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Research Article

Influence of the Freeze-Drying Process on the Physicochemical and Biological Properties of Pre-heated Amphotericin B Micellar Systems

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Abstract. The moderate heat treatment of amphotericin B (AmB) in its micellar form (M-AmB) results in superaggregates (H-AmB) that present a substantially lower toxicity and similar activity. The aim of this work was to evaluate the H-AmB behavior after a freeze-drying process. H-AmB and M-AmB micelles were evaluated before and after freeze-drying concerning their physicochemical and biological properties by spectrophotometry and activity/toxicity assay, respectively. Four concentrations of M-AmB and H-AmB were studied aiming to correlate their aggregation state and the respective biological behavior: 50 mg L^{-1}, 5 mg L^{-1}, 0.5 mg L^{-1}, and 0.05 mg L^{-1}. Then, potassium leakage and hemoglobin leakage from red blood cells were used to evaluate the acute and chronic toxicity, respectively. The efficacy of M-AmB and H-AmB formulations was assessed by potassium leakage from *Candida albicans* and by the broth microdilution method. After heating, in addition to an evident turbidity, a slight blueshift from 327 to 323 nm was also observed at the concentrations of 50 and 5 mg L^{-1} for H-AmB. Additionally, an increase in the absorbance at 323 nm at the concentration of 0.5 mg L^{-1} was detected. Concerning the toxicity, H-AmB caused significantly lower hemoglobin leakage than M-AmB. These results were observed for H-AmB before and after freeze-drying. However, there was no difference between H-AmB and M-AmB concerning their activity. Accordingly, the freeze-drying cycle did not show any influence on the behavior of heated formulations, highlighting the suitability of such a method to produce a new AmB product with a long shelf life and with both greater efficiency and less toxicity.

KEYWORDS: activity; amphotericin B; freeze-drying; heating; toxicity.

INTRODUCTION

Fungal infections such as candidemia and invasive candidiasis are responsible for a high index of mortality, especially in critically ill and immunocompromised patients. Moreover, they are responsible for a prolonged stay and an increase in the hospitalization costs. Amphotericin B (AmB) is a molecule widely used to treat that type of disease [1]. It contains hydrophilic polyhydroxyl and hydrophobic polyene domains, which confer particular spectrophotometric properties [2–6] and is commercialized as a micellar system, Fungizone® (M-AmB) [7–12].

In fact, M-AmB reduces the relative risk of invasive fungal infections by 80 to 50% and the overall patient mortality rate from 45 to 23% [13]. It is also highly effective against leishmaniasis [14]. For several decades, M-AmB has been used as the first choice in the treatment of leishmaniasis patients at greater risk such as those aged less than 6 years old or more than 65 years old [15]. Recently, its combination with miltefosine has demonstrated to be more cost-effective than most monotherapies in the treatment of visceral leishmaniasis [16]. Unfortunately, the M-AmB has its use limited by its toxic effects, mainly nephrotoxicity, which has been estimated to occur in up to 80% of patients [17]. Lipid-associated formulations of AmB such as AmBisome®, Amphotec®, and Abelcet® have been successfully developed. However, their high cost is a limiting factor for their use [18].

Moderate heat treatment of M-AmB has been found to induce a molecular rearrangement that yields structures known as superaggregates (H-AmB), which can be detected by changes in the AmB light scattering, its circular dichroism, and absorption spectra, and by cryo-transmission and electron microscopy [19–21]. Other important changes in the H-AmB, such as a larger size, a blueshift change on the spectrum, and a

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significantly lower toxicity, have been evidenced [19–21]. Recently, Silva-Filho *et al.* have also demonstrated that heated Fungizone® exhibits significantly lower *in vitro* toxicity against mammalian cells while keeping its antifungal activity [22]. However, the aqueous medium that surrounds this micellar system hinders their long-term stability in such a manner that its stability is decreased to 24 h at room temperature and to 1 week under refrigeration from 2 to 8°C [23].

In order to solve such stability issue, water has to be removed. The most common strategy is the freeze-drying process, which is a suitable drying method for AmB in that it is carried out under low temperatures, unlike other methods such as spray-drying, in which high temperatures are required, leading to instability problems [24]. The freeze-drying process comprises the sample freezing followed by primary and secondary drying [25]. These steps may trigger both chemical reactions and mechanical stress causing destabilization of micelle organization and probably secondary aggregation or fusion, hindering its micellar reorganization after reconstitution [26]. Thus, the use of a cryoprotector is generally required [25, 27].

To date, there is no report in the literature about the behavior of superaggregates under freeze-drying: whether this process could disturb the new organizational system and whether its lower toxicity remains after reconstitution. Therefore, considering the unquestionable importance of the freeze-drying to allow a commercial AmB stable product, the aim of this work was to evaluate the influence of the freeze-drying process on the physicochemical and biological properties of the H-AmB micellar system by a qualitative spectroscopy study and *in vitro* activity/toxicity assays, respectively.

MATERIALS AND METHODS

Materials

Micellar amphotericin B (M-AmB) was kindly provided as a gift from Cristália (Itapira, Brazil). Normal saline solution [NaCl at 0.9% (*w*/*v*)] was purchased from Braun (São Paulo, Brazil), and Sabouraud-Dextrose-Chloramphenicol (SDC) agar was provided by MicroMed (São Paulo, Brazil). All other chemicals were of analytical grade.

Preparation of the Samples

The commercial stock solution of M-AmB at the concentration of 5×10^{-3} M (5,000 mg L^{-1}) was prepared by adding 10 mL of water for injection into a vial containing 50 mg of AmB, approximately 41 mg of sodium deoxycholate, and phosphate buffer of pH 7.4. The mixture was subject to vortex shaking until dissolution. Subsequently, M-AmB was heated at 70°C for 20 min in order to obtain the formulation H-AmB. Both M-AmB and H-AmB were frozen at −80°C for 24 h in order to yield FM-AmB and FH-AmB, respectively (Fig. 1). Finally, the frozen samples (FM-AmB and FH-AmB) were dried by a freeze-dryer (Christ Alpha 1–2 LD, Germany) for 24 h under −65°C and 0.0018 mbar producing the samples DM-AmB and DH-AmB, respectively (Fig. 1).

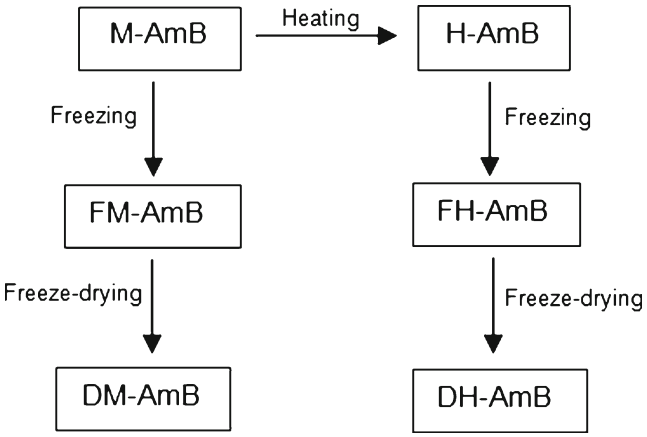

Fig. 1. Graphical representation of the studied formulations highlighting their method of production. Abbreviations: *AmB*=amphotericin B; *M-AmB*=AmB micellar system; *H-AmB*=heated M-AmB; *FM-AmB*=frozen M-AmB; *FH-AmB*=frozen H-AmB; *DM-AmB*=freeze-dried M-AmB; and *DH-AmB*=freeze-dried H-AmB

Physicochemical Analysis of AmB

The spectroscopy study was carried out using a UV–vis spectrophotometer (Biochrom Libra S32, UK). The analyzed concentrations were 5×10^{-5} M (50 mg L^{-1}), 5×10^{-6} M (5 mg L^{-1}), 5×10^{-7} M (0.5 mg L^{-1}), and 5×10^{-8} M (0.05 mg L^{-1}). These concentrations were obtained by successive dilutions 1:10 of each formulation (at 5×10^{-3} M) using water as solvent. Their molar extinction coefficients (ε) were calculated using the Beer–Lambert equation. All spectra were recorded at 25°C±0.1°C with a 300–450-nm range [4]. Temperature and time exposure were controlled by a built-in thermometer (Incoterm, Brazil) and a chronometer (Model Labor, Germany).

Toxicity Against Mammalian Cells

Preparation of Red Blood Cell Suspension

In accordance with the Ethical Research Committee of the Universidade Federal do Rio Grande do Norte, protocol number 002/2009, one healthy female adult donor signed the informed consent and provided all normal red blood cells (RBCs) for the *in vitro* experiments in order to minimize sources of variability. Five milliliters of venous blood were collected in sterile EDTA [1 mg/mL, ethylenediaminetetraacetate at 10% (*w*/*v*)] syringes and promptly centrifuged in a refrigerated centrifuge (ALC, Model PK121R, Italy) in tubes at 1,100×*g* for 10 min at 4°C.

Plasma and the exposed buffy coat were removed and discarded. The RBCs were washed three times under centrifugation (1,100×*g* for 5 min at 4°C) and suspended in five volumes of normal saline. They were then resuspended in 4 mL of saline, counted in a Neubauer™ chamber (Splabor, Brazil), and resuspended again until the desired concentration (5×10^{7} cells mL^{-1}) was achieved. The cells were used on the day of collection [28]. Assays were carried out in triplicate.

Evaluation of H-AmB Toxicity

Four milliliters of RBC (5×10^7 cells mL^{-1}) were incubated for 1 h at 37°C with the vehicle control and with 50, 5, 0.5, and 0.05 mg L^{-1} of M-AmB and H-AmB on its three studied forms—normal (M or H), frozen (F), and freeze-dried (D). The RBCs were then centrifuged for 5 min at 1,100×*g* and washed three times with normal saline. The final pellet was lysed with 4 mL of distilled water and then stirred and centrifuged (1,100×*g* for 10 min) for removing membranes. The potassium (K^+) content of the supernatant was determined using a Flame Photometer 7000 (910 M Analyser, Brazil) calibrated with K^+ reference at 5 mEq L^{-1}. Hemoglobin was determined from its absorption at 540 nm recorded on a UV–vis spectrophotometer (Biochrom Libra S32). The total amounts of K^+ and hemoglobin were measured from the control RBC tubes. Release of ions was calculated as the difference between control and treated cells, and expressed as a percentage of the total hemoglobin or K^+ content [28].

Activity Against *Candida albicans* Strain

Preparation of C. albicans Suspension

Inoculums of a strain of *C. albicans* ATCC (90027) were transferred to a Sabouraud-dextrose-agar scope and incubated at 37°C for 24 h. The desired concentration of 5×10^7 colony-forming units (CFU) mL^{-1} was obtained by counting in a Neubauer™ chamber (Splabor) [28].

Evaluation of H-AmB Efficacy

Two milliliters of a fungal suspension containing 5×10^7 CFU mL^{-1} was incubated for 1 h at 37°C with both M-AmB and H-AmB at the concentrations of 50, 5, 0.5, and 0.05 mg L^{-1}, separately. Cells were centrifuged for 10 min at 2,200×*g* and washed three times in normal saline, and 2 mL of purified water was added to the pellet of fungal cells. A fraction of this pellet was lysed by heating for 5 min at 100°C and centrifuged to remove membranes, and free K^+ was measured. The K^+ leakage was calculated similarly to the calculation of the RBCs [28].

The broth microdilution method was also conducted in order to evaluate the efficacy of H-AmB formulations. It was carried out using an adapted version of the guidelines developed by the Clinical Laboratory Standards Institute [29]. Briefly, serial two-fold dilution of the AmB formulations was performed in Muller Hinton medium in 96-well microtiter trays. Afterwards, wells were inoculated with the fungal cell suspension, previously adjusted to attain a final concentration of $0.5–2.5\times10^3$ CFU mL^{-1}. Then, the panels were incubated at 35°C for 48 h. The minimal inhibitory concentration was defined as the lowest concentration at which there was no growth. Assays were carried out in triplicate.

Statistical Analysis

The results were evaluated by means of one-way analysis of variance (ANOVA) and *t* test to analyze the variation response in the same group and in different groups, respectively, using Prism 4 for Windows 4.02 (GraphPad Software, San Diego, CA).

RESULTS

Physicochemical Evaluation of AmB

After the heating process of M-AmB, a prominent increase in the turbidity of the AmB micelle was observed suggesting a rearrangement in its structural organization. This was confirmed by the physicochemical evaluation performed by spectrophotometry and by the increase on the diameter size of the micelle aggregated system (results not shown).

In fact, as previously shown by Gaboriau *et al.* [19, 20] the spectrophotometry study reveals that the micellar AmB system has a very particular electronic absorption spectrum that was concentration dependent and changed following the aggregation state of the molecule into the media. At low concentrations (5×10^{-8} M), the AmB monomeric form was predominant and the absorption spectrum exhibits λ_{max} at 364, 385, and 408 nm, the latter one being assigned to AmB monomeric form (Fig. 2). This spectrum profile is comparable to that obtained with organic solvents of similar polarity to methanol.

However, as the concentrations increased, the spectra changed. At 5×10^{-5} and 5×10^{-6} M, in which there is a higher amount of aggregates, AmB presented a maximum peak centered at 327 nm (Fig. 2a–c). For H-AmB systems, a new band centered at 323 nm was observed (Fig. 3a).

As previously described by Silva-Filho *et al.*, the most important difference between M-AmB and H-AmB was found at the concentration of 5×10^{-7} M, at which the molar extinction coefficient was higher for all heated AmB samples (ε H-AmB = 60,800; ε FH-AmB = 59,800; and ε DH-AmB = 58,800) at 323 nm (Fig. 3a–c). On the other hand, for M-AmB, FM-AmB, and DM-AmB, these values were reduced to 44,600, 41,600, and 40,600, respectively, with λ_{max} centered at 327 nm (Fig. 2a–c). The spectrum recorded at 5×10^{-8} M was analyzed only to show the AmB concentration dependence since there was no difference between the studied formulations. In fact, this behavior was expected due to the predominance of the monomeric AmB form at this concentration previously described and due to the complete destruction of the micelle system (4). These results are in complete agreement with the ones found by Gaboriau *et al.* [19, 20].

Interestingly, after the freeze-drying process, these patterns remained unchanged (Fig. 3b–c). As expected, for M-AmB, no changes were observed once the product was already marketed in a freeze-dried form (Fig. 2a–c). The novelty found in this study was that the H-AmB micelle physicochemical properties remained unchanged after the freeze-drying process (Fig. 3). Taking into account that an isosbestic point was obtained at around 340 nm, the region of aggregates can be observed on the left of 340 nm and the monomers on its right (Figs. 2 and 3). At higher concentrations (5×10^{-5} and 5×10^{-6} M), the λ_{max} was detected at 327 nm for M-AmB and 323 nm for the heated one. As the AmB concentration decreased (5×10^{-7} and 5×10^{-8} M), the intensity of absorption changed to three peaks of absorption at 364, 385, and 408 nm, showing that an increase in the proportion of

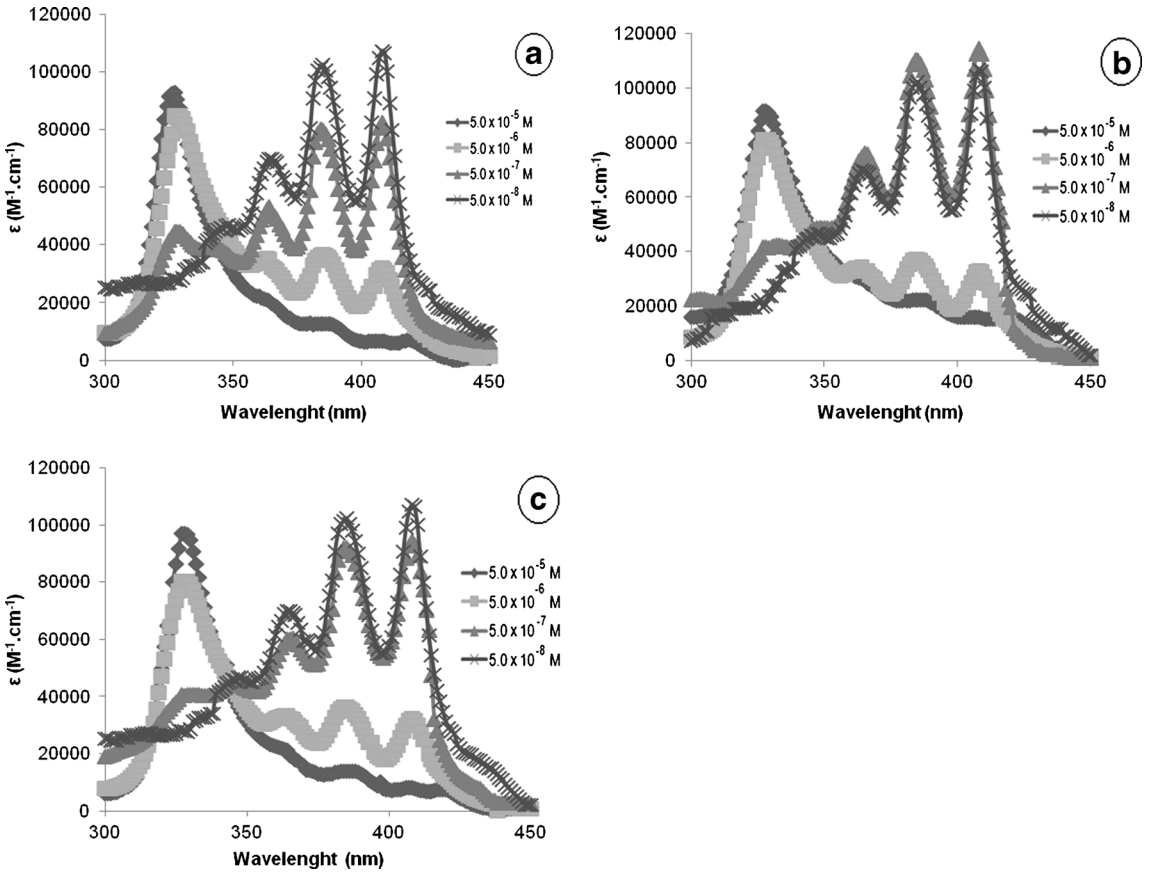

Fig. 2. Absorption spectra of M-AmB (**a**), FM-AmB (**b**), and DM-AmB (**c**) at 5×10^{-5}, 5×10^{-6}, 5×10^{-7}, and 5×10^{-8} M. Abbreviations: *AmB*=amphotericin B; *M-AmB*=AmB micellar system; *FM-AmB*=frozen M-AmB; *DM-AmB*=freeze-dried M-AmB

monomeric AmB forms occurs at the expense of the aggregated ones (Figs. 2 and 3). This variation is related to the shift in the equilibrium between monomeric and aggregated forms of AmB as shown in Eq.1:

$$AmB_{ag} \overset{K}{\rightleftarrows} AmB_m \quad (1)$$

where:

AmB_{ag} = AmB aggregates
AmB_m = AmB monomers
K = constant of equilibrium between AmB_{ag} and AmB_m

Toxicity Evaluation of H-AmB Micellar Systems

The K^+ and hemoglobin leakage following AmB administration has been extensively used as evidence of acute and chronic toxicity, respectively [22, 28]. The results reveal that the release of K^+, for all samples of M-AmB and H-AmB, presented a similar profile (Figs. 3 and 4). At the highest concentrations (50 and 5 mg L^{-1}), the K^+ leakage was found to be higher than 90% for the six analyzed AmB formulations (Fig. 4). It is important to note that the freeze-drying process did not modify this behavior.

On the other hand, concerning the hemoglobin leakage, a significant difference was found between M-AmB and H-AmB. While M-AmB induced the release of 99.10% ±0.02 and 81.78% ±0.01 of the hemoglobin for AmB concentrations of 50 and 5 mg L^{-1}, respectively, H-AmB formulations even at the highest concentration presented lower than 20% of hemoglobin release (Fig. 5a). Again, the same rate of chronic toxicity was found for the freeze-dried AmB formulations (Fig. 5b, c).

Activity of H-AmB and M-AmB Against *C. albicans* Strains

The *in vitro* activity against *C. albicans* was evaluated by means of the inhibitory concentration (IC 90) and K^+ release. The IC was found to be 0.5 μg mL^{-1} of AmB for the six formulations.

Such data are in agreement with the K^+ release study on *C. albicans*, in which all formulations showed the same profile of toxicity with no statistical significance difference among them and at the four AmB concentrations ($p>0.05$) (Fig. 4a–c). In fact, at the highest concentration (50 mg L^{-1}), a significant antifungal activity was reached, 81.80±0.06, 81.73 ±0.64, and 86.42% ±1.2 for H-AmB (Fig. 4a), FH-AmB (Fig. 4b), and DH-AmB (Fig. 4c), respectively. Additionally, for the unheated AmB formulations, M-AmB, FM-AmB, and DM-AmB, these values slightly changed to 84.21±3.17, 80.85 ±1.86, and 84.91% ±0.69, respectively.

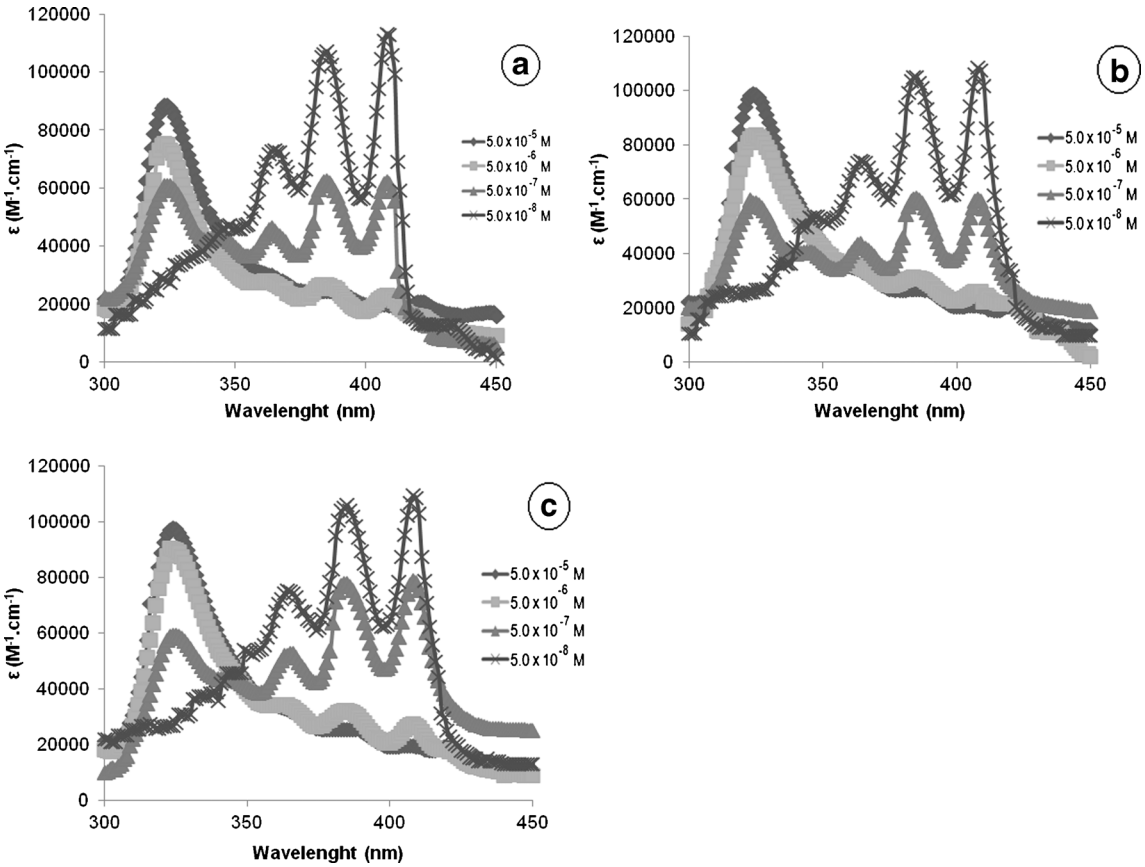

Fig. 3. Absorption spectra of H-AmB (**a**), FH-AmB (**b**), and DH-AmB (**c**) at 5×10^{-5}, 5×10^{-6}, 5×10^{-7}, and 5×10^{-8} M. Abbreviations: *AmB*=amphotericin B; *H-AmB*=heated AmB micellar system; *FH-AmB*=frozen H-AmB; *DH-AmB*=freeze-dried H-AmB

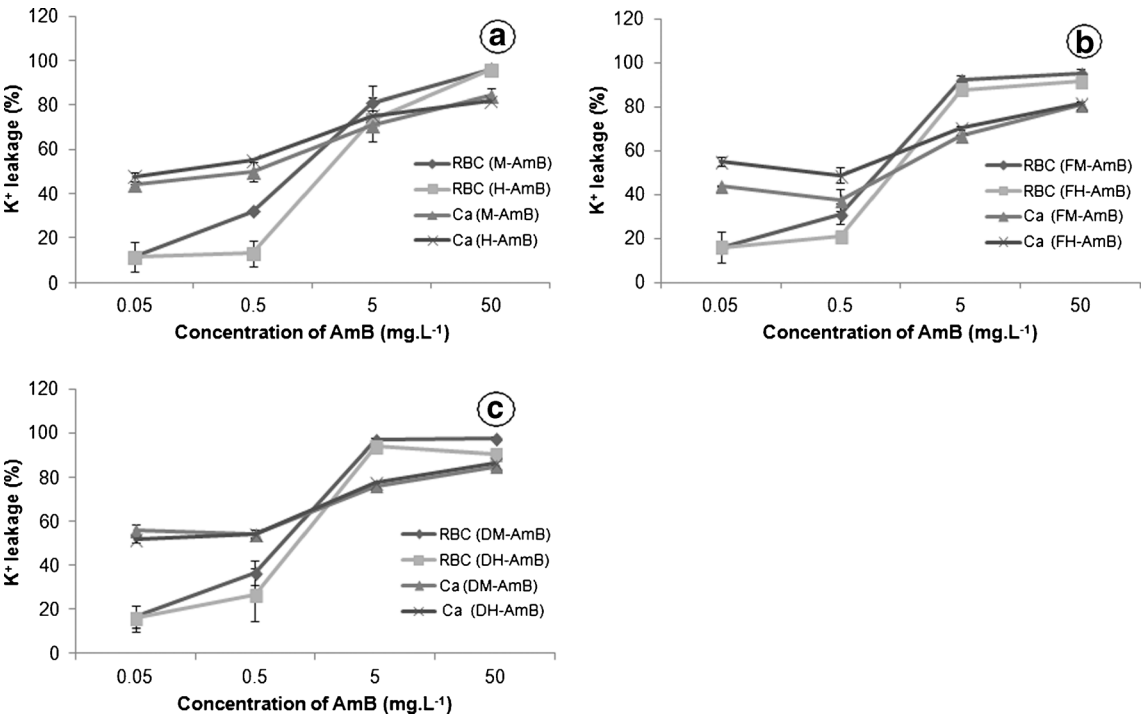

Fig. 4. *In vitro* release of K^+ from human red blood cells (*RBC*) and *C. albicans* (*Ca*) induced by M-AmB and H-AmB. **a** Before freeze-drying; **b** after freezing; and **c** after freeze-drying. Abbreviations: *AmB*=amphotericin B; *M-AmB*=AmB micellar system; *H-AmB*=heated M-AmB

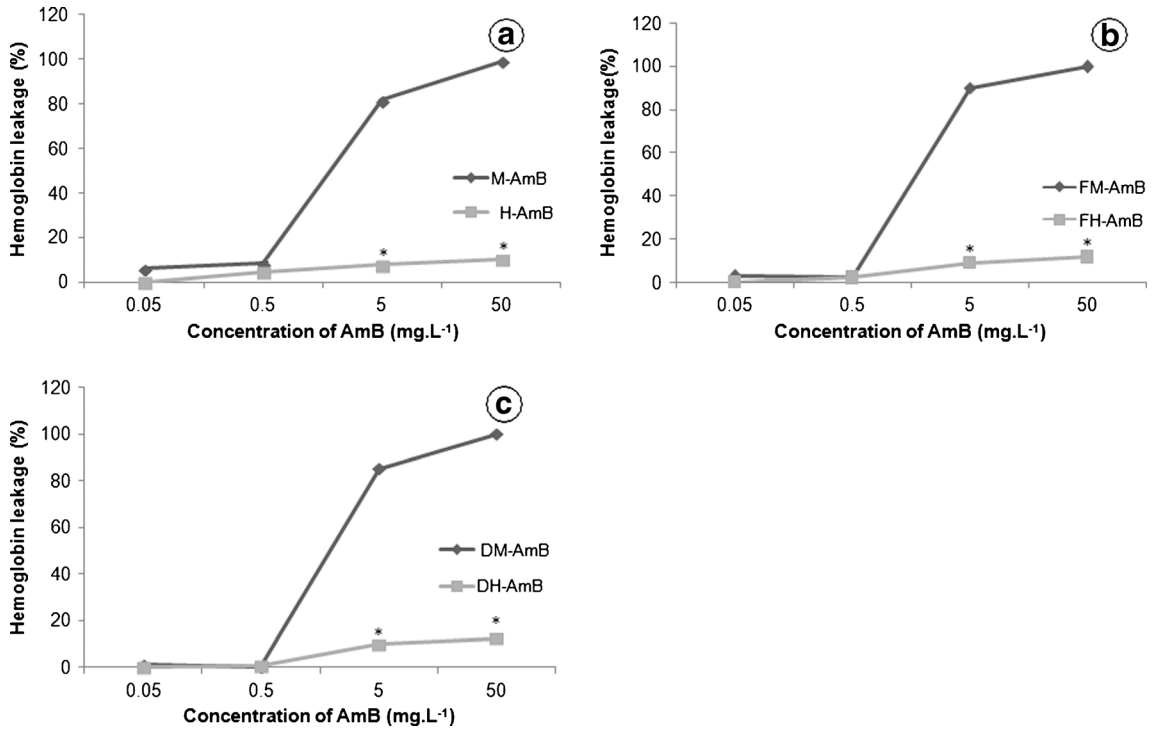

Fig. 5. *In vitro* release of hemoglobin from human red blood cells (*RBC*) induced by M-AmB and H-AmB. **a** before freeze-drying; **b** after freezing; and **c** after freeze-drying. Abbreviations: *AmB*=amphotericin B; *M-AmB*=AmB micellar system; *H-AmB*=heated M-AmB. *Significant difference between both products ($p<0.001$)

DISCUSSION

Organic molecules are subject to a variety of chemical reactions in aqueous media, many of which are unacceptable in terms of product stability. For instance, hydrolysis and oxidation are reactions which can reduce the biological activity [27]. This is completely true for the AmB molecule. For example, Manosroi *et al.* found that at 45°C, Fungizone® solution presented a shelf life of 2.5 days, while for the Fungizone® powder, this value changed to 9.8 days [30]. Additionally, it has been proved that at the dry state, such degradation reactions can be significantly delayed or reduced [27]. Therefore, a dried media is strongly recommended for therapeutically active formulations.

The freeze-drying process, which consists of the removal of the solvent from a frozen product by vacuum sublimation [27], can be divided into three steps: freezing, where the solidification occurs; primary drying, in which the freeze sublimates; and secondary drying, when desorption of unfrozen water happens [31]. The first direct consequence of freezing is a rapid solute concentration in the medium. A secondary consequence consists of a higher chance of chemical reactions such as oxidation [32]. The seven conjugated double bonds in the AmB molecule make it more susceptible to this process [33]. Radical autoxidation of AmB contained in Fungizone® was already demonstrated by Lamy-Freund *et al.*, and its kinetics is related to the aggregation state of the antibiotic [34]. However, Gaboriau and coworkers have suggested that superaggregates are more stable and can avoid the AmB autoxidation. They pointed out a decrease of 20% in the total AmB content of an unheated AmB micellar solution, as a consequence of the autoxidation processes, while a heated AmB micellar solution, after incubation for 1 h at 37°C, under stirring, did not undergo any relevant changes in the drug concentration [19, 20]. In another study, while the AmB autoxidation process involved less than 10% of a heated AmB micellar solution, the unheated one presented a 25% decrease, both after 2 h of incubation [19, 20]. In light of these findings, it can be partially explained why the superaggregates presented the same performance after the freeze-drying process. Concerning the oxidation process of M-AmB after its freeze-drying, this phenomenon was completely unknown and our results revealed that the eventual amount of oxidation that could happen was not sufficient to improve RBCs lysis. After heating, an increase in the turbidity of the system was observed. The turbidity of the AmB micellar suspension is related to the reduction in intensity of the transmitted beam because of scattering. Consequently, as the particle radius becomes larger, the turbidity increases. Therefore, the change in the turbidity of the system after heating is explained by the larger size of the H-AmB (approximately 300 nm, results not shown), according to van Etten *et al.* [36], as well as by its reservoir behavior [22, 35–37].

No difference in the UV–vis spectrum of H-AmB and M-AmB at 5×10^{-8} M was found. However, the absorption spectrum displays the direct influence of the drug concentration on the AmB state form presented in the solution. Accordingly, the monomeric form predominates as the concentration decreases with a maximum of absorption centered at 408 nm. Gaboriau *et al.* had demonstrated that after heating, the aggregates are condensed with the monomer yielding the superaggregates [19]. In fact, our results corroborate their findings because after heating, the three heated formulations, H-AMB, FH-AmB, and DH-AmB

(Fig. 3), showed a spectrum with a maxima at 323 nm, which is different from the characteristic wavelengths of aggregates for unheated AmB (Fig. 2).

Baas *et al.* demonstrated that after more than 10 h of M-AmB incubation, gelatinous aggregates are generated, consisting of large AmB aggregates, whereas the wholly deoxycholate remains in solution. By this way, they suggested that the heating process would accelerate the dissociation kinetics between the AmB micelles and the deoxycholate followed by the reassociation of AmB molecules [38]. In fact, the results presented here make one believe that the blueshift from 327 to 323 nm after heating is a consequence of a different pattern of organization of the AmB with the deoxycholate salt. Because of the seven double conjugated bonds of the AmB molecule and the larger size of the aggregates after heating [36], the amount of double bonds that are exposed to the spectrum light might have changed as could be seen by the blueshift and different pattern of molar absorptivity, especially at the concentration of 5×10^{-7} M.

The AmB main mechanism of action suggests that this molecule acts at the membrane level by binding to the membrane sterols, cholesterol, and ergosterol, and, as a consequence, it is toxic for both fungal and mammalian cells [39]. However, the degree of membrane interaction is dependent on the AmB concentration in the media. It is believed that AmB monomers, which exist only at low AmB concentration in the medium, are able to interact only with ergosterol, while AmB aggregates can interact with both cholesterol and ergosterol. When only a small amount of AmB aggregates is found in the media, a reversible permeation effect may occur on RBCs, whereas at higher levels, it causes lysis. These two mechanisms of action are not completely understood, but it is believed that a permeation effect is caused by AmB's ability to form transmembrane channels, while the lytic effect is related to the peroxidative action at the membrane level [40] by free radical production from the autoxidation process [19, 33, 34, 41]. The oxidation of unsaturated fatty acids leads to a change in the integrity of the membrane, which becomes more susceptible to the osmotic shock caused by the formation of channels [19]. Taking into consideration that superaggregates are more stable to the autoxidation process, the consequent lower production of free radicals would result in a reduced incidence of peroxidative and lytic actions of AmB as previously described. Therefore, this fact is believed to partially explain the lower ability of superaggregates at inducing lysis of RBC membranes.

In order to assess M-AmB and H-AmB activity, it was decided to replace the colony-forming units survival rate assay [22], which is laborious and time-consuming, with the broth microdilution method, a reference method used to evaluate the antimicrobial susceptibility when a more accurate result is required for clinical management [29]. The activity of both H-AmB and M-AmB, at their three studied formulations (normal, frozen, and freeze-dried), was similar with no statistical difference among them. These results demonstrated that the activity of H-AmB was maintained after the freeze-drying process, which is very important to keep the profile of activity of the superaggregates.

CONCLUSION

Several studies have demonstrated that the heating process of the AmB micelle systems is an excellent approach to reduce the toxicity of AmB while keeping its activity by a simple and low-cost procedure. However, to submit the AmB micelles to such a process, the dissolution of the formulation in water is a mandatory step. After heating, the newly produced micelle system has to be used in 24 h due to the instability phenomenon that starts to occur in the aqueous media. Therefore, a drying technology should be used after the heating process.

To the best of our knowledge, for the first time, it was demonstrated that the AmB micellar system can be freeze-dried and can maintain its physicochemical and activity/toxicity properties. Thus, the freeze-drying technique may be a suitable method to increase the long-term stability of H-AmB systems.

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