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Effect of aggregation state on the toxicity of different amphotericin B preparations

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ABSTRACT

The aim of this work was to study the effect of aggregation of amphotericin B (AMB) in their toxicity. The aggregation of AMB depends on different formulation factors such as pH and excipients, therefore three formulations with different AMB aggregation states were prepared: a monomeric form (M-AMB), a dimeric form (D-AMB) and a poly-aggregated form (P-AMB). The predominant aggregation state of each AMB formulation was characterized by spectrophotometry and their size by dynamic laser light scattering. Toxicity was evaluated by lethality in mice and hemolysis test from human erythrocytes and the experimental AMB formulations were compared with reference formulations of AmBisome®, Fungizone® and heated Fungizone®. The less toxic aggregation form of AMB was the poly-aggregated one which was similar to AmBisome®. Moreover, the P-AMB and heated Fungizone® were centrifuged to isolate different size fractions. The toxicity of these two heterogeneous formulations was related to their size, so the smaller the aggregation size fraction the higher the toxicity determined by hemolysis. It can be concluded that the aggregation state of AMB and their size affects critically the toxicity of AMB. The low toxic P-AMB formulations contain a different poly-aggregated state to that of AmBisome®, heated Fungizone® and the other studied AMB aggregation states.

1. Introduction

Amphotericin B (AMB) is a broad antimycotic agent with low incidence of resistances although its usefulness is limited by its toxicity. A large amount of data indicates that the antibiotic toxicity is related to its low aqueous solubility (Torrado et al., 2008). Due to its amphipathic nature, AMB self-associates and aggregates. Therefore, AMB in water form a mixture of water-soluble monomers and aggregates. The water-soluble monomer is usually related as a low toxic form of AMB (Barwicz et al., 1992; Legrand et al., 1992) and once intravenously administered is bound to serum proteins (Ridente et al., 1999). Several strategies have been applied to obtain AMB drug formulations predominantly in its monomeric form (Adams and Kwon, 2003, 2004; Toledo-Grijalba et al., 2006; Nishi et al., 2007). In relations to the aggregated forms of AMB, two forms have been usually defined: water-soluble oligomers and non-water-soluble aggregates. Clearly, water-soluble oligomers have been defined as the most toxic form of AMB (Barwicz et al., 1992; Legrand et al., 1992). Curiously, this is the AMB disposition in the Fungizone® preparation. Fungizone® is the AMB drug reference formulation and it was the only AMB formulation in the market for approximately 35 years. Different strategies were studied to decrease the toxicity of the Fungizone® preparation. One of them was to heat the drug in order to induce aggregation of AMB and decrease its toxicity (Gaboriau et al., 1997; van Etten et al., 2000; Hartsel et al., 2001). Another approach to decrease toxicity of AMB preparations is to elaborate AMB formulations with most of AMB in a non-water-soluble multi-aggregated form (Sánchez-Brunete et al., 2004). AMB in a multi-aggregated form is especially interesting to target the drug to the reticuloendothelial system in order to treat visceral leishmaniasis (Mullen et al., 1997).

The topic of AMB aggregation is complex because different species may be present at the same time in the preparation and also the aggregation state can change by dilution (Brajtburg and Bolard, 1996). Moreover, the *in vitro* disposition of the AMB molecules may change once the drug is administered *in vivo*. For this reason it is important to combine in the same work *in vitro* characterization studies with different biological toxicity tests.

The aim of this work was to study the effect of AMB aggregation on its toxicity. With this purpose three AMB formulations of different aggregation state were prepared: a monomeric form (M-AMB),

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a water-soluble aggregated or dimeric form of AMB (D-AMB) and a non-water-soluble multi-aggregated or poly-aggregated form (P-AMB). The P-AMB formulation was then centrifuged to isolate different fractions in order to study the heterogeneity of this preparation. The predominant aggregation state of AMB was characterized by spectrophotometry and the size of the particles by dynamic laser light scattering. Toxicity was evaluated by two methods: acute lethality in mice and a hemolysis test from human erythrocytes. The experimental AMB formulations were compared with reference formulations of AmBisome®, Fungizone®, heated Fungizone® and centrifuged heated Fungizone®.

2. Materials and methods

2.1. Materials

AMB was a gift from Bristol-Myers Squibb, Barcelona, Spain. Sodium deoxycholate was purchased from Fluka Chemie A.G., Buchs, Switzerland. Dibasic sodium phosphate, monobasic sodium phosphate, orthophosphoric acid, sodium hydroxide and dimethylsulfoxide were supplied by Panreac S.A., Barcelona, Spain. γ-Cyclodextrine (γ-CD) was obtained from Cerestar Ibérica, Martorell, Spain. Proprietary AMB formulations, AmBisome® and Fungizone®, were purchased from wholesalers.

2.2. Preparation of AMB experimental formulations

Three AMB formulations with AMB molecules in different aggregation state were prepared as reported previously (Sánchez-Brunete et al., 2004).

M-AMB was prepared by spiking AMB (50 mg) in 10 mL of a γ-CD solution in water (45 mg/mL) previously adjusted at pH 12.0 with sodium hydroxide. Once the drug was dissolved, it was neutralized to pH 7.4 with orthophosphoric acid.

D-AMB had similar qualitative and quantitative composition to the commercialized medicine Fungizone® (reference formulation), and was prepared as follows. AMB (50 mg) was dispersed in 5 mL of a water solution formed by sodium deoxycholate, dibasic sodium phosphate and monobasic sodium phosphate previously adjusted to pH 12.0 with sodium hydroxide. Once the drug was homogeneously dispersed, it was acidified to pH 7.4 employing orthophosphoric acid. Water was added to the resulting mixture up to a final volume of 10 mL.

P-AMB was prepared similarly to D-AMB formulation, but without the initial pH of 12.0. P-AMB was centrifuged for several cycles in a Hettich Universal 32 centrifuge. The five initial cycles were of 5 min at $700 \times g$ and the last cycle was of 5 min at $4500 \times g$. After every cycle the supernatant was removed for its characterization of aggregation state, size and toxicity by hemolysis.

Heated Fungizone: Fungizone® was heated for 1 h at 70 °C. This formulation was then centrifuged in the same conditions as P-AMB.

2.3. AMB molecular organization: determination of aggregation state

In order to determine the predominant aggregation state of AMB, all AMB formulations were reconstituted, if required, and diluted as necessary with deionized water. The resulting dilutions were immediately analyzed by spectrophotometry. Spectra between 300 and 450 nm were recorded employing a Simadzu UV-1700 spectrophotometer (Sánchez-Brunete et al., 2004).

2.4. DLS size determination

Dynamic light scattering experiments (DLS) were performed with a Protein Solutions DynaPro MS/X apparatus equipped with an 824.7 nm laser wavelength. A monochromatic beam of light illuminates a cuvette containing the sample, and the fluctuation of intensity in the scattered light (microsecond timescale) is detected by the photo diode at 90°. The fluctuation rate is related to the diffusion coefficient (D) of the scattering species that is calculated by the method previously reported by Papish et al. (2002).

In dynamic light scattering experiments, the radius (R) of the particle is calculated from the diffusion coefficient (D) via the Stokes–Einstein equation, where k is the Boltzmann constant, T is the temperature, η is the solvent viscosity, and $f = 6\pi\eta R_H$ is the frictional coefficient for a compact sphere in a viscous medium.

$$D = \frac{kT}{f} = \frac{kT}{6\pi\eta R_H}$$

The DLS measured radius is the radius of a hypothetical hard sphere that diffuses with the same speed as the particle under examination.

On the other hand, DLS is used to calculate the polydispersity index (PDI), that is a measure of the distribution of D (R_H) in a given polymeric sample. The PDI calculated is the weight average D divided by the number average of D.

DLS technique was used to measure the hydrodynamic size and polydispersity of the samples and the possible presence of high molecular weight aggregates.

2.5. Hemolysis test from human erythrocytes

Venous blood obtained from healthy volunteers was collected in tubes containing heparin (50 units/mL). Whole blood was centrifuged for 10 min at $1600 \times g$ and the supernatant and buffy coat were pipetted off and discarded. Erythrocytes (RBCs) were then washed twice with isotonic phosphate buffer solution of pH 7.4 (PBS) and were finely dispersed in PBS at a cell density of 7×10^9 cells/mL. They were used on the same day that they were obtained. Subsequently, 2 mL of the RCBs suspension was mixed with 2 mL of buffer containing several AMB formulations at various concentrations in triplicate. The dose level of AMB used in the *in vitro* assay was ranged from 0 to 200 μg/mL. Each sample was then incubated at 37 °C in a shaking water bath (100 strokes/min). After a 1 h incubation, hemolysis was stopped by reduced temperature (0 °C) and unlysed RBCs removed by centrifugation for 10 min at $2900 \times g$. The supernatants were collected for analysis of the extent of hemolysis by reading the absorption of the hemoglobin at 575 nm. Results from triplicate experiments were expressed as a percentage of hemolysis with respect to the amount of hemoglobin released in the presence of water, which was taken as measure of complete (100%) lysis. Percent hemolysis is reported by $100(Abs_s - Abs_b)/(Abs_1 - Abs_b)$ where Abs_s is the absorbance of the sample, Abs_b is the average absorbance of the buffer, and Abs_1 is the average absorbance of the lysed samples. Differences in hemolysis depending on type of formulation were studied statistically using a Student's t paired test (Excel, Office 2003). Significance was set at a P value <0.05.

2.6. Acute toxicity

Mice were divided into groups containing six animals each. Every group was injected by intracardiac route with one of the formulations (M-AMB, D-AMB or P-AMB), increasingly at 2, 5, 10,

Table 1
Aggregation state, hydrodynamic mean size and hemolysis of different AMB formulations (M-AMB, D-AMB, P-AMB, Fungizone®, heated Fungizone® and AmBisome®)

AMB formulation	Aggregation state	Hydrodynamic mean size[a] (nm)	Hemolysis[b] (%) at 100 μg/mL AMB
M-AMB	Monomer	594.5 (71.5)[c]	33.1 ± 2.04[c]
D-AMB	Dimer	11.9 (3.2)	5.07 ± 1.52
P-AMB	Poly-aggregates	1692.1 (615.5)	0.04 ± 0.01
Fungizone®	Dimer	138.7 (41.2)	9.03 ± 0.55
Heated Fungizone®	Mixture of different states	1909.0 (535.4)	3.17 ± 0.49
AmBisome®	Mixture of different states	35.0 (17.4)	0.06 ± 0.02

[a] Mean value of two different samples (polydispersion in parenthesis).
[b] Mean value of three different samples ± standard deviation.
[c] Same as γ-CD control without AMB.

15 or 40 mg of AMB per kilogram. The animals were examined for mortality over the following 48 h.

3. Results

Fig. 1 shows the absorption spectra of the three different aggregation states of AMB of the experimental preparations (M-AMB, D-AMB and P-AMB). Fig. 2 shows the absorption spectra of the marketed preparations AmBisome® and Fungizone®. In our experiment the AMB disposition in Fungizone® is the same as the experimental D-AMB formulation which corresponds to the water-soluble oligomer form. Once heated the AMB disposition in the Fungizone® preparation is changed. Fig. 2 shows the change in the absorption spectra which is similar to the spectra of the AmBisome® formulation. The wavelength of the maximum peak in the heated Fungizone® in relation to Fungizone® is displaced from 327 to 324 nm and this new peak has a lower intensity than the maximum of unheated Fungizone®. It has previously been reported that the heating of Fungizone® induces the formation of a mixture of monomer and superaggregated forms of AMB (Gaboriau et al., 1997; van Etten et al., 2000).

Table 1 summarized the aggregation state of the different tested formulations and their hydrodynamic mean size (R_H). The mean R_H is obtained by dynamic laser scattering technique and not necessarily corresponds to the true size of the particles (Alliance protein laboratories, 2007). The biggest mean size has been obtained with the heated Fungizone® while the smaller particles are obtained in the D-AMB preparation. Although it has not been included in the Table 1 different control samples have also been tested corresponding to the excipients sodium deoxycholate and γ-CD. The mean size of the sodium deoxycholate micelles was 1.2 nm while for the γ-CD system it was 594.7 nm. The hydrodynamic mean size of these two excipients in water may explain the differences in size of the corresponding AMB preparations: M-AMB and D-AMB. The P-AMB preparation has a bigger size than the M-AMB and D-AMB formulations and it is comparable to the heated Fungizone®. The D-AMB preparation has the same composition than the Fungizone® formulation. However, Fungizone® formulation has a bigger mean size than D-AMB, probably due to same agglomeration process of the micelles. The marketed Fungizone® preparation is a suspension which requires a vigorous shaking for its reconstitution. The experimental D-AMB formulation is elaborated a few hours before its use and maintains its initial appearance without signs of precipitation for several days. The liposomes of the AmBisome® formulation have a small hydrodynamic mean size of 35.0 nm.

Table 1 and Fig. 3 show the results of the hemolysis experiments at different AMB concentrations. In our experimental conditions between 10 and 40 μg/mL, D-AMB was the aggregation state of significantly highest hemolytic effect ($P < 0.05$). At 10 and 40 μg/mL no statistical differences were observed between M-AMB and P-AMB ($P > 0.05$). Nevertheless, at 100 μg/mL M-AMB formulation has the highest hemolytic effect among the tested formulations. This hemolytic effect is the same as observed with the pure excipient, γ-CD, which has been previously reported as hemolytic (Ohtani et

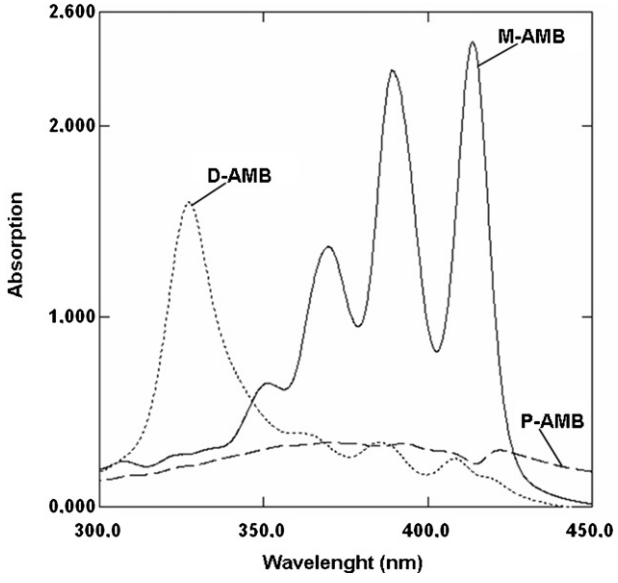

Fig. 1. Absorption spectra corresponding to formulations M-AMB (——), D-AMB (. . . . . .) and P-AMB (- - -).

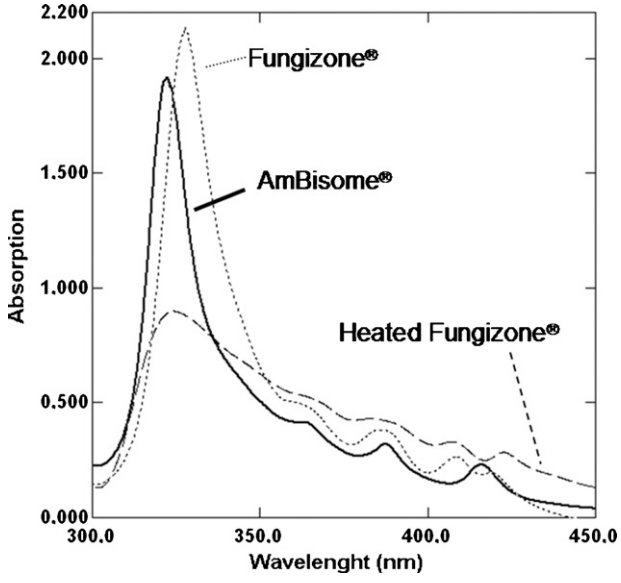

Fig. 2. Absorption spectra corresponding to Fungizone® (. . . . . .), AmBisome® (——) and heated Fungizone® (- - -).

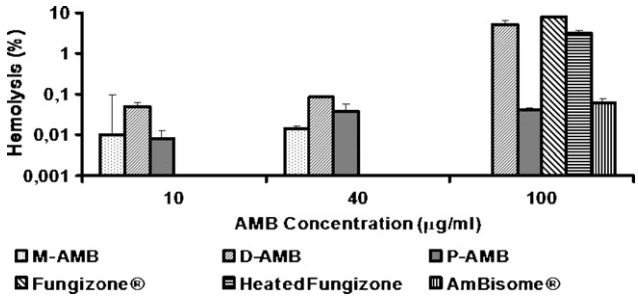

Fig. 3. Hemolysis of human erythrocytes at various concentrations (10, 40 and 100 μg/mL) of AmB as M-AMB, D-AMB, P-AMB, Fungizone®, heated Fungizone® and AmBisome® in semi-logarithmic scale.

al., 1989), although the use of this excipient at intravenous doses below 120 mg/kg has been reported as safe (Donaubauer et al., 1998). Clearly, the less hemolytic state is the poly-aggregated one (P-AMB) which has a hemolytic effect similar to that observed by the AmBisome® formulation. The reference Fungizone® formulation has a high hemolytic effect which is significantly decreased ($P < 0.05$) by a previous heating of the preparation.

The toxicity of the different aggregation states of AMB has also been studied in an acute mortality assay in mice. Table 2 shows the results at different doses. It is clear that P-AMB is the safest form of AMB followed by M-AMB. D-AMB is the most toxic form of AMB.

Due to the low toxicity observed by the P-AMB formulation it was interesting to study this preparation in more detail and compare it with the heated Fungizone®. Therefore, different centrifugation cycles were used to separate supernatants of both formulations. The aggregation state of the different AMB supernatant samples of P-AMB formulation is shown in Fig. 4. All the samples have the same spectra profile of P-AMB and they differ in their absorption intensity, which is related to their concentration. In the same way, the spectra profile of heated Fungizone® did not change after the centrifugation cycles (data not shown). At each centrifugation cycle different mass losses were observed and at the same time there was a decrease in the hydrodynamic mean size (see Fig. 5). This shows that P-AMB and heated Fungizone® are not homogeneous preparations. Especially relevant is the decrease in size observed after the third centrifugation cycle of the P-AMB formulation.

Fig. 6 shows the effect of centrifugation cycles on the hemolysis of human erythrocytes at 200 μg/mL of AMB. Fig. 6 shows how hemolysis increases with the number of centrifugation cycles. Therefore, in the P-AMB and heated Fungizone® formulations there is a relation between size of the aggregated AMB and hemolysis. In our experimental conditions the lower the size of the AMB aggregated the highest its toxicity by hemolysis.

Table 2
In vivo acute toxicity of different formulations of AMB in mice after a single bolus injection

AMB dose (mg/kg)	No. of animals that died/total No. of animals tested (%)		
	M-AMB	D-AMB	P-AMB
2	0/6 (0%)	1/6 (16.7%)	0/6 (0%)
5	0/6 (0%)	3/6 (50%)	0/6 (0%)
10	0/6 (0%)	6/6 (100%)	0/6 (0%)
15	6/6 (100%)		0/6 (0%)
40			0/6 (0%)

Values are calculated from the number of mice that died during the 48 h after the injection.

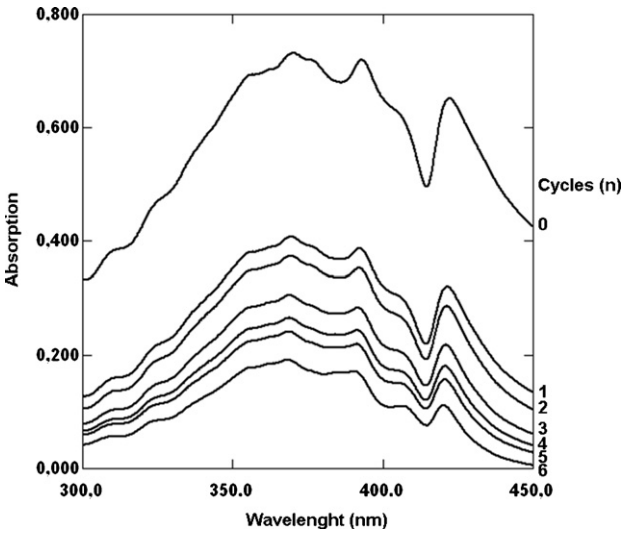

Fig. 4. Effect of centrifugation cycles number (n) on the absorption spectra of the P-AMB formulation.

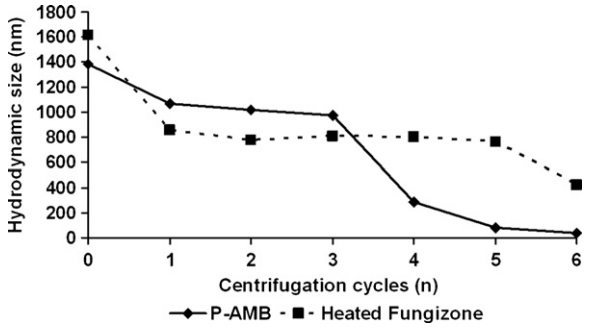

Fig. 5. Effect of number of centrifugation cycles on the hydrodynamic mean size of the P-AMB and the heated Fungizone® formulations.

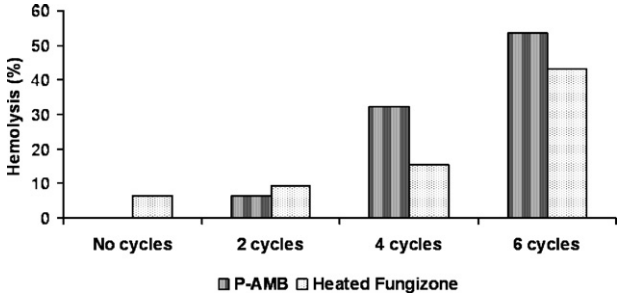

Fig. 6. Effect of centrifugation cycles number of the P-AMB and the heated Fungizone® formulations on the hemolysis from human erythrocytes at 200 μg/mL of AMB concentration in semi-logarithmic scale.

4. Discussion

The molecular state of AMB, particularly its self-aggregation in aqueous medium, depends on different factors such as: its final concentration, pH, temperature and ionic strength of the aqueous phase and excipients of the preparations. Among the excipients especially relevant are surfactants, tensoactive and cosolvents (Torrado et al., 2008). In most of the previous reported papers the monomer state of AMB has been obtained by previous solubilization of AMB in an organic solvent such as methanol (Legrand et al., 1992) or dimethyl sulfoxide (Nishi et al., 2007). This technical approach is perfectly valid for physical–chemical studies of

AMB formulations but when biological studies of toxicity such as hemolysis test or acute toxicity are going to be performed the effect of those remnant solvents in the sample is a question of concern. This effect can be partially known by adequate controls, which can provide the toxicity of those excipients. For instance, in our experimental conditions of hemolysis, γ-CD was hemolytic when used at 100 μg/mL of AMB in the M-AMB preparation while this effect was not observed at lower AMB concentrations of 10 and 40 μg/mL. Table 2 shows how the acute toxicity of M-AMB is lower than with D-AMB, which agrees with the results previously reported by other authors (Barwicz et al., 1992; Legrand et al., 1992). Other excipients have proved to be useful to achieve AMB formulations of low toxicity containing AMB in a monomer form (Lavasanifar et al., 2002; Larabi et al., 2004; Nishi et al., 2007).

The water-soluble aggregate state of AMB (D-AMB) seems to be formed by AMB in a stable dimeric form (Caillet et al., 1995; Mazerski and Borowski, 1996). The absorption spectrum of our D-AMB preparation coincides with that of the marketed formulation of Fungizone®. The absorption spectra of Fungizone® changed after heating (Fig. 2). Once heated the Fungizone® spectra changed in a similar way as it has been previously reported (Gaboriau et al., 1997). According to Gaboriau et al. (1997) heating of Fungizone® induces the formation of monomers and super-aggregates and so the heated Fungizone® is less toxic than Fungizone®. In our experimental conditions, it is clear that there is a change in the AMB conformation state that produces an increase in size and a decrease in its toxicity. Curiously, van Etten et al. (2000) centrifuged the heated Fungizone® and they did not found differences among the sizes or the biological characteristics (toxicity and antifungal activity) between centrifuged and non-centrifuged heated Fungizone®. However, in our experimental conditions when we have centrifuged the poly-aggregated AMB (P-AMB) and the heated Fungizone® different size fractions were separated (see Fig. 5) and different hemolysis toxicity was observed (Fig. 6). Nevertheless, the absorption spectrum of the P-AMB (shown in Fig. 1) is different from that of the heated Fungizone® (Fig. 2).

Fig. 2 shows how the aggregation state of AmBisome® coincides with that of the heated Fungizone®. The low toxicity by hemolysis of AmBisome® is well known and has been related to its aggregation state, the rate at which they release the AMB (Brajtburg and Bolard, 1996) and the presence of cholesterol in the composition of AmBisome® (Patankar and Wasan, 2006). In the AmBisome® formulation the AMB remains firmly associated with the liposome structure (Veerareddy and Vobalaboina, 2004) and the lysis of erythrocytes requires considerably longer incubation period than with the Fungizone® preparation (Wiehart et al., 2006).

The P-AMB preparation is a heterogeneous mixture of different non-water-soluble aggregates of AMB. In order to correctly characterize this system the aggregation state determined by spectroscopy is not enough and it should be complemented by size analysis because there is a clear relationship between size and biological activity as measured by the hemolysis test (shown in Figs. 5 and 6). Probably, the lower hemolysis of the P-AMB and the heated Fungizone® preparations can be related to its solubility and the higher the size of the fractions the lower their solubilization. In this way, the P-AMB and the heated Fungizone® probably act as a reservoir of AMB. The activity of the P-AMB preparation has been previously tested in a leishmaniasis experimental model with good results (Sánchez-Brunete et al., 2004). More recently, in an experimental candidiasis model the effect of aggregation state of AMB on different pharmacokinetic distributions and activities was observed (Espada et al., 2008).

In conclusion, as it has been previously described, the aggregation state of AMB is directly related to its toxicity. The non-water-soluble forms are the lowest toxic aggregation forms of AMB. Therefore, aggregation state of AMB has to be evaluated. The low toxic P-AMB formulation contains a poly-aggregated state different to that of AmBisome®, heated Fungizone® and the other studied AMB aggregation states. For this P-AMB preparation its evaluation should be done not only by spectrophotometry but also by a complementary size analysis technique because P-AMB is a heterogeneous size preparation. In our experimental conditions the lower the size of the aggregates the higher its toxicity by hemolysis.

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