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Abstract: The aim of this work was to develop a freeze-dried microemulsion using Design of Experiment and loaded with Amphotericin B, which is quite unstable in aqueous media, as a drug model. Different types of cryoprotectants were studied. The microemulsion containing Maltose at 5% (w/w), frozen at the temperature of –80 °C, and performed with a 24h of freeze-drying time yielded the best results. The freeze-drying process reduced the microemulsion droplet size and don't change the AmB content. Therefore, microemulsions containing Maltose are suitable for drug incorporation and the freeze-drying was able to enhance the drug stability in the system.

Keywords: Amphotericin B; statistical experimental design; lyophilization; dispersed system.

1. Introduction

Colloidal drug delivery systems are become more attractive in pharmaceutical technology because they are able to control drug release and to improve bioavailability [1]. Microemulsions (MEs), optically transparent systems with low viscosity, are thermodynamically stable dispersions of two immiscible liquids, stabilized by an interfacial film of a surfactant, usually in combination with a co-surfactant [2]. They have shown several advantages over conventional formulations, including enhanced drug solubility, good thermodynamic stability, an increased surface area improving absorption and enhancement of transdermal passage [3]. These properties give MEs potential applications in the food, cosmetic and pharmaceutical industries when solubilization of lipophilic or hydrophilic ingredients is necessary [4]. In pharmaceutics, MEs are used as vehicles to deliver a number of drugs due to their thermodynamic stability, simple preparation and good appearance [2].

Amphotericin B (AmB) is the drug of choice for the treatment in immunodeficient patients affected by systemic fungal infections [5]. However, due to the high incidence of adverse drug reactions, i.e. cardiotoxicity and hepatotoxicity of conventional formulations [6], AmB has been incorporated into a number of colloidal drug delivery systems, including MEs, in order to improve its therapeutic index [7, 8].

Generally, pharmaceutical MEs are produced with water as the continuous phase, which carries some risks such as microbiological contamination, degradation by hydrolysis and loss of pharmacological activity of the drug. A possible solution to these problems consists of the use of freeze-drying process, also known as lyophilization. Freeze-dried products have good stability and are easy to transport and store [9]. However, the freeze-drying process for MEs has rarely been studied in pharmaceutical technology due to the difficulties that arise during freezing of colloidal systems. Lyophilization consists in removing water by sublimation through three steps: freezing, primary drying and secondary drying [10]. During this process

stress may be generated that could destabilize the colloidal structure of the MEs, in particular the freezing stage, during which the crystallization of ice may produce mechanical stress. However, cryoprotectants (CP) can be used in order to protect these systems against damage [11]. Among the compounds that can exert cryoprotective effects, carbohydrates are interesting because are chemically innocuous and can be easily vitrified during freezing, supporting their use as CPs in the freeze-drying process [8].

The freeze-drying process has been used for several years in the pharmaceutical and biotechnology industries, leading to products destined for various administration routes such as parenteral, oral, nasal or pulmonary. However, the freeze-drying of colloidal systems is delicate due to the tendency of the droplets to interact and due to the damage caused by the freezing process, which can increase the droplet size and destabilize the system [9, 12]. In fact, there are very few reports in the literature of the successful freeze-drying of colloidal systems such as ME [12, 13].

The quality of the final freeze-dried product can be influenced by several factors relating to the formulation, to the container, to the equipment and to the freeze-drying process [9]. Therefore, the process efficiency must be optimized through adjustment of these factors. Indeed, process monitoring is crucial in order to obtain a product with the desired quality. It is necessary to select suitable freeze-drying parameters, evaluate the effects of these parameters and their possible interactions with the entire process. Therefore, Design of Experiment (DOE) has been used for the optimization, modeling and characterization of the freeze-drying process of some pharmaceutical products that are not colloidal systems [14].

DOE is a statistical approach used to determine the influence of several independents variables on the dependent variable of the process. The optimal design allows the time and cost of the experimentation to be reduced, as well as improving the process yield [15]. Therefore, this method is used much more often than the One Factor at a Time Method, which is

time-consuming and expensive because it requires a large number of experiments and does not examine interactions between the variables [16]. The response surface methodology is a technique of DOE that combines mathematics and statistics to analyze the relative significance of different parameters, finding the optimal working conditions, by combining a small number of variables, resulting in fewer experiments [17].

The aim of this work was to develop a freeze-dried ME product as a model to design freeze-dried ME systems containing drugs and, thereby, improve their stability. Therefore, AmB was used as a model drug incorporated within the ME. A DOE approach was used in order to establish the ideal CP content and the technical procedure to produce freeze-dried colloidal systems. Important parameters, which can influence the droplet size of this system, such as concentration and type of CP (Mannitol (MN), Glucose (GC), Lactose (LT) Sorbitol (ST) and Maltose (MT)), freezing temperature and freeze-drying time were also evaluated.

2. Materials and Methods

2.1 Materials

Miglyol® 812 was obtained from CONDEA Chemie GmbH (Hamburg, Germany), Lipoid® S100 was purchased from LIPOID GMBH (Ludwigshafen, Germany), Tween® 80, Mannitol (MN), Glucose (GC), Lactose (LT) Sorbitol (ST) and Maltose (MT) were obtained from Sigma Aldrich Inc (St. Louis, USA). The Na_2HPO_4 and the $NaHPO_4$, used to produce phosphate buffer pH 7.4, were purchased from Vetec Química Fina Ltda (Rio de Janeiro, Brazil). Ultra-pure water obtained by a Milli Q water purification system (Merck Millipore, Massachusetts, U.S.A.) was used along the experiments.

2.2 ME preparation

The MEs were prepared by mixing 68% (w/w) of phosphate buffer pH 7.4, 14.7% (w/w) of Tween® 80, 6.3% (w/w) of Lipoid® S100 and 11% (w/w) of Miglyol® 812 using magnetic

stirring, following by three cycles of sonication (40 watts power output for 1.5 min) using a Vibra Cell 75041 (Bioblock scientific) and ultrasound bath (120 watts power for 3 min) using a USC-1800A (Unique). The MN, MT, GC and LT were added to the MEs prior to the freeze-drying process for the DOE study.

2.3 Sample characterization

2.3.1 Macroscopic aspect, pH evaluation, isotropy and conductivity analysis

The color and homogeneity of the freeze-dried products (cake) were evaluated with the naked eye. The isotropy of the samples was evaluated by polarized light microscopy using an Olympus BX4 (Olympus Corporation, Tokyo, Japan) apparatus. The electrical conductivity was measured using a DM-32 conductivity meter (Digicrom Analytical, SP, Brazil), with a cell constant of 0.11 cm^{-1}. The pH values were measured by a PG-2000 pHmeter (GEHAKA, SP, Brazil). All analyses were performed in triplicate at 25 ± 2 °C.

2.3.2 Dynamic Light Scattering (DLS)

In order to evaluate the influence of the different CP types and concentration on the ME droplet size before the DOE study, MT, MN, GC, LT and ST, as a powder, were added (and dissolved) to the MEs at 5% (w/w), 10% (w/w), 15% (w/w) and 20% (w/w) and the droplet size of all preparations was analyzed. The samples were previously diluted with water in a ratio of 1:20 and their droplet size distribution was evaluated using a DLS, ZetaPlus (Brookhaven Instruments Corporation, NY, USA). Previous experiments revealed that this dilution process has no influence on the DLS measurements.

2.3.3 Differential Scanning Calorimetry (DSC)

In order to characterize the type of system (water-in-oil, bicontinuous or oil-in-water) [18], the thermal behavior of the ME and its components were analyzed by DSC using a DSC-60

Shimadzu (Shimadzu Scientific Instruments, Kyoto, Japan). The samples (5-15 mg) were weighed into aluminum pans. The thermal analyses were performed by a cooling step on a TAC-60i system (Shimadzu Scientific Instruments, Kyoto, Japan) at a predetermined rate from 25 °C to -40 °C. After 10 min at -40 °C, the samples were heated at 5 °C/min to 110 °C. A flow of nitrogen atmosphere of 50 ml/min was used.

2.4 DOE methodology for freeze-drying conditions

In order to determine the optimum parameters for the freeze-drying process, the DOE methodology was performed for ME systems for which no changes in droplet size were observed after CP incorporation. The freezing temperature (x_1), the CP type and concentrations (x_2), and the freeze-drying time (x_3) were chosen as independent variables. The ME droplet size after freeze-drying process, on the other hand, was chosen as the dependent output response variable. The experimental levels of independent variables for ME droplet size after freeze-drying are given in Table 1.

Table 1.

The levels of the variables, the CP concentration and the freeze-drying time were chosen in accordance with the values commonly found on the literature. On the other hand, the freezing temperature used was selected according to the temperature attained by the use of liquid nitrogen (-196 °C – the minimum temperature), the temperature produced by a regular freezer (Frost Free 260 (Brastemp, São Paulo, Brazil)) (-20 °C – the maximum temperature) and the temperature produced by a ultra-freezer Glacier NU-9438 ULT Freezer (NuAire,Inc., Minnesota, U.S.A.) (-80 °C – which is the DOE replicated point). A two-level three-factor full-factorial design with three replicated points leading to 8 experimental randomizations runs was performed for each CP (Table 2).

Table 2.

Freeze-drying was performed using a Christ Alpha 1-2 LD freeze-dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). After the freeze-drying process, the MEs were reconstituted by adding the same amount of water as that lost during the process, followed by vortex mixing and one cycle of sonication (40 watts power output for 1.5 min), after which the droplet size was measured by DLS.

The effect of the studied variables was graphically and statistically interpreted using the Statistic software version 7.0 (StatSoft Inc., Oklahoma, USA). Based on the statistical results, the parameters that would produce a dry powder ME after freeze-drying and that would present the smallest droplet size values after reconstitution were determined. Thereafter, the ME systems that showed the best aspect and concentration of CP, and the best freezing temperature and the freeze-drying time, was produced and thermodynamically characterized for their pH, conductivity and droplet size before and after the freeze-drying process. The optimized formulation was then used for further studies, including drug loading.

2.5 Incorporation of AmB to the ME system

AmB was incorporated, as a model drug, into the optimal ME system determined by the DOE methodology. The aim was to observe the efficiency of the freeze-drying process on the production of dried ME systems containing drugs. The production of a dried powder for this system was verified and pH, conductivity and droplet size before and after freeze-drying were measured. Furthermore, the influence of drug loading on the thermodynamic stability of the ME was evaluated.

The incorporation of AmB was performed by adding the drug to the ME to a final concentration of 2×10^{-3} M, under continuous stirring. In order to improve the AmB dissolution, the pH of the ME was increased ($pH \geq 10.0$) by the addition of sodium hydroxide

solution (NaOH 1 N). Afterwards, the pH was reduced using hydrochloric acid solution (HCl 1 N) to physiological pH (pH 7.4) [7].

2.6 Spectrophotometric analysis

A spectrophotometric assay was performed to analyze and compare the amount of AmB before and after the freeze-drying process, and to evaluate its stability. The experiment was carried out using a Biochrom Libra S32 UV-VIS spectrophotometer (Biochorom US, Massachusetts, USA), at the wavelength of 405 nm, by measuring the absorbances of the reference solution (methanol) and of the ME with and without AmB. The assay was performed in triplicate. The ME samples were first diluted with DMSO: methanol (1:9) at the ratio of 1:10 and, then, 50 µL of this solution was diluted with 20 ml of methanol. The drug loading efficiency was calculated by comparing the AmB concentration before (AmBb) and after (AmBa) centrifugation (Centrifuge 5410, Eppendorf, Hamburg, Germany), as shown in Eq. (1). The method was performed in triplicate by centrifuging 1 ml of ME with and without AmB at 14,000 g for 20 min to precipitate any insoluble content. Moreover, an aliquot of 100 µL of the supernatant of each sample was used to evaluate the AmB content as previously described.

$$Loading\ efficiency = 100 \times \frac{AmBa}{AmBb} \quad (1)$$

2.7 Statistical Analysis

The statistical tests used in the experiments were the Analysis of Variance (ANOVA) performed to analyze the statistical significance between 3 groups, followed by the Dunnet post-test, to compare with the control or the Bonferroni to compare all samples. Finally, a paired t-test was used in case of 2 unpaired groups. p values less than 0.05 (p <0.05) were considered to indicate significance.

3. Results and Discussion

3.1 Characterization of the ME system before adding the CP

3.1.1. Macroscopic aspect, pH evaluation, isotropy and conductivity analysis

The ME, a homogeneous and clear yellowish product that appeared dark under cross-polarized light microscopy (no birefringence), was classified as an isotropic system. Its pH value was 7.1 ± 0.09, which is considered physiologically acceptable.

In general, MEs can be divided into 3 types: water-in-oil (W/O), bicontinuous, and oil-in-water (O/W) [19]. A correlation between ME structure and electrical conductivity has been demonstrated and can be used as a tool to assess its properties [20]. The electrical conductivity of the studied ME was 832 ± 22 µS cm^{-1}. It is well known that O/W MEs have conductivity values similar to the aqueous phase [21, 22]. This high value is probably due to the large volume percentage of water in the system, from which can be inferred that the ME is of the O/W type [23, 24].

3.1.2. DSC analysis of ME components

The thermal behavior of the ME and its individual components (phosphate buffer solution alone, oil phase and surfactants) was evaluated by DSC analysis (Figure 1A and Figure 1B). Figure 1A shows thermal events associated with the ME: a large exothermic peak at -13.54 °C during the cooling curve, which was due to water freezing, following by an endothermic peak at 4.26 °C during the heating curve explained by the melting of water. A second, broad endothermic peak for ME at a temperature between 50 °C and 100 °C with an enthalpy value of -1.57 kJ/g probably represents water loss. It was also possible to observe small exothermic peaks for Tween® 80 and Miglyol® 812, following by only one endothermic peak for Miglyol® 812.

Figure 1.

Since it represents the freezing of water during the cooling curve, the position of the exothermic peak may indicate the state of water in the ME. In fact, the strong interaction among water and surfactant molecules compared to the weaker interactions among the water molecules themselves or water molecules and phosphate buffer may induce a large change in the temperature at which the water freezes [25]. The exothermic peak on Figure 1A indicates the freezing of water with less molecular interaction with surfactant molecules, since the onset freezing temperature of ME is approximately, at -18 °C, which is near to that of phosphate buffer solution (-19.09 °C). Moreover, no peaks characteristic of Lipoid or Tween® 80 were observed in the ME thermal profile, which suggests not only a strong interaction between the surfactants and other components of the system, but also that they could be located at the internal phase or at the interface of the ME droplet and not in contact with the continuous phase.

Therefore, the results of DSC measurements lead to the conclusion that water is the continuous phase [3], thus the ME is of the O/W type, confirming the results of the electrical conductivity.

3.1.3. Droplet size evaluation after addition of CP

Based on the hypothesis that ME stability could be modified by the type and concentration of the CP used, while ,on the other hand, the CP could affect both the droplet size distribution and the freeze-drying process, the influence of different CP types and concentrations in the ME systems were evaluated.

Macroscopically, all the ME systems, with and without CP, were clear, isotropic and homogeneous. On the other hand, those containing LT and the MN presented a turbid aspect when used at 15% (w/w) and at 20% (w/w), respectively, showing that these additives are not very soluble in the ME system.

The influence of CP addition on the droplet size can be observed in Figure 2. MEs showed an average diameter of 22 nm that do not change with the addition of MT, MN, GC and LT

(p>0.05). However, in the case of ST, the addition of even a small amount caused an increase in the ME droplet size (28 nm) and with the highest concentration it reached 62 nm. Those variations, compared with the ME without CP, were statistically significant (p<0.05).

Figure 2.

Therefore ST was excluded from the DOE, while the other CPs: MN, MT, GC and LT, at concentrations from 5% (w/w) to 20% (w/w) may be used to protect MEs during the freezing and drying steps required for the freeze-drying process, since they did not cause significant changes in the ME droplet size.

3.2 DOE for freeze-drying parameters

It has known that many factors, mainly related to the process, can influence the quality of the freeze-dried product. On this study three of them were evaluated: the freezing temperature, the freeze-drying time and the cryoprotectant concentration. The analysis of the freezing temperature is important because small and numerous ice crystals are formed using fast freezing whereas larger and less numerous crystals are produced when applying slow freezing. Such phenomena influence the further freeze-drying steps, since diffusivity is related to the pore size (the movement of water vapor are facilitated in large ice crystals). On the other hand, the freeze-drying time also influences on the final product. Indeed, if the time is not sufficient, the water would not evaporate properly, and the product will collapse. Furthermore, the cryoprotectant type and concentration is a key factor to be evaluated since these components could avoid the droplet aggregation or fusion during the freezing. Also, it could avoid phase transition of hydrated phospholipid during the drying steps. Therefore, a suitable amount and type of cryoprotectant is mandatory to stabilize the system [12].

Among the samples submitted to the freeze-drying process, those containing MT and LT produced dry powders. Disaccharides, the class to which these CPs belong, are more effective

freeze-drying protectants then other types of molecule because they are able to form an amorphous sugar glass [9].

Although the ME-LT at the lower level (5% (w/w)) produced a powder, it did not appear to be completely dry. This is probably because the concentration of CP was not high enough to completely stabilize the ME [26]. On the other hand, all MEs containing MT produced dry powders, showing the high capacity of this CP to stabilize freeze-dried MEs.

The use of the monosaccharide GC resulted in freeze-dried cakes, which might been generated due to the primary drying temperature, during the freeze-drying process, being above the glass transition temperature (Tg) (transition temperature range in which an amorphous sample in the glassy state change to a rubbery state or the reverse [27]) of the MEs containing GC, causing the collapse of the product [28].

Only the ME containing MN at 20% (w/w), frozen at the highest level (-196 °C), yielded dry powders after freeze-drying, probably because only this MN concentration was sufficient to stabilize the system while the rapid cooling inhibited the MN crystallization [29]. However, at this concentration, this CP was not soluble in the system and after a period of time at room temperature the dry powder changed to a rubbery state, revealing that MN was not the best choice as CP. The MEs containing MN at the other DOE levels (5% (w/w) and 12.5% (w/w)) did not produce powders, probably due to the tendency of MN to partly crystallize into MN hemi-hydrate. This could release water on conversion to the anhydrous crystal form and, thereby, cause phase separation in the cryo-concentrated portion of the frozen samples, resulting in the loss of CP activity and destabilization of the system [16, 30].

Formulations in which a dry powder was generated, ME containing MN 20% (w/w) and all samples of MEs containing MT and LT, were reconstituted with the same amount of water as that lost during the freeze-drying process and presented a Winsor IV ME system. Moreover, the droplet sizes of these samples were evaluated. Also, a Pareto's Chart, to investigate the

standardized effect of the independent variables and their interaction with the ME droplet size, was constructed. The results for MEs containing MT showed that the “square freezing” is the most important factor influencing the droplet size. The negative coefficients for the square freezing variables indicated an unfavorable or antagonistic effect on the droplet size. Therefore, the closer to the lower limit (-1 level) of the square freezing temperature (-20 °C), larger the droplet sizes. In other words, the higher the freezing temperatures, the larger the ME droplet sizes (Figure 3a) [31].

Freezing is the stage at which most of the water is separated from the solutes to form ice and concentrated solution [10, 32]. In the specific case of MEs, the “concentrated solution” is composed of oil, surfactants and buffer salts. Increasing the concentration may enhance the interaction between the droplets, leading to their aggregation or fusion and increasing the droplet sizes [26].

The Pareto’s Chart analysis of the MEs containing LT (Figure 3b) showed positive coefficients, for model components (x_2, x_3, x_2x_3, x_1x_3), and a synergistic or favorable effect on the droplet size. Additionally, the CP concentration was the most significant variable for droplet size variation [31].

Figure 3.

Date et al. also observed that the droplet size of MEs increased with CP concentration [33]. Additionally, Abdelwahed et al. demonstrated that certain amount of CP was necessary to effectively preserve the droplet size and ensure a maximum stabilization of nanoparticulate systems [9].

An empirical second order polynomial equation was developed for the response variable, droplet size, in terms of the three independent variables. This equation for MEs containing MT is expressed by the Eq. (1), and for MEs containing LT by the Eq. (2) in which **yi** (i=1, 2)

represents the response variable (droplet size). The coded values of the test variables are represented by x_1 (freezing), x_2 (CP concentration) and x_3 (freeze-drying time), respectively.

$$y_1 = 23.4 + 6.11x_1^2 \quad \text{Eq. (1)}$$

$$y_2 = 30.93 + 9.86x_2 + 4.19x_3 + 2.26x_1x_2 + 3.19x_1x_3 + 4.16x_2x_3 \quad \text{Eq. (2)}$$

The statistical significance, evaluated using the Fisher's F-test and ANOVA, showed, based on probability values inferior than 0.05, that it was indicated for the quadratic term of freezing (x_1^2) in MEs containing MT. On the other hand, MEs containing LT presented statistical significance for concentration (x_2), freeze-drying (x_3), interaction between them (x_2x_3), and interaction between freezing and freeze-drying (x_1x_3). Furthermore, the ANOVA results (Table 3) for both MEs showed that the calculated F value was found to be greater than the tabulated F value at the CP 5% (w/w) level, indicating that the treatment combinations are significant. Moreover, concerning the lack of fit and pure error, the calculated F < tabulated F demonstrated that the model was predictive [17]. The value of the coefficient of determination for MEs containing MT and LT were, respectively, $R^2 = 0.9236$ and $R^2 = 0.9120$, indicating that 92.36% and 91.2% of the response variability could be explained by the previously discussed models.

Table 3.

The effect of CP concentration (for ME with MT) at the lowest level of freeze-drying time showed that the lowest droplet size was observed using the lowest CP level (5% (w/w) of MT), regardless of the freezing temperature. However, at 48 h of freeze-drying time, the droplet size varies depending on the freezing temperature. In fact, freezing at -196 °C generates smaller ME droplet size when 5% (w/w) of MT was used, while for high freezing temperatures (-20 °C) a 20% (w/w) of MT was necessary to achieve the same result. It was also observed that the

droplet size increased with the CP concentration when the rapid freezing process (-196 °C) was used.

The analysis of the marginal means of ME containing LT showed that for both the high and the low level of freeze-drying times (48 h and 24 h) the ME droplet size increased with increasing CP concentration, regardless of the freezing temperature. Nevertheless, at the minimum freeze-drying time level (20 h) the freezing temperature of -20 °C produced a larger droplet size than the one at -196 °C. Otherwise, the freeze-drying time of 48 h led to a lower droplet size at this longer freezing time. Both marginal levels of freezing temperature showed decreased droplet size with decreasing CP concentration.

The relationship between the dependent and independent variables was further elucidated by the analysis of response surface diagrams, which was able to evaluate the relative significance of all factors involved in the process (Figure 4). Throughout this analysis it was possible to determine the optimum operational conditions for the system and the process [34]. In this study, the constant value of the remaining variables were maintained at the level 0 (central point). It was observed that the ME droplet size decreased with the reduction on the freeze-drying time and MT concentration in a linear way. However, when freezing time and other independent variables were compared, no direct linear relationship among them was observed. Nevertheless, it was possible to identify the smaller droplet size values of ME-MT at - 80 °C of freezing temperature (Figure 4a and Figure 4b).

The response surface analysis revealed that the droplet size increased with increasing LT concentration and freeze-drying time. In fact, the smallest droplet size was found at the central point of freezing temperature (Figure 4c and Figure 4d).

Figure 4.

Therefore, the best parameters for producing small droplets for freeze-dried MEs using either MT or LT as CP were a concentration of 5% (w/w), a freezing temperature of -80 °C and

a freeze-drying time of 24 h. Since at this concentration ME-LT become rubbery few minutes after the freeze-drying process and at a higher concentration the CP could not be dissolved in the system, MT was chosen as the best CP for this ME.

3.3 Characterization of the ME before and after freeze-drying

The general physicochemical characteristics of the ME-MT at 5% (w/w) showed some changes before and after the freeze drying process. Before freeze drying, the ME-MT presented a droplet size of 48.13 nm ± 1.57, a pH value of 7.23 ± 0.04 and a conductivity value of 749.1 $\mu S\ cm^{-1}$ ± 25.65. After the freeze drying process and subsequent dispersion with water, its macroscopic appearance remained homogeneous and a clear yellowish product was obtained. However its pH, conductivity values and droplet size were slightly different (Table 4).

Table 4.

The pH value decreased, probably due to the crystallization of the buffer salts during the freezing drying process [10]. However, even at this pH (6.86), the ME-MT is physiologically acceptable.

The reduction on the droplet size of the ME-MT might be due to the co-surfactant role that CP could play. The short poly-hydroxyl chain-alcohol could be adsorbed and intercalated into the interfacial film of the ME after the freeze-drying process and reconstitution, thereby, decreasing its surface tension [35, 36]. However, at this droplet size (20.13 nm) the sample remains within the ME droplet size range of 10 – 100 nm [37]. Furthermore, the droplet size result after freeze-drying corroborates experimentally with the Eq. (1) previously obtained from the DOE, since replacing the used coded value ($\mathbf{y_1 = 23.4 + 6.11x_1^2}$) a droplet size close to 23.4 nm, which were within the range obtained in the experiment (16.83 nm – 23.43 nm), was expected.

The decrease of conductivity just after CP addition and before the freeze drying process was probably due to its protective mechanism that enhances the viscosity of the system due to the interaction between the hydroxyl from the CP and the water molecules. In fact, it is well known that the crystallization of ice can be suppressed by the increase on the viscosity of the medium, which limits the mechanical damage [26]. Therefore, a decrease in the amount of ions can induce a reduction in the conductivity value of the ME-MT. On the other hand, the increase of this conductivity value in the reconstituted ME-MT may be due to the presence of maltose in aqueous media, or due to the H^+ from hydrolysis of maltose.

The protective mechanism of the CPs may be also explained by (i) the formation of an eutectic in the presence of water, due the multi-hydroxyl compounds, that leads to the formation of amorphous or imperfect ice crystalloids; (ii) the maintenance of the spatial orientation and distance between the droplets when the ice sublimes during the freeze-drying process, preventing the formation of aggregates [38]; and (iii) the amorphous glass formation into which the cryo-concentrated solution may vitrify at its Tg. The immobilization of the droplets within a glassy matrix of CP can prevent their aggregation and protect them from the mechanical stress of ice crystals [28].

3.4 Incorporation and freeze-drying of ME containing AmB

The incorporation of AmB into the ME generated a product with a homogeneous and clear yellowish aspect. The addition of MT at 5% (w/w) did not cause any significant changes in the ME appearance neither in the droplet size (from 57.4 ± 4.1 nm to 54.1 ± 2.4 nm). However, the freeze-drying process (24 h of freezing at - 80 °C and freeze-drying for 24 h) altered the AmB-ME formulation, although a dry powder was produced. In fact, the reconstituted AmB-ME, which was made by adding the same amount of water as that lost during the freeze-drying process and using a probe sonicator with an iced bath for fast reconstitution, showed changes in its electrical conductivity, pH and droplet size (Table 5). The

aforementioned reasons for changes in unloaded ME-MT formulations can also explain such changes in the AmB-ME product.

Table 5.

The addition of AmB increased the ME droplet size and the conductivity value (Table 4 and Table 5). The diameter of the droplet size was changed, probably, due to the AmB organization in the oil-water interface, increasing the volume of the droplets [39]. In fact, the ionization of functional groups on AmB (carboxyl and/or amino groups) as well as the NaOH and HCl added to solubilize and recover the neutral pH, may increase the amount of ions in the system, thereby, increasing the electrical conductivity [40]. The increase in the droplet size may be explained by the partitioning of AmB molecules into the oil phase since it is insoluble in water, increasing the oily phase volume. Furthermore, due its amphiphilic properties, the molecule might be located in the system interface, also increasing the droplet size [41].

The UV-Vis spectrophotometric method showed to be robust, efficient and sensitive. Thus, the linear equation ($y = 1.2565x - 0.0257$ and $R^2 = 0.9992$) could fit more than 99% of the experimental data. Therefore, the methodology may be confidently used for quantitative analysis of AmB. The AmB content of the ME remained unchanged after the freeze-drying process ($p>0.05$). Therefore, MT at 5% (w/w) was effective in preventing ME diameter changes and loss of AmB.

Although the AmB loading efficiency (encapsulation of the drug) was low (24.4%), the freeze-drying process did not change the concentration of entrapped drug ($p>0.05$). Therefore, no loss of drug occurred during the process. Despite adding a set amount of drug per mL of microemulsion, this result represents the amount of AmB that remained soluble on the system after centrifugation. Thus, about 75.6 % of the drug added to the system was not properly incorporated.

MT was an efficient CP avoiding drug loss, probably as a result of its ability to form hydrogen bonds to phospholipid head groups and replacing the water by reducing the gel to liquid crystalline phase transition that occurs when the dried phospholipids are rehydrated. In this way, the MT did not lead to inhomogeneous rearrangement of phospholipids that could result in particle aggregation and loss of incorporated drug into the aqueous medium [9].

4. Conclusions

Research and development of freeze-dried microemulsion systems has been a challenger for several research teams. In fact, several parameters may affect the system stability during the freeze-drying process. However, this work revealed that DOE can be a successful tool allowing determining the optimal freeze-drying conditions to achieve this task. By taking in account the type and concentration of CP, the freeze-drying time and freezing time temperature on the DOE, and using the droplet size as variable of response, a ME dried product can be produced using a low number of experiments. MT at 5% (w/w) as CP, - 80 °C of freezing time temperature and 24 h of freeze-drying time were the best parameters to produce a good aspect brick of freeze-drying ME. The incorporation of AmB did not dramatically change the ME physicochemical properties. Moreover, freeze-dried AmB-ME can became a new antifungal and antiprotozoal product to treat not only systemic candidiasis, but also leishmaniasis infection.

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Figure captions

Figure 1: DSC thermograms of ME and its components: (A) (-- --) Phosphate Buffer; (Black continuous line) Microemulsion; (B) (·····) Lipoid® S100; (Blue continuous line) Miglyol® 812; (- - -) Tween® 80.

Figure 2: Influence of the type and concentration of CP on the droplet size of the ME formulations before the freeze-drying process.

Figure 3: Pareto Chart with Linear (L) and Quadratic (Q) standardized effects of the factors on the (A) ME containing MT droplet size and (B) ME containing LT droplet size.

Figure 4: Response surface plot showing the effects of the mutual interactions between two independent variables on the droplet size. The effect of the freezing temperature and CP concentration on the MEs containing MT (a) and the effect of the freezing time and CP concentration on MEs containing LT (c). The effect of the freezing temperature and freeze-drying time on the MEs containing MT (b) and MEs containing LT (d). The constant value of the remaining variables was maintained at the level 0 corresponding at the central point.

List of tables

Table 1: Experimental levels of independent variables of the DOE for droplet size evaluation of ME after freeze-drying process.

Independent variable		Level		
i	X_i	-1	Replicated (0)	+1
1	Freezing temperature (°C)	- 20	- 80	-196
2	CP concentration (%w/w)	5	12.5	20
3	Freeze- drying time (h)	24	36	48

Table 2: DOE for the freeze-drying process of MEs for each CP type.

Samples	Freeze-drying temperature	CP concentration	Freeze – drying time
1	- 1	- 1	- 1
2	+ 1	- 1	- 1
3	- 1	+ 1	- 1
4	+ 1	+ 1	- 1
5	- 1	- 1	+ 1
6	+ 1	- 1	+ 1
7	- 1	+ 1	+ 1
8	+ 1	+ 1	+ 1
9	0	0	0
10	0	0	0
11	0	0	0

Table 3: Statistical Analysis (ANOVA) of results of DOE for ME-MT and ME-LT samples.

Source	ME – MT				ME –LT			
	Mean square	Calculated F	Tabulated F	R^2	Mean square	Calculated F	Tabulated F	R^2
Regression	81.518	9.9861	5.117	0.9236	25.315	12.5735	5.050	0.9120
Residue	8.163				19.908			
Lack of fit	9.755	3.7666	19.35		31.284	11.0027	19.16	
Pure Error	2.590				2.843			
Total	15.498				135.111			

Table 4: Physicochemical characterization of the ME-MT before and after the freeze-drying process (FDP).

Samples	Appearance	pH	Electrical conductivity ($\mu S\ cm^{-1}$)	Droplet size (nm)
ME + MT at 5% (w/w) before FDP	Homogeneous and clear yellowish	7.23 ± 0.04	749.1 ± 25.65	48.13 ± 1.57
ME + MT at 5% (w/w) after FDP	Homogeneous and clear yellowish	6.86 ± 0.01*	876.5 ± 14.80*	20.13 ± 3.30*

Average values ± SD

*$p < 0.05$ compared with the same sample before freeze-drying.

Table 5: Physicochemical characterization of ME with AmB containing 5% (w/w) of MT before and after freeze-drying process.

Samples	Appearance	pH	Electrical conductivity ($\mu S\ cm^{-1}$)	Droplet size (nm)	AmB concentration (M)
Before freeze-drying	Homogeneous and clear yellowish	7.59 ± 0.10	1583 ± 39.55	54.07 ± 2.35	1.97×10^{-3} ± 0.03
After freeze-drying	Homogeneous and clear yellowish	7.4 ± 0.11*	1639 ± 53.75*	32.30 ± 4.20*	1.94×10^{-3} ± 0.02**

Average values ± SD reported

*$p < 0.05$ compared with the same sample before freeze-drying process.

**$p > 0.05$ compared with the same sample before freeze-drying process.