

AMPHOTERICIN B ASSOCIATED WITH TRIGLYCERIDE-RICH NANOEMULSION: STABILITY STUDIES AND *IN VITRO* ANTIFUNGAL ACTIVITY

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Recebido em 16/5/07; aceito em 27/9/07; publicado na web em 2/4/08

Amphotericin B (AB) is the standard drug for invasive fungal infection therapy. It has a broad spectrum of activity and it is the best antifungal available against most yeasts and molds. Its therapeutic use, however, is limited by significant side effects, leading to a low therapeutic index when it is used as the traditional formulation (Fungizone®). Due to self-association, AB can form pores in cholesterol-containing membranes. We propose a triglyceride-rich nanoemulsion as a delivery system for AB in low levels of aggregation to reduce the toxicity against host cells.

Keywords: amphotericin B; lipid nanoemulsion; delivery system.

INTRODUCTION

Amphotericin B (AB) is the standard drug for therapy of invasive fungal infections. AB has a broad spectrum of activity against most yeasts and molds, but its parenteral administration is limited by toxic effects that include fever, chills, nausea, vomiting and hypokalaemia, resulted from treatment with traditional preparations (micelles mixed with sodium deoxycholate).^{1,2} In addition, symptoms of nephrotoxicity, including elevated serum creatinine and urea levels, often become apparent upon prolonged administration, which leads to a low therapeutic index.^{1,3} AB exerts a fungicidal effect by binding irreversibly to ergosterol, a component found in fungal cell, disrupting the membrane and causing cell death.⁴ Due to its broad spectrum of activity, AB has been considered the treatment of choice for invasive fungal infections for the past 30 years.

The therapeutic potential of AB and other polyene antibiotics is determined by their affinity for cholesterol and ergosterol, the principal sterols in eucaryote cell membranes. The selectivity for these sterols depends on the aggregation state of AB. Monomeric AB associates with the sterols in fungal cell membranes, whereas self-associated AB can also form pores in cholesterol-containing membranes, leading to toxicity towards host cells.⁵

Lipid formulations commercially available share the same spectrum of action and reduce most side effects representing a significant advance in AB delivery. These formulations include AB lipid complex, AB colloidal dispersion and small unilamellar vesicle formulations of AB. Despite the fact that activity seems superior to the traditional formulation, the dose and cost effectiveness need further study.⁶

The development of new lipid formulation for intravenous administration of AB might therefore be desirable, presenting monomeric forms and preserved activity against yeasts and moulds. In this study, we developed a stable triglyceride-rich nanoemulsion containing AB, with strong evidence that its applicability could be more successful than conventional formulations. We have hypothesized that caprylic/capric triglyceride-rich nanoemulsion

could solubilize AB and reduce its self-aggregation state in solution. In particular, the present paper describes the production of a nanoemulsion containing lipid components, its physicochemical characterization, *in vitro* activity against *Candida albicans* and stability during 6-month storage.

EXPERIMENTAL

Materials

AB powder (86.04% pure) was purchased from Bristol-Myers-Squibb (São Paulo, Brazil), AB in deoxycholate (AB-DOC) (Fungizone®, Bristol-Myers-Squibb) was purchased from a hospital pharmacy, soya bean lecithin, and cholesterol was obtained from Sigma Chemicals (St. Louis, MO). Miglyol 812 N® was kindly provided by Sasol Corp. (Germany). The solvents and reagents used were purchased from Sigma Chemicals (St. Louis, MO) and Merck (Rio de Janeiro, Brazil) and were the highest commercially available grade and were used without further purification. RPM1 1640 medium and morpholinepropane sulfonic acid (MOPS) were from Sigma Chemical (St. Louis, MO). *Candida albicans* serotype A (3153) was obtained from The London School of Hygiene of Tropical Medicine (UK).

Preparation of the triglyceride-rich nanoemulsion containing AB (AB-N)

AB-N was prepared as previously described by Souza *et al.*⁷ with some modifications. Briefly, caprylic/capric triglyceride (70%), soya bean lecithin (27%), and cholesterol (3%) were dissolved in chloroform-methanol (2:1, v/v) and AB powder was added (100 mg) to perform AB: lipids at a molar ratio of 1:11. The mixture was dried under nitrogen flow and kept under vacuum overnight in order to remove organic solvent. Follow, 40 ml of TRIS- HCl buffer (0.01 M, pH 8.05) was added and emulsified with a probe-type sonicator for 30 min at 50 – 60 °C. The final dispersion was obtained after centrifugation at 150,000 g for 30 min to precipitate titanium and non-incorporated drug. The nanoemulsion was filtrated through

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a 0.22 μm membrane and storage at 4 and 25 °C protected from light. The non-incorporated drug was used in order to quantify the concentration of AB into AB-N.

Physicochemical characterization of AB-N

In order to evaluate the solubilizing power of the produced formulations, the non-incorporated drug was quantitative determined by UV spectrophotometry, using methanol as organic solvent. AB incorporation into nanoemulsion was calculated by subtracting the initial amount of AB added from the quantity of insoluble drug recovery. Particle size of AB-N (mean diameter) was performed by using a ZetaPlus Analyser 90Plus (Brookhaven Instruments Corporation, NY, USA) equipped with a 35 mW laser light scattering at 90°. Aliquots were diluted in TRIS-HCl buffer (1:250) for all determinations. pH measurements were performed by a selective ion pH meter (Athos Instrumentação Ind, São Paulo) monthly. The particle size and pH measurements were determined just after AB-N preparation and then at intervals until up to 6 months. Absorption spectra (300–450 nm) were obtained by dispersing the formulations in glucose 5%, resembling injectable administration, using a Perkin-Elmer Lambda UV-visible spectrometer. For AB-DOC spectrum, the formulation was prepared according to the manufacturer. All spectra, particle sizing and pH measurements were obtained at room temperature (25 °C).

Chemical stability during storage

Stability studies were conducted at 0, 1, 2, 3, 4, 5 and 6 months in independent experiments with aliquots storage at 4 and 25 °C protected from light. AB content was evaluated by quantitative determination of non-incorporated drug (insoluble drug in the aqueous phase) extracted by centrifugation at 15,000 g for 20 min. After centrifugation, the supernatant was discarded and the remaining insoluble drug, if any, washed three times with 2 mL of distilled water. The washed drug crystals were dissolved in methanol and quantified by UV spectrophotometry (404 nm) using calibration curves. The system was validated by adding a defined amount of drug suspension to the nanoemulsion and separating the particles again, being the recovery 102%.

Determination of *in vitro* activity of AB-N against *Candida albicans*

Susceptibility testing was performed in RPMI 1640 medium without L-glutamine and without sodium bicarbonate and buffered to pH 7.0 with 0.165 M MOPS. Sterile 96-well microplates were used. Stock solutions of the AB-N was diluted with RPMI 1640 to obtain the final concentrations as a recommendation to minimize systematic pipetting error.⁸ Final drug concentrations were 0.005 to 12.5 mg/L. Some wells served as the growth control and sterility check. The *Candida* isolates (n°3153 serotype A) were cultured on Sabouraud dextrose agar for 24 h at 36 °C. The inoculum suspension was prepared by suspending colonies in 5 mL of sterile 0.85% saline. The resulting suspension was vortexed for 15 s, and the cell density was adjusted to 0.5 McFarland Standard. The suspensions were diluted 1:100 with sterile saline, followed by dilution 1:100 with RPMI 1640. One hundred microliters of the yeast suspension was added to each well, resulting in the desired final drug concentration and inoculum size. The microplates were incubated at 36 °C without agitation and read after 24 h. A solvent control, organism control and medium control were performed simultaneously to check the growth inhibiting activity, organisms and sterility of broth medium.

The MIC was defined as the minimum concentration of AB that shows a full inhibition of the fungus in the well, examined by naked eyes and confirmed by an inverted microscope (40 x). Susceptibility testing of AB-N, AB-DOC, and AB powder in dimethyl sulfoxide (DMSO) were performed in triplicate. For this test, formulations were storage over a 6-month period at 4 °C and were compared monthly with freshly prepared commercial preparations of AB-DOC and AB powder in DMSO. AB dissolved in DMSO was also diluted with phosphate buffered saline (PBS) pH 6.8, giving DMSO content less than 1%.

RESULTS

Physicochemical characterization of AB-N

The prepared AB-N formulation aimed for parenteral administration. Thus particle size and particle size distribution are crucial parameters for safe administration of such formulation. The mean particle size and size distribution of AB-N nanoparticles were determined by dynamic light scattering in fresh and in 6-month-storage formulations (4 and 25 °C). The results are showed in Figure 1 and 2. The mean particle size diameter was 137.2 nm just after preparation and mean size of droplets showed an increase

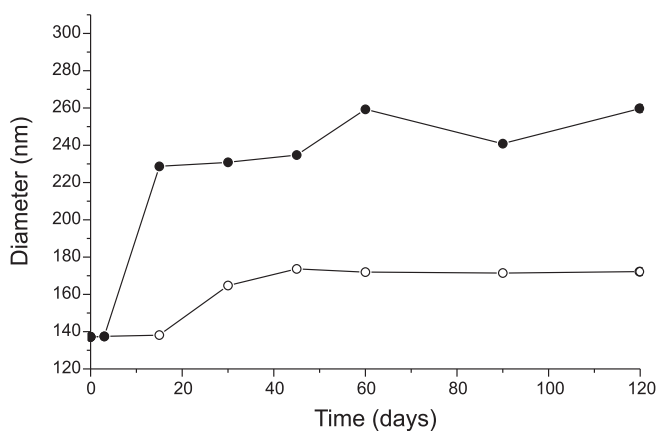


Figure 1. Particle diameter performed by laser light scattering. Nanoemulsions were analyzed just after preparation and in intervals until 6 months storage at 4 °C (○) and at 25 °C (●)

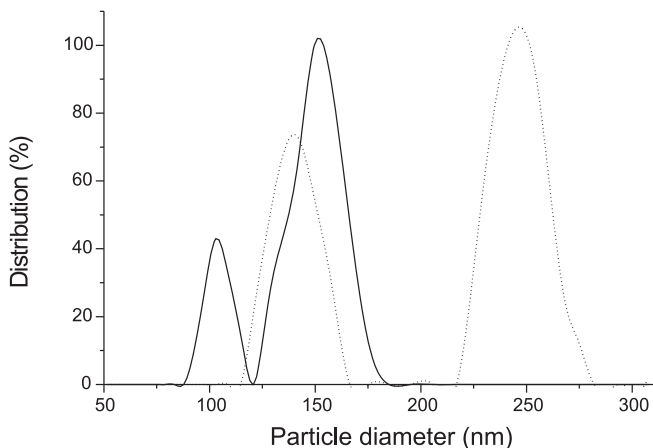


Figure 2. Dimensional distributions of AB-N just after preparation (straight line), and 6-month storage at 4 °C (dot line) expressed as percentage of distribution, determined by laser light scattering

($p=0.001$) after 6 month-storage at 4 (26.2%) and at 25 °C (115.1%). The pH measurements were performed on AB-N just after preparation and then monthly until 6 months, illustrated in Figure 3. No differences was found in pH values that ranged from 6.2 to 6.6 during storage at 4 and 25 °C ($p>0.05$).

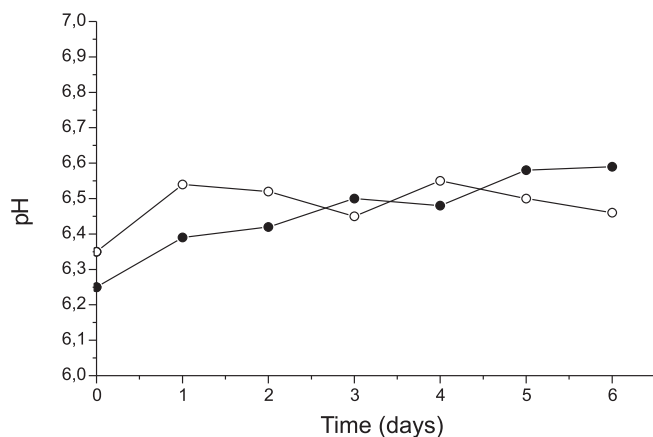


Figure 3. pH values of AB-N storage at 4 °C (○) and 25 °C (●) in function of time

Dispersion of AB-N in glucose 5%

The UV-vis spectroscopy was used to investigate the aggregation state of AB associated to the particles of the nanoemulsion (Figure 4). The absorbance spectrum of AB in methanol representing the monomeric form, showed high intensity peaks at 405, 382, and 363 nm and lower intensity peak at 345 nm. The absorbance spectrum of AB-DOC (Fungizone®), which represents the self-aggregation state of AB, showed a broad peak with high intensity at 326 nm and less intense peaks at 365, 385, and 408 nm. The absorbance spectrum of AB-N in glucose 5% showed a peak at 368 and 425 nm.

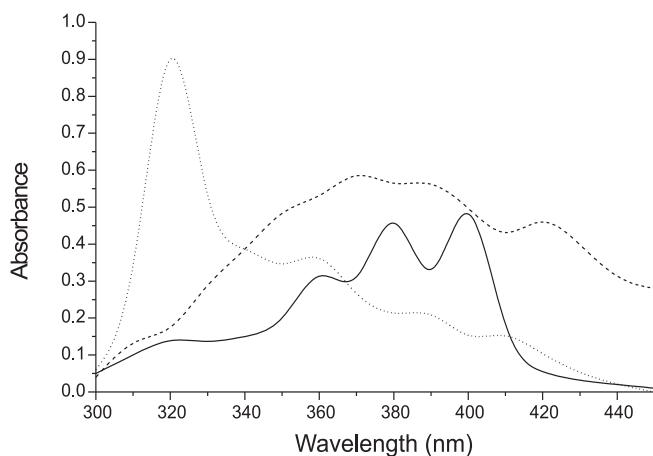


Figure 4. Evolution of absorbance spectra of AB in different formulations at $1.5 \times 10^{-5} M$: AB in methanol (straight line), and AB-DOC (dash line) and AB-N in glucose 5% (dot line)

AB content

In order to evaluate the solubilizing power of the produced formulation and its chemical stability, the AB solubility was expressed by AB as function of time, expressed as percentage of initial drug content. The fresh preparation incorporated 98% of AB

initial concentration and could maintain about 90% of AB in 6 months. Figure 5 shows the lost of AB content in formulations storage at 4 and 25 °C. In both storage conditions, no AB lost were detected at sixth month showing a chemical equilibrium between AB and lipid components. Finally, it was found that in the case of AB-N at 4 °C, drug decrease was higher than AB-N at 25 °C.

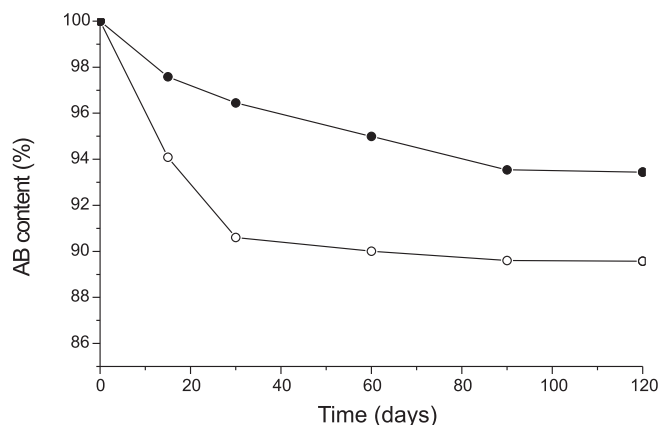


Figure 5. Lost of AB content in AB-N storage at 4 °C (○) and 25 °C (●) as a function of time. Data represent percentage determinations performed by UV spectroscopy at 404 nm

Susceptibility test of AB-N

The *in vitro* antifungal activity of AB-N was evaluated during 6-month storage at 4 °C. The MIC data of AB in DMSO, AB-DOC and AB-N is summarized in Table 1. Our results showed that AB-N could maintain the activity against *Candida albicans* during 6-month storage (MIC 0.03 µg/mL), showing a greater activity than AB-DOC (MIC 0.06 µg/mL). The basis for the low MIC of AB-N compared with that of AB-DOC is unclear, but the lipid components may be enhanced interaction of AB at a membrane level of fungi.

Table 1. Minimal inhibitory concentration (MIC) of AB in different formulations against *Candida albicans*

Sample	MIC (µg/mL)	
	Fresh preparation	6-month storage
AB in DMSO	0.12	^a
AB-DOC ^b	0.06	^a
AB-N	0.03	0.03

^a not performed

DISCUSSION

Amphotericin B is poorly soluble in water and can be successfully incorporated into the lecithin layer of a lipid carrier system by sonication. The aim of new AB formulation is to reduce the toxicity of this potentially useful antifungal drug. Many studies have suggested that this toxicity is related to the physicochemical state of the drug, and particularly to self-associated forms, which are able to complex cholesterol in mammalian cell membranes. We have previously demonstrated an effective incorporation of AB in a chylomicron-like nanoemulsion with favorable findings.^{7,9} Our previously formulation was designed with high-cost lipid components dissolved in DMSO whereas in AB-N it was used

alternative and biodegradable materials. AB-N can act as carrier system composed by coconut triglyceride, cholesterol and soybean lecithin in order to reduce self-aggregation state.

Experimental evidence gathered has shown that a close correlation exists between the aggregation state of AB in the vehicle and its toxicity in the site of infection.^{10,11} Therefore, various formulations of monomeric or preferably, slightly aggregated AB have been developed. Kawabata *et al.*¹² have previously demonstrated that a new formulation, micelles containing lysophosphatidylcholine and AB, monomerized more favorably than formulations such as liposomal formulations.

Spectra were recorded for the AB-N diluted in glucose 5% to give a final AB concentration of 1.5×10^{-5} M. For that ratio, bands between 425 and 368 nm were obtained and it was similar to monomeric states of AB bound with phospholipids reported by Tabosa do Egito *et al.*¹³ (Figure 4). These spectra showed qualitative differences as those reported by Bolard *et al.*⁵ for AB in the monomeric and aggregate form. Using diluted AB in DMSO, they observed peaks in the 409, 385 and 347 nm correspondent to monomeric form, whereas AB-N shift these bands to others ascribed in the presence of lipids. A difference in the aggregation state between AB-DOC and AB-N was observed with evidence of AB self-association (absorption band at 344 nm) for AB-DOC.

The phospholipids, or lecithins, used in the preparation of most parenteral emulsions are quite heterogeneous materials, acting as emulsifiers leading to long-term stability formulations.¹⁴ The presence of a drug in the emulsion can considerably alter its behavior. Fortunately, the drug effect can often be predicted with knowledge of the physicochemical properties of the drug. AB may adsorb to the interface but will not normally influence surface charge. Therefore, this behavior is only valid at specific pH, which suggests that pH measurements are the major tool to control this aspect of drug-containing nanoemulsion.¹⁵ According to Floyd,¹⁶ the optimum pH of the finished nanoemulsion is in the general range of 6-7 and low pH (values lower than 5) should be avoided as the electrostatic repulsion between particles is decreased, resulting in an increased globule size and coalescence. In addition, neutral pH minimizes lecithin hydrolysis. The pH values ranged from 6.2 to 6.6 during storage at 4 and 25 °C and particle size analysis confirmed the stability of the AB-N during 6-month storage. In general, nanoemulsions containing the smallest globules (usually 100- 500 nm) tend to be most physically stable.¹⁷ Our data demonstrated a stable formulation that could maintain the size even in critical storage conditions (25 °C).

Chemical stability studies were performed on formulations stored at room temperature and at 4 °C evaluating drug precipitation. Figure 5 reports AB content in the different storage conditions of AB-N as a function of time, expressed as percentage of initial drug content. It was found that AB-N could maintain 90% of AB stability for 6 months. It is to be underlined that in the present study, AB-N were stable both physically and chemically, probably because of the use of high purity soybean phosphatidylcholine.

The *in vitro* efficacy of AB-N, AB-DOC, and AB solubilized by DMSO was evaluated by the inhibition of *Candida albicans* growth. AB in DMSO and AB-DOC showed weaker antifungal activity than did AB-N (Table 1). This suggests that AB-N retains the potent activity of AB against fungal cells even though the AB is incorporated into nanoemulsion particles. Therefore, AB-N in the presence of fungal cells might be expected to release AB in sufficient amounts. Carrillo-Munoz *et al.*¹⁸ showed that the release of AB

from a liposomal formulation (AmBisome®) was slow and slight and the AB retained by liposomes might be unable to show its strong antifungal activity, even though AmBisome® can deliver high levels of AB to the infected site. The mechanism of AB transfer from lipid formulations to fungal cells is not well understood. Fukui *et al.*¹⁹ assumed that the difference in antifungal activity between AmBisome® and lipid nano-sphere containing AB results from a selective transfer of AB regulated by the physicochemical characteristics of the carrier and the target cells. According to our results, AB-N in the presence of fungal cells readily releases AB because the drug has a higher affinity for ergosterol than for lipid components of the nanoemulsion.

The physicochemical data obtained in the present study suggest that this nanoemulsion system shows an attractive potential as drug delivery system. Advantages of this technique are ease of manufacturing, use of biocompatible components for parenteral administration, and production in aqueous media avoiding organic solvents. We therefore believe that further evaluation of this new AB delivery system is merited.

ACKNOWLEDGEMENTS

The authors thank Dr. J. I. dos Santos from the Mycology Laboratory of the Centro de Ciências da Saúde, Universidade Federal de Santa Catarina, for kindly supplying the *C. albicans* inoculum and Bristol – Meyers Squibb (São Paulo, Brazil) for amphotericin B. This work received financial support from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes).

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