ORIGINAL ARTICLE

FORMULATION OPTIMIZATION OF PRAVASTATIN LOADED LONG-CIRCULATING LIPOSOMES USING A DESIGN OF EXPERIMENTS

BIANCA SYLVESTER, ALINA PORFIRE*, DANA-MARIA MUNTEAN, LAURIAN VLASE, IOAN TOMUȚĂ

Department of Pharmaceutical Technology and Biopharmaceutics, Faculty of Pharmacy, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, 400012, Romania

**corresponding author: aporfire@umfcluj.ro*

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Abstract

Pravastatin (PRAV) is a hydrophilic statin which has been reported to have antiangiogenic and pro-apoptotic effects. However, the beneficial effects of statins on tumour growth are obtained at high doses, the systemic administration of those doses being associated with severe toxicity. Thus, site-specific delivery with liposomal systems may be a novel approach in order to enrich its therapeutic effects, while reducing the overall doses required. The objective of this study was to optimize the formulation of PRAV-loaded long circulating liposomes (LCL-PRAV) by using a D-optimal experimental design. The influence of seven formulation and process factors i.e. phospholipids molar concentration (mM), the molar ratio of phospholipids to cholesterol, the PRAV molar concentration (mM), the hydration temperature (°C), the extrusion temperature (°C), the rotation speed at the formation of the lipid film (rot/min) and the rotation speed at the hydration of the film (rot/min) was studied on PRAV liposomal concentration, the encapsulation efficiency (EE %), liposomal size and the Polydispersity Index (PDI). The desired characteristics of LCL-PRAV are the relatively high drug encapsulation efficiency (> 45%), the low and predictable variation in the drug encapsulation efficiency, the particle size range of 180 - 200 nm and the low PDI value (< 0.100). The optimized formulation had liposomal PRAV concentration of 6128 ± 237 µg/mL, an encapsulation efficiency of 47 ± 13%, 192.3 ± 5 nm size and a PDI of 0,098 ± 0.006. The overall results showed that PRAV can be successfully incorporated into long-circulating liposomes.

Rezumat

Pravastatina (PRAV) este o statină hidrofilă, cu efecte proapoptotice și antiangiogenice. Cu toate acestea, efectele benefice ale statinelor asupra creșterii tumorale sunt obținute la doze mari, administrarea sistemică a acestora fiind asociată cu toxicitate severă. Astfel, utilizarea unui sistem lipozomal pentru transportul și eliberarea la țintă poate constitui o nouă abordare în scopul diversificării efectelor terapeutice și minimizării, în același timp, a dozele necesare.
Acest studiu a urmărit optimizarea formulării PRAV în lipozomi cu durată lungă de circulație (LCL-PRAV), utilizând un design experimental de tip D-optimal. A fost studiată influența a șapte factori de formulare, respectiv de proces: concentrația molară a fosfolipidelor (mM), raportul molar fosfolipide:colesterol, concentrația molară a soluției de PRAV (mM), temperatura de hidratare (°C), temperatura de extrudere (°C), viteza de rotație la formarea filmului lipidic (rot/min) și viteza de rotație la hidratarea filmului lipidic (rot/min), asupra concentrației lipozomale de PRAV, a eficienței de încapsulare (EE %), a mărimii lipozomilor și a indicelui de polidispersie (PDI). Caracteristicile dorite ale LCL-PRAV au fost: eficiență relativ ridicată a încapsulării PRAV (> 45%), variație scăzută și previzibilă în eficiența încapsulării, mărime medie a lipozomilor între 180 și 200 nm și valoare scăzută a PDI (< 0,100). Formularea optimizată a lipozomilor a fost caracterizată printr-o concentrație de 6.128 ± 237 µg/mL, o eficiență de încapsulare de 47 ± 13%, o mărime de 192,3 ± 5 nm și o valoare a PDI de 0,098 ± 0,006. Rezultatele obținute au arătat că PRAV poate fi încorporată cu succes în lipozomi cu durată lungă de circulație.

Keywords: pravastatin, liposomes, experimental design, optimization

Introduction

The use of statins as cholesterol-lowering agents for the primary and secondary prevention of coronary heart disease has been firmly established in current medical practice [2, 10, 13]. Statins have been designed as competitive inhibitors of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase, the rate-limiting enzyme in the mevalonate pathway leading to *de novo* cholesterol synthesis [23].

Besides these effects, studies have proved that statins have pleiotropic actions, including cancer prevention [6]. The carcinoma-preventive effect of statins can be explained by several mechanisms, such as induction of cancer cells' apoptosis and angiogenesis restraint, inhibition of malignant cell proliferation and expression of an inhibitory effect on chronic bowel inflammation [2]. The effect is mainly dependent on the statin potency, but also a biphasic dose-dependent effect has been described.

Thus, at high doses, statins have antiangiogenic and pro-apoptotic effects, whereas at low doses can favour angiogenesis [7]. The doses for the cancer prevention and treatment have been proved to be 100 to 500 fold higher than those needed for cholesterol lowering activity, the systemic administration of those doses being associated with severe toxicity, including rhabdomyolysis or even death [20]. As the beneficial effects of statins on tumour growth are obtained at high doses, site-specific delivery with liposomal systems may be an interesting approach to intensifying therapeutic effects, while reducing the overall doses required. Also, controlling the tissue distribution of statins with such drug delivery systems is important to promote the desired activity and to limit the adverse effects [1, 4]. Due to non-specific drug distribution, the parenteral administration of a pravastatin solution leads to a quick distribution of the drug in the blood stream to every major organ, contrarily to pravastatin loaded long-circulating liposomes which are preferentially targeted to the tumour tissue and macrophage rich regions [4].

Polyethylene glycol (PEG) has been shown to protect liposomes from recognition and rapid removal from the circulation by the mononuclear phagocyte system (MPS), enabling the liposomes to stay in the circulation for a prolonged period of time and allowing them to substantially accumulate in tumours, and hence giving the liposomes long-circulating properties [1, 4, 12].

Hydrophilic drug encapsulation in nanoparticles can provide a better pharmacokinetic profile and bioavailability, enhance the anticancer effect and reduce toxicity compared with drug administration without a carrier [8].

In the past few decades, liposomes have become very promising drug-delivery systems, due to their unique biological and physicochemical properties. Due to the structural similarity of liposome bilayers to cellular membrane, liposomes have been used as drug-carriers to deliver therapeutics to specific regions of the body since the early 1970s [14]. However, despite their multiple advantages, relatively few therapeutic products are available on the market. Several factors may be responsible for that, including the time consuming and complex nature of the preparation method, the difficulty to scale-up [15], high manufacturing costs due to low reproducibility, low entrapment of therapeutic agents and difficulties associated with the identification and control of the critical formulation and process design factors [30].

Hydrophilic drugs, such as pravastatin, are more challenging to encapsulate into liposomes, the high water solubility making it difficult to achieve a high degree of drug entrapped [15]. Furthermore, manufacturing variability can be the result of a lack of understanding of the preparation process, meaning that utilizing a design of experiments to assist formulation and process design is a promising way to find an optimum and robust method to successfully incorporate PRAV into long-circulating liposomes [29].

The desired characteristics of the long-circulating PRAV loaded liposomes are the relatively high drug encapsulation efficiency (> 45%), the low and predictable variation in the drug encapsulation efficiency, the particle size range of 180 - 200 nm and the low Polydispersity Index (PDI) value (< 0.100). To obtain the above target profile, a D-optimal experimental design (DoE) was successfully used.

A D-optimal experimental design with seven factors and two levels was employed in order to optimize the formulation of long-circulating PRAV loaded liposomes. The influence of seven formulation and process factors i.e. phospholipids molar concentration (mM), the molar ratio of phospholipids to cholesterol, the PRAV molar concentration (mM), the hydration temperature (°C), the extrusion temperature (°C), the rotation speed at the formation of the lipid film (rot/min) and the rotation speed at the hydration of the film (rot/min) was studied on PRAV liposomal concentration, the encapsulation efficiency (EE %), the liposomal size and PDI. The formulation was consequently optimized using Modde 10 software, in order to obtain the desired quality attributes of the final product.

The traditional process optimization is based on analysing one factor at time (OFAT), while keeping the other factors constant, allowing the detection of some factor effects without detection of interactions between the factors [17]. Contrarily to OFAT, DoE allows varying multiple factors at different levels simultaneously, making it possible this way to detect both the main effects and interactions between factors, and thus providing valuable information with minimal number of runs and without sacrificing the quality of the results [5, 11].

Materials and Methods

Materials

Pravastatin sodium salt was purchased from Biocon Limited (India). The phospholipids used for liposome preparation: 1,2-dipalmitoyl-sn-glycero-3-phospho-choline (DPPC) and N-(carbonyl methoxypoly-ethylenglycol-2000)-1,2-distearoylsn-glycero-3-phosphoethanolamine (Na^+-salt; MPEG-2000-DSPE) were purchased from Lipoid GmbH (Germany). Cholesterol (CHO) from sheep wool was provided by Sigma-Aldrich (Germany). All the other reagents used were of analytic grade purity, commercially available.

Methods

Liposome preparation

Pravastatin-loaded long-circulating liposomes (LCL-PRAV) were prepared using the film hydration method, as described by Schiffelers *et al.* [22]. Briefly, phospholipids (DPPC and MPEG-2000-DSPE in a molar ratio of 19:1) and cholesterol were dissolved in an appropriate amount of ethanol, in a round-bottomed flask. The solvent was evaporated under reduced pressure, at temperatures ranging from 40°C to 60°C in a rotary evaporator, leading to the formation of a thin film at the bottom of the flask. The remaining residual solvent was removed by maintaining the flask under a stream of nitrogen for 1 hour. Liposomes were formed by hydrating the film with 5 mL solution of phosphate buffered saline (PBS, pH = 7.4), in which the water soluble pravastatin sodium salt was dissolved, for 20 minutes at variable temperatures (40 - 60°C). Liposomal dispersion was subsequently extruded under high pressure three times through a 0.8 μm polycarbonate membrane and five times through a 0.2 μm polycarbonate membrane using LiposoFast LF-50 equipment (Avestin Europe GmbH, Germany). Unencapsulated drug was removed by dialysis in a Slide-A-Lyzer cassette, with a molecular weight cut-off of 10 kDa at 4°C, with repeated changes of buffer, over a period of 24 hours. Liposomes were stored at a temperature of 4°C, until analysis.

Measurement of liposomal size

Liposomal size and PDI value were determined by dynamic light scattering method, using Zetasizer Nano ZS analyser (Malvern Instruments Co., Malvern, UK). The measurement was performed at 25°C with a scattering angle of 90°. The dynamic light scattering data was collected using a helium laser source and mean results were provided by photon correlation spectroscopy (PCS).

Determination of PRAV content and encapsulation-efficiency

The pravastatin content of the liposomes was determined through an HPLC/UV method, after complete dissolution of liposomes in methanol [3, 24, 25]. Analyses were performed on a Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA), equipped with an ultraviolet (UV) detector. Cromatographic separation was carried out using a Gemini C18 column (50 x 2 mm, internal diameter 3 μm) from Phenomenex (Phenomenex, Torrance, CA). The mobile phase was 25% acetonitrile and 75% 0.1 % phosphoric acid solution. Cromatographic conditions set for the method were: flow rate 1 mL/min, column temperature 30°C, UV detection at 237 nm and injection volume 5 μL [28]. The retention time for PRAV was 1.6 min. Liposomal PRAV was expressed both as concentration (μg/mL) and encapsulation efficiency (%). The EE was calculated using the following equation, and represents the percentage of entrapped drug:

$$EE\ (\%) = (\text{Entrapped PRAV}/\ \text{Total PRAV}) \times 100 \quad (1)$$

Optimization of the liposomal formulation

A D-optimal experimental design with seven factors and two levels was employed to study the influence of formulation and process parameters on the preparation of LCL-PRAV, as seen in Table I. The design of the study was developed using Modde 10 software (Umetrics, Sweden).

Considering the costs for a single experiment, minimizing the amount of performed experiments, while choosing the most informative combination of factors, is always an aim [9]. Traditional experimental designs (Full Factorial Designs, Fractional Factorial Designs and Response Surface Designs) would require, in our case, too many runs (2^7) for the amount of resources and time allocated for the experiment. However, by employing a D-optimal design we could address this limitation. Given the total number of treatment runs for an experiment and a specified model, the D-optimal algorithm chooses the optimal set of design runs from a candidate set of possible design treatment runs. This candidate set of treatment runs consists of all possible combinations of various factor levels used in the experiment [19]. The optimal set of design runs is generated by an iterative search algorithm with the purpose of minimizing the covariance of the parameter estimates for the specified model. This is equivalent to maximizing the determinant $D = |X^T X|$, where X represents the design matrix of model terms (the columns) evaluated at specific treatments in the design space (the rows). Three centre point runs were added with the purpose of providing a measure of process stability and inherent variability [9, 19].

The matrix of the experimental design, obtained by applying this algorithm in MODDE software and comprising 19 formulations, is presented in Table II.

The parameters considered to have significant effect on liposomal properties have been selected as independent variables to be investigated. They can be divided into two categories: the formulation parameters- phospholipids molar concentration (X_1), the molar ratio of phospholipids to cholesterol (X_2) and PRAV molar concentration (X_3) and the process parameters-temperature used at the formation/ hydration of the lipid film (X_4), the temperature used in the extrusion step (X_5), the rotation speed at the formation of the lipid film (X_6) and the rotation speed at the hydration of the film (X_7). Three responses (dependent variables) evaluated were: the liposomal concentration of SIM (Y_1), EE (Y_2), the liposomal size after extrusion (Y_3) and PDI (Y_4).

The preparation method was previously tested during preliminary experiments, in order to establish if the method is adequate for PRAV encapsulation. The levels of independent variables, shown in Table I, were established within the range suggested in literature.

Table I

Independent and dependent variables of the experimental design used for the preparation of PRAV liposomes

	Level used	
Variable	**Low (-1)**	**High (+1)**
Independent variables		
X_1 = Phospholipids molar concentration (mM)	20	80
X_2 = Molar ratio of phospholipids to cholesterol	5	10
X_3 = PRAV molar concentration (mM)	10	25
X_4 = Hydration temperature (°C)	40	60
X_5 = Extrusion temperature (°C)	40	60
X_6 = Rotation speed at the formation of the lipid film (rot/min)	40	120
X_7 = Rotation speed at the hydration of the lipid film (rot/min)	60	180
Dependent variables		
Y_1 = Liposomal concentration of PRAV (mM)		
Y_2 = Encapsulation efficiency (%)		
Y_3 = Liposomal size (nm)		
Y_4 = Polydispersity Index		

Table II

The matrix of the experimental design

Formulation code	Run order	X_1 (mM))	X_2	X_3 (mM)	X_4 (°C)	X_5 (°C)	X_6 (rot/min)	X_7 (rot/min)
N1	18	20	5	10	40	40	40	60
N2	7	80	5	10	40	60	40	180
N3	6	20	10	10	40	60	120	60
N4	3	80	10	10	40	40	120	180
N5	2	20	5	25	40	60	120	180
N6	11	80	5	25	40	40	120	60
N7	1	20	10	25	40	40	40	180
N8	4	80	10	25	40	60	40	60
N9	8	20	5	10	60	40	120	180
N10	19	80	5	10	60	60	120	60
N11	15	20	10	10	60	60	40	180
N12	14	80	10	10	60	40	40	60
N13	9	20	5	25	60	60	40	60
N14	17	80	5	25	60	40	40	180
N15	16	20	10	25	60	40	120	60
N16	10	80	10	25	60	60	120	180
N17	5	50	7,5	17,5	50	50	80	120
N18	13	50	7,5	17,5	50	50	80	120
N19	12	50	7,5	17,5	50	50	80	120

X_1: the phospholipids molar concentration (mM), X_2: the molar ratio of phospholipids to cholesterol, X_3: the PRAV molar concentration (mM), X_4: the hydration temperature (°C), X_5: the extrusion temperature (°C), X_6: the rotation speed at the formation of the lipid film (rot/min), X_7: the rotation speed at the hydration of the film (rot/min).

In order to fit the experimental data with the chosen experimental design and to calculate the statistical parameters, the statistical module from Modde 10 software (Umetrics, Sweden) was used. Partial least squares (PLS) method was employed for data fitting and for calculation of the statistical parameters.

The regression coefficients of each investigated factor were determined using the following equation:

$$Y_n = b_0 + \sum b_i X_j + \sum b_{ij} X_i X_j \quad (2),$$

where Y_n is the dependent variable; b_0 is the model constant; b_i are the linear coefficients; $b_{ij,}$ are the interaction coefficients; and $X_{i,}$ X_j are the coded levels of independent variables.

R^2, which represents the explained variation and Q^2, representing the fraction of the variation of the response that can be predicted, were determined. The validity of the experimental design was determined by performing the analysis of variance (ANOVA) test [21]. Finally, the model was expressed graphically, in terms of response surface model.

The optimization step was performed using the desirability function, f(ds), that searches for the best

possible combination of factor settings, that predicts a result inside the response specifications and as close as possible to the targets [9]. The optimizer is used to find an experimental set point that fulfils all the required criteria (high PRAV liposomal concentration, good encapsulation efficiency, desired size of the liposomes and low PDI). The optimum formulation of PRAV-loaded long-circulating liposomes (LCL-PRAV-OPT) was selected based on the criteria of maximizing liposomal SIM concentration with an EE% of 50 ± 10%, for vesicles size of 180 ± 20 nm and a PDI < 0.100.

Results and Discussion

For long-circulating liposomes, drug concentration, encapsulation efficiency, liposomal size and PDI are the main properties affecting their therapeutic potential and targeting ability, making the formulation qualities critical. Achieving a higher percentage of encapsulation, especially for water-soluble active principle ingredients like pravastatin, represents an advantage for both manufacturers and patients. A higher percentage of encapsulated drug can increase drug concentration in the final product allowing greater flexibility in dosing, increased dosing intervals and hence improved patient compliance [30]. Based on this knowledge, the current study's aim was to use a DoE in order to optimize the formulation of long circulating PRAV liposomes in terms of drug content, EE, size and PDI.

We studied the influence of seven high risk factors, three of them being formulation factors (phosphor-lipids molar concentration, the molar ratio of phospholipids to cholesterol and PRAV molar concentration) and the other four were process related factors (the hydration temperature, the extrusion temperature, the number of rotations/ minute at the formation of the lipid film and the number of rotations/minute at the hydration of the film). The results are presented in Table III.

Table III

The experimental results of D-optimal design

Formulation code	Run order	Y_1 (µg/mL)	Y_2 (%)	Y_3 (nm)	Y_4
N1	18	336.6	7.54	209.4	0.226
N2	7	514.4	11.52	174.1	0.197
N3	6	980.2	21.95	177.1	0.109
N4	3	1203.7	26.95	210.5	0.233
N5	2	2845.8	25.49	160.9	0.102
N6	11	3624.3	32.46	202.9	0.206
N7	1	1683.6	15.08	201.5	0.201
N8	4	1943.6	17.41	162.4	0.089
N9	8	1491.1	33.39	207.3	0.219
N10	19	1433.4	32.10	176.5	0.110
N11	15	507.3	11.36	188.9	0.121
N12	14	900.8	20.17	209.2	0.208
N13	9	3419.8	30.63	155.7	0.101
N14	17	3675.6	32.92	170.2	0.205
N15	16	6777.8	60.72	203.1	0.199
N16	10	6224.4	55.75	174.3	0.078
N17	5	3370.6	43.13	191.1	0.147
N18	13	4239.8	54.26	182.5	0.118
N19	12	2909.1	37.23	199.2	0.177

The influence of various factors on liposomal PRAV concentration

As shown in Table III, liposomal PRAV concentration varied from 336.6 to 6777.8 µg/mL for the various factor combinations. The most significant factors that influenced liposomal PRAV concentration were: the PRAV molar concentration (X_3; $p < 0.02$), the hydration temperature (X_4; $p < 0.001$) and the number of rotations/minute at the formation of the lipid film (X_6; $p < 0.001$), as can be seen from the equation describing the influence of formulation and process parameters on liposomal PRAV concentration:

$$Y_1 = 2530.65 + 1426.69\ X_3 + 706.118\ X_4 + 724.913\ X_6 + 543.895\ X_3X_4 \quad (3).$$

A positive value of the regression coefficient means a positive influence on the response, meaning that increasing the values of all the significant factors (X_3, X_4, X_6) leads to an increase in the liposomal PRAV concentration.

PRAV molar concentration had the highest impact on the evaluated response. Liposomal concentration of PRAV was positively influenced by the PRAV molar concentration, which can be explained by the fact that more PRAV is available to be incorporated into the liposomes.

The hydration process is a vital step in the preparation of liposomes, the temperature used at the hydration of the lipid film and the rotation

speed at the formation, respectively hydration of the lipid film, being critical factors.

The hydration temperature had a positive effect on PRAV liposomal concentration. Also, an interaction between PRAV concentration and hydration temperature had been evidenced ($p < 0.005$), therefore the increase in liposomal PRAV concentration with a PRAV molar concentration (X_3) increase proved more significant, when the hydration temperature was higher (X_3) (Figure 1). Since hydrations only occurs at temperatures above the T_m (liquid crystal transition temperature) of the lipid, an increase in the hydration temperature leads to an increase in lipid bilayer fluidity and hence permeability, leading to better incorporation of the drug [16].

According to our findings, using a higher rotation speed at the formation of the lipid film had a positive effect on PRAV liposomal concentration. A possible explanation might be that a higher intake of mechanical energy may lead to the formation of a thinner, more homogenous film which is easier to hydrate.

The prediction confidence level of the model was 95% and the statistical analysis showed a good fitting of the model proposed ($R^2 = 0.910$ and $Q^2 = 0.842$). The results of the ANOVA test, presented in Table IV, showed a significant influence of variables on the response (liposomal PRAV concentration) ($p < 0.01$) and that the model did not present a significant lack of fit ($p = 0.598$).

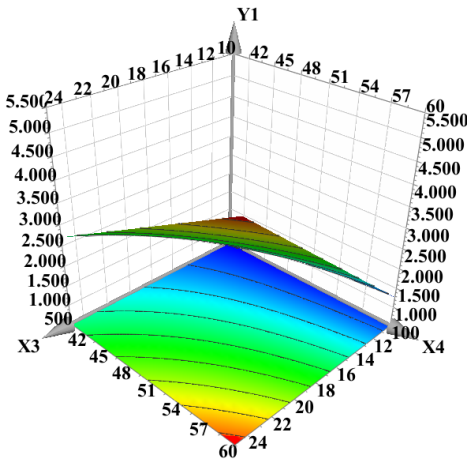

Figure 1.
Response surface for predicting liposomal PRAV concentration (Y_1) with respect to X_3 (the PRAV molar concentration) and X_4 (the hydration temperature)

Table IV
Analysis of variance for liposomal PRAV concentration

Source	Degrees of freedom	Sum of squares	Mean square	F value	p value
Total corrected	18	61987400	3443740		
Regression	6	56379900	9396640	20.1086	0.002
Residual	12	5607530	467294		
Lack of fit	10	4694320	469432	1.02809	**0.589**
Pure error	2	913207	456604		

The influence of various factors on encapsulation efficiency (EE %)

According to the data presented in Table III, EE % varied from 7.54 to 60.72 % for the various factor combinations. The equation describing the influence of formulation and process parameters on EE % is the following:

$$Y_2 = 1.416 + 0.110\ X_3 + 0.117\ X_4 + 0.157\ X_6 \quad (4).$$

The most significant factors that influenced EE % were, according to the equation (4), the PRAV molar concentration (X_3; $p < 0.01$), the hydration temperature (X_4; $p < 0.01$) and the rotation speed at the formation of the lipid film (X_6; $p < 0.001$).

The equation shows a positive influence of formulation factor X_3 and of the process parameters X_4 and X_6 on the studied response, EE %. This can also be observed from the response surface diagrams in Figure 2.

An increase in PRAV molar concentration leads to higher encapsulation efficiency, which can be due to a higher amount of PRAV available to be incorporated into the liposomes and also considering the interactions between drug and phospholipids, a small portion of the free drug associates with the liposome surfaces, causing an increase in EE. This additional increase is dependent on the free drug concentration in the medium, hence a higher concentration of PRAV can lead to an increase in EE. At very high drug concentrations, any additional increase in drug concentration does not make a significant difference in drug encapsulation, due to the fact that the surface attached drug percentage becomes negligible [30].

As mentioned earlier, the method of preparation can influence significantly the characteristics of the liposomes. Drug entrapment can be enhanced by hydrating a thinner film of dry lipids, at temperatures above the T_m (liquid crystal transition temperature) of the lipids. This could be the explanation for the increase in EE with the increase of hydration temperature and rotation speed at the formation of the lipid film, since a higher rotation speed leads to the formation of a thinner, more homogenous and easier to hydrate lipid film [27].

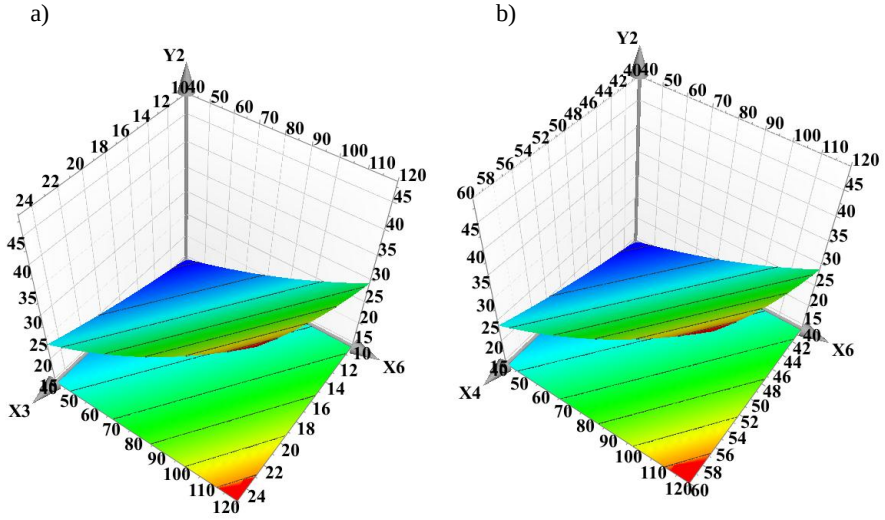

Figure 2.

Response surface for predicting EE (Y_2) with respect to:

a) X_3 (the PRAV molar concentration) and X_6 (the rotation speed at the formation of the lipid film);

b) X_4 (the hydration temperature) and X_6 (the rotation speed at the formation of the lipid film).

Furthermore, statistical analysis was applied, showing a good fit of the model, based on $R^2 = 0.749$ and $Q^2 = 0.590$ values and a prediction confidence level of 95%.

The ANOVA test results, illustrated in Table V, showed a significant influence of variables on PRAV encapsulation efficiency ($p < 0.001$). The value of $p = 0.240$ shows that the proposed model did not present a significant lack of fit.

Table V

The analysis of variance for encapsulation efficiency

Source	Degrees of freedom	Sum of squares	Mean square	F value	p value
Total corrected	18	1.11257	0.0618093		
Regression	5	0.833760	0.1667521	7.77518	0.001
Residual	13	0.278807	0.0214467		
Lack of fit	11	0.265212	0.0241102	3.54693	**0.240**
Pure error	2	0.013595	0.0067974		

The influence of various factors on liposomal size and PDI

The size of the liposomes ranged from 155.7 to 210.5, as seen in Table III. The equation showing the influence of formulation and process parameters on liposomal size is the following:

$$Y_3 = 187.2 + 4.375\ X_2 - 7.625\ X_3 - 15.262\ X_5 \quad (5).$$

The molar ratio of phospholipids to cholesterol (X_2; $p < 0.02$), the PRAV molar concentration (X_3; $p < 0.001$), and the extrusion temperature (X_5; $p < 0.05$) were the most significant factors that influenced the liposomal size. Out of these studied factors, X_2- the molar ratio of phospholipids to cholesterol had a positive impact on liposomal size, while X_3- the PRAV molar concentration and X_5- the extrusion temperature had a negative significant influence. Thus, the increase of phospholipids concentration and the decrease of cholesterol content will result in a greater size of the liposomes, while increasing PRAV molar concentration and extrusion temperature result in a decrease in the size, as seen in Figure 3.

According to the data presented in Table III, the PDI varied between 0.078 and 0.233. Out of all studied factors only the PRAV molar concentration (X_3; $p < 0.02$) and the extrusion temperature (X_5; $p < 0.001$) had a significant influence on PDI, as it is illustrated in the equation describing the influence of formulation and process parameters on the liposomal PRAV concentration:

$$Y_4 = 0.160 - 0.015\ X_3 - 0.049\ X_5 \quad (6).$$

Both factors had a negative impact on PDI, meaning that an increase in both PRAV molar concentration and extrusion temperature leads to a decrease in PDI, which can also be observed in the response surface diagrams in Figure 4.

a) b)

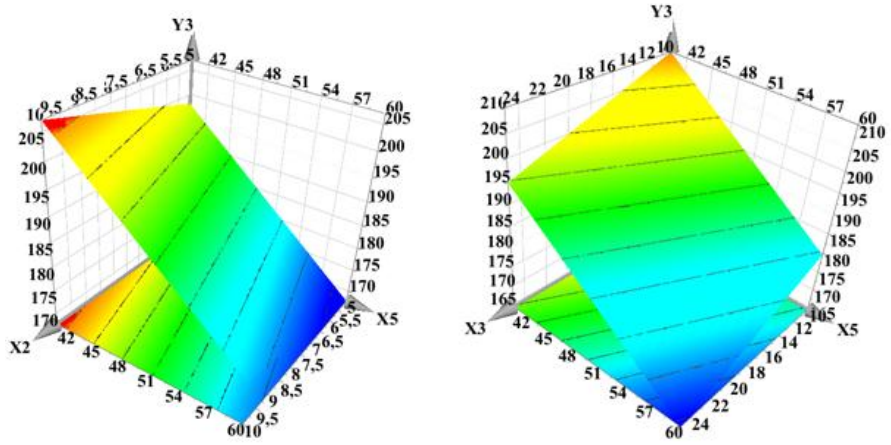

Figure 3.
Response surface for predicting size of liposomes (Y_3) with respect to:
a) X_2 (the molar ratio of phospholipids to cholesterol) and X_5 (the extrusion temperature);
b) X_3 (the PRAV molar concentration) and X_5 (the extrusion temperature).

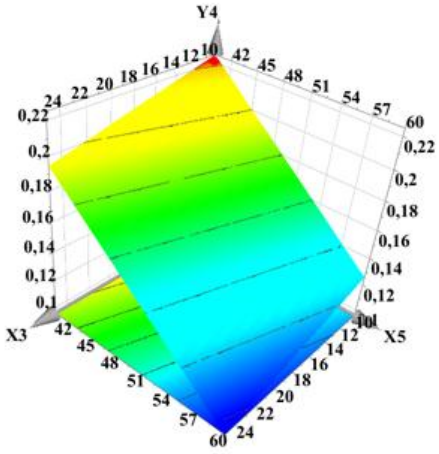

Figure 4.
Response surface for predicting PDI (Y_4) with respect to X_3 (the PRAV molar concentration) and X_5 (the extrusion temperature)

Lipids concentration and the presence of cholesterol in the liposome formulation have been recognized to contribute to the long-circulating properties of liposomal carriers [12]. Cholesterol is a membrane constituent, widely found in biological systems which serve the purpose of modulating membrane fluidity, elasticity, and permeability [26]. Incorporation of cholesterol into liposomes can increase their stability and reduce membrane fluidity and permeability [18]. A higher concentration of cholesterol, respectively a lower molar ratio between phospholipids and cholesterol, has been previously reported to have a positive effect on the liposomal size, due to an increased stability of the vesicles to disruption in the homogenization step of the preparation process [20]. The possible explanation for our contrary finding, that a decrease of cholesterol content will result in a greater size of the liposomes, could be due to interactions between cholesterol and the active ingredient, PRAV.

The PRAV molar concentration had a significant negative impact on liposomal size, meaning that at higher concentrations of PRAV a decrease in the size of the liposomes was observed. This could be explained by the interactions between PRAV and the phospholipids from the lipid bilayer which may lead to a smaller internal-to-external volume ratio of the liposomes.

A negative impact of PRAV molar concentration has been noticed on PDI, as well. PDI, or heterogeneity index, is a measure of the heterogeneity of sizes of molecules or particles in a mixture. A possible explanation for our finding is that, at higher PRAV molar concentrations, the population of obtained liposomes is more homogenous, leading to a lower PDI, considering that the number of passages through the polycarbonate membrane during the extrusion step was kept constant.

The extrusion temperature had a negative influence on both size and PDI of liposomes, meaning that a higher extrusion temperature led to a final product with smaller size and more homogenous distribution (lower PDI). For constant pressure and membrane pore size, the increase in temperature in the extrusion step led to an increase in lipid bilayer fluidity, the liposomes being more susceptible to the conversion from multi-lamellar vesicles (MLV), which were obtained using the method of preparation described, to large uni-lamellar vesicles (LUV), leading to a reduction of the final size of liposomes and a lower PDI.

A good fit of the models was proved by statistical analysis, based on R^2 = 0.930 and Q^2 = 0.770 values for liposomal size and R^2 = 0.866 and Q^2 =

0.728 for PDI, respectively. The ANOVA test results, illustrated in Tables VI and VII, showed a significant influence of variables on the liposomal size ($p < 0.001$). The values of $p = 0.350$, respectively 0.407, showed that the proposed models did not present a significant lack of fit.

Table VI

Analysis of variance for liposomal size

Source	Degrees of freedom	Sum of squares	Mean square	F value	p value
Total corrected	19	671787	35357.2		
Regression	8	5538.70	692.338	16.662	0,001
Residual	10	415.520	41.5519		
Lack of fit	8	276.030	34.5041	0.4947	**0.350**
Pure error	2	139.487	69.7374		

Table VII

Analysis of variance for Polydispersity Index (PDI)

Source	Degrees of freedom	Sum of squares	Mean square	F value	p value
Total corrected	19	0.53995	0.02841		
Regression	5	0.04473	0.00894	16.662	0,001
Residual	13	0.00690	0.00053		
Lack of fit	11	0.00516	0.00046	0.5390	**0.407**
Pure error	2	0.00174	0.00087		

Out of the seven risk factors that were taken into account for this experiment, the phospholipids molar concentration (mM)- X_1 and the rotation speed at the hydration of the film (rot/min)- X_7 did not have a significant influence on the studied responses. Lipid concentration has been previously reported to have a positive effect on drug encapsulation, due to the larger population of vesicles in the system and consequently, larger internal volume for the drug encapsulation [20, 31]. The possible explanation for our finding is that at relatively low lipid concentrations, an increase in the lipid concentration causes a proportional increase in the encapsulation efficiency, but as the concentration continues to increase, a plateau is reached, which might be the case for our formulations.

Assay of the optimum formulation

The goal of this design was to obtain the maximum PRAV concentration in liposomes having the size of 190 ± 10 nm, with EE (%) greater than 45% and low PDI (< 0.100). Based on this goal, the optimum conditions for the preparation of liposomes were determined. The formulation with the following composition: phospholipids molar concentration (X_1) = 55 mM, the molar ratio of phospholipids to cholesterol (X_2) = 6, the PRAV molar concentration (X_3) = 25 mM and the following process parameters: hydration temperature (X_4) = 60°C, extrusion temperature (X_5) = 60°C, the rotation speed at the formation of the lipid film (X_6) = 120 rot/min and the rotation speed at the hydration of the film (X_7) = 78 rot/min fulfilled the conditions of an optimal formulation.

The optimum formulation was prepared in triplicate and its characteristics were compared with the predicted values. The formulation had a liposomal PRAV concentration of 6,128 ± 237 µg/mL, an encapsulation efficiency of 47 ± 13%, 192.3 ± 5 nm size and a PDI of 0.098 ± 0.006. The measured responses were not significantly different from the calculated ones ($p < 0.01$) and the percentage error between the observed and predicted responses was lower that 6%, in all cases.

Conclusions

The results of this study showed that pravastatin can be successfully encapsulated into long circulating liposomes. The use of a design of experiments in order to gain a comprehensive understanding of formulation and process parameters affecting liposome formulation proved to be the best approach in this case. Using DoE software, the analysis of more than one response was possible. The PRAV liposomal concentration, EE, the size and PDI could be analysed together. Compared to the traditional process optimization, DoE was able to detect both the main effects and the interaction between factors, providing a better understanding of the process [5]. An overview of factorial analysis revealed that the liposomal properties were highly influenced by both formulations and process parameters, identification of these risk factors and the attempt to find the optimal levels of these factors leading to a final product with optimum properties.

References

1. Banciu M., Schiffelers R.M., Metselaar J.M., Storm G., Utility of targeted glucocorticoids in cancer therapy. *J. Liposome Res.*, 2008; 18(1): 47-57.
2. Butu A., Rodino S., Golea D., Butu M., Butnariu M., Negoescu C., Dinu-Pîrvu C.E., Liposomal nanodelivery

system for proteasome inhibitor anticancer drug bortezomib. *Farmacia*, 2015; 63(2): 224-229.
3. Carje A.G., Valentin I., Muntean D.L., Hancu G., Balint A., Imre S., Enantioseparation of Indapamide by High Performance Liquid Chromatography using Ovomucoid Glycoprotein as Chiral Selector. *Farmacia*, 2016; in press.
4. Coimbra M., Banciu M., Fens M.H., de Smet L, Cabaj M., Metselaar J.M., Storm G., Schiffelers R.M., Liposomal pravastatin inhibits tumor growth by targeting cancer-related inflammation. *J. Con. Rel.*, 2010; 148: 303-310.
5. Curic A., Reula R., Möschwitzera J., Frickerb G., Formulation optimization of itraconazole loaded PEGylated liposomes for parenteral administration by using design of experiments. *I. J. Pharm.*, 2013; 448: 189-197.
6. Demiere M.F., Higgins P.D., Gruber S.B., Hawk E., Lippman S.M., Statins and cancer prevention. *Nat. Rev. Cancer*, 2005; 5: 930-942.
7. Elewa H.F., El-Remessy A.B., Somanath P.R., Fagan S.C., Diverse effects of statins on angiogenesis: new therapeutic avenues. *Pharmacotherapy*, 2010; 30(2): 169-176.
8. Eloy J.O., Claro de Souza M., Petrilli R., Barcellos J.P.A., Lee R.J., Maldonado Marchetti J., Liposomes as carriers of hydrophilic small molecule drugs: Stategies to enhance encapsulation and delivery. *Colloids and surfaces B: Biointerfaces*, 2014; 123: 345-363.
9. Eriksson L., Johansson E., Kettaneh-Wold N., Wikström C., Wold S., Design of Experiments-principles and Applications: Umetrics AB, Umeå, Sweden, 2008.
10. Farkas S.Z., Imre S., Muntean D.L.,Tero-Vescan A., Analysis of Drug Related Impurities by Infrared Spectrometry in The Class Of Statins. *Farmacia*, 2013; 61(6): 1091-1101.
11. Ferencz L., Muntean D.L., Possible Substitutes For Nimesulide: the Results of a Comprehensive Screening Based on Structural Similarity and Docking Simulation on the Surface of Enzymes. *Farmacia*, 2015; 63(2): 189-195.
12. Gabizon A.A., Stealth liposomes and tumor targeting: one step further in the quest for the magic bullet. *Clin. Cancer Res.*, 2001; 7: 223-225.
13. Graafa M.R., Richelb D. J., van Noordenc C.J.F., Guchelaard H.J., Effects of statins and farnesyltransferase inhibitors on the development and progression of cancer. *Cancer Treat. Rev.*, 2004; 30: 609–641.
14. Gregoridias G., The carrier potential of liposomes in biology and medicine (second of two parts). *N. Engl. J. Med.*, 1976; 295: 765-770.
15. Guan T., Miao Y., Xu L., Yang S, Wang J., He H.,Tang X., Cai C., Xu H., Injectable nimodipine-loaded nanoliposomes: Preparation, lyophilization and characteristics. *I. J. Pharm.*, 2011; 410: 180-187.
16. Kirby C., Clarke J., Gregoriadis G., Effect of the cholesterol content of small unilamellar liposomes on their stability *in vivo* and in vitro. *Biochem. J.*, 1980; 186(2): 591-598.
17. Kleppmann W., Taschenbuch Versuchsplanung, Produkte und Prozesse optimieren sixth ed., *Carl Hanser Verlag*, München, 2009.
18. Lee S.C., Lee K.E., Kim J.J., Lim S.H., The effect of cholesterol in the liposome bilayer on the stabilization of incorporated retinol. *J. Liposome Res.*, 2005; 15: 157-166.
19. Mathworks. D-optimal designs, available from: http://www.mathworks.com/help/stats/d-optimal-designs.html.
20. Porfire A., Tomuță I., Muntean D., Luca L., Licarete E., Alupei M.C., Achim M., Vlase L., Banciu M., Optimizing long circulating liposomes for delivery of simvastatin to C26 colon carcinoma cells. *J. Liposome. Res.*, 2014; 9: 1-9.
21. Porfire A.S., Tomuță I., Leucuța S.E., Achim M., Superoxide Dismutase Loaded Liposomes. The influence of Formulation Factors on Enzyme Encapsulation and Release. *Farmacia*, 2013; 61(5): 865-873.
22. Schiffelers R.M., Metselaar J.M., Fens M.H., Janssen A.P., Molema G., Storm G., Liposome-encapsulated prednisolone phosphate inhibits growth of established tumors in mice. *Neoplasia*, 2005; 7(2): 118-127.
23. Slater E.E., MacDonald J.S., Mechanism of action and biological profile of HMG CoA reductase inhibitors. A new therapeutic alternative. *Drugs*, 1998; 36(3): 72-82.
24. Szabo Z.I., Toth C., Hancu G., Muntean D.L., Simultaneous Chiral Separation of Four H1-Anti-histamines by Capillary Zone Electrophoresis Using a Dual Cyclodextrin System. *Chromatographia*, 2015; 78(21-22): 1377-1384.
25. Szabo Z.I., Szocs L., Muntean D.L., Noszál B., Tóth G., Chiral Separation of Uncharged Pomalidomide Enantiomers Using Carboxymethyl-Cyclodextrin: A Validated Capillary Electrophoretic Method. Chirality, 2016;28(3):199-203.
26. Tefas L.R., Muntean D.M., Vlase L., Porfire A., Achim M., Tomuță I., Quercetin-loaded liposomes: Formulation optimization through a D-Optimal experimental design. *Farmacia*, 2015; 63(1): 26-33.
27. Vemuri S., Rhodes C.T., Preparation and characterization of liposomes as therapeutic delivery systems: a review. *Pharmaceutics Acta Helvetiae*, 1995; 70: 95-111.
28. Vlase L., Imre S., Muntean D., Achim M., Muntean D.L., Determination of Spironolactone and Canrenone in Human Plasma by High-performance Liquid Chromatography with Mass Spectrometry Detection. *Croatica Chem. Acta*, 2011; 84(3SI): 361-366.
29. Xu X., Costa A.P., Khan M.A., Burgess D.J., Application of quality by design to formulation and processing of protein liposomes. *I. J. Pharm.*, 2012; 434: 349-359.
30. Xu X., Khnan M.A., Burgess D.J., A quality by design (QbD) case study on liposomes containing hydrophilic API: I. Formulation, Processing design and risk assessment. *I. J. Pharm.*, 2011; 419: 52-59.
31. Xu X., Khnan M.A., Burgess D.J., A quality by design (QbD) case study on liposomes containing hydrophilic API: II. Screening of critical variables, and establishment of design space at laboratory scale. *I. J. Pharm.*, 2012; 423: 543-553.