Physicochemical stability evaluation of the lyophilized amphotericin B superaggregates

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INTRODUCTION AND OBJECTIVE

The amphotericin B on its micellar form (M-AmB) is a formulation widely used to treat systemic fungal infections. Unfortunately, its use is limited because of its toxicity. In aqueous solution, this micellar system presents monomeric and aggregated forms, these latter being responsible for its side effects. In order to solve this problem some lipid formulations has been developed, but they present high cost (Gaboriau 1997).

It has been demonstrated that controlled heat treatment of AmB (H-AmB) leads to a micellar rearrangement, commonly known as "superaggregates" that can be observed by changes in light scattering, circular dichroism, absorption spectra, cryo-transmission and electron microscopy (Gaboriau 1997). The aim of this work was to evaluate the H-AmB behavior after a freeze-drying process by means of its spectrophotometric behavior before and after freeze-drying.

MATERIALS AND METHODS

Preparation of the H-AmB and M-AmB samples
Stock solution of M-AmB was prepared at the $5x10^{-3}$ M concentration. The H-AmB was prepared under controlled heat of the M-AmB stock solution at 70ºC for 20 min. The samples were subsequently diluted to $5x10^{-5}$ M, $5x10^{-6}$ M, $5x10^{-7}$ M and $5x10^{-8}$ M in order to proceed with the assay.

Lyophilization procedure
Each step of the lyophilization process the samples were monitored by their physicochemical and toxicity/activity aspects. After freezing (-80ºC by 24 hr), frozen M-AmB (FM-AmB) and frozen H-AmB (FH-AmB) were obtained. After drying (-65ºC and 0.0018 mbar by 24hr), dry M-AmB (DM-AmB) and dry H-AmB (DH-AmB) were obtained.

Physicochemical analysis
The spectroscopic study was performed for the four previously mentioned concentrations by means of a UV-VIS Spectrophotometer. Their molar extinction coefficients (ε) were calculated using the Beer–Lambert equation.

Evaluation of toxicity
Four milliliter of RBC (5×10^7 cells. mL^{-1}) were incubated for 1 hour at 37°C with the control and with 50, 5, 0.5, and 0.05 mg. L^{-1} of M-AmB and H-AmB. The RBCs were then centrifuged for 5 min and washed three times with normal saline. The pellet was lysed by distilled water and, then, stirred and centrifuged for removing membranes. The total K^+ and hemoglobin content was measured from the control samples (Araújo 2005).

Evaluation of activity
Two milliliter of a fungal suspension containing $5 x10^7$ cfu.mL^{-1} were incubated for 1 hour at 37ºC with both M-AmB and H-AmB at the concentrations of 50, 5, 0.5, and 0.05 mg.L^{-1}. Cells were centrifuged and washed three times with normal saline, and, then, 2 mL of purified water was added to pellet the fungal cells. An aliquot of this pellet was lysed and free K^+ was measured (Araújo 2005). A MIC by the microdillution method was also conducted in order to evaluate the H-AmB formulations efficacy.

RESULTS AND DISCUSSION

All absorption spectra of the AmB showed to be concentration-dependent (Figure 1). At low concentrations ($5x10^{-8}$ M), the monomeric form predominates with absorption spectrum exhibiting maxima at 364, 385, and 408 nm, which is similar to that obtained with polar organic solvents such as methanol. At the concentrations of $5x10^{-5}$ and $5x10^{-6}$ M, where there is a higher amount of aggregates, AmB presented a maximum peak centered at 327 nm (Figure 1 A).

For H-AmB systems, it was observed a new band centered at 323 nm (Figure 1 D-F). The most important difference between M-AmB and H-AmB was found at the concentration of $5x10^{-7}$ M, in which there was a higher molar extinction coefficient for all heated AmB samples ($\varepsilon_{H\text{-}AmB}$= 60,800; $\varepsilon_{HF\text{-}AmB}$ = 59,800 and $\varepsilon_{HD\text{-}AmB}$= 58,800) at 323 nm (Figure 1D-F). On the other hand, for M-AmB, these values changed to 44,600, 41,600 and 40,600, respectively, with a maximum wavelength centered at 327 nm (Figure 1 A-C).

Organic molecules are subject to a variety of chemical reactions in aqueous solutions. It has been shown that at the dry state such degradation reaction can be significantly retarded. Therefore, freeze-drying is a procedure widely used aiming a better stability (Abdelwahed 2006). Interestingly, the M-AmB and H-AmB maintain both the physiochemical and biological

unchanged after the freezing and drying process (Figure 1).

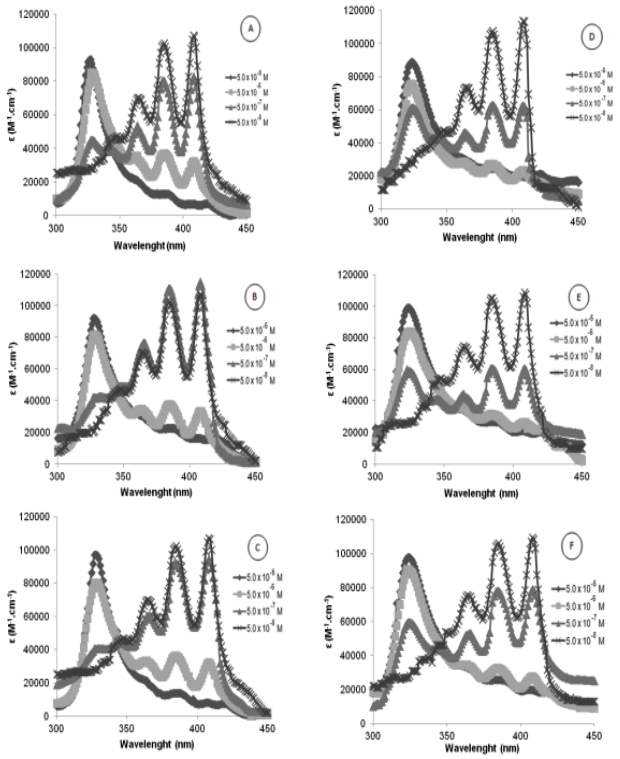

Figure 1: Spectra of M-AmB and H-AmB before and after freeze-drying. (A) M-AmB, (B) FM-AmB (C) DM-AmB, (D) H-AmB, (E) FH-AmB, (F) DF-AmB.

For K^+ leakage, all M-AmB and H-AmB samples presented similar profile. The freezing-drying did not alter the behavior of H-AmB samples: at the highest concentrations (50 $mg.L^{-1}$ and 5 $mg.L^{-1}$) a strong K^+ release, higher than 90%, was found for the six analyzed AmB preparations (Figure 2 D to F).

However, it was found a significant difference on the hemoglobin leakage before the freeze-drying process between M-AmB and H-AmB. While M-AmB presented 99.10% ± 0.02 and 81.78% ± 0.01 for the concentrations of 50 and 5 $mg.L^{-1}$, respectively, H-AmB formulations even at the higher concentration, presented lower than 20% of Hb release (Figure 2 A and D). The same profile was found to the freeze-dried formulations (Figure 2 E and F).

The MIC was found to be 0.5 $\mu g.mL^{-1}$ of AmB for the six analyzed preparations (M-AmB and H-AmB before freezing and after freeze and drying) (Figure 2). These data are in agreement with the K^+ release in which the six preparations showed the same profile with no statistically significant difference between them for the four analysed concentrations ($p>0.05$).

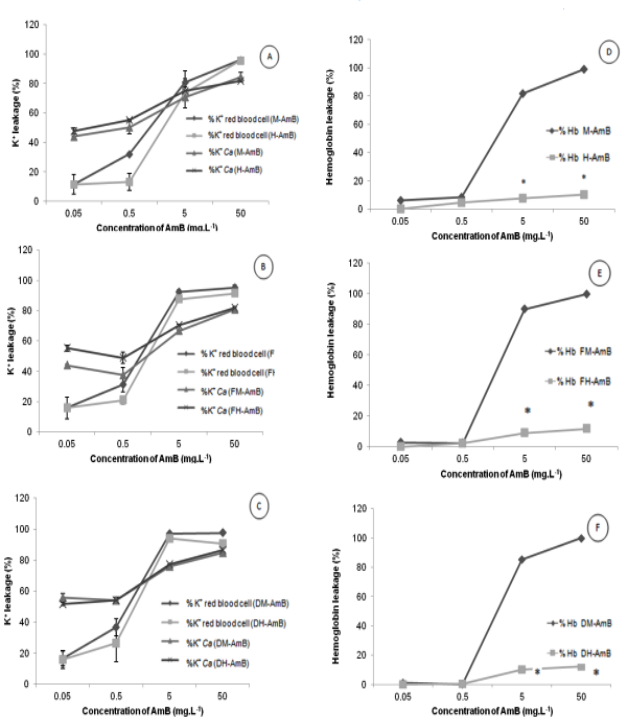

Figure 2: In vitro release of potassium and hemoglobin from human RBC and *Candida albicans* induced by M-AmB and H-AmB. (A) M-AmB, (B) FM-AmB (C) DM-AmB, (D) H-AmB, (E) FH-AmB, (F) DF-AmB.

CONCLUSION

From this study it can be inferred that the freeze-drying process did not alter the caracteristics of the M-AmB superaggregates. Thus, these results can be the base of a new AmB formulation.

REFERENCES

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