Evaluation of the antifungal activity of microemulsions containing Amphotericin B after the lyophilization process

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INTRODUCTION AND OBJECTIVE

Recently, the use of colloidal systems as pharmaceutical carriers has been changing the way as drugs act in the human organism. The reasons are based on their ability to improve the drug bioavailability, even decreasing the systemic toxicity (Pouton 2000). A widely used colloidal carrier is the microemulsion (ME) system (Date 2008).

Studies have been carried out on the incorporation of the antifungal Amphotericin B (AmB) in colloidal systems. This drug is widely used in the treatment of systemic fungal infections. However, its poor solubility, even as its related side effects, may interfere in the pharmacotherapy accession. Although the use of ME systems be an important scientific advent in order to improve the therapy efficacy of the AmB, it has a poor microbiological stability caused by the presence of water in the system medium (Darole 2008).

The freeze-drying (FD) is often used to remove the water content of pharmaceuticals formulations (Fissore 2011). During this process, some mechanical stresses produced on droplet surface of the system may cause the drug release of the carrier, interfering in its pharmacological response. The cryoprotectants are used to avoid such problem (Abdelwahed 2006). However, a way to validate the antimicrobial effectiveness of drug delivery systems is through its minimum inhibitory concentration (MIC) capable to inhibit the growth of the studied microorganism (Fernandez Campos 2012).

The objective of this work was to analyze the MIC of non-freeze-dried and freeze-dried MEs containing AmB, in addition to compare their antifungal activity with the commercial AmB Fungizone®, using *Candida albicans* ATCC 90027 as fungal strain.

MATERIALS AND METHODS

Materials

The Miglyol 812® was obtained from CONDEA Chemie GMBH (Hamburg, Germany). Lipoid S100® was purchased from LIPOID GMBH (Ludwigshafen, Germany) and the Tween 80® was obtained from Sigma Aldrich Inc (St. Louis, USA). The Fungizone® was obtained from Cristália (São Paulo, Brazil). The 96-well microdilution plates were purchased from TPP (Trasadingen, Switzerland).

Methods

The ME was composed of 68 % of phosphate buffer pH 7.4, 14.7 % of Tween 80®, 6.3 % of Lipoid S100® and 11 % of Miglyol 812®. A mixed under magnetic stirring was processed followed by three cycles of sonication and ultrasonic bath. The AmB incorporation in the ME systems was proceeded at concentrations of 1.5 and 5 mg/mL, followed by the addition of Maltose at 5%. The samples for the FD were frozen at -80 °C for 24 h.

The samples studied in this work were composed of ME unloaded and loaded with AmB at different concentrations, in which part of these samples were freeze-dried and the other part were not freeze-dried. A reconstitution of the Fungizone® was made using bi-distilled water and solutions of the drug were prepared in the same concentrations of the AmB containing in the MEs to compare the antimicrobial activity.

The MIC was developed by the method of broth microdilution. Previously, the strain of *Candida albicans* ATCC 90027 was first cultured on Sabouraud dextrose agar at 35 °C for 48 h. Based on the 0.5 McFarland standard, a yeast inoculum was prepared by suspending colonies in sterile sodium chloride solution. This suspension was used to prepare further dilutions in Mueller-Hinton broth, containing 0.5 to 2.5 x 10^3 CFU/mL.

The tests were processed using a 96-well microdilution plate from 750 to 1.5 mg/L for the AmB concentration of 1.5 mg/mL, and 2500 to 5.5 mg/L for the concentration of 5 mg/mL. The same concentrations were used for the Fungizone® solutions. Moreover, 100 µL of microorganism cell suspension was added to each well.

All plates were incubated for 48 h at 35 °C in an orbital shaker (TE – 420 Tecnal, Piracicaba, SP, Brazil) at 150 rpm. The positive and negative growth control wells were composed of Muller-Hinton broth with the inoculum and the pure Muller-Hinton broth, respectively. There tests were performed in triplicate.

RESULTS AND DISCUSSION

The macroscopic analysis at room temperature showed the formation of clear and homogeneous ME systems with a dark yellow color when the incorporation of AmB was processed. After the

lyophilization, were formed powders on the lyophilized samples, in which were achieved by adding ultra-pure water. Thus, after the reconstitution step it was possible to observe that the macroscopic characteristics were preserved. The MIC results analyzed after the incubation at 35 °C for 48 h of the 96-well microdilution plates were summarized in Table 1.

Table 1 : The MIC of the ME samples, non-freeze-dried and freeze-dried , containing AmB tested againsta *Candida albicans* strain.

Formulation	*Candida albicans* ATCC 90027 (mg/L)
ME with AmB 1.5 mg/mL N.L.	< 1.5 ± 0.0
ME with AmB 5 mg/mL N.L.	< 5.5 ± 0.0
ME with AmB 1.5 mg/mL L.	< 1.5 ± 0.0
ME with AmB 5 mg/mL L.	< 5.5 ± 0.0
Fungizone® 1.5 mg/mL	< 1.5 ± 0.0
Fungizone® 5 mg/mL	< 5.5 ± 0.0

Results after incubation at 35 °C for 48 h.

The AmB entrapped in the ME systems, on concentrations of 1.5 and 5 mg/mL, showed the same antifungal activity that the Fungizone® solutions at equivalent concentrations. Others studies obtained a higher inhibitory action against *Candida albicans* strains for the drug delivery systems, compared to the commercial formulation (Jung 2009). Nevertheless, all the ME systems loaded with the drug presented the lowest value of MIC in this work, supporting the regular release of the drug by the carrier. However, the *in vitro* assay demonstrated that the MEs, freeze-dried and non- freeze-dried, unloaded with the drug have no antifungal activity against the yeast studied, even at the highest concentration.

Comparing the results obtained for all ME samples loaded with AmB, it was possible to observe that the MICs for the freeze-dried and non- freeze-dried samples on equals concentrations of the drug were the same. Therefore, it is possible to suggest that the FD process did not interfer on the carrier function of the ME, in which the colloidal systems may have kept the drug entrapped in its structure during the drying technique (Moreno 2001).

CONCLUSIONS

Both the freeze-dried ME formulations containing AmB showed an *in vitro* antifungal activity effectiveness, and the same MICs as the commercial AmB Fungizone®. However, based on the results of MIC, it is necessary to perfor a study with lower concentrations of AmB to identify the real value of the drug carrier MICs'. Therefore, this preliminary study proved that a successful use of the FD technique is a promising way to preserve the drug delivery system properties and its required qualities.

REFERENCES

- Abdelwahed W. et al. (2006) *Freeze-drying of nanoparticles: formulation, process and storage considerations*. Adv Drug Deliv Rev 58 (15) 1688-1713.
- Darole P. S. et al. (2008) *Formulation and evaluation of microemulsion based delivery system for amphotericin B*. AAPS PharmSciTech 9 (1) 122-128.
- Date A. A. et al. (2008) *Parenteral microemulsions: an overview*. Int J Pharm 355 (1-2) 19-30.
- Fernandez Campos F. et al. (2012) *Development and characterization of a novel nystatin-loaded nanoemulsion for the buccal treatment of candidosis: ultrastructural effects and release studies*. J Pharm Sci 101 (10) 3739-3752.
- Fissore D. et al. (2011) *Advanced approach to build the design space for the primary drying of a pharmaceutical freeze-drying process*. J Pharm Sci 100 (11) 4922-4933.
- Jung S. H. et al. (2009) *Amphotericin B-entrapping lipid nanoparticles and their in vitro and in vivo characteristics*. Eur J Pharm Sci 37 (3-4) 313-320.
- Moreno M. A. et al. (2001) *Lyophilized lecithin based oil-water microemulsions as a new and low toxic delivery system for amphotericin B*. Pharm Res 18 (3) 344-351.
- Pouton C. W. (2000) *Lipid formulations for oral administration of drugs: non-emulsifying, self-emulsifying and 'self-microemulsifying' drug delivery systems*. Eur J Pharm Sci 11 Suppl 2 S93-98.

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