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# Poly(ε-caprolacton) nanospheres as an alternative way to reduce amphotericin B toxicity

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**Abstract**

A new stable amphotericin B nanosphere-based delivery system was obtained by a solvent displacement process. A reproducible and monodisperse size distribution centered on 220 nm was obtained, when AmB and poly(ε-caprolacton) were dissolved in an appropriate solvent mixture. UV-Visible and circular dichroism spectroscopy suggested that nanoparticles modified the aggregation state of AmB, probably due to a weak interaction between the drug and the polymer of the nanospheres. Zeta potential measurements indicated that amphiphilic amphotericin B could be adsorbed onto the nanospheres. Fourier transformed infrared spectroscopy (FT-IR) seemed to confirm the absence of drug incorporation into the core of these carriers and that no chemical interaction between the drug and the polymer occurred. The reduction of the acute toxicity of AmB in healthy mice by association of the antibiotic with nanospheres warrants further investigation of the antifungal activity of these systems. © 1997 Elsevier Science B.V.

*Keywords:* Amphotericin B (AmB); Poly(ε-caprolacton) nanospheres; Zeta potential; Infrared spectra; Absorption spectra; Circular dichroism spectra

## 1. Introduction.

Amphotericin B (AmB), an amphiphilic polyene antibiotic, is the drug of choice for the treatment of the frequently fatal disseminated fungal infections which occur as a result of reduced

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immunocompetence in chemotherapy and AIDS patients.

Unfortunately, the administration of AmB is severely limited due to its toxic side-effects, which include acute chills and fever, and chronic nephrotoxicity. In order to minimize these drawbacks and in an attempt to circumvent the poor solubility of the drug in water, several drug carrier systems have been developed. Most of these have been liposomes (AmBisome®) or lipid complexes (Abelcet®) (Amphocil®) (Szoka and Tang, 1993). All these formulations enhance the therapeutic index of AmB by decreasing its toxicity. However, recently, several infrequent side-effects have been detected in patients; including allergic reactions (Ringden et al., 1991), cardiopulmonary toxicity (Levine et al., 1991) and severe systemic side-effects (Sanders et al., 1991).

Another suitable approach to overcome some of the problems associated with AmB use could be association with biodegradable polymeric carriers such as nanoparticles. These systems have been shown to be a good strategy for drug delivery in the treatment of liver diseases (Couvreur and Vauthier, 1994) and parasitic infections when loaded with primaquine (Gaspar et al., 1992; Rodriguez et al., 1995), pentamidine (Mbela et al., 1992) or amphotericin B (Vernier-Julienne et al., 1995).

As compared with phospholipid vesicles, nanoparticles are expected to be more stable during storage and in biological fluids. Amphiphilic AmB has already proved to be an excellent candidate for incorporation within liposomes or in lipid carriers, in many studies carried out over the past decade. Nevertheless, the particular physicochemical properties of AmB: insolubility in water with formation of aggregates of different size as a function of drug concentration, low solubility in oil and in the most of organic solvents, have limited its association with biodegradable nanoparticles prepared from co-poly(lactic acid)–poly(glycolic acid) (PLGA) (Vernier-Julienne and Benoit, 1996).

In order to improve this system, we have tried to associate this drug with nanospheres of poly(ε-caprolacton), another polyester, much more hydrophobic than PLGA. Therefore, the aim of this work was to prepare and characterize amphotericin B-loaded poly(ε-caprolacton) nanospheres. Its acute and renal toxicity was tested in mice to know if this formulation was able to reduce AmB toxicity, as a first approach before to focus on more precise system characterization.

## 2. Material and methods

### 2.1. Materials

Poly(ε-caprolacton) (MW 42 500) and poloxamer 188 (Pluronic F68®) were supplied by Sigma (Madrid, Spain). AmB was kindly given by Bristol-Myers Squibb (Madrid, Spain). All other chemicals were reagent grade.

### 2.2. Methods

#### 2.2.1. Preparation of AmB dispersion and AmB-nanospheres (AmB-Nps)

The preparation of empty and AmB-loaded poly(ε-caprolacton) nanospheres was based on a solvent displacement process (Fessi et al., 1987). The different formulations tested are summarized in Table 1. Briefly, 125 mg of poly(ε-caprolacton) and 1 mg of AmB were first dissolved, by heating and sonication, in 20 ml of acetone and a cosolvent. This organic phase was adjusted to pH 4 with HCl in order to promote AmB solubilization. This solution was poured into 40 ml of water containing 125 mg Pluronic F68® as the hydrophilic surfactant under moderate magnetic stirring. Finally, the organic solvents were evaporated under reduced pressure at 58°C and the final volume of the aqueous suspension was adjusted to 10–20 ml.

A dispersion of AmB was prepared as described above for AmB-Nps, omitting surfactant and polymer.

#### 2.2.2. Physicochemical characterization

*2.2.2.1. Particle size and zeta potential analysis.* The mean particle size of free dispersed AmB, empty nanospheres and AmB-Nps was determined, just after preparation and after a 1-month

Table 1
Influence of the cosolvent and its concentration in the average size of Amphotericin B micelles, AmB-nanospheres and AmB-free nanospheres: 20 ml acetone, 125 mg PF68® and 10 mg AmB used in AmB preparations

| | AmB-nanospheres | | | | | | AmB micelles | AmB-free nanospheres |
|---|---|---|---|---|---|---|---|---|
| Cosolvent | | None | DMSO | Propylene glycol | Ethanol | Methanol | | |
| $V$ (ml) | | | 0.5 | 2 | 10[a] | 10[a] | | |
| $J_0$ | Mean diameter (nm) | >1 μm[b] | 236 | 656 | 740 | 216 | 370 | 222 |
| | Polydispersity index | | 0.20 | >0.5 | >0.5 | 0.11 | >0.5 | 0.084 |
| $J_{1\ month}$ | Mean diameter (nm) | — | >1 μm[b] | 251 | 235 | 218 | >1 μm[b] | 224 |
| | Polydispersity index | | | 0.13 | 0.13 | 0.098 | | 0.090 |

[a] Solvents eliminated under reduced pressure.
[b] Amorphous precipitated.
$J_0$, size determination after preparation.
$J_{1\ month}$, size determination after 1 month storage at 4°C.

storage at 4°C, by Photon Correlation Spectroscopy (PCS) on a Zetamaster Instrument (Zetasizer 4, UK). The zeta potential measurements of all batches were carried out after sample dilution (ratio 1:100 v/v) in distilled water, adjusted to desired pH with HCl or NaOH.

*2.2.2.2. UV-Visible and circular dichroism (CD) spectroscopy.* Absorption and circular dichroism spectra of free AmB and AmB-Nps were analyzed by diluting stock preparations (1 mg/ml AmB) to a final concentration of AmB of 10 μg/ml, at room temperature, in a 1-cm cuvette. CD spectra were recorded with a Jobin-Yvon Mark V dichrograph at room temperature. CD measurements were expressed in $\Delta\varepsilon$, the differential molar dichroic absorption coefficient ($10^3 \cdot cm^2 \cdot mol^{-1}$), characteristic of the AmB autoassociation.

UV-visible absorption spectra were recorded with a Varian Cary 219 spectrophotometer.

*2.2.2.3. Differential scanning calorimetry (DSC).* The thermograms of AmB powder, empty nanospheres, AmB-Nps and the mixture of AmB powder with empty nanospheres were recorded on a Setaram DSC-92 differential scanning calorimeter calibrated with indium and zinc. The thermal behaviour was studied by heating $5.0 \pm 2.0$ mg of the samples in a covered sample pan under nitrogen gas flow. The temperature range used was between 25 and 250°C, with a heating rate of 10°C/min.

In all cases, the different preparations were freeze-dried (without cryoprotector) prior to the analysis.

*2.2.2.4. Fourier transformed infrared spectroscopy (FT-IR).* FT-IR spectra of the freeze-dried formulations were recorded on a double-beam infrared Perkin Elmer 681 spectrophotometer using the conventional KBr pellet method.

### *2.2.3. Acute toxicity*

Single bolus injections (0.4 ml) containing various doses of fungizone® (0.8/1.6/3.2/6.4 mg/kg), Ambisome® and AmB-Nps (5/10/20/40 mg/kg) were given to groups of six female Swiss mice, weighing 20–25 g, in the tail vein.

Dead mice were counted daily for 7 days and the $LD_{50}$ was determined by the method of Litchfield and Wilcoxon (1949). In order to investigate the effects of the different formulations on kidney function, the urea concentration in blood, blood urea nitrogen (BUN), was determined in

blood samples obtained from retroorbital plexus of survivors at Day 7.

The results were compared between two groups by using the Mann–Whitney *U*-rank sum test, when the Kruskal–Wallis test, performed on more than two groups, was significant. Differences where $p < 0.05$ were considered significant.

## 3. Results and discussion

### *3.1. AmB-nanospheres preparation*

Nanospheres containing AmB were prepared by a nanoprecipitation method avoiding the use of toxic chlorinated organic solvents. Nevertheless, several difficulties have to be overcome in order to incorporate the drug into the nanospheres. The main one was to select an organic phase which was able to solubilize both AmB and the polyester polymer. For polyester nanosphere preparation, acetone (a water-miscible and low-boiling-point solvent) is the solvent of choice. However, the preparation of AmB-Nps in acetone yielded an amorphous precipitate of non-associated drug. One possible solution was the use of cosolvents. Moreover, it has been described that AmB solubility in different solvents can be increased by acidification with dilute HCl. Therefore, we used a cosolvent, and acidic conditions to optimize the solubility of both AmB and the polymer. The mean size of AmB-Nps prepared with four different cosolvents was measured just after their preparation and after storage at 4°C (Table 1). In all cases, the volume of cosolvent used was the minimal amount necessary to dissolve the drug.

We prepared AmB dispersions with or without polymer at 1 mg/ml AmB. The formulation including methanol as cosolvent was chosen because this cosolvent allowed the preparation of reproducible nanospheres, small ($220 \pm 10$ nm) and stable in size over time (1 month), with a high pay-load of AmB (maximum 3 mg/ml). Ethanol and propylene glycol, not as good as methanol for AmB and polymer solubilization respectively, led to the formation of more polydisperse nanoparticles. AmB-Nps with DMSO as cosolvent were not stable more than 24 h. One possible reason of this instability could be the presence of oligomeric AmB species in the solvent (acetone/DMSO) mixture, as described by Brittain (1994).

The association of AmB with nanospheres did not modify the size as compared to empty nanospheres, whatever the concentration of the drug (average diameter $220 \pm 10$ nm). On the other hand, the size of AmB micelles was polydisperse, concentration-dependent and changed with time (Table 1).

### *3.2. Physicochemical characterization*

Usual separation methods for removing free drug like ultrafiltration, dialysis and ultracentrifugation are adapted to the elimination of aqueous soluble drugs. AmB is water insoluble and it makes self-aggregated very difficult to separate from drug loaded nanospheres. As we could not calculate the drug loading in our formulation, we tried to develop other strategies to characterize the system.

The characteristic optical properties of AmB due to the presence of conjugated double bonds make the study of environmental modifications by spectroscopic methods particularly appealing. In fact, Hemenger et al. (1983), Rinnert et al. (1977), Ernst et al. (1981), Gruda et al. (1988) have demonstrated that electronic absorption, and especially CD spectra of AmB are very sensitive to conformational changes induced either by modifications in the aggregation state of the polyene or by its association with other compounds. Therefore, these techniques were used to investigate the association with the nanosphere polymer without prior free drug removal.

In fact, at a submicellar AmB concentration (below 0.1 μg/ml), the antibiotic exhibits four positive bands, at 409, 385, 365 and 347 nm in circular dichroism and in absorption spectra. These bands are characteristic of the monomeric form of AmB.

At higher concentrations, aggregation of AmB occurs and a bathochromic shift produces a new absorption spectra with a broad intense single band at 340 nm and other lower intensity bands at 360, 385 and 420 nm. Furthermore, the CD spectra exhibit a very intense negative doublet

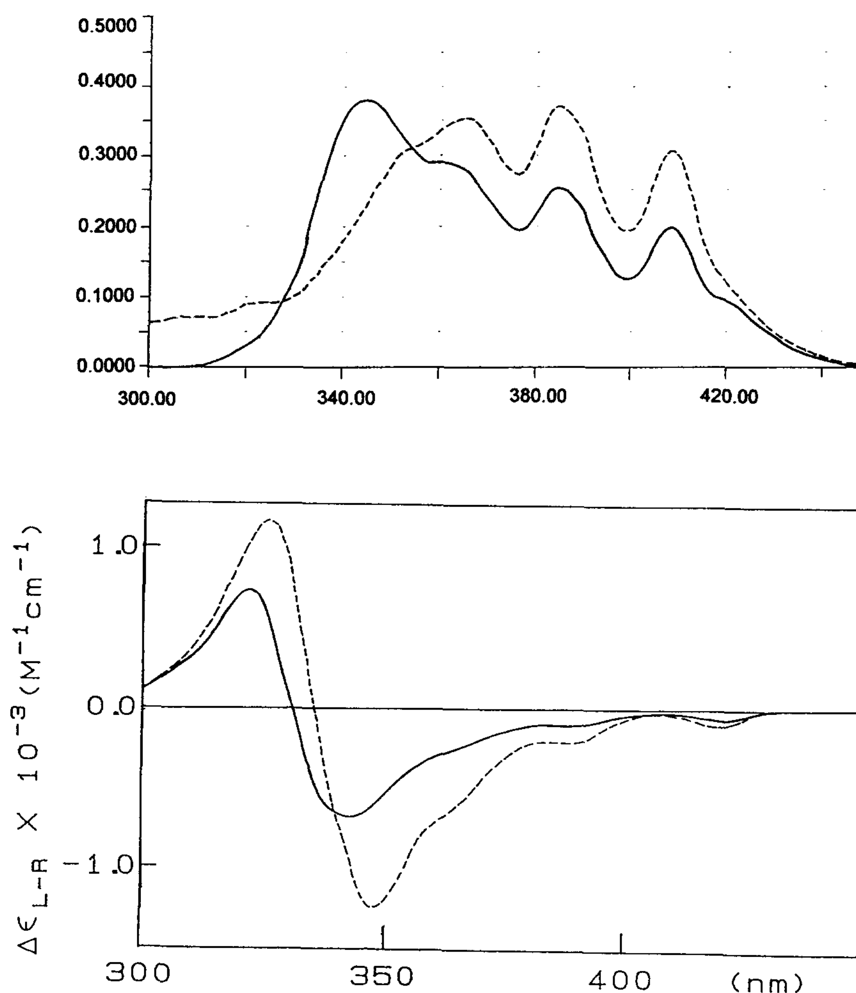


Fig. 1. Absorption and CD spectra of AmB micelles (——) and AmB-nanospheres (· · ·) at 10 μg/ml by dilution from a stock solution at 1 mg/ml.

centered around 340 nm, which is characteristic of aggregated AmB species, and three negative bands at 368, 393 and 423 nm.

At 10 μg/ml AmB, UV-visible spectra of AmB-Nps (Fig. 1) revealed a red shift of the 340 nm peak of free AmB towards 350 nm. CD spectra confirmed this shift accompanied by an increase of $\Delta\varepsilon$. These results suggest that the AmB aggregation state is modified by its association with nanospheres during the solvent displacement process. Moreover, the electronic absorption and circular dichroism spectra of AmB-Nps were as concentration-dependent as free AmB in aqueous suspension (Bolard et al., 1991). In fact, the dilution of AmB-Nps below 5 μg/ml drug led to the dissociation of AmB aggregates and the release of free monomeric AmB which could be detected by decreasing the intensity of doublet corresponding self-aggregated for the drug (Fig. 2). It seems that the interaction between AmB and nanospheres was relatively weak and could have been due to the association of AmB with nanospheres by adsorption.

FT-IR analysis was used to ensure that no chemical interactions between the drug and the polymer had occurred (Fig. 3). The AmB spectra had no bands of high intensity. It showed a C=O band near 1692 $cm^{-1}$, at 1566 $cm^{-1}$ C=C stretch

band and a poorly resolved substructure at 800–950 $cm^{-1}$.

On the other hand, FT-IR spectra corresponding to AmB-Nps and a physical mixture of AmB with nanospheres were identical to polymer spectra in AmB-free nanospheres, without any changes or appearance of new bands. This seems to indicate the absence of chemical interaction between the polymer and the drug in AmB-nanosphere preparations. These spectra did not display the bands characteristic of the drug because these were of low intensity and were hidden by the bands produced by the polymer (Sanchez et al., 1993).

DSC thermograms of free AmB, empty nanospheres, polymer, AmB-Nps, and a physical mixture of the drug powder and empty nanospheres in the same proportion as in the formulation, were made in an attempt to define the physical state of the drug in these carriers and the possibility of interactions between the drug and polymer within the network of the polymer in the nanoparticles (Dubernet, 1995; Ford et al., 1989). The results are illustrated in Fig. 4.

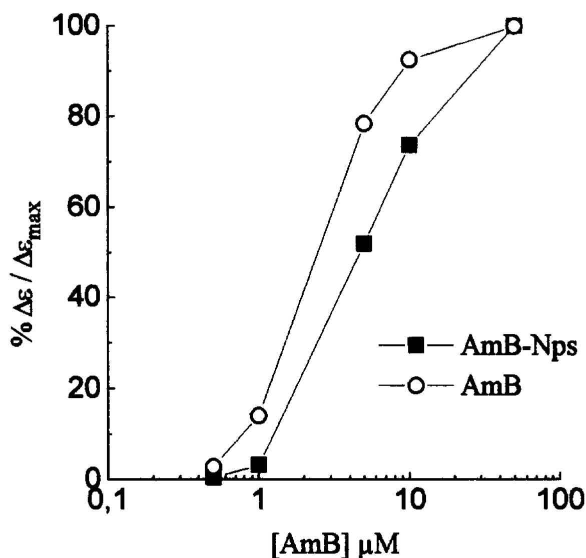


Fig. 2. Evolution of % $\Delta\varepsilon/\Delta\varepsilon_{max}$ for free Amphotericin B (AmB) (○) and AmB-nanospheres (AmB-NPs) (□) as a function of AmB concentration prepared by dilution from a stock solution at concentration 1 mg/ml.

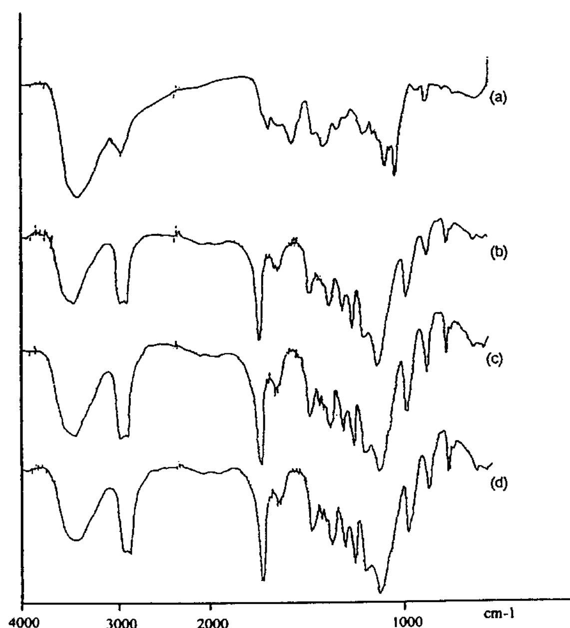


Fig. 3. FT-IR of free AmB micelles (a), unloaded nanospheres (b), AmB-nanospheres (c) and a physical mixture (d).

The thermogram of AmB powder showed an endothermic transition which began around 130°C with the maximum slope near 205°C. These changes could reflect drug decomposition, which appeared to happen without melting.

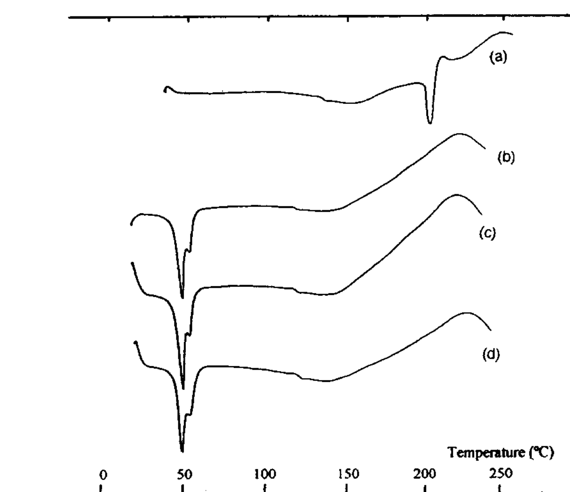


Fig. 4. Thermograms of free AmB micelles (a), unloaded nanospheres (b), AmB-nanospheres (c) and a physical mixture (d).

The thermograms of empty, AmB-Nps and its physical mixture were identical, with an endothermic peak at 58°C corresponding to the melting temperature ($t_m$) of the polymer used, poly($\varepsilon$-caprolacton). At 200–210°C there was an exothermic transition corresponding to the decomposition of the polymer.

Unfortunately, the polymer poly($\varepsilon$-caprolacton) has a glass transition temperature ($t_g$) of $-60$°C and for this reason, no information about the influence of AmB on the $t_g$ of the polymer could be obtained.

The only result which would definitively establish the physical state of the drug would be a comparison of the fusion heats of free and associated AmB. However, firstly AmB seemed to be degraded before its melting temperature was reached. Secondly, even in the physical mixture of AmB and empty nanospheres no signal related to the thermal behaviour of AmB could be detected, probably because of the low drug/polymer ratio in the preparation, 1:12.5 w/w (Dubernet et al., 1987).

To find out whether adsorption occurred, we measured the zeta potential of AmB micelles, AmB-Nps (concentration 1 mg/ml of the drug) and empty nanospheres in aqueous suspensions as a function of pH (adjusted with HCl 0.1 M) and at low ionic strength (Fig. 5). For empty nanospheres, the zeta potential was always negative and its value increased with increasing pH. For AmB micelles, the zeta potential was strongly positive at pH 2. Nevertheless, it changed from weakly positive to negative between pH 4 and 5. Above pH 5, it remained negative. These changes resulted from the behaviour of the 2 ionizable groups of the polyenic antibiotic: the amino group which has a p$K_a$ of 5.7 and the carboxylic group which has a p$K_a$ of 10 (Hung et al., 1988; Mazerski et al., 1990). In fact between pH 4 and 9, intramolecular electrostatic interactions between charges of opposite signs on the AmB molecule could maintain high level of aggregation, as described by Mazerski et al. (1990). This phenomena could explain the absence of change in potential zeta between pH 5 and 12. Below pH 4, AmB solubility increases as a result of decreasing intramolecular interactions and positively charged AmB species can be detected.

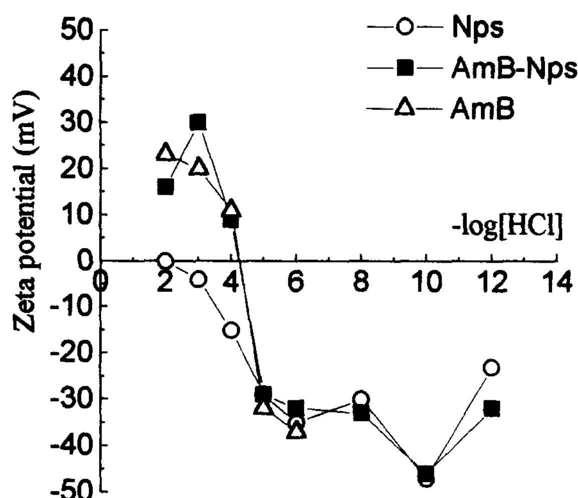


Fig. 5. Evolution of zeta potential of free AmB micelles (AmB) (△), unloaded nanospheres (NPs) (○) and AmB-nanospheres (AmB-NPs) (□) as a function of the pH in aqueous solutions adjusted with different concentrations of HCl or NaOH. The concentration of the drug was 0.01 mg/ml after the dilution necessary to perform measurement by PCS.

The zeta potential of AmB-Nps changed in the same way as free amphotericin B at the same concentration. The drug therefore seems to be responsible for the superficial charge of the nanospheres.

### 3.3. Acute toxicity.

The results of the acute lethal toxicity studies are shown in Fig. 6. The acute $LD_{50}$ of AmB-Nps was found to be 27.95 mg/kg, as compared with 3.81 mg/kg for Fungizone® and $>40$ mg/kg for AmBisome®. Clearly, nanoparticles formulation is less toxic than Fungizone® and more toxic than AmBisome®. However, except AmBisome®, acute toxicity for nanoparticles preparation is similar to the other lipid preparations (. And it has to be noticed Fungizone® still remains the preparation administrated as a first choice treatment. Considering that the drug is not truly included within nanospheres but simply adsorbed onto their surface, the differences found in toxicity studies between liposomal (AmBisome®) and AmB-Nps formulations may be in part explained by lower stability of AmB-Nps upon dilution. However,

preparations with lower stability than AmBisome®, such as AmB-emulsions (Tabosa do Egito et al., 1996) showed a less marked decrease of acute toxicity compared with micellar AmB ($LD_{50}$ = 7.82 mg/kg for emulsions). That suggests the stability upon dilution is not the only factor determining the toxicity of the different formulations.

BUN levels for the different treatment groups are shown in Table 2. The groups treated with Fungizone® (0.8 and 3.6 mg/kg), AmBisome® and AmB-Nps (20 mg/kg) showed nephrotoxicity compared with control animals. However, those treated with AmBisome® and AmB-Nps at 5 mg/kg remained within the normal range. These results indicate that formulation of AmB as nanospheres reduces the renal toxicity of the drug with respect to similar doses of Fungizone® (F 3.6 mg/kg versus AmB-Nps 5 mg/kg).

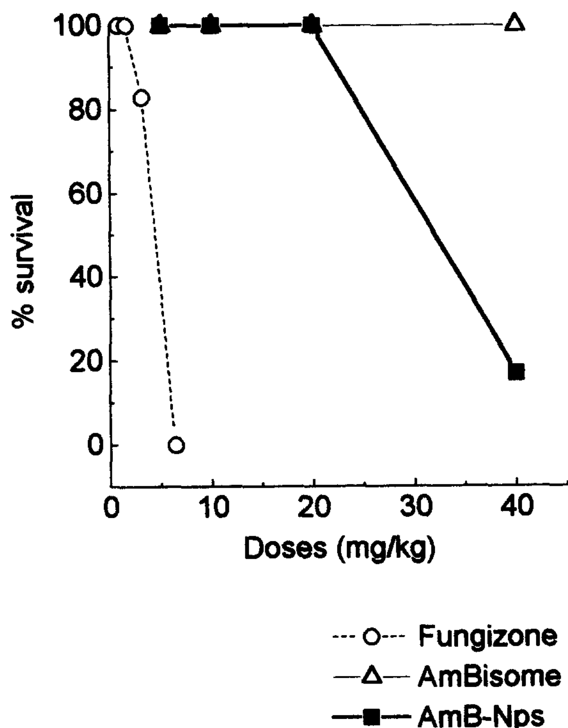


Fig. 6. Acute lethal toxicity of Fungizone® (○), AmBisome® (△) and AmB-nanospheres (□) after a single IV administration.

Table 2
Effect of Fungizone®, AmBisome® and AmB-nanospheres (AmB-NPs) on blood urea nitrogen levels (BUN) (g/L) after a single bolus administration

| Treatment | Doses (mg/kg) | BUN (g/L) |
|---|---|---|
| Fungizone® | 0.8 | 0.46 ± 0.02* |
| | 3.6 | 0.80 ± 0.34* |
| AmBisome® | 5 | 0.44 ± 0.03** |
| | 20 | 0.69 ± 0.06* |
| AmB-NPs | 5 | 0.35 ± 0.06** |
| | 20 | 0.68 ± 0.44* |
| Control | | 0.39 ± 0.04 |

* $P<0.05$ as compared to control group using U Mann Whitney rank sum test.
** $P<0.05$ as compared to 3.6 mg/kg Fungizone® group using U Mann Whitney rank sum test.

On the other hand, if the high stability of AmBisome® can explain its lower toxicity, it is also responsible for the decreased efficacy reported by several authors (Pahls and Schaffner, 1994; Van Etten et al., 1993; Legrand et al., 1992, 1996). Taking into account, the lower stability of our formulation, we think it would be interesting to test its antifungal activity in vitro and in infected mice in further studies. In fact, preliminary results showed AmB-nanoparticles were more potent in vitro (MIC, MFC) against clinical isolates of *Candida albicans* than AmB. These results suggest this new AmB formulation could improve the therapeutic index of the drug by a different way of the other lipid preparations.

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