# Lyophilized Lecithin Based Oil-Water Microemulsions as a New and Low Toxic Delivery System for Amphotericin B

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***Purpose.*** To develop and investigate lecithin based oil-water microemulsions as potential amphotericin B (AmB) delivery systems and to evaluate their *in vivo* acute toxicity.
***Methods.*** AmB was added to the microemulsion and its location was evaluated by partitioning studies and UV-visible spectrophotometric analysis of the drug. Both, non-lyophilized and reconstituted microemulsions were characterised and assessed for their stability. Single-dose acute toxicity of the AmB microemulsion was studied on male albino Webster-derived CD-1 mice and compared with Fungizone®.
***Results.*** The studies performed showed that AmB was intercalated on the oil-water interface of the microemulsion as a complex formed with lecithin molecules. AmB addition did not seem to modify the rheological properties of the original system, but had an effect on its particle size distribution. Lyophilization of the microemulsion led to an oily cake, easily reconstituted and stable at the conditions studied. Single-dose acute toxicity studies proved that the $LD_{50}$ of AmB microemulsions was of 4 mg $kg^{-1}$ of animal weight, compared with 1 mg $kg^{-1}$ found for Fungizone®.
***Conclusions.*** Lyophilized lecithin based oil-water microemulsions appear to be valuable systems for the delivery of AmB in terms of easy and low-cost manufacturing, stability and safety compared with the formulations already in market.

**KEY WORDS:** amphotericin B; lecithin-based oil-water microemulsions; lyophilization; stability; single-dose acute toxicity.

## INTRODUCTION

Amphotericin B is a membrane-active polyene antibiotic with strong antifungal activity. It is the drug of choice for the treatment of disseminated mycosis in immunodepressed patients (AIDS, organ transplants, cancer chemotherapy) (1–3). Its low solubility in most solvents leads to poor bioavailability by the oral route, and it is therefore parenterally administered as a solubilizate in sodium deoxycholate (Fungizone®, Bristol-Myers Squibb). Unfortunately, a wide range of adverse effects are associated to this formulation, and limit its usefulness. These include infusion-related reactions, such as fever and chills, rigors, nausea and vomiting, and general malaise (4), as well as more serious complications, the most important of which are haematological intolerance (5) and, above all, considerable nephrotoxicity (6,7). Consequently, two different approaches have been adopted to reduce the toxicity of the drug while maintaining its antifungal efficacy: the first is the synthesis of new derivatives, without much success (8), and the second is the development of new pharmaceutical forms. Among them are:

- Emulsions: the use of lipid emulsions of amphotericin B has been considered to diminish the side effects associated with the drug. Two methods of emulsion preparation were originally suggested, which were *de novo* emulsification, and extemporaneous incorporation of Fungizone® into the commercial parenteral emulsion Intralipid®. *De novo* emulsification was first proposed by Davis and Washington (9) by use of ultrasonication, but this technique had two main drawbacks, which were the difficulty of industrial scale-up and the risk of AmB degradation. Extemporaneous incorporation is a common practice in hospitals (10,11), although a study of the resulting mixtures has shown that amphotericin B is not included in the lipid droplets and that phase separation with precipitation of the drug soon occurs (12). One of the most interesting as well as easiest approaches made in this field was the development of an AmB parenteral emulsion by spontaneous emulsification (13), although further studies and considerations should be made on this formulation.
- Liposomes: the first liposomal formulation of amphotericin B was proposed by Lopez-Berestein *et al.* (14–16); a form based on small unilamellar vesicles is marketed in the United States by Vestar Ltd. under the trade name of AmBisome® (17,18).
- Lipid complexes: an amphotericin B/cholesterol complex has been developed by Guo *et al.* (19,20), which is a colloidal dispersion of a stable 1:1 molar complex with cholesteryl sulphate (Amphocil®). In the same line, another amphotericin B lipid complex has recently been launched commercially under the trade name of Abelcet®.

Although these products have demonstrated good efficacy with a greatly reduced toxicity, their extremely high cost, due to the technology involved on their manufacture and the stability problems associated to the dosage forms, have made them unaffordable to less developed countries, their use being strictly restricted to infants and patients highly intolerant to Fungizone® in many others.

In this work, we have developed and evaluated the use of lyophilized lecithin-based oil-water microemulsion as new and less toxic delivery systems for amphotericin B, in terms of physico-chemical characterisation, accelerated stability and *in vivo* single dose acute toxicity.

## MATERIALS AND METHODS

### Chemicals

Amphotericin B was supplied by Dumex (Denmark). Microemulsion oil phase consisted on isopropyl myristate (IPM), that was obtained from Merk Chemicals (Madrid, Spain). The lipophilic surfactant was soybean lecithin (20%

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phosphatidil-choline content) and was purchased from Sigma Chemical Co. (Madrid, Spain). The hydrophilic surfactant was a polyoxy-ethylene sorbitan fatty acid ester derivative, Polysorbate 80 (Tween 80), and was from Sigma Chemical Co. (Madrid, Spain). Deionised ultra-pure distilled water was used as the external phase of the microemulsions, and obtained with a Milli-Q Plus Equipment, Millipore (Barcelona, Spain). Mannitol was used as a bulky agent for microemulsion lyophilization, and was supplied by Merk Chemicals (Madrid, Spain).

### Microemulsion Preparation

A polysorbate 80/water solution (1:3, w/w) was added to a lecithin/IPM dispersion (1:1, w/w), and the mixture was magnetically stirred at 25°C until equilibrium was reached. The resulting system was analysed for homogeneity, isotropy (with cross polarizers) and visual transparency, in order to be considered as a true microemulsion. All microemulsions used in this work had the same composition (Table I), with a polysorbate 80/lecithin relationship of 2:1, w/w.

### Amphotericin B Addition

Amphotericin B was incorporated at a concentration of 2 mg/ml, to the previously obtained microemulsion. Briefly, amphotericin B was dissolved (20 mg/ml) in sodium hydroxide solution (1 M) and added to the microemulsion at a temperature of 80°C, over the phase transition temperature of the emulsifiers. pH was adjusted to 8.5–9.5, with 1N ortophosphoric acid. AmB microemulsions were finally filtered through 0.45 μm pore size cellulose filters (Millipore, Madrid, Spain) in order to eliminate AmB suspended, and hence, not properly incorporated to the disperse system. AmB recovery from microemulsion was determined at the end of the addition process, following the extraction and HPLC procedures validated and described by Moreno *et al.* (21), in order to assess the real amount of the drug incorporated to the disperse system.

### Interactions Between Amphotericin B and the Oil Phase of the Microemulsion

Possible interactions between amphotericin B and the oil phase, as well as its location into the disperse system (aqueous phase, oil phase, or water/oil interface), were determined by UV-visible absorption spectrophotometry and partitioning studies.

**Table I.** Composition (% w/w) of Placebo and Amphotericin B Microemulsions

| | Placebo | Amphotericin B microemulsion |
|---|---|---|
| Drug | | |
| Amphotericin B | — | 0.2 |
| Excipients | | |
| Isopropyl myristate | 10.0 | 10.0 |
| Distilled water | 60.0 | 59.8 |
| Polysorbate 80 | 20.0 | 20.0 |
| Soybean lecithin | 10.0 | 10.0 |

#### *Spectrophotometric Studies*

UV-visible spectra of AmB in microemulsions were recorded over the wavelength range of 200–600 nm with a Beckman DU-6 spectrophotometer. AmB microemulsions were diluted with phosphate saline buffer (PBS), pH = 7.4, to yield AmB concentrations spanning 15.0–1.5 μg/ml, and their spectra were recorded in order to determine the influence of AmB concentration on its interaction with the internal phase of the microemulsion.

#### *Partitioning Studies*

Partition coefficients of AmB between water and n-octanol, IPM, and IPM:lecithin mixtures (2:1; w/w) were determined by adding a known amount of the drug (50 mg) to 50 ml of the aqueous/organic mixtures (1:1, v/v). The samples were mixed vigorously by vortexing for 5 minutes, and centrifuged at 8000 g for 45 minutes at room temperature. The amount of AmB present in each phase was determined, after appropriate dilution in methanol, by high performance liquid chromatography in a Hewlett- Packard (Madrid, Spain) equipment consisting of a 1050 series pump, a Teknokroma (Madrid, Spain) C18 column (200 × 4.6 mm), and a 1050 series UV-visible detector set at 405 nm. The mobile phase was a mixture of methanol and a 0.005M sodium EDTA solution, 80:20 (v/v), at a flow rate of 1.8 ml/min. A calibration curve (10 to 50 μg/ml) was prepared in triplicate from a stock solution of amphotericin B in dimethylsulphoxide, further diluted in methanol ($r^2 = 0.996$; RSD = 2.92%). The injection volume was 20 μl.

### Amphotericin B Microemulsion Lyophilization

Amphotericin B microemulsions were lyophilized in order to avoid hydrolysis reactions of lecithin phosphatide groups (22,23), and hence to prevent these disperse systems from any decomposition phenomena related to such processes. Due to the fluid nature of most of the excipients, 5% (w/v) of mannitol was added to the external phase of the formulation as a bulky agent. Vials were filled with 10 ml of microemulsion each and placed inside the lyophilizer chamber. Lyophilization of the samples was performed with a Telstar (Tarrasa, Spain) equipment. Conditions of the whole process are shown in Table II. The resulting product was evaluated for its macroscopic appearance, reconstitution and water content by the Karl-Fischer method (Metrohm 658 Processor with 665 Dossimat. Metrohm, Barcelona, Spain).

### Microemulsion Characterisation

Microemulsions with and without AmB, as well as those obtained after reconstitution of the AmB lyophilized product with 6 ml of water (microemulsion aqueous phase), were characterised in terms of macroscopic appearance, particle size, viscosity and rheology, pH, and drug content (where appropriate).

#### *Macroscopic Appearance*

The colour, isotropy and homogeneity of the microemulsions, and the presence of precipitates or phase separation were scored after visual and cross polarizers examination at room temperature.

**Table II.** Lyophilization Conditions for Amphotericin B Microemulsions

| Step in process | Duration | Product final temperature | Condenser final temperature | Pressure in chamber |
|---|---|---|---|---|
| Freezing | 2 hours | ≈−40°C | ≈19°C | Atmospheric |
| 1ry Dessication | 24 hours | ≈−36°C | ≈−50°C | 30 microns |
| 2ry Dessication | 40 hours | ≈30°C | ≈−50°C | 30 microns |

*Size Distribution Analysis*

The droplet size distribution of the oil phase and its polydispersity were estimated by photon correlation spectroscopy (PCS) using a Malvern Zetamaster-S®. The emulsions were diluted 5-fold in water, filtered (0.22 μm pore size) before use, and analysed at 20 ± 2°C. All analysis were done in triplicate.

*Rheology and Viscosity of Microemulsions*

Rheology and viscosity of microemulsions were determined in triplicate using a rotate-spindle Brookfield HB viscometer, equipped with a Rheocalc V1.1 data processing software. All the microemulsions studied were submitted, in triplicate, to up and down cycles (0 to 25 rpm spindle rotation speed) at 25 ± 1°C, and the rheological behaviour of each disperse system was evaluated by plotting the shear stress ($\sigma$) vs. the shear rate ($\gamma$) values obtained. Systems that showed proportionality in both parameters ($r^2$ values $\geq 0.99$), were considered to be as Newtonian fluids and their viscosity ($\eta$) was determined from the slope of the curve aforementioned. All other systems were deemed to be non-Newtonian fluids, and their viscosity values were obtained by the viscometer at the highest spindle rotation speed (25 rpm).

*pH Determination*

pH values of AmB microemulsions were determined in triplicate at room temperature with a Crison micro pH 2000 pHmeter. pH values of AmB microemulsions and AmB reconstituted microemulsions should be in the range 8.5–9.5 as stated above.

*Amphotericin B Content of Microemulsions*

Drug content of recently prepared and reconstituted AmB microemulsions were determined in triplicate by HPLC (21).

**Stability Studies**

Since AmB lyophilized microemulsions are drug products intended for storage in a refrigerator, the accelerated stability study of the formulation was carried out in closed semi-permeable containers, at 30°C and 60% relative humidity (RH) (ICH Steering Committee just recommends 25°C and 60% relative humidity) (24) for a period of 6 months.

Samples were withdrawn in triplicate at 0, 1, 3 and 6 months, and assessed for their:

*i)* Chemical stability: expressed as AmB content, determined by HPLC. Samples were deemed to be stable if presented an AmB content between 95–105% of the amount declared at the beginning of the study.

*ii)* Physical stability: Water content of the lyophilized product should be less than 3%; organoleptic properties of the lyophilized product should be dark yellow coloured with a characteristic odour; macroscopic appearance of the reconstituted microemulsion should be an homogeneous, transparent, optically isotrope disperse system; pH of the reconstituted microemulsion should be in the range 8.5–9.5; droplet size of the reconstituted microemulsion: should have a diameter smaller than 200 nm; rheology and viscosity of the reconstituted microemulsions should behave as Newtonian fluids with low viscosity values that may not have an effect on the physical integrity of the system.

Stability studies of AmB non-lyophilized microemulsions were also carried out in closed semi-permeable containers, at 30°C for a period of 21 days.

Samples were withdrawn in triplicate at 0, 7, 14, and 21 days and assessed for their chemical and physical stability by evaluating the same parameters aforementioned for reconstituted microemulsions.

**Single Dose Acute Toxicity Studies**

Male albino Webster-derived CD-1 mice weighing 20g were injected through the tail vein with various doses of AmB either as Fungizone® (0.5, 1.0, 1.5 and 2.0 mg/kg) or as various microemulsion formulations at different concentrations (1.0, 2.0, 3.0, and 4.0 mg/kg), obtained by dilution of the reconstituted AmB microemulsion with 5% sterile glucose solution. Each AmB dosage form was administered intravenously (IV) by a single bolus injection (0.1 ml) to groups of 10 mice. Fungizone® was prepared according to the manufacturer's instructions using sterile water for resuspension, followed by dilution with 5% sterile glucose solution. Since this is a novel AmB formulation, the toxicity of the placebo microemulsion (without AmB) was tested by IV injection of 0.1 ml to one group of 10 mice (control group). Survival was followed up to 45 days.

Sterility of the different formulations was achieved by sterile filtration through sterile 0.22 μm pore size pyrogen-free cellulose filters (Sartorius, Madrid, Spain). Possible AmB lost on filtration was firstly assessed by HPLC determination of the drug content in filtered reconstituted microemulsions.

## RESULTS AND DISCUSSION

**Microemulsion Preparation**

Figure 1 shows the pseudo-ternary phase diagram and the area of existence of oil-in-water (o/w) microemulsions (ME) for IPM/soybean lecithin/polysorbate 80/water systems at the polysorbate/lecithin mass ratio of 2:1, w/w. As it can be clearly seen, the systems described in this work had a composition within the o/w microemulsion area and seemed to be homogeneous, transparent as well as optically isotropic, for which they were deemed to be true microemulsions.

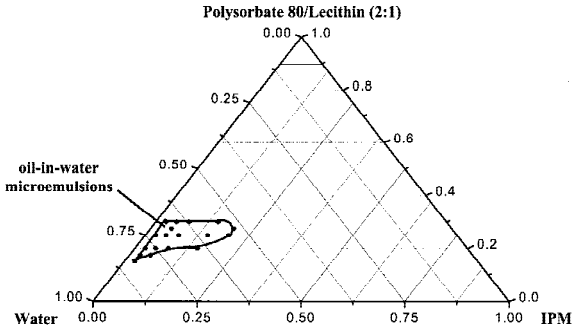


**Fig. 1.** Pseudo-ternary phase diagram of the system, isopropyl myristate (IPM), water, Polisorbate 80 and Lecithin showing the area of existence of oil-in-water microemulsions at the polysorbate/lecithin weight ratio indicated (2:1).

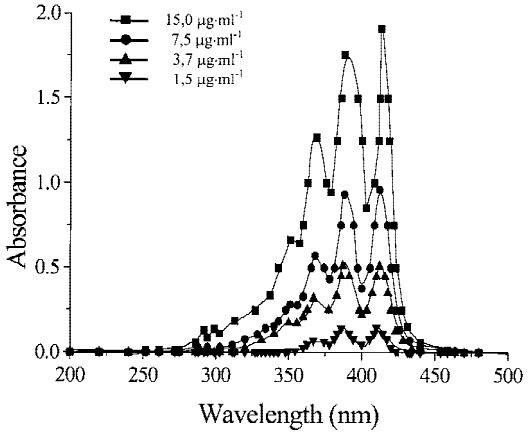


**Fig. 2.** UV-Visible absorption spectra of AmB microemulsions at a drug concentration range of 15.0–1.5 μg $ml^{-1}$.

## Amphotericin B Addition and Interactions with the Oil Phase of the Microemulsion

Amphotericin B was successfully incorporated to microemulsions at a concentration of 2 mg/ml by the method already described, since the HPLC analysis for the drug content of the systems gave a mean recovery percentage ($n=6$) of 97.6% with a RSD value of 0.60%, with no indication of any decomposition of amphotericin B in the samples analysed.

The n-octanol/water and IPM/water partition coefficients of AmB (1.35 ± 0.05 and 1.46 ± 0.08 respectively) proved its amphiphilic behaviour, since the drug did not show any special preference between water and the organic/oleous solvents studied. Nevertheless, the addition of lecithin to IPM (2:1, w/w) led to an increase on the IPM-lecithin/water partition coefficient of the drug to a value of 12.39±0.02, probably due to an AmB-lecithin complex formation.

To verify such hypothesis, UV-visible absorption spectrophotometric studies were performed on AmB microemulsions, as stated above. As it is well known, amphotericin B has an hydrophobic pole which consists of a series of seven double bonds in the *trans* configuration that lead to an intense absorption spectra around 400 nm, the shape of the spectra depending on the state of aggregation of the molecules (25). In water, amphotericin B is poorly soluble and shows several forms: monomeric, oligomeric, water-soluble and aggregates non water insoluble. The relative proportion of such different forms depends on concentration, and it is then possible to follow the aggregation state of amphotericin B by its absorption spectra (26). At low concentrations, the absorption spectra of an aqueous suspension of amphotericin B shows four bands between 420 and 320 nm (405, 385, 365, and 347) (13). The amphotericin B monomeric form is responsible for the band at 405 nm which is as well the absorption maximum.

In contrast, at high concentrations, the molecules self-associate to form oligomers and then aggregates of oligomers, and a new spectra is then observed. The bands near 400 nm are replaced by 420, 385, and 320 bands, and a new intense but flattened band is observed at approximately 340 nm (13). These bands are characteristic of the changes in the molecular state of AmB due to aggregation.

Figure 2 shows the UV-Visible absorption spectra of amphotericin B microemulsions. As it can be seen, and in contrast with the aqueous suspensions spectra, there was no amphotericin B self-association band and the spectra was found to be poorly concentration dependent, since the same bands were observed at low and high AmB concentrations. At high concentrations, the band at 420 nm is replaced by a 413 nm band that was assumed to be characteristic of monomeric AmB complexed with the phospholipids of the microemulsion.

These results, as well as the partition coefficients obtained before, suggest that AmB is located in the microemulsion droplets and is not free in the continuous phase. The drug could either be at the interface between the two phases or within the oil phase of the disperse system. In the later case, given the insolubility of AmB in IPM (3.3 ± 0.6 μg $ml^{-1}$ as determined), the presence of oligomers would have been apparent from their characteristic band at 340 nm. It is therefore highly probable that the drug is to be found at the interface of the disperse system, as an AmB-lecithin complex.

## Amphotericin B Microemulsion Lyophilization

Amphotericin B microemulsions were lyophilized as previously described, and all lyophilized products were characterised in terms of macroscopically appearance, organoleptic characteristics, water content and reconstitution.

**Table III.** Characterisation of Placebo, AmB and Reconstituted AmB Microemulsions

| Microemulsion | Macroscopical appearance | Mean particle size (nm) | Rheological behaviour | Viscosity (cPs) | pH | AmB content (%) |
|---|---|---|---|---|---|---|
| Placebo | Yellowish, isotropic and homogeneous | 12.0 ± 1.6 (90%)[a]<br>12.5 ± 15.4 (10%)[a] | Newtonian | 87.7 ± 1.2 | — | — |
| AmB | Yellowish, isotropic and homogeneous | 41.3 ± 5.7 | Newtonian | 87.0 ± 0.7 | 8.89 ± 0.06 | 99.0 ± 1.8 |
| Reconstituted AmB | Yellowish, isotropic and homogeneous | 21.0 ± 6.1 | Newtonian | 42.4 ± 6.1 | 9.12 ± 0.08 | 95.6 ± 1.5 |

[a] Values in parenthesis represent the percentage of each droplet population within the total droplet size distribution of the disperse system.

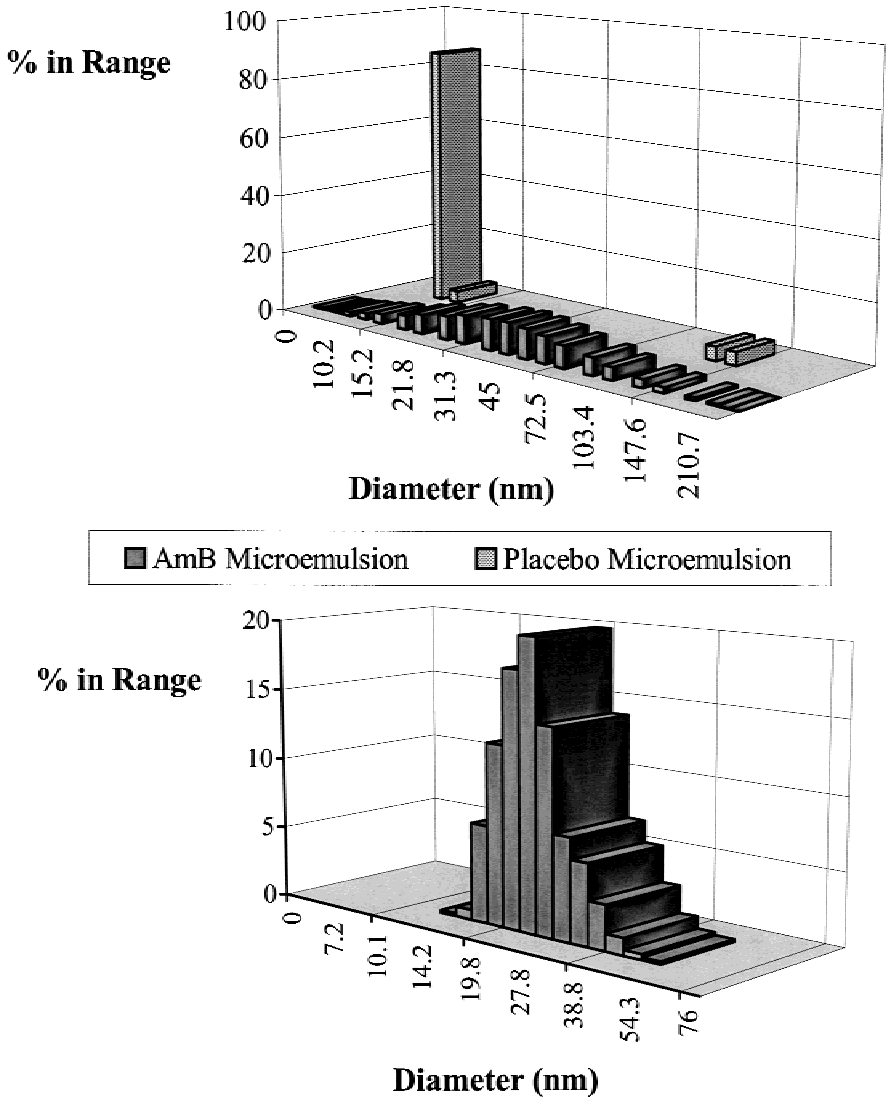


**Fig. 3.** **a.** Particle size distibutions of AmB and placebo microemulsions. **b.** Particle size distribution of reconstituted AmB microemulsion.

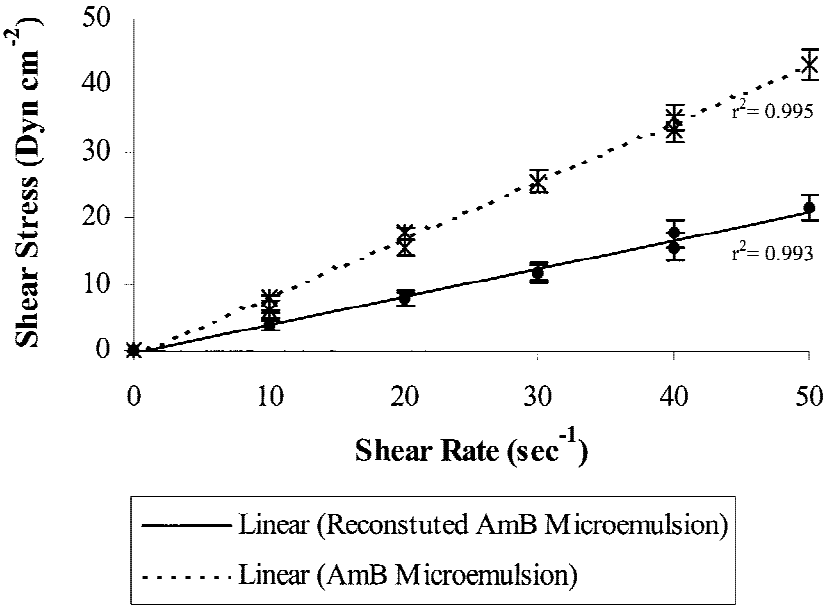


**Fig. 4.** Shear stress vs. shear rate plots and determination coefficients for AmB and reconstituted AmB microemulsions.

**Table IV.** Results from Stability Evaluation of AmB Non-Lyophilized Microemulsions

| Parameters studied | Time (days) | | | |
|---|---|---|---|---|
| | 0 | 7 | 14 | 21 |
| Reconstituted microemulsions appearance | As specified[a] | AmB precipitated | Phase[b] separation | Phase[b] separation |
| pH value | 8.97 ± 0.03 | 8.20 ± 0.11 | — | — |
| Droplet size (nm) | 41.3 ± 5.7 | 175.1 ± 32.4 | — | — |
| Rheological behaviour | Newtonian | Newtonian | — | — |
| Viscosity (cPs) | 87.0 ± 0.7 | 62.3 ± 8.0 | — | — |
| AmB content (%) | 99.0 ± 1.8 | 63.3 ± 0.9 | — | — |

[a] Stable AmB microemulsion macroscopical characteristics described in text (stability studies).
[b] After phase separation systems were no longer considered as microemulsions.

All lyophilized microemulsions showed an homogeneous, consistent and low fluid oily aspect, with a dark yellow colour and a characteristic odour due to presence of soybean lecithin and polysorbate 80 in the dosage form.

The humidity content was determined in order to quantify the residual water of the products obtained right after the lyophilization process. Such content was of just 0.75 ± 0.02%, as expected considering the low hygroscopicity of all the formulation constituents.

Reconstitution of the lyophilized AmB microemulsions was achieved by adding 6 ml of ultrapure distilled water, followed by magnetic stirring. The systems so obtained seemed to be homogeneous, transparent, optically isotropic and free from precipitates, for which they were deemed to be true microemulsions.

### Microemulsion Characterisation

Table III shows the results obtained from the characterisation of the different microemulsions studied in this work. As it can be observed, the three disperse systems were macroscopically identical, *i.e.,* homogeneous, transparent without any precipitates, optically isotropic, yellow coloured and with a characteristic odour.

AmB addition to the originally obtained microemulsion did not have an effect on the rheology and viscosity of the disperse system (Table III), but did modified the particle size distribution of the microemulsions. As it can be seen in Figure 3.a, AmB microemulsions showed a mono-modal droplet size distribution, in contrast with the original disperse system, which clearly showed two well differentiated droplet populations that may fit to a bi-modal distribution. The reason for this could be attributed to the co-surfactant role that AmB, an amphiphilic compound with a terminal amino- group, may plays, since it is located at the water/oil interface of the microemulsion as an AmB-lecithin complex that could contribute to the stabilisation of the disperse system.

As it can also be observed in Table III, liophilization of AmB microemulsions reduced both the particle size and viscosity of the reconstituted disperse system, although it still showed a mono-modal droplet size distribution (Figure 3.b) and a Newtonian rheological behaviour since the lineal regression of the shear strength vs. shear rate plot gave a $r^2$ = 0.993 (>0.990), as it can be seen in Figure 4.

Two facts may contribute to the particle size reduction:

- The co-surfactant role of mannitol (a short-chain polyhydroxy-alcohol), which was adsorbed and intercalated onto the interfacial film of the microemulsion after the lyophilization and reconstitution of such disperse system.
- Sublimation of the IPM water of saturation might have created a vacuum effect that could have clearly reduced the microemulsion mean droplet size.

The viscosity reduction observed on the reconstituted microemulsions could be explained by the polysorbate 80 lost (approximately 2% w/w of the total amount employed) on the lyophilization process, that was observed when condensed products were analysed. Such a small lost did not obviously have a negative effect on the physical integrity of the microemulsion, since the presence of mannitol might have been enough to reduced the interfacial tension at the water/oil interface and hence to stabilise the system (27).

**Table V.** Results from Stability Evaluation of AmB Lyophilized Microemulsions

| Parameters studied | Time (months) | | | |
|---|---|---|---|---|
| | 0 | 1 | 3 | 6 |
| Lyophilized products appearance | As specified[1] | As specified[1] | As specified[a] | As specified[a] |
| Humidity content (%) | 0.75 ± 0.03 | 0.52 ± 0.01 | 0.57 ± 0.04 | 0.47 ± 0.02 |
| Reconstituted microemulsions appearance | As specified[b] | As specified[b] | As specified[b] | As specified[b] |
| pH value | 9.12 ± 0.08 | 9.20 ± 0.02 | 8.96 ± 0.07 | 8.98 ± 0.06 |
| Droplet size (nm) | 21.0 ± 11.6 | 45.9 ± 24.1 | 47.9 ± 23.8 | 41.5 ± 21.3 |
| Rheological behaviour | Newtonian | Newtonian | Newtonian | Newtonian |
| Viscosity (cPs) | 42.4 ± 6.1 | 53.5 ± 5.2 | 74.7 ± 2.9 | 77.1 ± 7.4 |
| AmB content (%) | 95.6 ± 1.5 | 99.2 ± 2.2 | 96.5 ± 1.4 | 97.6 ± 1.0 |

[a] Stable lyophilized product macroscopical characteristics described in text (stability studies).
[b] Stable reconstituted microemulsion macroscopical characteristics described in text (stability studies).

**Table VI.** AmB Lost on Microemulsion Sterile Filtration (0.22 μm pore size)

| Formulation | Theoretical dose (mg/kg animal weight) | Recovery (%) |
|---|---|---|
| Fungizone® | 0.5 | 96.7 ± 0.2 |
| | 1.0 | 97.9 ± 0.3 |
| | 1.5 | 96.5 ± 0.2 |
| | 2.0 | 96.3 ± 0.0 |
| Microemulsion | 1.0 | 91.5 ± 1.1 |
| | 2.0 | 90.1 ± 0.2 |
| | 3.0 | 90.8 ± 0.2 |
| | 4.0 | 89.9 ± 0.4 |

**Table VII.** Acute Toxicity of Microemulsion Vehicle and AmB Dosage Forms in BALB/c Mice

| Group | % Survival after 45 days | Mortality |
|---|---|---|
| Saline control | 100 | — |
| Microemulsion control | 100 | — |
| Fungizone® | | |
| 0.5 mg $kg^{-1}$ | 100 | — |
| 1.0 mg $kg^{-1}$ | 50 | Immediate |
| 1.5 mg $kg^{-1}$ | 0 | Immediate |
| 2.0 mg $kg^{-1}$ | 0 | Immediate |
| Microemulsion | | |
| 1.0 mg $kg^{-1}$ | 100 | — |
| 2.0 mg $kg^{-1}$ | 100 | — |
| 3.0 mg $kg^{-1}$ | 100 | — |
| 4.0 mg $kg^{-1}$ | 50 | Immediate |

### Stability Studies

Tables IV and V show the results obtained from the stability evaluation of non-lyophilized and lyophilized AmB microemulsions, respectively, at the conditions studied. Those results proved the great stability of the lyophilized microemulsions, when compared with the non-lyophilized systems, since all the parameters evaluated at the end of the study remained within the limits established for such formulations and the variations observed on viscosity did not seem to modify the physical integrity of the disperse system and so were not considered as degradation phenomena. On the contrary, non-lyophilized microemulsions started to suffer from remarkable degradation phenomena after just 7 days of storage.

### Single Dose Acute Toxicity Studies

Table VI shows the percentages of AmB recovered from the sterilised formulations at the different doses studied. As it can be seen, AmB content of Fungizone® did not vary after sterile filtration and hence the different doses of such pharmaceutical product could be administered with a total injection volume of 0.1 ml.

Nevertheless, the amount of AmB found on sterilised microemulsions was of about 90% and was not dependent of the dose assessed, so in order to inject the appropriate doses of the formulation, a total volume of 0.11 ml of the correspondent microemulsion was administered at the injection site.

The acute toxicity results (Table VII) were obtained from three independent single dose experiments. $LD_{50}$ and $LD_{100}$ for Fungizone® were found to be 1.0 and 1.5 mg $kg^{-1}$ respectively, whereas no deaths occurred at 0.5 mg $kg^{-1}$. In all cases animal death was immediate, a few seconds after the administration of such dosage form. Mice that survived Fungizone®injection, showed episodes of nervous shaking followed by an increase on the respiratory rhythm of the animal, both transient and related to the dose administered.

$LD_{50}$ for AmB microemulsions was of 4.0 mg $kg^{-1}$, whereas no deaths were found at doses of 3.0 mg $kg^{-1}$. As for Fungizone®, animal death was immediate but could not be attributed to the excipients of the dosage form since the plain microemulsion was well tolerated at a dose of 0.1 ml (Table 7). All surviving animals suffered from transient nervous shaking, unrelated to the dose administered.

The results obtained clearly demonstrated that AmB formulated as a microemulsion delivery system was six times safer than commercial Fungizone®, which might permit the administration of much higher doses of the drug, leading to an increase on the efficacy of the pharmaceutical product in the treatment of systemic fungal infections, as well as to a reduction on resistance development.

## CONCLUSION

Lyophilized lecithin based oil-water microemulsions have proved to be valuable systems for the delivery of AmB in terms of easy and low-cost manufacturing, as well as high stability, which are essential for their viability in pharmaceutical industry and for their extended use world-wide.

The rheological and particle size characteristics of such dosage forms make them suitable for its intravenous administration, and the lower level of toxicity exhibited by these parenteral lipids formulations permits the administration of larger doses, which consequently increase their efficacy and diminish the risk of resistance development by the pathogen fungal agent.

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