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Application of statistical experimental design to study the formulation variables influencing the coating process of lidocaine liposomes

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Abstract

In this paper, we have used statistical experimental design to investigate the effect of several factors in coating process of lidocaine hydrochloride (LID) liposomes by a biodegradable polymer (chitosan, CH). These variables were the concentration of CH coating solution, the dripping rate of this solution on the liposome colloidal dispersion, the stirring rate, the time since the liposome production to the liposome coating and finally the amount of drug entrapped into liposomes. The selected response variables were drug encapsulation efficiency (EE, %), coating efficiency (CE, %) and zeta potential. Liposomes were obtained by thin-layer evaporation method. They were subsequently coated with CH according the experimental plan provided by a fractional factorial (2^{5-1}) screening matrix. We have used spectroscopic methods to determine the zeta potential values. The EE (%) assay was carried out in dialysis bags and the brilliant red probe was used to determine CE (%) due to its property of forming molecular complexes with CH. The graphic analysis of the effects allowed the identification of the main formulation and technological factors by the analysis of the selected responses and permitted the determination of the proper level of these factors for the response improvement. Moreover, fractional design allowed quantifying the interactions between the factors, which will consider in next experiments.

The results obtained pointed out that LID amount was the predominant factor that increased the drug entrapment capacity (EE). The CE (%) response was mainly affected by the concentration of the CH solution and the stirring rate, although all the interactions between the main factors have statistical significance.

Keywords: Chitosome; Experimental design; Liposome; Lidocaine; Confocal; Chitosan

1. Introduction

In Pharmaceutical Technology, the development and optimization of the different pharmaceutical dosage forms present a high number of factors influencing the formulation. Normally, in any experiment, one or more factors deliberately modify in the process with the objective to observe its effects in one or more answer variables. Therefore, a complex, expensive and time-consuming formulation studies are often necessary for the development of a product with the required stability properties.

Experimental design methodology is the strategy to project, select or to establish the smaller number of experiments in such a way that the information required is obtained from the most effective and precise way, carrying out the necessary experimentation (Lewis et al., 1999).

The screening experiments are useful to reduce the number of variables to a manageable size so that further experiments can be performed using these key variables for a better understanding of the process (Lewis et al., 1999). This reduction strategy in the number of variables allows experiments to focus process improvement efforts on the key factors and permits the union of various factors of interest in a unique formulation.

Local anaesthetic agents (LA) can be defined as drugs that clinically have been used to produce a reversible loss of sensation

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in a circumscribed area of the body. Many attempts provide an efficient anaesthesia of the skin or mucosal tissues; however, several studies have indicated the limited efficiency on intact skin of available anaesthetic preparations and the need of prolonged applications and high drug concentrations (Morganti et al., 2001; Zempsky et al., 2004).

Lidocaine, the first modern amide LA is an effective and reliable LA of rapid onset, intermediate action and low systemic toxicity. In percutaneous or dental applications, the drug should remain in the skin surface as its uncharged, lipophilic form for a substantial time, so that it penetrates the stratum corneum and desensitize the underlying pain receptors within the skin (Hou and Yu, 1997).

As the stratum corneum is the major obstacle when attempting to deliver drugs transdermally, one of the methods to enhance drug penetration across the skin is to encapsulate the drug in vesicular carriers, like liposomes. In previous papers, we have encapsulated minoxidil (López-Pinto et al., 2005) and ketoprofen–cyclodextrin complexes (Maestrelli et al., 2005), to enhance the permeation depth and rate of these substances across the skin. Local anaesthetics incorporated into liposomes showed greater effectiveness, providing shorter application periods as well as a decrease of side effects (Schmid and Korting, 1996).

Other authors have studied liposome formulations containing lidocaine hydrochloride (LID) (Ottiger and Wunderli-Allenspach, 1997; Glavas-Dodov et al., 2002); nevertheless, the great interest in these delivery systems, prompted us to evaluate the possibility to optimize a liposome formulation containing LID, inputting other factors and mixing variables.

CH is a biopolymer, hydrophilic and biocompatible. In general, it is obtained by alcaline deacetylation of chitin, which is the main component of the exoskeleton of crustaceans. CH is known for being biocompatible allowing its use in various medical applications, such as implantation (Patashnik et al., 1997), injection (Song et al., 2001), or topical ocular application (Felt et al., 1999). In addition, it has been reported that this polymer acts as a penetration enhancer by opening epithelial tight-junctions (Kotzé et al., 1999).

One of the most interesting properties of CH is the capacity of interact with water causing the medium to thicken and after drying forming biodegradable films and membranes with good mechanical properties (García et al., 2004).

Owing to the amino/ammonium groups, the CH shows a natural attraction for some components of the skin and the hair. Due to its positive charge at physiological pH, CH is also bioadhesive, which increases the retention at the site of application. Especially, this property makes CH as a possible good coating agent for transdermal delivery systems.

Several authors have used this biopolymer as liposome coating, to increase the stability towards drug release (Alamelu and Panduranga, 1991; Dong and Rogers, 1991; Henriksen et al., 1997) and for targeting purposes (Takeuchi et al., 1994). It is accepted that the chitosan-coated liposomes have been formed via ionic interaction between the positively charged CH and negatively charged phosphatidylcholine on the surface of the liposomes (Takeuchi et al., 1996; Kozlova et al., 2001).

Taking into account the promising applications of a delivery system carrying LID and the CH coat properties, in this work, we seek to optimize a formulation of LID liposomes coated with CH.

The number of factors that can influence in such a system is large and a small change in one factor may seriously alter the characteristics of the whole system. In this work, we have used a multifactor experimental design methodology to evaluate the influence of five factors on several responses. Two formulation factors: CH concentration and LID amount, and three technological variables: dripping rate of CH solution, stirring rate of colloidal dispersion and time passed to coating process were evaluated. The selected responses were: encapsulation efficiency (EE), coating efficiency (CE) and the liposome surface charge.

The proposed experimental design strategy will allow a rapid evaluation and identification of the most important parameters in determining the stability of vesicles, providing a powerful support for their rational selection during formulation studies and thus, shortening the time necessary for the development of effective dosage forms.

2. Materials and methods

2.1. Materials

L-α-Phosphatidylcholine, PC (60%, w/w purity), cholesterol, CHOL (99%, w/w purity) and α-tocopherol, were purchased from Sigma (Barcelone, Spain). LID was obtained from Genox Farma (Barcelone, Spain) and the chloroform was received from Panreac Chemistry (Barcelone, Spain).

CH samples (viscosity: 50 cP; molecular weight: 70,000 Da) were kind gift from Padetec Interprice (Brazil). Cibacron brilliant red 3B-A was purchased from Sigma (Barcelone, Spain). All other chemicals were at least reagent grade and used as received.

2.2. Preparation of chitosan-coated liposomes

Each sample was prepared according to the experimental matrix. Multilamellar vesicles (MLV) were prepared by the thin-layer evaporation (TLE) method. In a typical procedure, 60 mg (78.94 μmol) PC, 40 mg (103.46 μmol) CHOL and 1 mg (2.32 μmol) α-tocopherol, were dissolved in a small amount of chloroform. The solution was placed in a rotary evaporator (*Büchi R 200/205*) at 58 °C until a thin lipid film was obtained. This film was then hydrated with 4 mL of Sorensen phosphate buffer pH 5.0 containing 10 or 125 mg/mL of LID following six cycles of vortex and the incubation for 30 min.

For liposome coating, appropriate amounts of CH were dissolved in a solution containing 0.5% (v/v) of glacial acetic acid. CH solution (containing 0.1%, w/v or 1%, w/v) was added drop wise into the respective liposome receive suspension under controlled stirring rate at room temperature, followed by incubation at 10 °C for 1 h. The dropping and stirring rates were varied from 0.17 to 0.67 mL/min and 30 to 100 rpm, respectively. The final concentrations of the lipids and CH were half of the original

solutions. The time passed from the production of the vesicles to the coating process was selected 30 min or 24 h.

2.3. Characterization of chitosomes

2.3.1. Encapsulation efficiency (EE)

The entrapment capacity was determined by an indirect method, by using dialysis bags of cellulose acetate (Trotta et al., 2002; Foco et al., 2005). In a previous paper, we have demonstrated the suitability of this method, whose results do not show statistically significant differences in comparison with those obtained by ultracentrifugation technique (López-Pinto et al., 2005). These membranes (*Spectra/Por MWCO 12-14.000*) were kept into a saline solution (0.9%, w/v NaCl) for 1 h before dialysis to ensure the thorough wetting of the membrane. After that, 3 mL of the drug-loaded vesicles were placed into the dialysis bag which was then transferred into 150 mL of a cold phosphate buffer solution (PBS) adjusted to pH 5.0. The receiver medium was stirred for 3 h with a magnetic stirrer. Samples of 10 mL were withdrawn at fixed times from the receiver solution and replaced with equal volumes of PBS solution. Samples were analyzed by UV–visible spectrophotometry (*Hitachi U-2000)* at 263 nm.

The entrapment capacity was calculated from the relationship:

$$\mathrm{EE} = 100 - \frac{Q_t - Q_d}{Q_t} \times 100$$

where *EE* is the entrapment efficiency, Q_t the theoretical amount of LID that is added and Q_d is the amount of LID dialyzed.

2.3.2. Coating efficiency (CE)

The coating efficiency was determined as the difference between the initial amount of CH added to the formulation in the coating process and the amount of CH free in solution after the centrifugation of the coated liposomes.

Samples of 4 mL of LID liposomes coated by CH solution were centrifuged at 3000 rpm for 45 min to separate the fraction of CH adhered and non-adhered to the vesicles. The amount of CH coated in the liposomes was estimated by the difference of the concentration of CH in the separated supernatant.

To calculate the amount of CH free in the supernatant, a colorimetric method was applied (Muzzarelli, 1998). This method is based on the capacity of the protonated amino groups of CH to act as cationic sites for anionic dyes. We use the Cibacron Brilliant Red 3B-A dye (also known as Reactive Red 4).

Initially the dye solution was prepared by dissolving 150 mg of Cibacron brilliant powder in 100 mL of distilled water. An aliquot of 5 mL of this solution was made up to 100 mL with citrate buffer and then adjusted to pH 3.2, to obtain a dye concentration of 0.075 g/L.

The calibration curve was performed by the following procedure: 15, 30, 45, 60, 80, 100, 150, 200 and 250 μL of CH solution were introduced into test tubes. Each tube was completed by different amounts of buffer, to reach the total of 300 μL. Then, aliquots of 3 mL of dye solution were added to all tubes. A tube containing citrate buffer (0.3 mL) and dye solution (3 mL) were used as reference. The absorbance values were measured at 575 nm.

After centrifugation, samples containing 200 μL of the supernatant were added to 3 mL of dye solution. After, they were measured at 575 nm by UV–visible spectrophotometry in a Hitachi U-2000 spectrophotometer. Confocal laser scanning microscopy (CLSM) visualized this parameter.

2.3.3. Confocal laser scanning microscopy (CLSM)

This technique was used to visualize the liposome structure, before and after the coating process. The objective of this procedure was to observe the aspect of the liposomes structures and its integrity after coating.

The confocal microscope used was a Leica TCS SP II confocal unit (Leica, Heidelberg, Germany) equipped with a Krypton–Argon–Helio/Neon laser. This unit was mounted on a Leica DM IRE 2 inverted microscope (Leica, Heidelberg) using HC PL Fluotar Leica lens with magnifications of 10, 20 (dry) and HCX PLAN APO Leica lens 40× (0.85 multi-immersion objective) on its oil position. For excitation of the label, the 488 nm laser line was used and the fluorescence emission was detected above 520 nm.

The following parameters were used for the confocal microscope starts: magnification 10, 20 and 40, laser power 5%, scan modus slow, gain values 400–600, *xyz* mode, 488 nm emission line at 31% maximun power, transmission light channel activated and bidirectional scanning pattern.

Samples were analyzed by using the transmitted light. The samples were compared with the images obtained by fluorescence emission of the dye.

2.3.4. Photonic correlation spectroscopy (PCS)

Surface charges of chitosomes were measured on a Malvern Zetasizer 2000 using a dip cell. The zeta potential (ζ) is deduced from the mobility (U) based on the equation: $\zeta = 4\pi\eta U/\varepsilon$, where η is the viscosity of the medium and ε denotes the dielectric constant.

Samples were diluted with citrate buffer pH 5.0 to achieve sufficient signal with scatter less than 1.5×10^5 counts per second. Measurements were made in triplicate, using the automated procedure provided by the instrument.

2.4. Experimental design

2.4.1. Screening study

In this study, a two-level 2^{k-p} fractional factorial design was employed. The experimental matrix for this design is shown in Table 1. The factors were defined as follows:

- *Factor A*: CH concentration in the coating solution
  High level (+): 1%, w/v
  Low level (−): 0.1%, w/v
- *Factor B*: dropping rate of the chitosan coating solution to the liposome receive medium
  High level (+): 0.67 mL/min
  Low level (−): 0.16 mL/min

Table 1
Experimental matrix corresponding to a fractional factorial design (2^{5-1}) with five factors at two levels each factor

Test	A	B	C	D	E = ABCD
1	−	−	−	−	+
2	+	−	−	−	−
3	−	+	−	−	−
4	+	+	−	−	+
5	−	−	+	−	−
6	+	−	+	−	+
7	−	+	+	−	+
8	+	+	+	−	−
9	−	−	−	+	−
10	+	−	−	+	+
11	−	+	−	+	+
12	+	+	−	+	−
13	−	−	+	+	+
14	+	−	+	+	−
15	−	+	+	+	−
16	+	+	+	+	+

- *Factor C*: stirring rate of the receive medium during the coating process
  High level (+): 100 rpm
  Low level (−): 30 rpm
- *Factor D*: Time period from the liposome production to the coating process
  High level (+): 24 h
  Low level (−): 0.5 h
- *Factor E*: Amount of LID per milliliter of liposome (mg/mL)
  High level (+): 25 mg/mL
  Low level (−): 10 mg/mL

This specific design, comprising 16 runs, is described as a $2^{(5-1)}$ design of resolution *V* (5). This means that we studied $k = 5$ factors overall (the first number in parentheses). However, $p = 1$ of those factors (the second number in parentheses) was generated from the interactions of a full factorial design. As a result, the design does not give full resolution, that is, there are certain interaction effects that are confounded with other effects. In this design of resolution *V* no main effects of the examined factors are confounded with any other interaction of order less than $R = 5 - 1 = 4$. Main effects are not confounded with three-way interactions, but only with four-way interactions. Thus, the three-way interactions in this design are confounded with each other.

The 16 formulations listed in Table 2 were evaluated in random order to nullify the effect of extraneous of nuisance variables. After the three responses had been collected, the system was ready for analysis.

2.4.2. Statistical analysis

The software DOE pack 2000 was used for computation of the main effects and the interactions of the control factors. The main effect of a control factor is the difference in average response observed due to a change in the level of the factor, that is, the change from level − to level + in this case. The application of main effect plots is to determine which set of factors influence the response and to compare the relative strength of the effects. In a main effect graph, we plot the mean response at each factor level and then connect the points by a straight line. If the slope of the line connecting the average responses for a factor is parallel to the *x*-axis, then it implies that there is no main effect present. The greater the slope of the line, the stronger the main effect is.

In order to determine whether these main effects and the interactions among them are statistically significant, it was decided to use the Pareto plot.

This is a useful plot for identifying factors being statistically significant. This graph will show the factor main effect estimates plotted against the factors in the vertical axis. The factors main effects are ordered from A to Z, according its nomenclature, with the interactions between them. This design allows us to separate the factor size and its sign. Then, standard deviation of the test

Table 2
Experimental plan, sample compositions and responses of the different formulations

Trial	CH (%)	DR (mL/min)	SR (rpm)	Time (h)	LID (mg)	EE (%)	CE (%)	Zeta
1	0.1	0.17	30	0.5	100	81.41 ± 4.58	29.97 ± 4.61	34.9 ± 1.8
2	1	0.17	30	0.5	40	36.03 ± 1.97	88.86 ± 0.51	42.7 ± 0.1
3	0.1	0.67	30	0.5	40	60.25 ± 0.60	73.87 ± 0.39	29.6 ± 1.1
4	1	0.67	30	0.5	100	73.42 ± 4.21	97.72 ± 0.04	41.5 ± 0.5
5	0.1	0.17	100	0.5	40	64.47 ± 1.66	42.25 ± 1.68	41.7 ± 0.2
6	1	0.17	100	0.5	100	68.55 ± 0.68	95.97 ± 1.38	36.7 ± 1.9
7	0.1	0.67	100	0.5	100	69.8 ± 5.51	50.12 ± 0.68	36.1 ± 0.2
8	1	0.67	100	0.5	40	52.59 ± 1.08	94.18 ± 0.06	38.7 ± 0.7
9	0.1	0.17	30	24	40	68.4 ± 4.48	35.40 ± 1.53	41.5 ± 1.1
10	1	0.17	30	24	100	66.89 ± 0.15	96.78 ± 0.13	39.4 ± 0.7
11	0.1	0.67	30	24	100	71.12 ± 0.17	20.46 ± 4.31	38.2 ± 1.0
12	1	0.67	30	24	40	63.48 ± 0.90	94.69 ± 0.43	41.5 ± 1.1
13	0.1	0.17	100	24	100	35.57 ± 4.41	82.15 ± 0.84	34.7 ± 0.5
14	1	0.17	100	24	40	64.18 ± 0.37	92.55 ± 0.11	34.6 ± 0.4
15	0.1	0.67	100	24	40	64.94 ± 2.33	63.16 ± 4.55	35.7 ± 1.0
16	1	0.67	100	24	100	71.42 ± 4.56	89.46 ± 1.23	42.3 ± 0.1

CH: chitosan concentration; DR: dropping rate; SR: stirring rate; Time: time from the fabrication of the liposome dispersion until coating process; LID: lidocaine chlorhidrate amound added. Response values for the studied formulations. EE: encapsulation efficiency; CE: coating efficiency; Zeta: zeta potential values ($n = 3$).

(S_e) and of the effect (S_{eff}), and the t-statistic were determined.

$$S_e = \sqrt{\frac{\sum \text{S.D.}^2}{n}}, \qquad S_{eff} = S_e\sqrt{\frac{4}{N}}, \qquad \text{d.f.} = (r-1)n,$$

$$\text{DL} = \pm t S_{eff}$$

where n is the experiment number, $N = nr$, r is the replicate number in each test, d.f. the degree freedom and DL represents the decision limits for each response.

From these results, two regions (lower and upper bounds) were calculated, to indicate the threshold for statistical significance.

3. Results and discussion

The application of the experimental design methodology had the objective of identifying the most significant factors affecting the lidocaine liposome coating process and establishing the best levels for optimizing the considered experimental responses. With this aim, the effects of the different levels of each factor on the considered responses were studied. In order to evaluate these effects, Pareto charts were applied. This graphs shows, in the vertical axis, the calculated effects, and in the abscise axis, the different factors evaluated and their interactions. According to the Pareto charts, the effects that pass through the reference lines (decision limits) are significant.

3.1. Effects on drug encapsulation efficiency

Liposomes have been formulated by incorporating LID into the aqueous compartment of vesicles. The effects of the factors on the encapsulation efficiency are plotted in Fig. 1.

The Pareto plot of the effects shows that the main effects A (CH concentration) and E (LID amount), and the interactions AB (CH concentration–dripping rate), AD (CH concentration–stirring rate), CD (stirring rate–time from liposome production to coating) and DE (time from liposome production to coating–LID amount) are statistically significant at 99% confidence range.

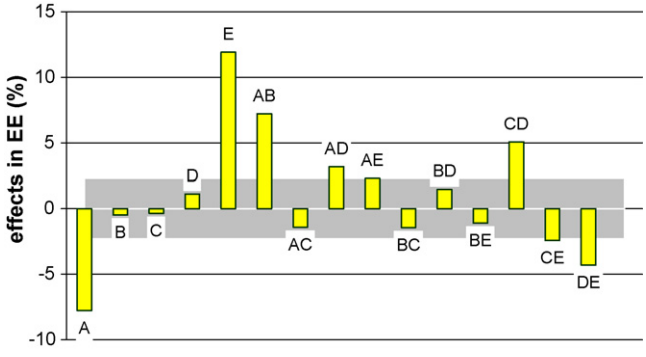

Fig. 1. Graphic analysis of effects for EE (%) by means of Pareto chart. The A–DE nomenclature were the coefficient relative to the change of level of the factors and the second order interactions. (A) CH concentration (%); (B) dripping rate (mL/min); (C) stirring rate (rpm); (D) time from liposome fabrication to coating process (h); (E) LID amount (mg).

The Pareto chart revealed that the factors LID amount (positive effect) and CH concentration (negative effect) considerably affected drug encapsulation capacity. When the LID amount was increased from the lower level (40 mg) to the higher level (100 mg), a positive effect on EE value was obtained. This means that to maximize this response is advisable fix this factor in the higher level, because the formulation allows the inclusion of higher dose per volume unit of formulation.

The degree or efficiency of drug loading into liposomes, assessed as percentage of drug incorporation, depends on a number of factors, including liposome size and liposome composition. In this paper, the influence of the drug-to-lipid ratio on the encapsulation efficiency has been evaluated. We have found that increasing the drug-to-lipid ratio significantly enhanced the retention of the drug. Cationic formulations containing PC, Chol, α-tocopherol and chitosan as the coating agent, had drug-to-lipid ratios of 1:1 and 10:1, representing encapsulation efficiencies of 35.57–81.41%. Several authors (Mohammed et al., 2004; Zhigaltsev et al., 2005) have previously reported this fact.

Lidocaine HCl is a water-soluble drug that is added to the aqueous compartment of liposomes. The pK_a value of lidocaine is 7.87, and thus a high percentage of the molecules are charged at pH 5.0 (Moorman et al., 1986). The ionized LID fraction is localized in the membrane bilayer, partially penetrating into the hydrophobic core of the membrane because of its amphiphilic nature (Jutila et al., 1998). At these conditions, this drug binds avidly to membranes containing phospholipids negatively charged. When the drug-to-lipid ratio is 1:1, chitosan, which also is ionized at this pH, significantly competes with lidocaine in their binding to phosphadidylcholine, making difficult the retention of drug into the vesicles (Lutwyche et al., 1998). However, when the ratio is 10:1, an excess of charged lidocaine exists, minimizing the competence of chitosan. This result was corroborated when the coating efficiency was analyzed: an inverse relationship was showed when the amount of lidocaine was increased, as will be discussed in next section.

On the contrary, the CH concentration had a negative effect on the response studied and the lower level must be chosen for this factor.

These two conclusions have been corroborated by analyzing the dialysis profiles obtained for the different batches. The EE (%) will be obtained from the difference between 100% and the percent of drug dialyzed. As an example, in Fig. 2, we have plotted the LID amount (%) dialyzed for 2 h through the cellulose acetate bags. These figures correspond to four samples whose compositions are described in Table 2. From these profiles, it is possible to see that formulations containing high levels of LID and low levels of CH show an increase in the EE (%), while for the batches with high levels of CH and low levels of LID, the EE (%) has been reduced. So, the effect of these two variables on the drug entrapment capacity has been noted.

This result can be explained taking into account that several authors have been reported that the interaction of positively charged substances with lipid membranes depends on electrostatic attraction at the head group level (Lo and Rahman, 1995).

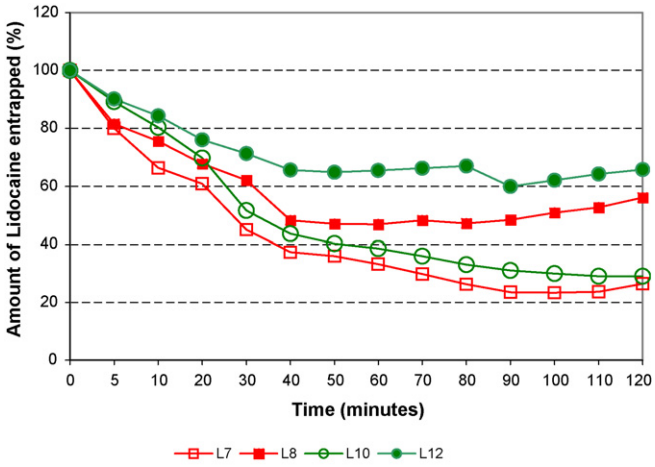

Fig. 2. Dialysis profiles of LID (%) from liposome formulations. In this graph, the amount of LID entrapped into vesicles vs. time, is plotted. L7 and L10 were formulated with 0.1% CH and 100 mg LID. L8 and L12 contained 1% CH and 40 mg LID.

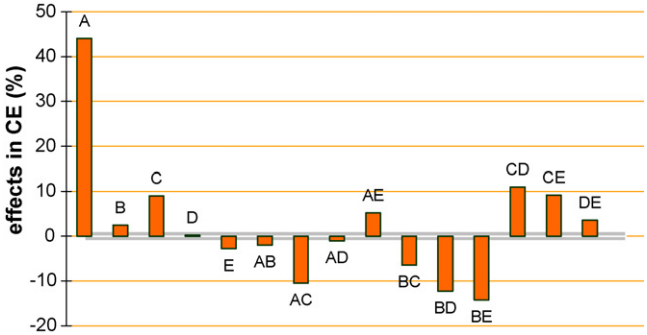

Fig. 4. Graphic analysis of effects for CE (%) by means of Pareto chart. The A–DE nomenclature were the coefficient relative to the change of level of the factors and the second order interactions. (A) CH concentration (%); (B) dripping rate (mL/min); (C) stirring rate (rpm); (D) time from liposome fabrication to coating process (h); (E) LID amount (mg).

CH has strong affinity for the phospholipids of the vesicle bilayer. Due to these conditions both, LID and CH have positive charge and they compete with each other to interact with the phospholipids of the bilayer. This kind of competition has already been reported by other authors with others drugs (Guo et al., 2003).

The mutual repulsion between the cationic drug molecules and the positively charged CH matrix prevents any kind of interaction between them. The presence of a large number of protons resulted from the dissociation of LID also contributes to this repulsive force, reducing the drug encapsulation efficiency, although it is somewhat offset by the chloride ions which are attracted towards the polymer matrix (Ramanathan and Block, 2001).

Two factors are said to interact with each other if the effect of one factor in the response is different at different levels of the other factor. In order to interpret the interactions, it is better to construct the interaction plots (Fig. 3). The lines are non-parallel to each other, so, an interaction exists between the factors. This implies that the change in the mean response from low to high level of a factor depends on the level of the other factor. The

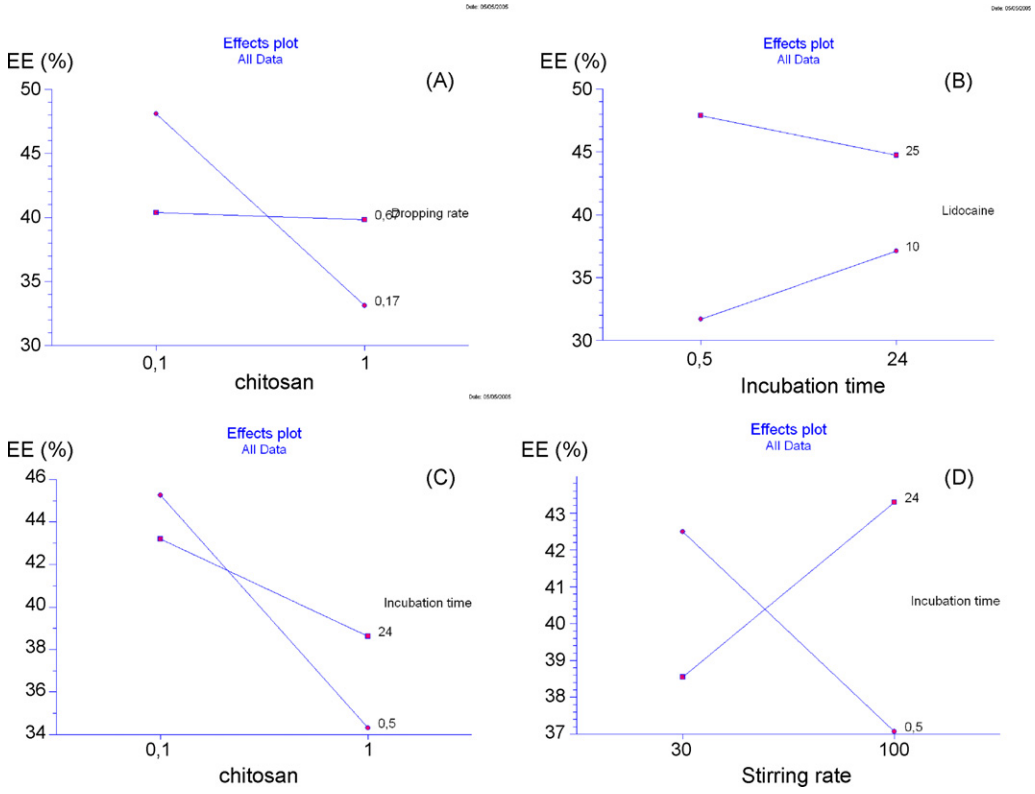

Fig. 3. Main second order interactions of the effects between the factors for the EE (%). (A) AB interaction; (B) DE interaction; (C) AD interaction; (D) CD interaction.

greater the degree of departure from being parallel, the stronger the interaction effect is. In this study, some first-order interactions shown a medium effect (AB, AD, CD and DE) and they could be considered in the planning of next experiments. This means that, although the main effects (dropping rate-B, stirring rate-C and time until coating-D) were no significant, the interaction with other factors produce significant effects on this response.

3.2. Effects on CH coating efficiency

Liposomes or phospholipid vesicles are spherical, self-closed structures composed by curved lipid bilayers that entrap part of the water phase in its interior. In the lipid bilayer, hydrophilic head groups of the phospholipid molecules are positioned outward to the water phase and the hydrophobic chains inward tail to tail. There are numerous reports on the effect of surface coating by polymers to preserve the liposome stability (Feng et al., 2004). The protective effect of hydrophilic polymer coating would depend on the adhesive ability of the polymer onto the lipid bilayers. We tested the effect of a polymer, CH, on the liposome stability.

The main effects and the interactions of the factors affecting the coating efficiency response were plotted in the Pareto charts (Fig. 4). It was observed that the CH concentration is the main factor exercising a high positive effect on the coating efficiency.

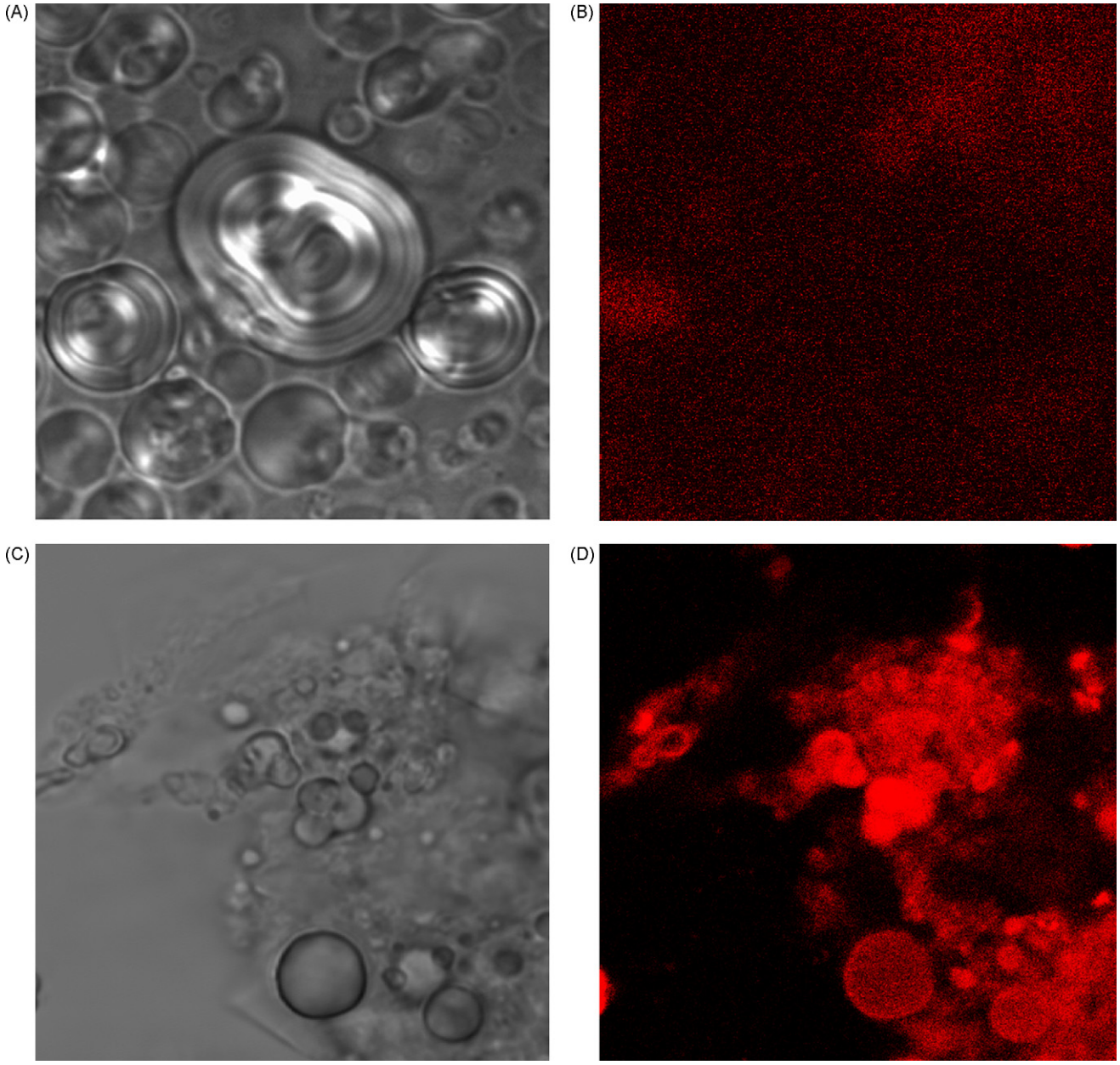

Fig. 5. Representative CLSM images of two formulations of liposomes. (A and B) Liposomes without CH coating and stained with brilliant red. Images were obtained by transmitted light and fluorescent emission probe at 520 nm, respectively. (C and D) Liposomes coated wit CH and treated with the dye. Images were obtained by transmitted light and fluorescent emission probe at 520 nm, respectively.

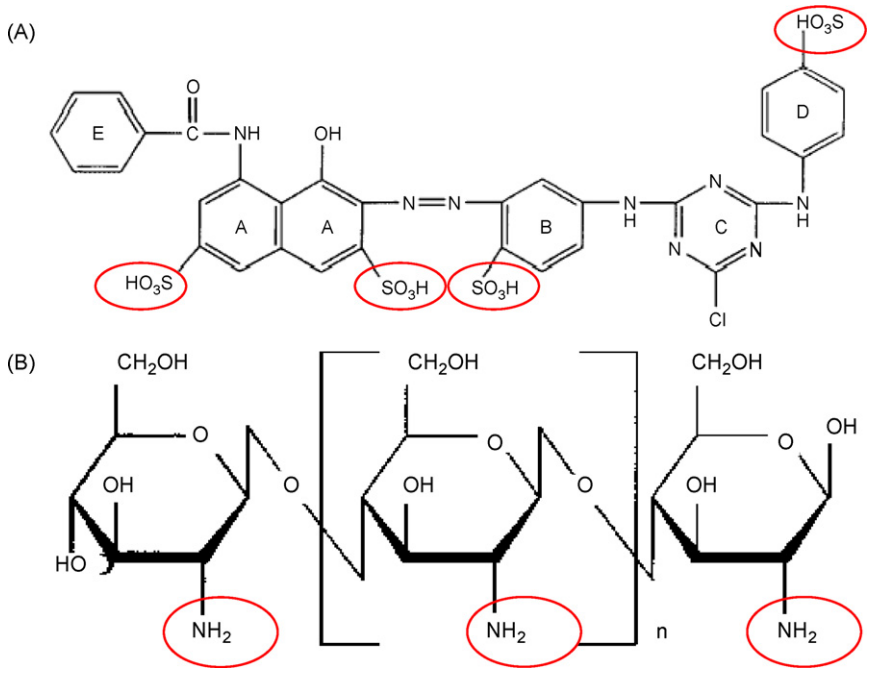

Fig. 6. Molecular structures of: (A) Brilliant Red 4B and (B) chitosan.

This information means that the coating process is favoured by passing from the lower CH concentration (0.1%, w/v) to the higher CH concentration (1%, w/v).

When the CH solution was added to the colloidal dispersion, the CH adhered to the liposome surface. Polymer-coated vesicles were produced and it was probably formed by electrostatic interaction between positively charged CH and the opposite charge on liposome surface (Filipovic-Grcic et al., 2001). The CH covers the surface of the liposomes by forming the ion-complex with PC in the liposome formulation.

On the other hand, the sedimentation volumes of all the samples were determined as a probe of formulation stability. From the zero values obtained, we suppose that at these CH concentrations, all liposomes were individual and uniformly dispersed in the solution. The cationic surface charge probably contributes to the stability of the formulations.

The high positive charge might play an important role in the flocculation and coagulation resistance. The structured adsorbed polymer film now serves to stabilize the particles against particle–particle interaction, presumably through a mechanism of steric stabilization (Guo et al., 2003).

From the Pareto charts, we can conclude that the stirring rate affects significantly the CE response in a positive sense. Therefore, to obtain a good CE, the higher level for this factor must be used (100 rpm).

Although the other main factors (B, D and E) have a low or do not have significance on the coating efficiency, the interactions between them affect this response. Therefore, they must be considered in next optimization designs. It was observed that the effects with minor strength in the Pareto's graphs change the sign when they interact with the other factors, increasing their weight in the influence on the response.

In addition, to elucidate the behaviour of the red dye–CH complex and the affinity of this structure to the phospholipids of liposome, several formulations were visualized by CLSM. Fig. 5A shows an image obtained by transmitted light of a liposome formulation without polymeric coating and treated with the brilliant red. It is possible to observe multilamellar structures and some aggregates among them. When the sample was submitted to the emission range, the affinity of this dye to the membrane phospholipids was negligible (Fig. 5B). However, the reaction between the probe and CH has been shown in Fig. 5C and D. From the images obtained, we can conclude that a complex has been formed between the coating polymer and the dye. The emission of fluorescence throughout the vesicle surface indicates the affinity of the dye to the coating polymer. In Fig. 6, the molecular structure of the dye and CH was shown. The negative charge providing from the sulphurous radical of the red brilliant dye reacts with the cationic charge of CH provided by the ionization of the amino groups, giving rise to an ionic complex.

3.3. Effects on the zeta potential

Zeta potential gives us the information concerning the charge beyond the hydrodynamically stagnant layer. The movement of a charged surface with respect to an adjacent liquid phase is the basic principle underlying this technique.

The effects of CH coating on the characteristics of liposomes, as the surface charge, are shown in Table 2. We could observe that coating the negative-charged surface of liposomes with CH shifted the zeta potential from negative value to a positive value, which is an indicator of surface charge. This motive justifies the use of chitosan-coated liposomes as mucoadhesive delivery

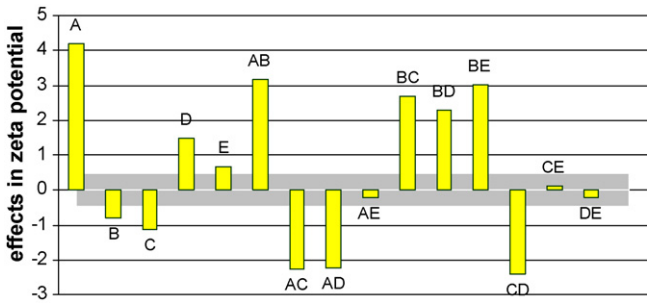

Fig. 7. Pareto chart to analyze the effects for the zeta potential. The A–DE nomenclature were the coefficient relative to the change of level of the factors and the second order interactions. (A) CH concentration (%); (B) dripping rate (mL/min); (C) stirring rate (rpm); (D) time from liposome fabrication to coating process (h); (E) LID amount (mg).

system: their positively charged surface favours the adhesion to the cells membranes, which are normally negatively charged (Feng et al., 2004).

The coating process significantly affects the surface charge of the vesicles. This response must be maximized, since a good adhesive reaction between the chitosomes and the skin may be produced. The interaction between a charged particle and a strong polyelectrolyte of an opposite charge has in most cases interpreted in terms of a "patch" or "charge mosaic" model (Henriksen et al., 1997).More recently, the most accepted theory with respect to liposome–chitosan interaction is that CH covers the surface of the liposomal formulation (Takeuchi et al., 2003; Guo et al., 2003). In this paper, the cationic liposomes were prepared by incorporating two different concentrations of CH. The positive values of the zeta potential of the resultant liposomes increased proportionally to the increase of the amount of CH in the liposome formulation (Fig. 7).

On the other hand, an inverse relationship between the stirring rate (factor C) and the zeta potential has been shown. It is logical that higher stirring rates confer to the colloidal dispersion particle-to-particle collision, making difficult the coating process (Henriksen et al., 1996).

In general, physical stability has been increased when liposomes were coated with chitosan. This stability has been proved by measuring the zeta potential for 3 months. Formulations containing chitosan do not exhibit statistically significant differences in zeta potential data, whereas formulations without chitosan showed lower zeta potential values versus time.

4. Conclusion

Experimental design strategy is a very useful tool in liposome formulation studies. The screening assay is especially important to identify and quantitatively define the significant factors influencing the stability of the colloidal system. Graphic analysis of the effects enabled identification of the factors influencing the EE, EC and zeta potential responses and allowed the determination of the best level for the response optimization.

The different results obtained underlined the role of the CH presence in modifying the encapsulated drug amount and the surface charge density of vesicles.

According to our results, to prepare a liposome formulation with the maximum drug trapping efficiency, the coating solution must be prepared with the lower CH level (0.1%, w/v) and with the higher LID level (100 mg). However, to maximize the CE response, it is necessary to use the higher level of CH (1%, w/v), to stir the solution at a rate of 100 rpm (high level) and to add the lower LID level (40 mg).

It is important to point out that these contradictory results give rise to planning other experiments to clarify and optimize the concentrations of both components that maximize the two responses studied.

The optimization process of the formulation combining all the factors affecting significantly these responses is currently in progress. Several optimization methods are being applied and they will be the purpose of the next papers.

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