

Application of Response Surface Methodology in Development of Sirolimus Liposomes Prepared by Thin Film Hydration Technique

Saeed Ghanbarzadeh^{1,2}, Hadi Valizadeh^{3*}, Parvin Zakeri-Milani⁴

¹Drug Applied Research Center and Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran

²Students' Research Committee, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran

³Research Center for Pharmaceutical Nanotechnology and Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran

⁴Liver and Gastrointestinal Diseases Research Center and Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran

ARTICLE INFO

Article Type:

Research Article

Article History:

Received: 1 Dec. 2012

Revised: 12 Feb. 2013

Accepted: 16 Feb. 2013

ePublished: 30 Apr. 2013

Keywords:

Liposome

Sirolimus

Central Composite Design

Response Surface Methodology

ABSTRACT

Introduction: The present investigation was aimed to optimize the formulating process of sirolimus liposomes by thin film hydration method. **Methods:** In this study, a 3² factorial design method was used to investigate the influence of two independent variables in the preparation of sirolimus liposomes. The dipalmitoylphosphatidylcholine (DPPC) / Cholesterol (Chol) and dioleoyl phosphoethanolamine(DOPE) /DPPC molar ratios were selected as the independent variables. Particle size (PS) and Encapsulation Efficiency (EE %) were selected as the dependent variables. To separate the un-encapsulated drug, dialysis method was used. Drug analysis was performed with a validated RP-HPLC method. **Results:** Using response surface methodology and based on the coefficient values obtained for independent variables in the regression equations, it was clear that the DPPC/Chol molar ratio was the major contributing variable in particle size and EE %. The use of a statistical approach allowed us to see individual and/or interaction effects of influencing parameters in order to obtain liposomes with desired properties and to determine the optimum experimental conditions that lead to the enhancement of characteristics. In the prediction of PS and EE % values, the average percent errors are found to be as 3.59 and 4.09%. This value is sufficiently low to confirm the high predictive power of model. **Conclusion:** Experimental results show that the observed responses were in close agreement with the predicted values and this demonstrates the reliability of the optimization procedure in prediction of PS and EE % in sirolimus liposomes preparation.

Introduction

Sirolimus (SRL, formerly rapamycin; CAS No: 53123-88-9), a potent antitumor and immunosuppressive agent, is used to prevent acute renal allograft rejection and it may be given in conjunction with cyclosporine (Sandimmune, Neoral) in the management of renal transplant patients. SRL binds to an immunophilin, FKBP12, and is a competitive inhibitor of peptidyl-prolyl isomerase activity. SRL suppresses interleukin-driven T-cell proliferation by blocking post receptor events.¹⁻⁶ Since 1999, it has been approved by FDA for the prophylaxis of organ rejection in patients older than 13 years. However, a few studies and case reports have shown the use of SRL for patients with tuberous sclerosis, Kaposi's sarcoma and psoriasis. SRL is a substrate for the major drug-metabolizing enzyme cytochrome P450 3A4 and the efflux transporter P-glycoprotein.⁷⁻¹² SRL has

poor water solubility (2.6 µg/mL) and high lipophilicity (logP_{ow} = 5.77), and is the substrate of CYP450 3A. It has low oral bioavailability (<15%) from commercial formulations, such as oral solution and tablets. The low bioavailability is attributed to its sensitivity to gastric acid, partial intestinal absorption, and first-pass hepatic metabolism.¹³⁻¹⁷ In theory, encapsulation of the drug inside (e.g., liposomes) could improve its stability. Liposomes are spherical vesicles composed of amphiphilic phospholipids and cholesterol, which self-associate into bilayer to encapsulate an aqueous interior. Although liposome technology was discovered over 50 years ago, liposome-based drug formulations have not entered the market in great number. Some of the major problems limiting the manufacture and development of liposomes are their stability, poor batch-to-batch reproducibility, difficulties in sterilization and low drug loading. Many

*Corresponding author: Hadi Valizadeh, Email: valizadeh[at]tbzmed.ac.ir

attempts have been made to overcome such problems by methods such as improvements to the processes used for liposome preparation and incorporation of diverse lipids to improve the stability and entrapment efficiency.¹⁸⁻²⁵ In pharmaceutical technology, in the development and optimization of different pharmaceutical dosage forms, there are a high number of factors which influence the product characteristics. Therefore, complex, expensive and time-consuming formulation studies are often necessary for the development of a product with required and desired properties. Experimental design methodology is a strategy to use smaller number of experiments and to avoid unnecessary experiments.²⁶⁻³⁰

In this study, the effect of liposome composition (lipid type, cholesterol and their ratio) on the encapsulation efficiency and liposome size was evaluated in a full factorial design. The combined influence of lipids' type and their ratios were further studied by means of response surface methodology (RSM) using a central composite design (CCD) approach. The optimization approach was applied to obtain desired particle size and EE % for SRL liposomes.

Materials and methods

Materials

Sirolimus was obtained from Poli Company (Lazio, Italy). Dipalmitoylphosphatidylcholine (DPPC) and dioleoyl phosphoethanolamine (DOPE) were purchased from Lipoid GMBH Company (Ludwigshafen, Germany). Cholesterol was obtained from Merck Company (Darmstadt, Germany). All solvents were HPLC grade and all reagents were analytical grade and purchased from Merck Company (Darmstadt, Germany).

Methods

Liposome preparation

In this study, DPPC was used for conventional liposome. In order to make fusogenic liposomes, DOPE was added. Cholesterol in different molar ratios was used as a fluidity buffer. SRL liposomes were prepared using the modified thin film hydration technique. This method is the conventional and most common technique for liposome preparation. Different ratios of phospholipids and cholesterol were dissolved in an organic solvent consisting of chloroform and methanol (3:1 v/v %). The concentration of total lipids was 20 µm. Lipid solution was kept in a rotary evaporator (Buchi, Zurich, Switzerland) for 2 hr at 45°C and 150 rpm under vacuum 300 mmHg. Evaporation was continued for extra half an hour under vacuum 50 mmHg until a thin lipid layer was observed and all of organic solvents were evaporated. The dried thin film was hydrated with SRL500 µg/ml in phosphate buffered saline (PBS) at 45°C, above the gel-liquid crystal transition temperature (T_g) of phospholipids. The mixture was kept in the rotary for 3 hr at temperature 45°C and speed of 150 rpm. Finally, the latter suspension was kept in a sonicator bath for 10 min at 45°C, in order to reduce

particle size.

Measurement of particle size (PS) of liposome

Mean vesicle size and size distribution profile of liposome were determined by using particle size analyzer (SALD 2101, Shimadzu, Japan). All measurements were performed in triplicate.

Determination of SRL encapsulation efficiency (EE)

After the removal of unbound drug, the remaining drug in liposome was considered as encapsulated drug. Un-encapsulated drug was removed from liposome suspensions by the dialysis method after 24 h at 25°C (below phase-transition temperature of phospholipids) using PBS at sink condition in a receiver compartment.

EE % was calculated by the following equation:^{1, 31-41}

$$EE (\%) = [(C_{total} - C_{free}) / C_{total}] \times 100$$

Where, C_{total} is total drug which was added and C_{free} is un-entrapped drug.

Drug analysis

The amount of SRL was determined using our previously validated HPLC method.⁴² An HPLC system (Beckman, Florida, USA) with a variable wavelength ultraviolet spectrophotometric detector (166 gold) set at 278 nm was used. System Gold software was used for data acquisition and system Gold nouveau software was used for data reporting and analysis. The separation was achieved using a KNAUER column (C18, 5 µm, 4.6 × 150 mm). Mobile phase consisted of acetonitrile and ammonium acetate buffer (70:30, v/v %) at flow rate of 1.5 mL/min. The column temperature was kept at 54°C. A linear response was observed over a concentration range of 125–2000 ng/mL ($r^2 > 0.991$).

Optimization of formulation using a 3² full factorial design

A 3² randomized full factorial design was utilized in the present study. Two factors, each at three levels were evaluated. Experimental trials were carried out at all nine possible combinations. The factors and their limit were selected based on preliminary study. The molar ratio of DPPC/Chol and molar ratio of DOPE/DPPC were selected as independent variables. The size of liposomes and EE % were selected as dependent variables. The formulation composition of the factorial batches (F_1 to F_9) is shown in Table 1.

Response surface methodology approach for optimization of factors

Based on the RSM approach, the runs were conducted in CCD model-designed experiments to visualize the effects of independent factors on the response along with the experimental conditions. Response surface diagram was constructed using Minitab version 15. Formulation was optimized with the help of response surface diagrams.

Results

Effect of formulation ingredients on encapsulation efficiency

Based on the RSM approach, the runs were conducted in

Table 1. 3² Full factorial design, molar composition of DPPC, DOPE and cholesterol in formulations together with responses values [Particle size (PS), Encapsulation efficiency (EE %), Polydispersity index (PDI)]

Formulation code	DPPC	DOPE	Cholesterol	PS (nm)	PDI	EE (%)
F ₁	5	0	1	493	0.21	57.9
F ₂	5	2.5	1	486	0.19	62.7
F ₃	3	0	1	532	0.31	72.5
F ₄	3	3	1	556	0.32	68.1
F ₅	1	0.5	1	646	0.38	85.1
F ₆	5	5	1	474	0.22	59.3
F ₇	1	0	1	615	0.39	84.2
F ₈	3	1.5	1	549	0.31	76.2
F ₉	1	1	1	627	0.39	82.2

CCD model-designed experiments to visualize the effects of independent factors on the responses.

A general equation for the relation of affecting factors and response is: $Y_j = b_0 + b_1 X_1 + b_2 X_2 + b_{11} X_1 X_1 + b_{22} X_2 X_2 + b_{12} X_1 X_2$. Where Y is the dependent variable, b_0 is the arithmetic mean response of the nine runs and b_i is the estimated coefficient for the factor X_i . The main effects (X_1 and X_2) represent the average result of changing one factor at a time from its low to high value. The interaction terms ($X_1 X_2$) show how the response changes when two factors are simultaneously changed. The polynomial terms ($X_1 X_1$ and $X_2 X_2$) are included to investigate non-linearity. Magnitude and mathematical sign of coefficients show the effectiveness of dependent variables on responses.

Results of PS and EE % are illustrated in Table 1. The model equation derived for EE % was:

$$Y_{EE\%} = 92.08 - 6.58 X_1 + 4.33 X_2 + 1 X_1 X_2$$

The negative sign for coefficient of X_1 indicates the lowering effect of DPPC/Chol on EE % ($P < 0.05$). The EE % of different liposomal batches was in a range of 57.9 to 85.1%. The maximum entrapment was observed in batch F5 with the composition of DPPC/DOPE/Chol (1: 0.5: 1 molar ratio).

The relationship between the dependent and independent variables was further elucidated using contour and response surface plots. As shown in Fig. 1, at low level of DOPE/DPPC, EE % decreased from 84.2 % to 57.9 % when DPPC/Chol increased from 1 to 5. Similarly, at high level of DOPE/DPPC, EE % decreases from 82.2 % to 59.3 % when DPPC/Chol increases from 1 to 5.

Effect of formulation ingredients on particle size

One of the most important parameters, which need to be monitored during liposome preparation, is the vesicle size and the size distribution. From a number of reports, it is evident that the size and size distribution of the liposomes determine their *in vitro* or *in vivo* performance. The particle sizes of different batches of liposomes were in a range of 474 to 646 nm. The minimum and maximum size values correspond to formulations F6 and F5, respectively. The modified model for particle size is:

$$Y_{PS} = 665.08 - 49.12 X_1 + 28.91 X_2 + 2.79 X_1^2 - 7.75 X_1 X_2$$

The equation clearly indicates that the PS values are

strongly dependent on the selected independent variables. The negative sign for coefficient of X_1 indicates that DPPC/Chol has a decreasing effect on particle size ($p = 0.037$). The coefficient of X_1 was found to be significant at the level of $P < 0.05$. From the results obtained, it may be concluded that DOPE/DPPC molar ratio and its interaction term do not contribute significantly to the particle size of liposomes ($p > 0.05$).

As shown in Fig. 2, at low level of DOPE/DPPC, PS decreased from 615 nm to 493 nm when DPPC/Chol increased from 1 to 5. Correspondingly, at high level of DOPE/DPPC, PS decreased from 627 nm to 474 nm when DPPC/Chol increased from 1 to 5.

Overlaid contour plot with defined conditions for desired PS and EE % was obtained using RSM approach. The white area corresponds to conditions resulting in a particle size in the range between 500 nm to 550 nm and EE % range between 70 to 85% (Fig. 3).

Formulation optimization of liposomes

Validation: The accuracy of the proposed model was validated by conducting other reactions with different conditions and then comparing the obtained results with the model. Observed and calculated PS and EE % using these equations are illustrated in Table 2. Percent error (PE) was obtained using following equation:

$$PE = \frac{|\text{Calculated} - \text{observed}|}{\text{observed}} \times 100$$

Average percent error (APE) for particle size and EE % in train set were 1.27 % and 2.7 %, respectively.

The internal percent error (PE) of the proposed model can be calculated using obtained equations for the train experiments ($n = 9$). The average PE for all 9 experiments are 1.28 and 2.57 % for PS and PE, respectively (Table 2). To evaluate the external predictive performance of the model, three more experiments were carried out in duplicate as a test set. Table 3 shows conditions and results of these reactions. The results revealed that the average PE for these experiments is 3.52 and 4.09% for PS and PE, respectively. Considering the low internal and

external PE, it might be concluded that the model has a good predictive power in the studied range of variables. The proposed model was also used to obtain optimum conditions (Fig. 4). Desired PS and EE % were defined as 500 nm and 80 %, respectively. A new formulation was prepared according to proposed levels for independent factors. Proposed levels are in accordance with white

area in the overlaid plots. Observed responses values were close to the calculated values in the proposed formulations. Prediction error of EE % and PS were 6.3% and 5.9%, respectively. These results further demonstrate the suitability of the optimization procedure in developing SRL liposomes.

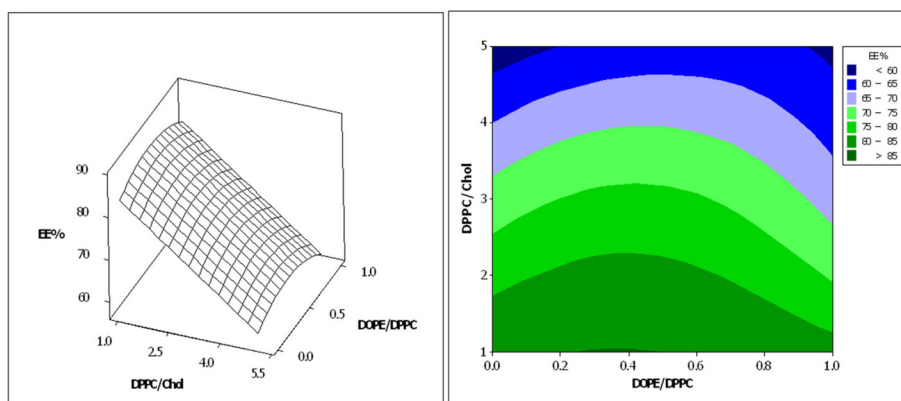


Fig. 1. Response surface plot (left) and Contour plot (right) of the effect of lipid content on encapsulation efficiency percent (EE %).

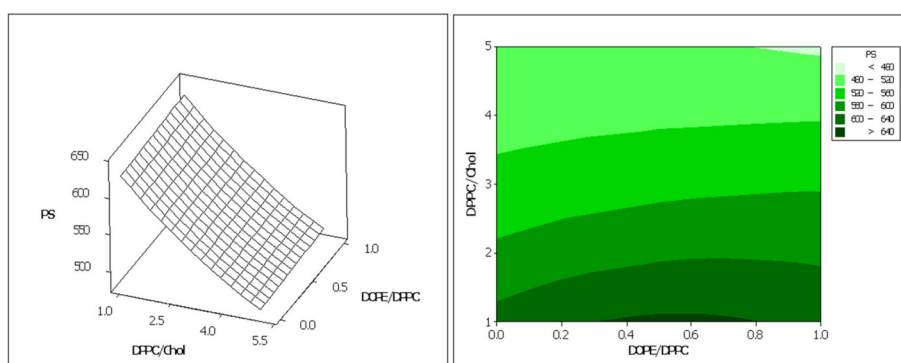


Fig. 2. Response surface plot (left) and contour plot (right) of the effect of lipid content on particle size (PS).

Table 2. Observed and calculated percent error (PE) values for particle size (PS) and encapsulation efficiency percent (EE %) in train set

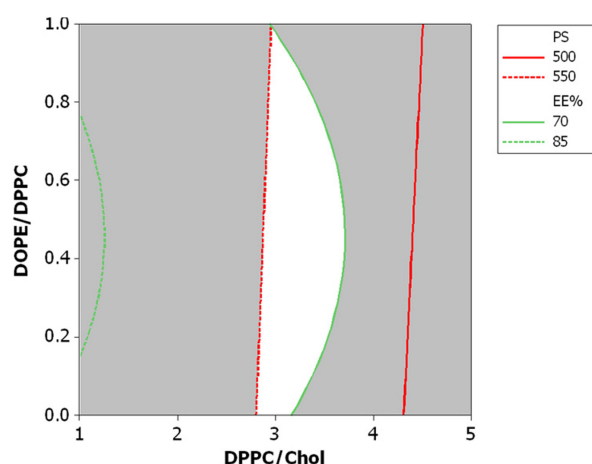
Formulation code	Obs PS (nm)	Calc PS (nm)	PS PE (%)	Obs EE%	Calc EE%	EE% PE (%)
F ₁	493	489	0.76	57	59	3.80
F ₂	486	484	0.34	62	59	4.03
F ₃	532	543	2.04	72	72	0.46
F ₄	556	549	1.34	68	71	4.41
F ₅	646	629	2.58	85	84	1.38
F ₆	474	479	1.16	59	60	1.41
F ₇	615	619	0.61	84	85	1.78
F ₈	549	546	0.60	76	72	5.70
F ₉	627	640	2.07	82	82	0.20
PS APE			1.28	EE% APE		2.57

Table 3. Observed and calculated percent error (PE) values for particle size (PS) and encapsulation efficiency percent (EE %) in test set

X1	X2	Obs PS (nm)	Calc PS (nm)	PS PE (%)	Obs EE%	Calc EE%	EE% PE (%)
4	1	507	511	0.83	63	67	3.83
2	0.5	562	585	4.05	74	59	5.06
3	0.75	580	547	5.67	69	70	3.38
PS APE				3.52	EE% APE		4.09

Discussion

It is desirable to develop an acceptable pharmaceutical formulation in shortest possible time, using minimum number of man-hours and raw materials. Traditionally, pharmaceutical formulations are developed by changing one variable at a time by trial and error method which is time consuming in nature requiring a lot of imaginative efforts. Moreover, it may be difficult to develop an ideal formulation using this classical technique, since the joint effects of independent variables are not considered. It is

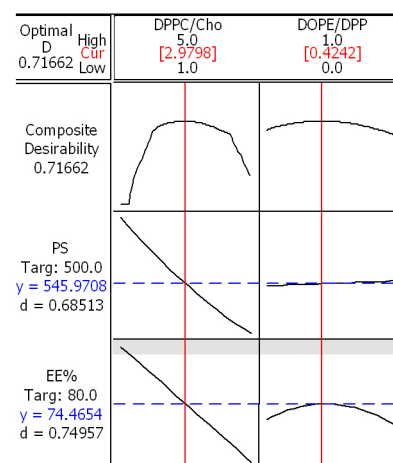
**Fig. 3.** Overlaid contour plot with defined conditions for desired particle size (PS) and encapsulation efficiency percent (EE %).

therefore very essential to understand the complexity of pharmaceutical formulations by using established statistical tools such as factorial design.^{27,30,43-45}

RSM is a collection of mathematical and statistical technique which quantifies the functional relationship between a number of measured response variables and several explanatory factors to obtain an optimal response by using a series of tests. The main advantage of RSM is to reduce the required experimental runs required and it is already widely applied to optimize formulation design in pharmaceuticals studies.

Conclusion

A full factorial design and central composite design of response surface methodology can be used to determine the significant variables and optimum condition for prep-

**Fig. 4.** Optimization plot for formulation with desired particle size (PS) and encapsulation efficiency percent (EE %) values.

aration of SRL liposomes. The present study focused on the preparation and characterization of SRL liposome using the thin film hydration method. Particle size and EE % are important characteristics in liposome formulations which have important effects on *in vitro* and *in vivo* properties. Percentage of encapsulation efficiency was optimized after studying the effect of various formulation variables. DPPC/Chol molar ratio had a profound effect on the entrapment efficiency and liposome size. The proposed model could be successfully used to predict and optimize both liposome size and EE %.

Acknowledgement

The authors would like to thank the authorities of Research Center for Pharmaceutical Nanotechnology, Tabriz University of Medical Sciences, for financial support. This article is a part of a thesis submitted for PhD degree (No. 54) in Faculty of Pharmacy, Tabriz University of Medical Sciences.

Ethical issues

Not applicable in this study.

Competing interests

The authors report no competing interests.

References

1. Agashe H, Lagisetty P, Awasthi S, Awasthi V. Improved formulation of liposome-encapsulated hemoglobin with an anionic non-phospholipid. *Colloids and Surfaces B: Biointerfaces* **2011**;75:573-83.
2. Aspeslet LJ, Yatscoff RW. Requirements for therapeutic drug monitoring of sirolimus, an immunosuppressive agent used in renal transplantation. *Clinical Therapeutics* **2000**;22:B86-B92.
3. Chen K, Tsai M, Lai IR, Wu F, Hu R, Lee P. Favorable results of concomitant tacrolimus and sirolimus therapy in Taiwanese renal transplant recipients at 12 months. *Journal of the Formosan Medical Association* **2008**;107:533-9.
4. Dichtl W, Stocker E, Mistlberger K, Debbage P, Yan Z, Alber HF, *et al.* Countervailing effects of rapamycin (sirolimus) on nuclear factor-B activities in neointimal and medial smooth muscle cells. *Atherosclerosis* **2006**;186:321-30.
5. Kandzari DE, Leon MB, Popma JJ, Fitzgerald PJ, OaShaughnessy C, Ball MW, *et al.* Comparison of zotarolimus-eluting and sirolimus-eluting stents in patients with native coronary artery disease: A Randomized Controlled Trial. *Journal of the American College of Cardiology* **2006**;48:2440-7.
6. Togni M, Windecker S, Cocchia R, Wenaweser P, Cook S, Billinger M, *et al.* Sirolimus-eluting stents associated with paradoxical coronary vasoconstriction. *Journal of the American College of Cardiology* **2005**;46:231-6.
7. Liistro F, Fineschi M, Angioli P, Sinicropi G, Falsini G, Gori T, *et al.* Effectiveness and safety of sirolimus stent implantation for coronary in-stent restenosis: The TRUE (Tuscany Registry of Sirolimus for Unselected In-Stent Restenosis) registry. *Journal of the American College of Cardiology* **2006**;48:270-5.
8. Masri M, Rizk S, Barbari A, Stephan A, Kamel G, Rost M. An assay for the determination of sirolimus levels in the lymphocyte of transplant patients. *Transplantation Proceedings* **2007**;39:1204-6.
9. Mano N, Sato M, Nozawa M, Matsumoto Y, Mori M, Yamaguchi H, *et al.* An accurate quantitative LC/ESI/MS/MS method for sirolimus in human whole blood. *Journal of Chromatography B* **2011**;879:987-92.
10. Mehilli J, Byrne RA, Tiroch K, Pinieck S, Schulz S, Kufner S, *et al.* Randomized trial of paclitaxel- versus sirolimus-eluting stents for treatment of coronary restenosis in sirolimus-eluting stents: The ISAR-DESIRE 2 (Intracoronary Stenting and Angiographic Results: Drug Eluting Stents for In-Stent Restenosis 2) study. *Journal of the American College of Cardiology* **2011**;55:2710-6.
11. Meier-Kriesche H, Kaplan B. Toxicity and efficacy of sirolimus: Relationship to whole-blood concentrations. *Clinical Therapeutics* **2000**;22:B93-B100.
12. Ong ATL, van Domburg RT, Aoki J, Sonnenschein K, Lemos PA, Serruys PW. Sirolimus-eluting stents remain superior to bare-metal stents at two years: Medium-Term Results From the Rapamycin-Eluting Stent Evaluated at Rotterdam Cardiology Hospital (RESEARCH) Registry. *Journal of the American College of Cardiology* **2006**;47:1356-60.
13. Trepanier DJ, Gallant H, Legatt DF, Yatscoff RW. Rapamycin: Distribution, pharmacokinetics and therapeutic range investigations: an update. *Clinical Biochemistry* **1998**;31:345-51.
14. Yanchar NL, Fedorak RN, Kneteman NM, Sigalet DL. Nutritional and intestinal effects of the novel immunosuppressive agents: Deoxyspergualin, rapamycin, and mycophenolate mofetil. *Clinical Biochemistry* **1996**;29:363-9.
15. Kahan BD, Napoli KL. Role of therapeutic drug monitoring of rapamycin. *Transplantation Proceedings* **1998**;30:2189-91.
16. Hu X, Lin C, Chen D, Zhang J, Liu Z, Wu W, *et al.* Sirolimus solid self-microemulsifying pellets: Formulation development, characterization and bioavailability evaluation. *International Journal of Pharmaceutics* **2012**; 438:123-33.
17. Rowland RN, Woodley JF. Uptake of free and liposome-entrapped horseradish peroxidase by rat intestinal sacs in vitro. *FEBS Letters* **1981**;123:41-4.
18. Franzen U, Nguyen TTTN, Vermehren C, Gammelgaard B, Astergaard J. Characterization of a liposome-based formulation of oxaliplatin using capillary electrophoresis: Encapsulation and leakage. *Journal of Pharmaceutical and Biomedical Analysis* **2011**;55:16-22.
19. Franz-Montan M, Silva ALR, Fraceto LF, Volpato MC, Paula Ed, Ranali J, *et al.* Liposomal encapsulation improves the duration of soft tissue anesthesia but does not induce pulpal anesthesia. *Journal of Clinical Anesthesia* **2011**;22:313-7.
20. Fritze A, Hens F, Kimpfner A, Schubert R, Peschka R. Remote loading of doxorubicin into liposomes driven by a transmembrane phosphate gradient. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **2006**;1758:1633-40.
21. Gabizon A, Shmeeda H, Horowitz AT, Zalipsky S. Tumor cell targeting of liposome-entrapped drugs with phospholipid-anchored folic acid-PEG conjugates. *Advanced Drug Delivery Reviews* **2004**;56:1177-92.
22. Giulio AD, Maurizi G, Odoardi P, Saletti MA, Amicosante G, Oratore A. Encapsulation of ampicillin in reverse-phase evaporation liposomes: a direct evaluation by derivative spectrophotometry. *International Journal of Pharmaceutics* **1991**;74:183-8.
23. Hwang KJ, Merriam JE, Beaumier PL, Luk KS. Encapsulation, with high efficiency, of radioactive metal ions in liposomes. *Biochimica et Biophysica Acta (BBA) - General Subjects* **1982**;716:101-9.
24. Kim TD, Sakon M, Kawasaki T, Kambayashi J, Ohshiro T, Mori T. Studies on liposome-encapsulated heparin. *Thrombosis Research* **1986**;43:603-12.
25. Maherani B, Arab-Tehrany E, Kheirloomoom A, Cleymand F, Linder M. Influence of lipid composition on physicochemical properties of nanoliposomes encapsulating natural dipeptide antioxidant l-carnosine. *Food Chemistry* **2010**;134:632-40.
26. Braun E, Wagner A, Farnschliel E, Katinger H, Vorauer-Uhl K. Experimental design for in vitro skin penetration study of liposomal superoxide dismutase. *Journal of Pharmaceutical and Biomedical Analysis* **2006**;40:1187-97.
27. Carbone C, Tomasello B, Ruozzi B, Renis M, Puglisi G. Preparation and optimization of PIT solid lipid nanoparticles via statistical factorial design. *European Journal of Medicinal Chemistry* **2010**;49:110-7.

28. Liu N, Park H. Factors effect on the loading efficiency of Vitamin C loaded chitosan-coated nanoliposomes. *Colloids and Surfaces B: Biointerfaces* **2011**;76:16-9.
29. Loukas YL. A computer-based expert system designs and analyzes a 2(k-p) fractional factorial design for the formulation optimization of novel multicomponent liposomes. *Journal of Pharmaceutical and Biomedical Analysis* **1998**;17:133-40.
30. Hermans K, Van den Plas D, Everaert A, Weyenberg W, Ludwig A. Full factorial design, physicochemical characterisation and biological assessment of cyclosporine A loaded cationic nanoparticles. *European Journal of Pharmaceutics and Biopharmaceutics* **2012**;27:35.
31. Meisner D, Mezei M. Liposome ocular delivery systems. *Advanced Drug Delivery Reviews* **1995**;16:75-93.
32. Niggemann B, Erdmann G, Wellhaner HH. Liposome-entrapped [125I]anti-tetanus immunoglobulin G: Evidence for entry into spinal cord neurons of the rat. *Neuroscience Letters* **1984**;51:231-4.
33. Nii T, Ishii F. Encapsulation efficiency of water-soluble and insoluble drugs in liposomes prepared by the microencapsulation vesicle method. *International Journal of Pharmaceutics* **2005**;298:198-205.
34. Perrie Y, Gregoriadis G. Liposome-entrapped plasmid DNA: characterisation studies. *Biochimica et Biophysica Acta (BBA) - General Subjects* **2000**;1475:125-32.
35. Piel G, Piette M, Barillaro V, Castagne D, Evrard B, Delattre L. Betamethasone-in-cyclodextrin-in-liposome: The effect of cyclodextrins on encapsulation efficiency and release kinetics. *International Journal of Pharmaceutics* **2006**;312:75-82.
36. Sanchez-Lopez V, Fernandez-Romero JM, Gamez-Hens A. Evaluation of liposome populations using a sucrose density gradient centrifugation approach coupled to a continuous flow system. *Analytica Chimica Acta* **2009**;645:79-85.
37. Storm G, van Bloois L, Steerenberg PA, van Etten E, de Groot G, Crommelin DJA. Liposome encapsulation of doxorubicin: Pharmaceutical and therapeutic aspects. *Journal of Controlled Release* **1989**;9:215-29.
38. Wu P, Tsai Y, Liao C, Chang J, Huang Y. The characterization and biodistribution of cefoxitin-loaded liposomes. *International Journal of Pharmaceutics* **2004**;271:31-9.
39. Yuba E, Kojima C, Harada A, Tana G, Watarai S, Kono K. pH-Sensitive fusogenic polymer-modified liposomes as a carrier of antigenic proteins for activation of cellular immunity. *Biomaterials* **2010**;31:943-51.
40. Zignani M, Drummond D, Meyer O, Hong K, Leroux J. In vitro characterization of a novel polymeric-based pH-sensitive liposome system. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **2000**;1463:383-94.
41. Modi S, Xiang T-X, Anderson BD. Enhanced active liposomal loading of a poorly soluble ionizable drug using supersaturated drug solutions. *Journal of Controlled Release* **2012**;162:330-9.
42. Islambulchilar Z, Ghanbarzadeh S, Emami S, Valizadeh H, Zakeri-Milani P. Development and validation of an HPLC method for the analysis of sirolimus in drug products. *Advanced Pharmaceutical Bulletin* **2012**;2:135-9.
43. Derakhshandeh K, Erfan M, Dadashzadeh S. Encapsulation of 9-nitrocamptothecin, a novel anticancer drug, in biodegradable nanoparticles: Factorial design, characterization and release kinetics. *European Journal of Pharmaceutics and Biopharmaceutics* **2007**;66:34-41.
44. Gonzalez-Rodriguez ML, Barros LB, Palma J, Gonzalez-Rodriguez PL, Rabasco AM. Application of statistical experimental design to study the formulation variables influencing the coating process of lidocaine liposomes. *International Journal of Pharmaceutics* **2007**;337:336-45.
45. Hao J, Wang F, Wang X, Zhang D, Bi Y, Gao Y, *et al.* Development and optimization of baicalin-loaded solid lipid nanoparticles prepared by coacervation method using central composite design. *European Journal of Pharmaceutical Sciences* **2010**;47:497-505.