reaction catalysed by the enzyme L-Asparaginase. L-Asparaginase is not found in the human blood but it breaks down the essential amino acid L-Asparagine into L-aspartic acid along with the release of ammonia when administered through intravenous route.[5] Unlike normal cells, leukemic cells do not have asparagine synthetase enzyme for the auto synthesis and subsequent replenishment of L-asparagine in cancer cells which leads to its apoptosis. This difference in cellular pathway was scientifically explored to bring selective cytotoxicity of leukemic cells and a breakthrough in the treatment of acute lymphocytic leukaemia (ALL). The enzyme L-Asparaginase was extracted and found effective against ALL for the first time in the year 1953. A chemist J.G. Kidd successfully extracted it from the serum of guinea pig and explore its hydrolytic characteristics [6]. Since then, it has been used to expand life expectancy of leukemic patients. The development of a new anti-cancer drug molecule is a very lengthy and costly venture so working on the betterment and safety efficacy of an existing drug molecule like L-Asparaginase is comparatively a better alternate move. Greater discoveries in the field of drug delivery are also projected in the immediate future. The clinical practice of drug delivery has reformed vividly with the time. Drug Delivery System is an engineered technology, preferably used to get sustained release (control the rate at which a drug is released) and a better targeted delivery (location in the body where it is released) of the anti-cancer drug

molecule in the body [7-9]. Next to it is the Carrier Based Drug Delivery System (CBDDS). It is a vast research in the field of medical science that explores new approaches of controlled and sustained drug delivery that commits maximum therapeutic effect with minimum side effects [10]. There are various forms of carrier-based delivery system which are already been explored in the different pharmaceutical labs. This includes nanoparticles, solid lipid nanoparticles, liposomes, microemulsions, phytosomes etc. This strategy of optimization also overlooks interface between factors and leads to many experimental runs [11]. Through these studies it is made clear that the choice of lipids and surfactants and their process parameters like processing speed time and temperature brought impact on the desired quality of SLN.[12] The desired optimal characteristics of SLN is dependent on the relative amount of lipid/oil, surfactant/s, the ratio of lipid:drugs etc. This states that the ingredients used in the optimised design of SLN significantly affect the physicochemical properties like mean particle size, zeta potential, PDI and drug-release profiles of the nanoparticles.[13] To develop a desired pharmaceutical formulation the application of statistical experimental design found to be efficient in acquiring the requisite information that would help in recognizing the relationship between dependent and independent variables. RSM is appropriate in doing parallel analysis of formulation variables in spite of enormous complexities.[14] Several experimental designs[15-17] are used to develop a pharmaceutical formulation that provides an estimate of the relative significance of different process variables and generally requires minimal experimentation. In this study an already established and marketed anticancer drug L-Asparaginase has been transformed into a new formulation through using SLN as carrier system (L-ASN loaded SLN). An RSM design Box-Behnken which requires 3-factor experimental design was applied to make the study more effective.[18] The objective of the study was to evaluate the effect of interactions of the process variables through Box-Behnken design and to further optimize the L-Asparaginase-loaded SLN formulation. Glyceryl monostearate (GMS) was selected as a source of solid lipid and Span 20 and Tween 20 were taken as emulsifying agents.[19-20] The model drug in this study was L-Asparaginase which was encapsulated in the SLN. L-ASN loaded SLN was prepared using double emulsion method and was further investigated in detail for its physicochemical characteristics. The design was fabricated using a pool of both independent as well as dependent factors. Lipid (X_1), Drug conc. (X_2), Surfactants (X_3) and Homogenisation Speed (X_4) were selected as the independent factors of the design whereas Size of the particle (nm) (Y_1), Drug Entrapment Efficiency (%) (Y_2) and Enzyme Loading (%) (Y_3) were the chosen as dependent variables. BBD found to be very effective in considering the effects of independent formulation variables to develop an optimised L-ASN loaded SLN formulation consisting of GMS (Lipid); Surfactants (Tween 20 and Span 20 used in 2:1 ratio) and L-Asparaginase (Drug) primarily prepared using double emulsion method at a speed 10000 rpm and at a constant temperature 40°C and a sufficient activity of the enzyme was found in the formulation.

2.0 MATERIAL AND METHODS

The standard drug L-Asparaginase in the lyophilised powder form was a kind gift received from United Biotech (P) Ltd. (India). Glyceryl Monosterate, Span 20 and Tween 20 were purchased from Himedia Laboratories (P) Ltd. (India). High-performance liquid chromatography (HPLC) grade acetone was used. Other solvents and reagents were of analytical reagent grade purchased from Himedia Laboratories (P) Ltd. (India). Homogeniser (Silent Crusher M, Heidolph Instruments, Germany) and Magnetic stirrer with hot plate (MR Hei-Tec, Heidoph Instruments, Germany) were used for the homogenisation, stirring and maintaining 40°C temperature during the entire formulation process

2.1 Preparation of L-Asparaginase loaded SLN and determination of Mean Particle Size and Zeta Potential

L-Asparaginase loaded solid lipid nanoparticles were prepared by already reported double emulsion method (w/o/w) with some modifications followed by solvent evaporation. At first a specific amount of GMS was melted at 70°C in the presence of acetone used as a solvent in a completely covered beaker followed by the addition of Span 20. The temperature was gradually lower down and maintained at 40°C. [18] The standard L-Asparaginase was dissolved in deionised sterile water (1mg/1ml) and was brought to a temperature of 40°C. The drug solution was added at a constantly slower pace into the melted lipid while homogenising at a speed of 10000 rpm using high speed homogeniser (Silent Crusher M, Heidolph Instruments, Germany) for 10 minutes and at a constant temperature to prepare the stable primary emulsion. The primary emulsion w/o thus prepared was added drop wise into deionised sterile water at 40°C which was already having dissolved tween 20 and was kept under continuous magnetic stirring (MR Hei-Tec, Heidoph Instruments, Germany) at a speed of 1400 rpm for rapid and complete solvent evaporation.[19] The magnetic agitation at a constant temperature was continued till a uniform dispersion of nanoparticles as w/o/w is obtained. The mean particle size and zeta potential of optimised L-Asparaginase loaded SLN formulation were measured by Zetasizer (Delsa™Nano Zeta Potential and Submicron Particle Size Analyzer by Beckman Coulter). A suitable concentration was achieved using deionised water before taking the readings of particle size and zeta potential[20].

2.2 Design of Experiment used for formulation development

A Box-Behnken design (29-run, 4-factor, 3-level) was applied in the study of the formulation optimization process. Polynomial models were constructed using Design-Expert software (Trial Version 11.1.2.0, Stat-Ease Inc., MN) in the formulation optimisation process. The quadratic response surface was suitably investigated with this design. Second order polynomial model was constructed with this design. The design identifies the main aspects of the study that was used to evaluate the major effects, quadratic effects and effects of several interactions of the formulation ingredients to optimize the formulation process**.** This design has generated the non-linear quadratic model as:

$$Y = A_0 + A_1X_1 + A_2X_2 + A_3X_3 + A_4X_4 + A_5X_1X_2 + A_6X_2X_3 + A_7X_3X_4 + A_8X_1X_3 + A_9X_1X_4 + A_{10}X_2X_4 + A_{11}X_1^2 + A_{12}X_2^2 + A_{13}X_3^2 + A_{14}X_4^2$$

Y is the measured response of the amount of the Lipid used, Drug conc., Surfactants % and Homogenisation Speed (dependent variables) associated with each factor-level combination; The regression coefficients of the respective variables was A_0-A_1. Their interaction factors were computed from the experimental values thus observed as Y; and independent variables were coded as X_1, X_2, X_3 and X_4. The independent factors evaluated in this study for their 03 values viz low, middle, and high values (−1, 0 and +1), as described in Table 1. The dependent responses were studies as Y_1, Y_2, Y_3 for the Mean Particle Size, entrapment efficiency (EE%) (Y_2), drug loading (DL%) (Y_3) with constraints applied as mentioned in Table 1. The design matrix generated by the software shown in Table 2.

Table 1: Variables and Levels in the Box-Behnken Design

	Levels		
	-1	**0**	**+1**
Independent Variables			
X_1 = Amount of lipid (mg)	225	275	325
X_2 = Amount of Drug (ml)	1	2	3
X_3= Surfactant (%)	4	6	8
X_4= Homogenisation Speed	6000	10000	14000
Dependent Variables			**Constraints**
Y_1= Mean Particle Size			Minimum
Y_2= Entrapment Efficiency (%)			Maximum
Y_3= Drug Loading (%)			Maximum

Table 2: Box-Behnken Design

Formulation	Lipid conc. (mg)	Surfactant Conc. (%)	Drug Conc. (ml)	Homogenisation Speed (rpm)
1.	225	6	1	10000
2.	325	4	2	10000
3.	275	4	2	6000
4.	275	8	2	14000
5.	325	6	2	6000
6.	225	4	2	10000
7.	325	6	1	10000
8.	275	4	2	14000
9.	275	6	2	10000
10.	275	6	1	6000
11.	275	6	1	14000
12.	225	6	2	6000
13.	275	6	3	6000
14.	325	8	2	10000
15.	225	6	3	10000
16.	275	6	2	10000
17.	225	6	2	14000
18.	325	6	2	14000
19.	275	8	1	10000
20.	275	8	3	10000
21.	275	6	2	10000
22.	275	6	2	10000
23.	275	8	2	6000
24.	275	6	3	14000
25.	275	4	3	10000
26.	275	6	2	10000
27.	225	8	2	10000
28.	275	4	1	10000
29.	325	6	3	10000

2.3 Determination of Entrapment Efficiency and Drug Loading

The activity of L-Asparaginase was confirmed by *in vitro* Nesslerization method estimated analytically using UV visible spectrophotometer (Perkin Elmer). To further estimate the percentage drug loading and percentage entrapment efficiency the formulation was pellet out using centrifugation at 15000 rpm using refrigerated centrifugation (Thermo Fischer) at 4°C for 20 min. The pellet was further treated with PEG 400 at 40°C. The treated formulation dispersed in 35 mL buffer (phosphate buffered saline at pH 7.4), to solubilise the drug absorbed on the

surface of nanoparticles followed by centrifugation. The free drug content in was labelled as W_{free}. The % of drug encapsulation (EE) and % drug loading were calculated as per the equation equations (1) and (2)

$$EE = \frac{W_{total} - W_{free}}{W_{total}} \times 100\%$$

$$DL = \frac{W_{total} - W_{free}}{W_{total} - W_{free} + W_{Lipid}} \times 100\%$$

Where W_{total} is the weight of total drug added, W_{lipid} were the weight of lipid added in the formulation.

3.0 RESULTS AND DISCUSSION

3.1 Analysis of the experimental data using DOE

Design-Expert software was used to statistically analyse the results of formulation parameters engaged in the experimental design. The independent variables that includes the quantity of lipid used, concentration of surfactants added, concentration of drug taken and homogenisation speed, reflects its significance in the the experimental outcomes as Entrapment Efficiency (%), Drug Loading (%) and Particle size (nm) as mentioned in Table 3. Based on the estimation of statistical parameters generated using Design-Expert software a polynomial equation was determined. The equations were validated established using statistical tool ANOVA. 3-D model graphs plotted for response surface measurement illustrating the effects of the factors on the % entrapment efficiency, and Particle size (nm) and % drug loading as presented in figures. Using RSM plots the effect of interactions between independent variables and dependent variables were studied and an qualitative effect was found for each variable. This analysis done using plots of DoE when observed carefully genuinely confirms the qualitative effect of each variable on each response which confirmed the its effectiveness in the experiments.

Table 3: Observed and predicted value of Particle Size (nm), % encapsulation efficiency and %drug loading of formulations using design

Formulation	Entrapment Efficiency (%)		Drug Loading (%)	
	Predicted	Observed	Predicted	Observed
1.	49.52	48.60	68.12	68.22
2.	65.66	67.40	79.59	80.50
3.	50.20	50.30	78.30	78.44
4.	72.87	72.80	84.11	84.04
5.	76.11	76.30	82.54	82.37
6.	42.28	49.80	78.85	81.49
7.	74.55	74.50	69.32	69.50
8.	49.98	49.80	78.28	78.29
9.	67.12	66.70	82.91	82.84
10.	59.64	62.70	68.03	69.40
11.	56.68	61.60	67.56	69.03
12.	47.03	42.30	80.68	78.87
13.	61.14	62.30	86.71	87.12
14.	87.84	86.40	84.86	84.10
15.	56.12	56.20	88.27	88.16
16.	67.12	65.40	82.91	82.53
17.	53.21	46.90	82.33	80.55
18.	73.29	71.90	81.64	81.50
19.	72.18	70.20	72.42	71.74
20.	75.38	74.30	88.41	88.90
21.	67.12	69.80	82.91	83.50
22.	67.12	68.90	82.91	83.28
23.	69.28	69.50	83.34	83.40
24.	67.48	70.50	87.93	88.44
25.	57.34	53.20	86.51	85.24
26.	67.12	64.80	82.91	82.42
27.	62.06	66.40	84.45	85.42
28.	48.24	43.20	63.44	61.00
29.	80.25	81.20	88.23	88.20

3.2 Effects on Entrapment efficiency (%) The entrapment efficiency estimated (%) as mentioned in Table 3 varies from 42.3% to 84.64% for formulation 12 and formulation 14 respectively. The independent factors specifically the amount of lipid and surfactants affecting the entrapment efficiency and this effect can be explained by the following quadratic regression equation:

$$Y_2 = 67.12 + 12.29X_1 + 3.07\ X_2 + 10.49\ X_3 + 0.8417X_4 - 0.2250X_1\ X_2 + 0.6000\ X_1\ X_3 - 2.25\ X_1X_4 - 1.48X_2\ X_3 + 2.33X_2\ X_4 + 0.9500X_3\ X_4 - 0.4183\ X_1^2 - 1.59X_2^2 - 2.24\ X_3^2 - 4.29\ X_4^2$$

The negative value before a factor in the quadratic equation depicted that the response decreases with the factor and positive value indicates increase in response. The value of the correlation coefficient (r^2) indicates a good fit as its value was found to be 0.9307. The result showed that with the increase in the quantity of lipid entrapment efficiency

increased gradually. The surfactant also has an important positive effect on the entrapment efficiency. Homogenisation speed doesn't produce positive effect on the entrapment efficiency. The results are predicted as 3D surface as Figure 1.

3.3 Effects on drug loading (%) The drug loading estimated (%) as mentioned in Table 3 varies from 61% to 88.90% for formulation 20 and formulation 28 respectively. The independent factors specifically the amount of lipid and surfactants affecting the drug loading and this effect can be explained by the following quadratic regression equation:

$$Y_2 = 82.91+0.2883X_1+9.76X_2 + 2.72X_3 +0.1875X_4 – 0.3100X_1X_2 - 0.0825X_1X_3 - 0.6375X_1X_4 -1.77X_2X_3 + 0.4225X_2X_4 + 0.1975X_3X_4 - 0.0937X_1^2 -4.33X_2^2 -1.02X_3^2 -0.8837X_4^2$$

The negative value before a factor in the quadratic equation depicted that the response decreases with the factor and positive value indicates increase in response. The value of the correlation coefficient (r^2) indicates a good fit as its value was found to be 0.9790. The result showed that with the increase in the quantity of drug and homogenisation speed drug loading increased gradually. The positive effect of increased surfactant conc. and homogenisation speed on the drug loading was observed. Lipid conc doesn't produce positive effect on the drug loading. The results are predicted as 3D surface as Figure 2.

4.0 DISCUSSION

SLN formulation optimisation is multifactorial complex research process. It involves many dependent variables and independent responses were studied. A complexity of interactions was observed. Conclusively this study demonstrates that the lipid 275 mg, Drug volume (2ml), Surfactants (6%) and Homogenisation speed 10,000 rpm were found to be the ingredients of an optimised formulation as per the Box-Behnken design. The response surface plots and polynomial equations thus obtained, predicted an optimised formulation with desired characteristics. The L-Asparaginase-SLN obtained in vitro release experiments exhibited a biphasic release pattern with burst release at the initial phase followed by sustained release.

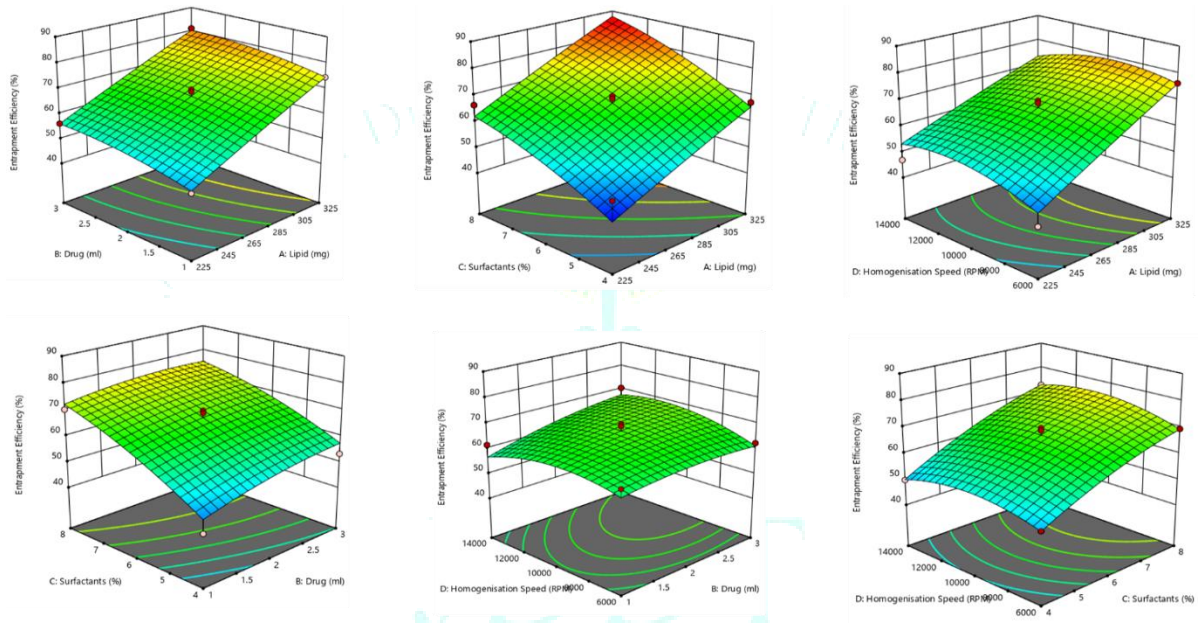

Figure 1: Response surface plot showing effect of the different variables on entrapment efficiency

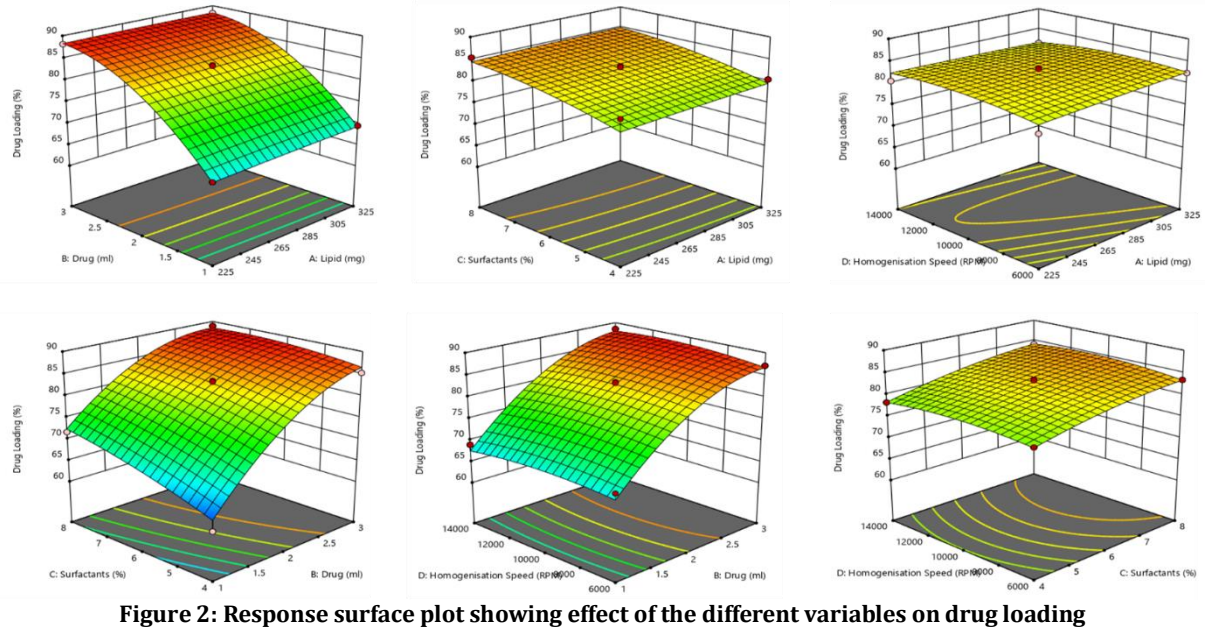

Figure 2: Response surface plot showing effect of the different variables on drug loading

ACKNOWLEDGMENTS

We thank the IK Gujral Punjab Technical University, Kapurthala, Punjab India the university where the research scholar is enrolled for PhD research and also thanks to ISF College of Pharmacy, Moga, Punjab for giving the infrastructure to do the research work.

REFERENCES

1. Panchagnula R. Transdermal delivery of drugs. Indian J Pharmacol. 1997; 29:140–56.
2. Rao PR, Diwan PV. Permeability studies of cellulose acetate free films for transdermal use: Influence of plasticizers. Pharm Acta Helv. 1997; 72:47–51.
3. Drug Delivery Systems: Getting Drugs to Their Targets in a Controlled Manner; NIH Turning Discovery in Health; 2016
4. De Jong W, Borm P. Drug delivery and nanoparticles: applications and hazards. Int J Nanomedicine. 2008; 3(2):133–149.
5. Huang Z, Hua S, Yang Y, Fang J. Development and evaluation of lipid nanoparticles for camptothecin delivery: a comparison of solid lipid nanoparticles, nanostructured lipid carriers, and lipid emulsion. Acta Pharmacologica Sinica. 2008; 29(9):1094–1102.
6. Üner M, Yener G. Importance of solid lipid nanoparticles (SLN) in various administration routes and future perspectives. Int J Nanomedicine. 2007; 2(3):289–300.
7. Freitas C, Müller R. Correlation between long-term stability of solid lipid nanoparticles (SLN (TM)) and crystallinity of the lipid phase. Eur J Pharm Biopharm. 1999; 47(2):125–132.
8. Rao PR, Diwan PV. Formulation and *in vitro* evaluation of polymeric films of diltiazem hydrochloride and indomethacin for transdermal administration. Drug Dev Indian Pharm. 1998; 24:327–36
9. Müller R, Rühl D, Runge S, Schulze-Forster K, Mehnert W. Cytotoxicity of solid lipid nanoparticles as a function of the lipid matrix and the surfactant. Pharm Res. 1997; 14(4):458–462.
10. Müller R, Mäder K, Gohla S. Solid lipid nanoparticles (SLN) for controlled drug delivery-a review of the state of the art. European Journal of Pharmaceutics and Biopharmaceutics. 2000; 50(1):161–177.
11. Derakhshandeh K, Erfan M, Dadashzadeh S. Encapsulation of 9-nitrocamptothecin, a novel anticancer drug, in biodegradable nanoparticles: factorial design, characterization and release kinetics. Eur J Pharm Biopharm. 2007; 66(1):34–41.
12. Gohel M, Amin A. Formulation optimization of controlled release diclofenac sodium microspheres using factorial design. J Control Release. 1998; 51(2–3):115–122.
13. Nazzal S, Khan M. Response surface methodology for the optimization of ubiquinone self-nanoemulsified drug delivery system. AAPS PharmSciTech. 2002; 3(1):23–31.
14. Chang J, Huang Y, Hou S, Wang R, Wu P, Tsai Y. Formulation optimization of meloxicam sodium gel using response surface methodology. Int J Pharm. 2007; 338(1–2):48–54.
15. Liu C, Wu C, Fang J. Characterization and formulation optimization of solid lipid nanoparticles in vitamin K1 delivery. Drug Dev Ind Pharm. 2010; 36(7):751–761.
16. Manjunath K, Reddy J, Venkateswarlu V. Solid lipid nanoparticles as drug delivery systems. Methods Find Exp Clin Pharmacol. 2005; 27(2):127–144.
17. Arai H, Suzuki T, Kaseda C, Takayama K. Effect of an Experimental Design for Evaluating the Nonlinear Optimal Formulation of Theophylline Tablets Using a Bootstrap Resampling Technique. Chem Pharm Bull. 2009; 57(6):572–579.
18. El-Malah Y, Nazzal S, Khanfar N. D-optimal mixture design: optimization of ternary matrix blends for controlled zero-order drug release from oral dosage forms. Drug Dev Ind Pharm. 2006; 32(10):1207–1218.
19. Bozkir A, Saka O. Formulation and investigation of 5-FU nanoparticles with factorial design-based studies. Il Farmaco. 2005; 60(10):840–846.
20. Bhavsar M, Tiwari S, Amiji M. Formulation optimization for the nanoparticles-in-microsphere hybrid oral delivery system using factorial design. J Control Release. 2006; 110(2):422–430.