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DEVELOPMENT AND OPTIMIZATION OF ALENDRONATE LOADED LIPOSOMES FOR ORAL ADMINISTRATION BY USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT. The aim of this study was to develop an optimal liposomal formulation for bisphosphonic compounds with appropriate in vitro stability and granulometric distribution. Liposomes were obtained by lipid film hydration method and analyzed using DLS and spectrophotometric assays. The Box Behnken design was used to study the influence of individual and combined effects of three factors (phosphatidylcholine: cholesterol ratio, the lipid component: active substance ratio and sonication time) on the responses. The selected dependent variables (responses) were drug encapsulation efficiency (DEE, %) and liposomes size (diameter). The optimum liposome formulation with alendronate was developed using surface response methodology for evaluating the effects of independent variables on the selected responses. The results obtained pointed out that lipid:drug ratio was the predominant factor that influenced drug encapsulation efficiency and liposome size distribution was mainly affected by the lipid:drug ratio and sonication time.

Keywords: liposome formulation, alendronate, design of experiments, oral administration, spectrophotometry

INTRODUCTION

Alendronate is a synthetic analog of pyrophosphate, with a high affinity for mineralized tissues, especially the solid phase of calcium phosphate that binds strongly with. Bisphosphonates, the family drugs of which alendronate belongs to, are widely used to treat diseases characterized by osteolysis, for their high affinity to hydroxyapatite, their rapid binding at sites of osteoclastic activity and their ability to inhibit bone resorption (Fisher *et al.*, 1999; Holmberg *et al.*, 2010). Alendronate has been approved for the treatment and prevention of osteoporosis, treatment of glucocorticoid induced osteoporosis in men and women, therapy of Paget's disease of bone (Silverman, 2008; Lambrinoudaki *et al.*, 2006; Ebetino Frank *et al.*, 2011). Several market products based on bisphosphonates (capsules- Neobon®, oral solution-Fosamax®, tablets-Fosamax®, Bifosa®, Alendros® etc.) are used frequently, instead of their low absorbtion and bioavailability after oral administration respect to intravenous route (Masarachia *et al.*, 1996; Shinkai, *et al.*, 1996; Povoroznyuk *et al.*, 2008; Nakhla *et al.*, 2011).

In order to obtain an advanced bioavailability following oral administration of a highly hydrophilic drug, such as alendronate, it is necessary that active substance or formulation can be absorbed in large quantities, spread and pass through biological membranes and be released in a controlled manner, without being inactivated. Liposome encapsulation of Alendronate should reduce renal clearance and, when formulated for long circulation, may increase accumulation of active substance in osteoclasts (Hosny *et al.*, 2013).

Liposomes are small, spherical vesicles which consist of amphiphilic lipids arranged concentrically and an equal number of aqueous spaces or compartments included inside of them. By controlling the physical-chemical double layer of lipid and biological interactions with the environment have been investigated and obtained various types of liposomes, which may be of interest for therapeutic applications. These new formulations are used for improving the delivery of therapeutic agents, enzymes, vaccines and genetic materials (Van Rooijen *et al.*, 1994; Budai *et al.*, 2001; Torchilin, 2005; Xu *et al.*, 2011).

Response surface methodology (RSM) is a widely practiced approach in the development and optimization of drug delivery systems (Hatambeygi *et al.*, 2011). It represents a collection of mathematical and statistical techniques which explores the relationships between several explanatory variables (factors) and one or more measured response variables with the main aim of process optimization. It uses the fitting of polynomial equation to the experimental data to describe the behavior of data sets including interactive effects among the examined variables. The main advantage of RSM is that allows to reduce the experimental runs that would be needed in a full factorial design or in a more traditional single parameter optimization (Ghanbarzadeh *et al.*, 2013; Ma *et al.*, 2014; Zhong *et al.*, 2007).

Box Behnken design (BBD) is a popular form of RSM, acknowledged as one of the best statistical and analytical models and often considered more effective than other response surface designs (Chopra *et al.*, 2007; Ferreira *et al.*, 2007). This design is characterized by a set of experiments lying at the midpoint of each edge of a multidimensional cube and center point replicates (n = 5), whereas the "missing corners" help the researcher to avoid the combined factor extremes (Box & Behnken, 1960).

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The aim of this study was to investigate how different important factors affect the ability to produce single stable Alendronate liposomes with a higher theoretical bioavailability after oral administration, a more comfortable approach for the treatment of osteoporosis.

MATERIALS AND METHODS

Materials

Working standard Alendronate monosodium (Teva LTD API Division, Israel), Chloroform solution (Fluka, Sigma-Aldrich Chemie GmbH, Germany), Phosphatidylcholine (Avanti Lipids, Germany), Cholesterol (PanReac AppliChem, Germany), $Cu(II)(NO_3)_2*3H_2O/HNO_3$ synthesized in the Physical Chemistry Department, University of Bucharest, Bucharest.

Formulation of liposomes

The small vescicles with different compositions (Table I) were prepared by lipid film hydration method, which was described previously (Szoka *et al.*, 1980). The mixture of lipids was dissolved in chloroform under continuous agitation and the solvent was removed by placing the vial on a vacuum pump, at room temperature. The lipid film resulted on the walls of a round-bottomed flask by evaporation of the residual organic solvent was hydrated with an aqueous solution (1mg/ml) containing active substance heated at 60°C (a temperature above the phase transition of lipids used into the formulation) using the Stuart Apparatus, UC152, Stirrerhotplate, ceramic plate, Staffordshire, UK. The suspension was sonicated in order to detach the thin film from the walls and to form multilamellar vesicles with different size. The sonication time was different, according to the specification of the formulation. Depending on the processing conditions and the chemical composition, liposomes could contain one or several concentric bilayers, with various dimensions from 30 to 150 nm.

Particle size analysis

Liposomes dimensions were measured by Dynamic Light Scattering (DLS), one of the most popular methods used to determine the size of particles in suspension, due to the short experiment duration, its automation and modest development costs (Berne *et al.*, 1976). The analysis was conducted using the Zetasizer Nano ZS, Malvern, Herrenberg, Germany. The particles diameter in nanometer was measured at room temperature for all preparations by diluting the liposomes 1:4 in distilled water.

Entrapment efficiency

Based on the affinity for metallic ions existing in solution, alendronate can form soluble or insoluble complexes depending on the solution pH and complexing metal. This property allows the dosage of the active substance from various formulations using spectrophotometric method.

The quantitative determination of alendronate is based on its property to form complexes with Cu (II), which determine a specific absorbance in UV/Vis at a path length of λ=234nm (Koba *et al.*, 2008). The analysis of the encapsulation efficiency of liposomes was conducted using a Shimadzu UVmini-1240 double beam UV-Visible Spectrophotometer, North America. After the separation of the liposomes using ultracentrifugation (20000 rpm, 15 minutes), the examined samples showed that it was not possible to make a direct determination because the Copper within the complexing agent (Solution of 1.5mmol/L of Cu $(II)(NO_3)_2*3H_2O/HNO_3$) gives himself an absorbance and interferes the experiment.

The drug encapsulation efficiency was calculated as follows:

DEE (%) = (Cencapsulated drug/ Ctotal drug)x100, where ”C” denotes the concentration (mg/ml) of the indicated substance.

Optimization

Three independent variables, namely ratio of lipid to drug w/w (X_1), ratio of phosphatidylcholine to cholesterol (X_2) and sonication time (X_3), were selected to optimize the preparation conditions of alendronate liposomes. The selected factors were subjected to response surface methodology (RSM) with a three-factor three-coded level Box-Behnken design (BBD) in order to study their individual and combined effects on the experimental responses (drug encapsulation efficiency and liposomes dimensions). The range and the levels of experimental variables investigated in this study are presented in Table I.

Table I

Factors and levels of Box-Behnnken experimental design

Factors	Code	Range and levels		
		-1	0	+1
Ratio of phosphatidylcholine to cholesterol (w/w)	X_1	3:1	4:1	5:1
Ratio of lipid to drug (w/w)	X_2	4:1	7:1	10:1
Sonication time (min)	X_3	2	4	6

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Experimental data were fitted according to the following second-order polynomial equation calculated by multiple regression analysis:

$$Y = b_0 + \sum_{i=0}^{n} b_i X_i + \sum_{i=0}^{n} b_{ii} X_i^2 + \sum_{i<j}^{n} b_{ij} X_i X_j + \varepsilon$$

where Y represents the measured response, "b" are coefficients calculated by multiple regression analysis, X_i represent the main effects of the independent variables, X_iX_j the interaction terms between variables, X_i^2 quadratic expressions of the independent variables (included into the model in order investigate nonlinearity) and ε is the random error.

The quality of the fitted model was expressed by the coefficient of determination (R^2) and its statistical significance was checked by F-test and P-value test. The statistical analysis of the model was performed in the form of analysis of variance (ANOVA).

The optimum experimental conditions were determined by using desirability functions.

The experimental design, data analysis and quadratic model building were computed by means of Design-Expert 7.0 software (Stat-Ease Inc, Minneapolis, MN, USA).

RESULTS AND DISCUSSIONS

According to BBD designs, a total of seventeen tests (including five replicates of the center point) were carried out in random order (Table II).

Table II

Variables and observed responses in Box–Behnken design for Alendronate liposomes

Run no.	Independent variables			Responses	
	X_1	X_2	X_3	Y_1 (DEE, %)	Y_2 (Size, nm)
1	4:1	7:1	4	74.30	150.8
2	3:1	10:1	4	67.46	44.5
3	4:1	10:1	6	71.13	96.7
4	5:1	10:1	4	71.08	138.3
5	3:1	7:1	2	63.28	63.5
6	4:1	7:1	4	74.33	146.5
7	3:1	4:1	4	81.24	91.1
8	5:1	4:1	4	80.85	34.6
9	4:1	7:1	4	74.26	145.7
10	4:1	7:1	4	74.26	141.2
11	4:1	4:1	6	78.56	53.1
12	5:1	7:1	6	60.34	74.3
13	4:1	10:1	2	60.47	31.9
14	3:1	7:1	6	67.77	93.1
15	5:1	7:1	2	68.20	53.9
16	4:1	4:1	2	80.95	45.2
17	4:1	7:1	4	74.22	132.8

The results showed that the DEE ranged from 60.34 to 81.24 %. The maximum DEE value (81.24%) was found in conditions of X_1 = 3:1, X_2 = 4:1 and X_3 = 4 min. The medium particle size values measured by DLS for the different formulations showed wide variation (i.e., values ranged from a minimum of 31.9 nm to a maximum of 150.8 nm). The results clearly indicate that the responses values are strongly affected by the variables selected for the study.

The coefficients of the polynomial equations were generated using multiple linear regression analysis. The response variables and the test variables were related by the following second-order polynomial equations:

$$Y_1(DEE\%) = 32.01 + 31.88X_1 - 12.47X_2 + 14.43X_3 + 0.33X_1X_2 - 1.54X_1X_3 + 0.54X_2X_3 - 3.49X_1^2 + 0.49X_2^2 - 1.47X_3^2$$

$$Y_2(Particle\ Size) = -337.98 + 125.17X_1 + 6.92X_2 + 88.25X_3 + 12.52X_1X_2 - 1.15X_1X_3 + 2.37X_2X_3 - 25.89X_1^2 - 4.49X_2^2 - 11.57X_3^2$$

The relationship between the dependent and independent variables is further illustrated using the response surfaces, which enable the visual checking of the effects in the three dimensional space (Fig. 1 and 2). In all representations, one factor was kept constant at its center value.

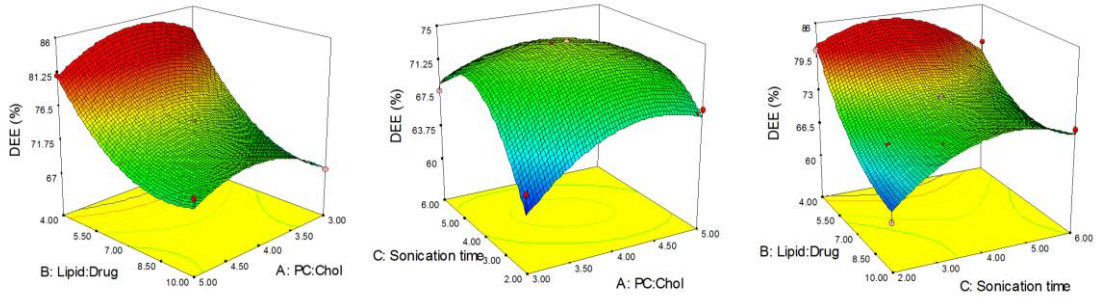

Fig. 1 Response surface plots for effect of independent variables on drug encapsulation efficiency (DEE %)

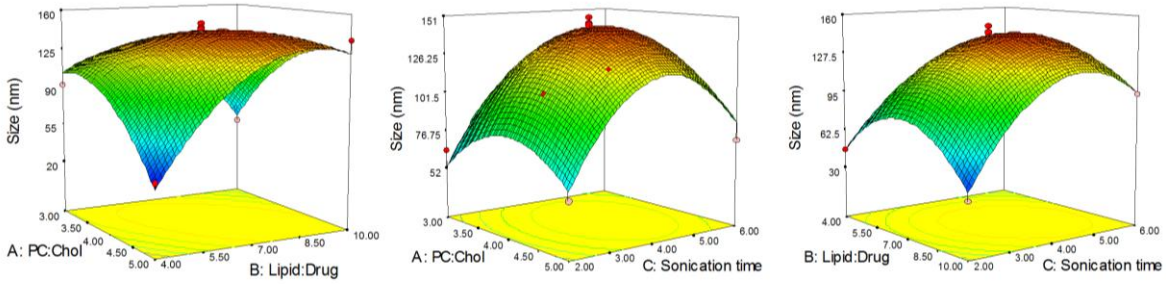

Fig. 2 Response surface plots for effect of independent variables on liposomes mean particle size

By applying ANOVA for the two regression equations, the models were found to be significant ($P < 0.05$), thus very useful in predicting the effects of the three different level factors on the selected responses.

The predicted and observed coefficients of determination (R^2) values for the above regressions were 0.9670 for DEE% and 0.9715 for particle size respectively, indicating that the model adequately fits the real relationship between the parameters chosen in this study and is adequate for prediction within the range of experimental variables.

The P value was used as a tool to check the significance of each coefficient, a P value smaller than 0.05 being considered significant.

Among the linear coefficients, only X_2 (lipid to drug ratio) was find to be significant on DEE, whereas X_1 and X_3 were not statistically significant (0.8921 and 0.3751 respectively). The negative value of X_2 coefficient indicates an unfavorable effect on DEE. However, two of the interaction coefficients (X_1X_3 and X_2X_3) and all quadratic terms are significant, suggesting nonlinear mixed effects on DEE (Table III). X_1X_3, X_1^2, X_3^2 have unfavorable effect on X_2 (negative coefficients), while X_2X_3 and X_2^2 have a favorable effect (positive coefficients).

For Y_2 two of the linear coefficients (X_2 and X_3), all quadratic term coefficients (X_1^2, X_2^2 and X_3^2) and all the interaction coefficients (X_1X_2, X_1X_3 and X_2X_3) were found significant with X_2, X_3, X_1X_2 and X_2X_3 favoring larger particle size, whereas X_1X_3 and all quadratic terms favoring obtaining of smaller size liposomes (Table IV).

Table III

Estimated regression model of relationship between DEE% (Y_1) and independent variables.

Source	SS	df	MSS	F	P-value Prob > F
Model	688.31	9	76.48	22.83	**0.0002**
X_1	0.07	1	0.07	0.02	0.8921
X_2	330.93	1	330.93	98.78	**< 0.0001**
X_3	3.01	1	3.01	0.90	0.3751
X_1X_2	4.02	1	4.02	1.20	**0.3095**
X_1X_3	38.12	1	38.12	11.38	**0.0119**
X_2X_3	42.57	1	42.57	12.71	**0.0092**
X_1^2	51.40	1	51.40	15.34	**0.0058**
X_2^2	80.77	1	80.77	24.11	**0.0017**
X_3^2	145.43	1	145.43	43.41	**0.0003**
Residual	23.45	7	3.35		
R^2	***0.9670***				
Adj R^2	***0.9247***				

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Table IV
Estimated regression model of relationship between liposomes mean particle size (Y_2) and independent variables.

Source	SS	df	MSS	F	P-value Prob > F
Model	30075.62	9	3341.74	26.51	**0.0001**
X_1	9.90	1	9.90	0.08	0.7874
X_2	954.41	1	954.41	7.57	**0.0284**
X_3	1881.30	1	1881.30	14.92	**0.0062**
X_1X_2	5643.77	1	5643.77	44.77	**0.0003**
X_1X_3	21.11	1	21.11	0.17	0.6946
X_2X_3	807.41	1	807.41	6.41	**0.0392**
X_1^2	2822.01	1	2822.01	22.39	**0.0021**
X_2^2	6870.13	1	6870.13	54.50	**0.0002**
X_3^2	9019.73	1	9019.73	71.55	**< 0.0001**
Residual	882.40	7	126.06		
R^2	***0.9715***				
Adj R^2	***0.9348***				

Using the polynomial equations describing the effect estimates on the dependent variables and the surface response methodology, an optimal formulation was developed. The target values for the responses were defined 84.13 to be maximum value for Y_1 and 50±10 nm for Y_2. A ratio of phosphatidylcholine to cholesterol of 4.53:1, a ratio of lipid to drug of 4:1 and 2.85 min sonication time were found to be the optimum values for the independent variables (desirability value=0.980) (Figure 3).

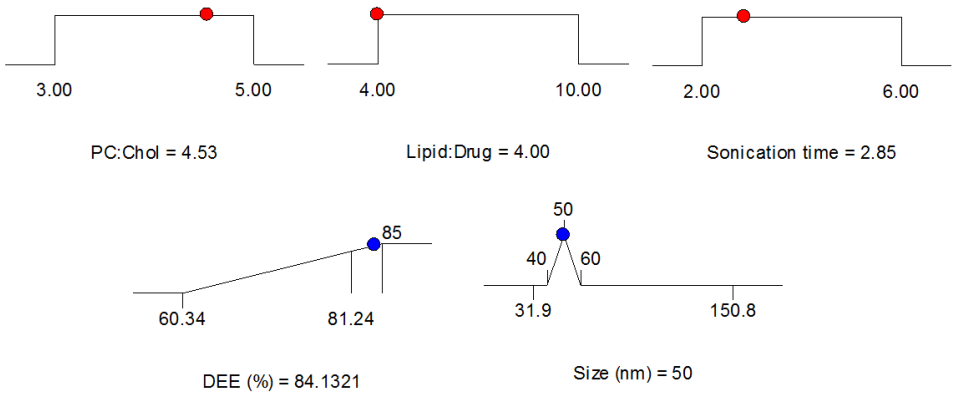

Fig. 3 Optimization of alendronate loaded liposomes by means of desirability function in ramp function graph reprezentation. Optimum values of the independent variables are presented as red dots, whereas the predicted values of responses at the optimum factor levels are dotted in blue.

The studied data set of the independent variables studied during the experiment showed no significant relevance for the measured results. This may suggest that a small variation of the parameters doesn't determine major changes in the entrapment efficacy and stability of the formulations.

As far as the value of variable X_2 (ratio of lipid to drug) is getting bigger, the resulted formulations have a higher turbidity. This is because more liposomes are formed intro suspension. Here is an example of four formulations, in which the ratio of Phosphatidylcholine to cholesterol is kept constant, and X_2 is varied (Figure 4).

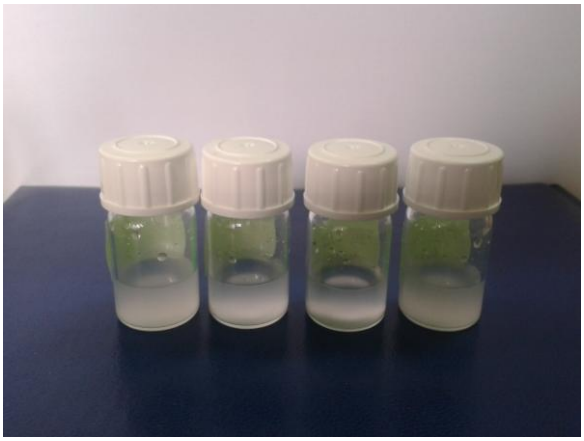

Fig. 4 Liposomes- Formulation I (X_2=10:1), Formulation II (X_2=7:1), Formulation III (X_2=4:1), Formulation IV (X_2=10:1) with different aspect and turbidity (formulations are numbered from left to right).

It was seen that the highest DEE% is attributed to formulations in which the ratio of Lipid to drug is 4:1 (Table V) and it is not affected by the ratio of phosphatidylcholine to cholesterol, even though it is known that cholesterol makes the vescicles of liposome more rigid, stable and more able to encapsulate the active substance.

The stability of the formulations depends on any variation of the three variables studied and it was characterized using the size distribution by number (Table V).

Table V

Stability of liposome formulations, given the size distribution by number: "+++" – high stability (%numb>99), "++"- moderate stability (95<%numb<99), "+"- low stability (%numb<95).

	Independent variables			Responses				
Run no.	X1	X2	X3	Y1 (DEE, %)	Entrapment efficacy	Y2 (Size, nm)	% Numb	Stability
1	4:01	7:01	4	74.3	++	150.8	95.9	++
2	3:01	10:01	4	67.46	+	44.5	98.7	++
3	4:01	10:01	6	71.13	++	96.7	97.2	++
4	5:01	10:01	4	71.08	++	138.3	98.5	++
5	3:01	7:01	2	63.28	+	63.5	100	+++
6	4:01	7:01	4	74.33	++	146.5	95.9	++
7	3:01	4:01	4	81.24	+++	91.1	99.1	+++
8	5:01	4:01	4	80.85	+++	34.6	98.6	++
9	4:01	7:01	4	74.26	++	145.7	96.1	++
10	4:01	7:01	4	74.26	++	141.2	97.3	++
11	4:01	4:01	6	78.56	+++	53.1	85.1	+
12	5:01	7:01	6	60.34	+	74.3	92.9	+
13	4:01	10:01	2	60.47	+	31.9	85.9	+
14	3:01	7:01	6	67.77	+	93.1	99.6	+++
15	5:01	7:01	2	68.2	+	53.9	95.5	++
16	4:01	4:01	2	80.95	+++	45.2	100	+++
17	4:01	7:01	4	74.22	++	132.8	95.9	++

CONCLUSIONS

Box Behnken design offered the possibility of analyzing 17 different models for drug delivery systems with alendronate, which showed a higher entrapment efficacy and theoretical bioavailability of the active substance than the market products. By using this approach, it is possible to create the appropriate experimental conditions in order to obtain an efficient and stable formulation for the alendronate liposomes. The results of the assay pointed out that lipid: drug ratio was the predominant factor that influenced drug encapsulation efficiency and liposome stability and size distribution were mainly affected by the lipid: drug ratio and sonication time. The optimum formulation developed by the design is defined by a ratio of phosphatidylcholine to cholesterol of 4.53:1, a ratio of lipid to drug of 4:1 and 2.85 min sonication time.

The suitability of the model predicting the optimum response values will be tested by using the selected optimal conditions and further experiments will reveal if a bigger variation and interactions between the independent variables will influence even more the optimum formulation.

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