International Journal of Pharmaceutics 454 (2013) 641–648

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International Journal of Pharmaceutics

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Development of oil-in-water microemulsions for the oral delivery of amphotericin B

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ARTICLE INFO

Article history:
Received 17 February 2013
Received in revised form 12 May 2013
Accepted 16 May 2013
Available online 31 May 2013

Keywords:
Amphotericin B
Lipids
Microemulsions
Surfactants
Rheology
In vitro toxicity

ABSTRACT

Amphotericin B (AmB) is a very efficient drug against serious diseases such as leishmaniasis and systemic fungal infections. However, its oral bioavailability is limited due to its poor solubility in water. Nevertheless, it is marketed as high-cost lipid parenteral formulations that may induce serious infusion-related side effects. In this study, oil-in-water (O/W) microemulsions (MEs) were developed and characterized with a view to their use as solubility enhancers and oral delivery systems for AmB. Therefore, different nonionic surfactants from the Tween® and Span® series were tested for their solubilization capacity in combination with several oils. Based on pseudoternary phase diagrams, AmB-loaded MEs with mean droplet sizes about 120 nm were successfully produced. They were able to improve the drug solubility up to 1000-fold. Rheological studies showed the MEs to be low-viscosity formulations with Newtonian behavior. Circular dichroism and absorption spectra revealed that part of the AmB in the MEs was aggregated as an AmB reservoir carrier. Cytotoxicity studies revealed limited toxicity to macrophage-like cells that may allow the formulations to be considered as suitable carriers for AmB.

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1. Introduction

Amphotericin B (AmB) is a polyene antibiotic with potent antifungal and leishmanicidal activities (Hartsel and Bolard, 1996; Ibrahim et al., 2012). Its chemical structure is characterized by a glycosylated lactone with an amphiphilic polyhydroxyl region, a conjugated heptane chromophore and an amphoteric ion pair (Fig. 1). As a consequence of both apolar and polar sides of its lactone ring and due to the presence of ionizable carboxyl and amine groups, AmB molecule presents both amphoteric and amphiphilic behavior (Damasceno et al., 2012). As a result, AmB is poorly soluble in aqueous media and in many organic solvents (Torrado et al., 2008). This low solubility leads to limited bioavailability and membrane permeability, which hinder the development of formulations for the oral route that is the most convenient and acceptable route for patients.

On the other hand, effective lipid parenteral formulations of AmB have been developed and marketed, but they have some serious limitations such as the inconvenience and complexity of the intravenous administration, the incidence of serious acute infusional side effects and the high cost that poses an important barrier for patients in developing countries (Wasan et al., 2009).

Recently, lipid-based formulations have been extensively investigated as a suitable approach to improve the bioavailability of poorly soluble drugs after oral administration (Han et al., 2009). When incorporated into these systems, the active molecule is believed to remain in solution throughout its period in the gastrointestinal tract (Pouton, 2006). Additionally, the absorption of the drug could be enhanced by the presence of lipids as a result of stimulation of biliary and pancreatic secretions by the gallbladder, an increase in the gastric residence time and others (Dahan and Hoffman, 2008).

Since microemulsions (MEs) are able to incorporate a wide range of drug molecules, increase their solubilization and bioavailability, and reduce their toxicity, they are promising delivery systems for oral administration of lipophilic molecules, such as AmB (Fanun, 2012; Pestana et al., 2008). Therefore, the aim of this work was to develop oil-in-water (O/W) MEs based on long- and medium-chain triglycerides in order to increase the solubility of AmB and enable its use by the oral route.

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http://dx.doi.org/10.1016/j.ijpharm.2013.05.044

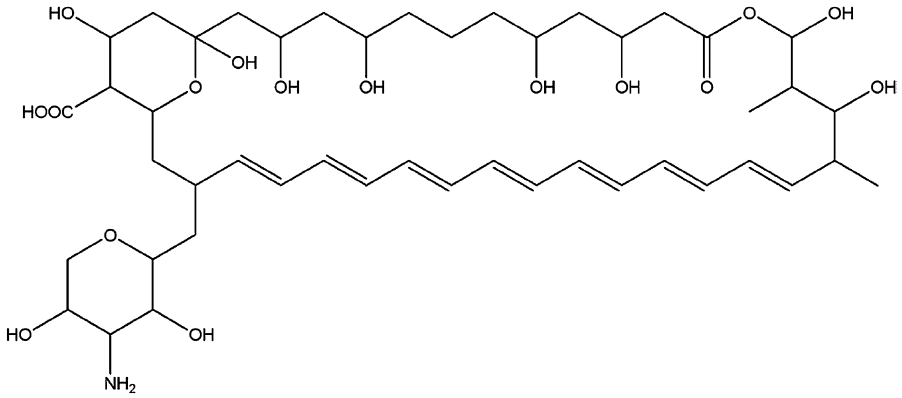

Fig. 1. Chemical structure of AmB (Santos et al., 2012).

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

Sodium hydroxide (NaOH), chloride acid (HCl), amphotericin B (AmB) and HPLC grade methanol were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France).

2.1.2. Surfactants

Span® 20, Span® 80, Span® 85, Tween® 20, Tween® 80 and Tween® 85 were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France).

2.1.3. Lipids

Capryol® 90 (C90), Capryol® PGMC (CPGMC), Lauroglycol® 90 (L90), Labrafac® lipophile WL 1349 (LWL), Labrafac® PG (LPG) and Peceol® (Pec) were kindly supplied by Gattefossé S.A. (Saint-Priest, France). Corn oil and olive oil were obtained from Sigma–Aldrich (Saint Quentin Fallavier, France).

2.2. Methods

2.2.1. Selection of oil and hydrophilic surfactant

Nonionic surfactants of the Tween® series (Tween® 20, 80 and 85) and the lipids mentioned in Section 2.1.3 were weighed and put into a series of screwcap test tubes in the ratios of 0.1:0.9, 0.2:0.8, 0.3:0.7, 0.4:0.6, and 0.5:0.5 (w/w) g of 1 g per batch, mixed together, and vortexed thoroughly. Afterwards, 100 μL of distilled water was added to each oil–surfactant mixture in 20–25 μL drops using a micropipette. After each drop of water was added, the system was vortexed for 15 s at room temperature.

Visual observations were made, and the clarity or turbidity of each sample was recorded. The isotropy of each system was also observed by light polarized microscopy through a Nikon E600 Eclipse direct microscope (Champigny/Marne, France) equipped with a long focus objective (LWD 40 × 0.55; 0–2 mm). A Nikon Coolpix 950 camera was used to record the images with a resolution of 1600 × 1200 pixels. The surfactant forming most clear systems was selected as the hydrophilic surfactant that best matched the tested lipid.

2.2.2. Selection of surfactant blends

The individual nonionic hydrophilic surfactant chosen in Section 2.2.1 was blended with the lipophilic surfactants of the Span® series (Span® 20, 80 and 85) in ratios of 3:2, 7:3, 4:1, and 9:1 (w/w) to produce blends of surfactants with various hydrophilic-lipophilic balances (HLBs) in the range of 9.7–14.4 (Table 1). The solubilization capacities of the blends of surfactants were studied using the same method as that used to study the other surfactants individually. The blend of surfactants forming a clear system at most of the ratios was selected as the blend that best matched the HLB of the tested lipid.

Table 1
Composition of the surfactant blends and their final HLB values.

Surfactant blend	Surfactants		Weight ratio	HLB
M1	Tween® 80	Span® 20	3:2	12.4
M2	Tween® 80	Span® 20	7:3	13.1
M3	Tween® 80	Span® 20	4:1	13.7
M4	Tween® 80	Span® 20	9:1	14.4
M5	Tween® 80	Span® 80	3:2	10.7
M6	Tween® 80	Span® 80	7:3	11.8
M7	Tween® 80	Span® 80	4:1	12.9
M8	Tween® 80	Span® 80	9:1	13.9
M9	Tween® 80	Span® 85	3:2	9.7
M10	Tween® 80	Span® 85	7:3	11.0
M11	Tween® 80	Span® 85	4:1	12.4
M12	Tween® 80	Span® 85	9:1	13.7

M, mixture; HLB, hydrophilic LIPOPHILIC balance, mixture; HLB, hydrophilic lipophilic balance.

2.2.3. Construction of pseudoternary phase diagrams

After selection of the most suitable surfactant blend, pseudoternary phase diagrams were constructed based on the types of systems formed when the mixtures of lipids and surfactant blend were serially titrated by water followed by sonication. The systems were characterized by visual observation as described by Mahdi et al. (2011) (Table 2). The systems were also assessed regarding their isotropy by polarized light microscopy as described in Section 2.2.1.

2.2.4. Preparation of microemulsions

Based on the pseudoternary phase diagrams, the most suitable ratios of oil, surfactant blend and water for the production of O/W microemulsions were selected. The lipid was mixed with the

Table 2
Classification of the systems generated on the pseudoternary diagrams.

Category	Description
Microemulsions (ME)	Transparent or translucent and can flow easily
Liquid crystal (LC)	Transparent or translucent nonflowable when inverted 90°
Emulsion (EM)	Milky or cloudy and can flow easily
Emollient gel or cream (EG or EC)	Milky or cloudy non flowable when inverted 90°
Bicontinuous phase (BP)	More than one type of dispersion existing in the mixture, as indicated by the presence of more than one abbreviation of dispersions

Table 3
Composition of the AmB-loaded and AmB-unloaded microemulsions.

Formulation	Oil	Oil:surfactant weight ratio	AmB
ME 1	C90	1:9	–
ME 2	C90	2:8	–
ME 3	CPGMC	1:9	–
ME 4	CPGMC	2:8	–
ME-AmB 1	C90	1:9	+
ME-AmB 2	C90	2:8	+
ME-AmB 3	CPGMC	1:9	+
ME-AmB 4	CPGMC	2:8	+

The surfactant blend selected for all the formulations comprised a mixture of Tween® 80:Span® 80 in the weight ratio of 9:1. ME 1, ME 2, ME 3 and ME 4 are AmB-unloaded formulations while ME-AmB 1, ME-AmB 2, ME-AmB 3 and ME-AmB 4 are AmB-loaded formulations. All formulations have 83.3% of water, 1.7% of oil and 15% of surfactant blend for ME 1, ME 3, ME-AmB 1 and ME-AmB 3, 3.4% of oil and 13.3% of surfactant blend for ME 2, ME 4, ME-AmB 2 and ME-AmB 4. The AmB containing formulations have 0.08% of AmB.

surfactant blend in the weight ratios of 1:9 and 2:8 and 5 mL of water (Table 3). The mixture was vortexed and subjected to sonication at 140 V for 60 s (Digital Sonifier, model 450, Branson Ultrasonic SA, France).

2.2.5. Drug incorporation

An excess of AmB was added to the blank MEs, and the systems were vortexed for 2 min. After stirring, the mixtures were left for 10, 30 and 60 min under magnetic stirring at pH 11 in order to evaluate the time necessary for the incorporation of AmB into the systems at 25 ± 0.1 °C. Thereafter, the pH was neutralized. The MEs were centrifuged at 10,000 × *g* in a Hitachi Himac CP-80 Ultracentrifuge (USA) for 15 min to remove the excess of drug. The supernatant was recovered and carefully filtered using a 0.22 μm membrane. The filtrate was diluted and dissolved in methanol for the quantitative analysis of the AmB by HPLC.

2.2.6. Microemulsion characterization

2.2.6.1. Droplet hydrodynamic size, distribution and morphology. The droplet hydrodynamic size and distribution were evaluated by dynamic light scattering (DLS) using a Malvern-Zetasizer Nano ZS (Malvern, Worcestershire, UK). The morphology of the droplets of selected O/W MEs was observed by transmission electron microscopy (TEM) using an electron microscope JEOL 1400 (SamX-Plus, France) equipped with a high resolution CCD Gatan digital camera (SC1000 Orius, France) and operated at 60 kV as the acceleration voltage.

2.2.6.2. Quantitative analysis of AmB. The incorporation of AmB was determined by high performance liquid chromatography (HPLC) using a Waters 2690 separations module, with a Waters 2487 dual absorbance detector (Waters, Guyancourt, France) and a C18 column (Interchim, 150 mm × 3 mm, 5 μm). The mobile phase consisted of a solution containing methanol/water (80:20). An isocratic elution was performed with a flow rate of 0.5 ml min^{-1}. UV detection was performed at a wavelength of 406 nm and 25 μL of sample was injected for each analysis. To determine the linearity of the method, different concentrations of AmB in the range 0.07–4 μg mL^{-1} were prepared and analyzed.

2.2.6.3. Rheological behavior. The rheological properties of AmB-loaded and unloaded MEs were determined using a controlled-stress ARG2 rheometer (TA instruments) with cone-plate geometry. The cone-plate geometry is a 1° Peltier plate aluminum cone with a 40 mm diameter and a truncation gap of 28 μm between the cone and plate. The analyses were carried out with a shear rate in the range of 10^{-3}–10^{5} s^{-1}. All rheological determinations were carried out in triplicate for all samples and at 25.0 ± 0.2 °C.

2.2.6.4. Absorption and circular dichroism (CD). Absorbance measurements were made by using a Perkin-Elmer Lambda 11 UV–vis spectrophotometer. The CD spectra were recorded with a Jasco J-810 dichrograph, and $\Delta\epsilon$ (M^{-1} cm^{-1}) is the differential molar absorption dichroic coefficient. These spectroscopic measurements were made at room temperature (around 20 °C) after dilution in water to a final AmB concentration of 0.3 mg/mL (path length of quartz cuvette: 1 cm) to evaluate the aggregation state of AmB in the formulations.

2.2.6.5. Toxicity of AmB-loaded microemulsions against macrophages. The cytotoxicity of the AmB-loaded and unloaded MEs was tested using the MTS [3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay. The conversion of this salt by mitochondrial enzymes reflects the number of viable cells. J774.A1 cells (ECACC catalog number 91051511) were cultivated in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (both from Lonza Sales Ltd., Basel, Switzerland). For experiments, they were seeded into 96-well plates at 5000 cells/well and incubated for 24 h to allow adhesion. Samples of freshly prepared MEs (50 μL) were added to groups of 6 wells at the following AmB concentrations: 1, 5, 10, 25, 50 and 100 μg/mL. Wells without cells, but containing the same concentration of unloaded MEs were used to estimate background absorbance due to light scattering. The cells were incubated for 1 and 3 h at 37 °C and 0.5% CO_2. Thereafter, 20 μL of MTS was added to the cells and they were incubated for a further 2 h, after which the absorbance was measured using a 492-nm high-pass filter in a Multiskan MS microwell plate reader (Labsystem, Ramat-Gan, Israel). The mitochondrial enzymatic activity was expressed as a percentage of that of untreated control cells, after correcting for background absorbance.

2.2.7. Statistical analysis

Statistical analysis of the raw data was conducted using one-way analysis of variance (ANOVA) in Graph Pad Prism (Graph Pad software for Mac v. 6.0, San Diego, CA). Statistical significance of differences between two conditions was assessed using Tukey's *post hoc* method. Differences between different groups were considered statistically significant at $p < 0.05$.

Table 4
Qualitative and quantitative composition and HLB values of the lipids tested as the oil phase.

Lipid	Composition	HLB
Capryol® 90	Propylene glycol mono- and diesters of caprylic acid (C8), the monoester fraction being predominant (>90%)	6
Capryol® PGMC	Propylene glycol mono- and diesters of caprylic acid (C8), the monoester fraction being predominant (55–80%)	5
Lauroglycol® 90	Propylene glycol mono- and diesters of lauric acid (C12), the monoester fraction being predominant (>90%)	5
Labrafac® PG	Dipropylene glycol esters of caprylic and capric (C10) acids	2
Labrafac® lipophile WL 1349	Triglycerides of caprylic and capric acids	1
Peceol®	Mono-, di- and triglycerides of oleic acid (C18:1), the monoester being predominant (32–52%)	3
Corn oil	Triglycerides of linoleic acid (18:2) (60%), oleic acid and linoleic acid (18:3) (<15%).	9
Olive oil	Triglycerides of oleic acid (55–85%), linoleic acid (7.5–20%) and palmitic acid (C16:0) (7.5–20%)	8

3. Results and discussion

3.1. Selection of surfactant and surfactant blends

C90, CPGMC, L90, LPG, LWL and Pec are liquid at room temperature and they are mainly composed of mono-, di- and triglycerides of caprylic, capric, lauric and oleic acids. Their exact composition is listed in Table 4, according to information from the supplier and the literature (Varka and Karapantsios, 2011).

In order to develop MEs, an important parameter take into account is the HLB of the surfactant or surfactant mixture (Feng et al., 2009). It is related to the contribution of both hydrophilic and hydrophobic fragments of a surfactant molecule. Generally, surfactants with HLB values between 8 and 20 are able to form O/W MEs, while W/O MEs are formed when the HLB range is 4–7 (Lawrence and Rees, 2012).

Tween® 80 was shown to be the hydrophilic surfactant with the highest solubilization capacity when compared with Tween® 20 and Tween® 85. These emulsifiers have the same polar head, but different hydrophobic tails (lauric, oleic and oleic acid, respectively in Tween 20, 80 and 85). The length of these hydrophobic chains determines the interactions with the oil phase (Mosca et al., 2013). In our experiments, we obtained clear mixtures of water and oil at the highest weight ratios for C90, CPGMC and L90. For the other oils tested, no clear mixture was obtained. Therefore, these were not used in further studies.

It is possible that the low HLBs of LPG, LWL and Pec: 2, 1 and 3 respectively, were responsible for the incompatibility between these lipids and the hydrophilic surfactants. Furthermore, the interactions between surfactants at an oil–water interphase are known to be highly dependent on the nature of the oil (Mosca et al., 2013). Corn and olive oils are composed of long-chain triglycerides and probably showed a weak interaction with the surfactant from the same fatty acid derivative, as stated in the literature (Mahdi et al., 2011).

It is known that a single surfactant is not sufficient to form single-phase microemulsions and an adequate mixture of surfactants may be required to optimize the microemulsion formation (Djekic et al., 2011). The use of mixtures of nonionic surfactants is an interesting approach from the pharmaceutical point of view, since such surfactants are generally regarded as having low toxicity and irritancy and therefore, considered to be acceptable for oral administration. Additionally, the use of mixtures allows the individual concentration of each surfactant to be decreased, which may increase the biocompatibility of the final formulations (Djekic et al., 2011; Pouton and Porter, 2008). Therefore, Tween® 80 was mixed with the hydrophobic surfactants of the Span® series to provide surfactant blends in order to screen and select the best surfactant mixture to prepare oil-in-water microemulsions.

The results obtained for the solubilization power of the surfactant blends revealed two mixtures as having the highest capacities: Tween® 80/Span® 20 7:3 (v/v) (M2) and Tween® 80/Span® 80 9:1 (v/v) (M8), the HLB values of the two blends being 13.1 and 13.9, respectively. Thus, these two surfactant blends were selected to study the phase diagram behavior of C90, CPGMC and L90.

3.2. Construction of pseudoternary phase diagrams

According to the pseudoternary phase diagrams, several types of dispersions could be produced by mixing C90, CPGMC and L90 with the surfactant blends M2 and M8 followed by titration with water. For instance, large areas of emulsions, microemulsions and some considerable areas of liquid crystal could be detected, as well as smaller areas of bicontinuous phase, cream and gel (Figs. 2 and 3).

Both the surfactant blends M2 and M8 were able to produce some microemulsion-forming regions for the three lipids tested

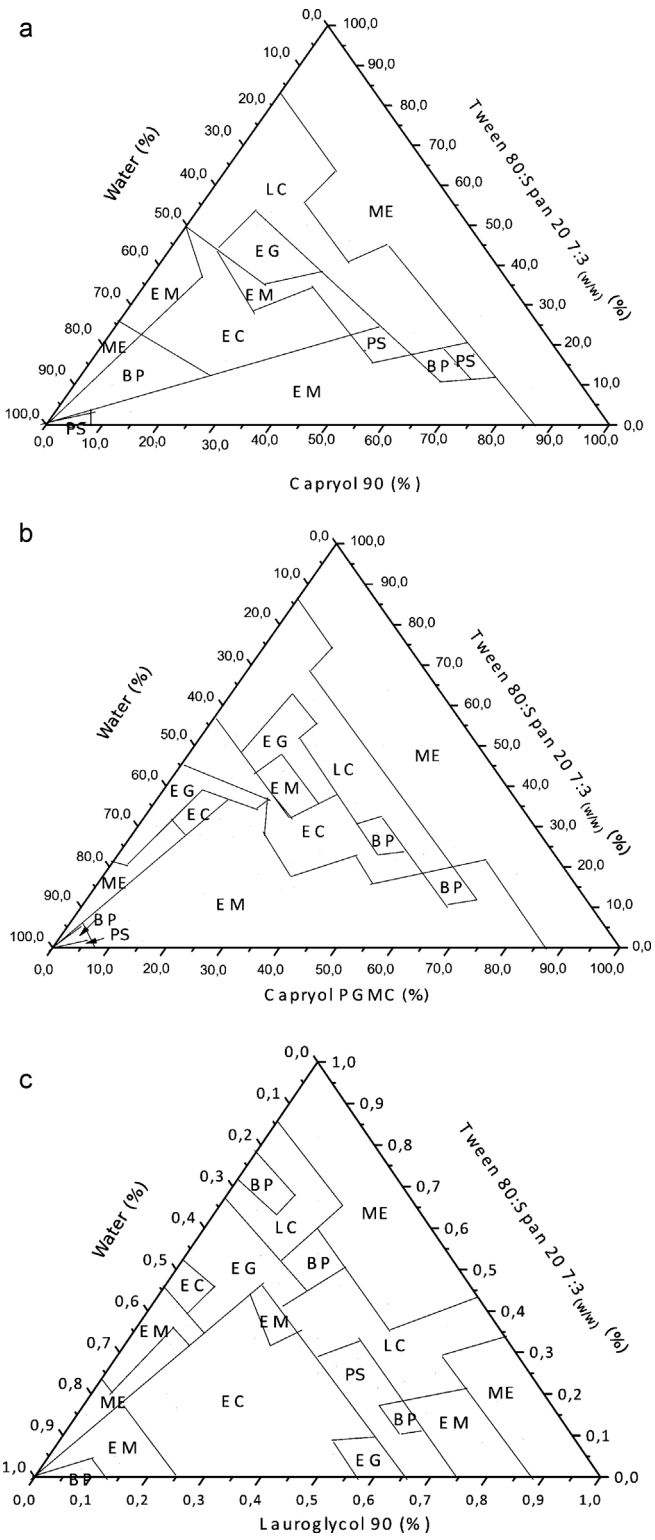

Fig. 2. Pseudoternary phase diagrams formed by (a) C90, (b) CPGMC and (c) L90 as the oil phase and Tween 80:Span 20 7:3 (w/w) as the surfactant blend and water. ME, microemulsions; LC, liquid crystal; EM, emulsion; EG, emollient gel; EC, emollient cream; BP, bicontinous phase; PS, phase separation.

(Figs. 2 and 3). This seems to be coherent with the HLB values of surfactants or mixture of surfactants reported to be optimal for the preparation of microemulsions, since M2 and M8 presented very similar HLB values: 13.1 and 13.9, respectively. However, it was evident that both C90 and CPGMC were able to produce larger areas of O/W emulsions and O/W microemulsions than L90. Thus, propylene

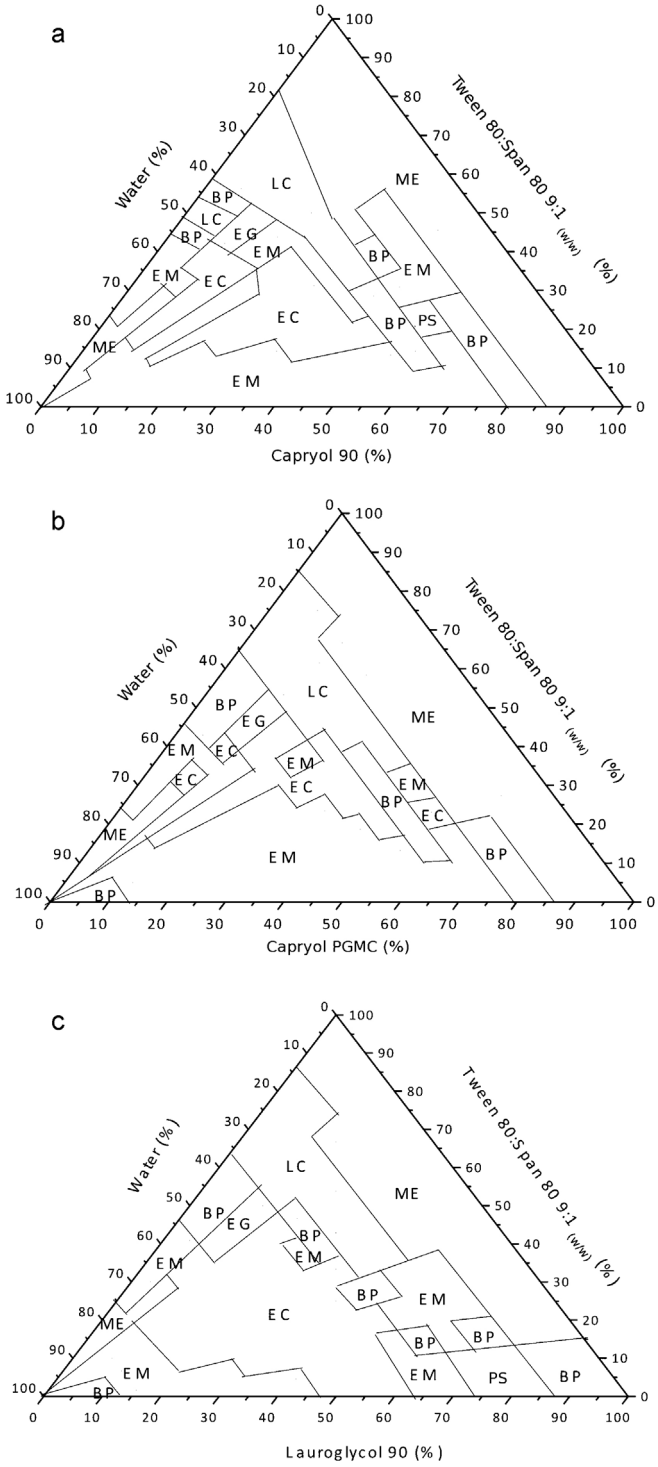

Fig. 3. Pseudoternary phase diagrams formed by (a) C90, (b) CPGMC and (c) L90 as the oil phase and Tween® 80:Span® 80 9:1 (w/w) as the surfactant blend and water. ME, microemulsions; LC, liquid crystal; EM, emulsion; EG, emollient gel; EC, emollient cream; BP, bicontinous phase; PS, phase separation.

glycol esters of caprylic acid seem to be more appropriate for the preparation of O/W emulsions and microemulsions than propylene glycol esters of lauric acid. Furthermore, the phase diagram behavior of those lipids was not only affected by the HLB value of the surfactant, but also by the structure of the co-surfactant.

Previous studies have observed that in general the most stable emulsions are formed when the two emulsifying agents have the same hydrocarbon chain length, such as the combination between Tween® 80 and Span® 80, because of their similar chemical structure (Schmidts et al., 2009). Mahdi and coworkers stated that high solubilization capacity can be obtained when surfactants with the lowest and highest HLB values are mixed. In our case, we believe that the three chains of oleic acid esters in the molecule of Span® 85 hinder the interaction with Tween® 80. On the other hand, the interaction between Span® 80 and Tween® 80 proved to be more effective in reducing the oil-water interfacial tension and producing MEs. These formulations were used for further studies.

3.3. Microemulsion characterization

Regardless of the oil phase, DLS and TEM analysis revealed that drug-free and drug-loaded MEs were composed of spherical non-aggregated droplets with mean sizes around 80 and 120 nm respectively (Fig. 4). This increase in the diameter of the ME droplets after the incorporation of AmB is explained by the fact that AmB has surface properties due to its amphiphilic behavior and is adsorbed at the oil-water interface (Franzini et al., 2012; Santos et al., 2012). The polydispersity index was shown to be around 0.25 and 0.31 for unloaded MEs and AmB-loaded MEs, respectively.

When the quantity of encapsulated AmB by was measured by HPLC, it was shown that the drug incorporation was dependent on the volume fraction of the dispersed phase and ranged between 70% and 90%. These data highlight the fact that the MEs are able to increase the AmB solubility up to 1000-fold when compared with its solubility in water. It also gives further evidence for the strong interaction between the hydrophobic AmB molecule and the oil present in the ME (Franzini et al., 2012). More precisely, the ME containing C90 as the oil phase at the weight ratio of 2:8 (oil:surfactant) gave the highest rate of incorporation of AmB. The incorporation time did not influence the amount of AmB incorporated, showing that the association occurred rapidly over 10 min.

3.4. Rheological behavior

The physicochemical characterization of delivery systems is an essential step in the pre-formulation process to predict the feasibility of the final products. Among the parameters for the characterization of MEs, rheology is a fundamental approach to investigate structural properties and acquire helpful information not only on the stability of such systems, but also on the handling, storage and pipeline transportation of MEs (Formariz et al., 2010; Pal, 2011).

Although the rheological analysis indicated that the viscosity was very low for all the samples, it appeared to decrease at low shear rates between 10^{-3} and $10\,s^{-1}$ and remained constant at higher shear rates than $10\,s^{-1}$ (data not shown). However, the flow curves revealed that all the ME systems showed a linear relationship between the shear stress and shear rate, which is a feature of Newtonian flow materials (Feng et al., 2009) (Fig. 5a and b). These results confirm that our samples are discontinuous MEs. As reported by previous studies, discontinuous MEs show constant viscosity over a wider range of shear rates than bicontinuous MEs (Acharya and Hartley, 2012). As a consequence of their low viscosity, such systems are considered suitable for oral delivery (Lawrence and Rees, 2012).

The influence of AmB on the micro-organization of the MEs was investigated. No change in the linear profile of the flow curves was observed (Fig. 5b), indicating that the drug did not influence the flow properties of the system.

3.5. Aggregate state of AmB

AmB self-associates in aqueous media, forming supramolecular aggregates. Monomers, and soluble and insoluble aggregates

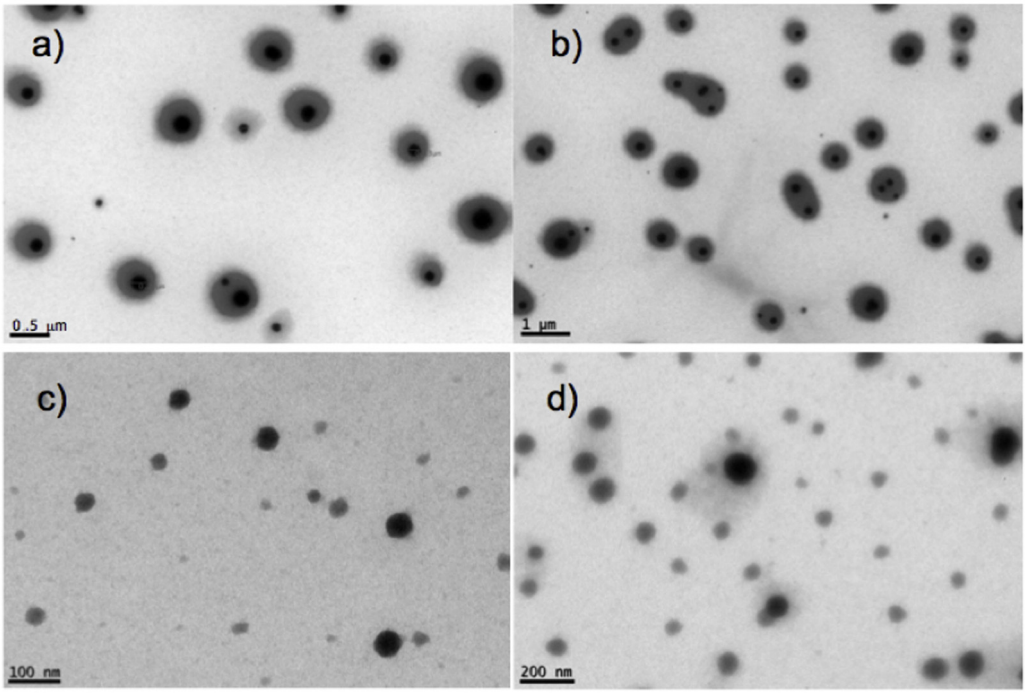

Fig. 4. TEM images of AmB-loaded microemulsions based on C90:M8 at 1:9 (a) and 2:8 (b) (w/w), and CPGMC at 1:9 (c) and 2:8 (d) (w/w), at magnification of 4000×, 2500×, 30,000× and 15,000×, respectively.

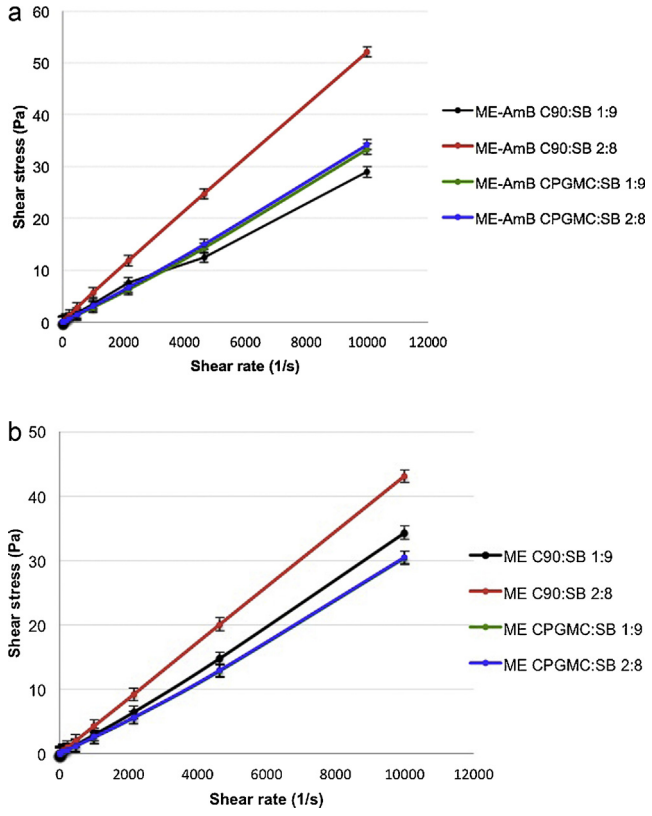

Fig. 5. Flow curves of (a) AmB-loaded and (b) AmB-unloaded MEs.

present in the dispersing medium determine the toxicity of AmB (Gaboriau et al., 1997; Silva-Filho et al., 2012). In order to study the aggregation state of AmB molecules after incorporation into the MEs, the formulations were analyzed by circular dichroism and electronic absorption. These methods are useful and sensitive tools to identify changes in the conformation of AmB (Bolard et al., 1981). The presence of 7 conjugated double bonds in this molecule leads to very characteristic spectra between 300 and 450 nm, which depend on conformational changes as a result of its self-association in water or its association with other compounds, such as lipids (Boudet and Bolard, 1979; Espuelas et al., 1998). These distinctive optical properties of AmB enable its study by both electronic absorption and circular dichroism spectroscopy (Barwicz et al., 2002; Boudet and Bolard, 1979).

As can be seen in Fig. 6a, the absorption spectrum of a stock solution of AmB in methanol after dilution in water to 0.3 mg/mL shows three bands at 410, which are characteristic of the monomeric form, 385, 365 nm and a smaller band at 344 nm. The absorption spectra for the MEs at the same concentration of AmB showed peaks at the same wavelengths, but with different intensities (Fig. 6a).

In addition, a peak at 318 nm was detected at different intensities for each formulation. This is believed to indicate the presence of self-associated molecules of AmB. Thus, a part of the AmB in the MEs was shown to be aggregated. The formulation containing CPGMC as the oil phase at the weight ratio of 1:9 (ME-AmB 3) between oil to surfactant mixture showed the lowest proportion of aggregates and the highest proportion of monomers. Its spectrum appears to be very similar to that of AmB in methanol (Fig. 6a).

These results are corroborated by the CD spectra of the MEs (Fig. 6b). They reveal the dichroic doublet, which is characteristic of the aggregation state of AmB, for all the formulations with the center at 317 nm, with different intensities depending on the system. It is important to note that the CD spectra of AmB in water and in methanol are completely different (Fig. 6c), with the latter one showing no dichroic doublet, which is characteristic of the monomer state of AmB.

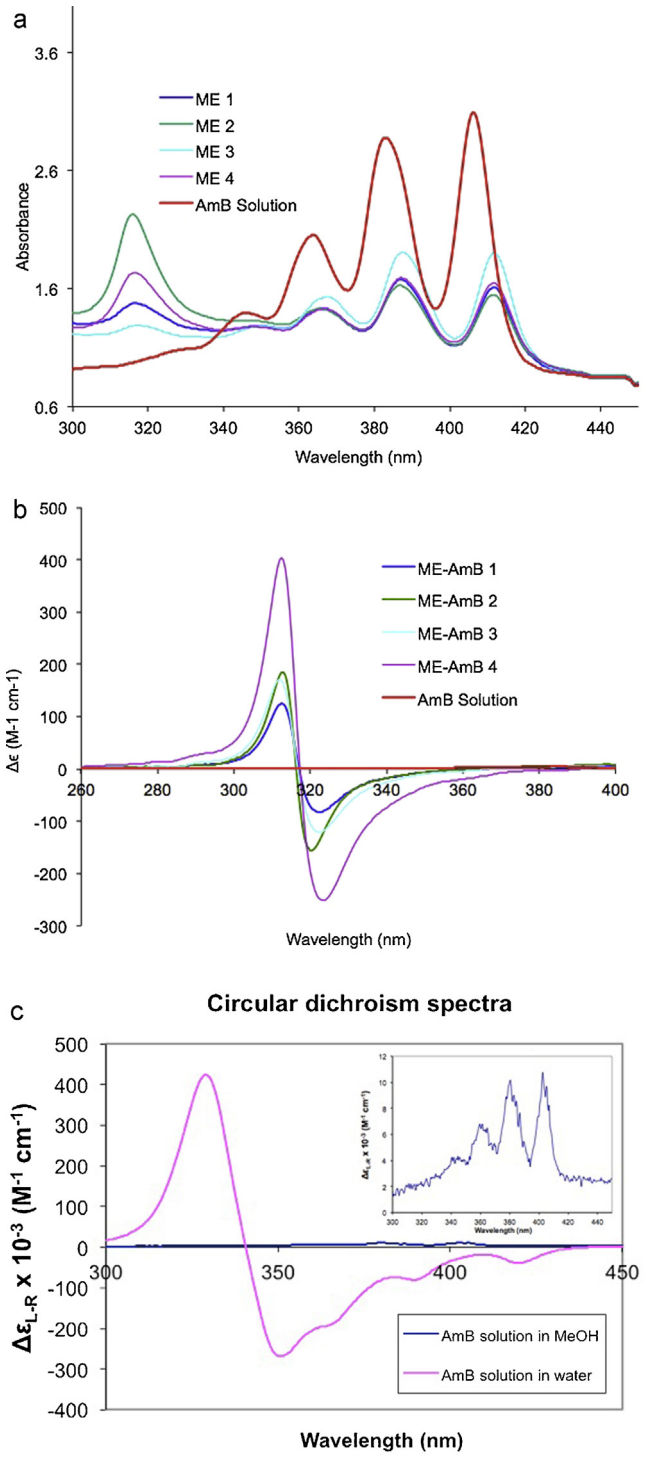

Fig. 6. (a) Electronic absorption and (b) CD spectra of an aqueous solution of AmB-loaded MEs and (c) CD spectra of an aqueous solution and a methanolic solution of AmB (insert: CD spectrum in methanol with an expanded ordinate).

3.6. Cytotoxicity profile

Fig. 7a and b show the viability of J774 macrophages as determined by MTS conversion after contact with AmB-unloaded ME (ME 2), AmB-loaded ME (ME-AmB 2) and a solution of AmB in DMSO (AmB-DMSO) for 1 h and 3 h, respectively. The chosen formulation corresponds to the ME with C90 as the oil phase and 2:8 as the weight ratio between C90 and the surfactant mixture Tween® 80:Span® 80 (9:1) (w/w) (M4 on Table 1).

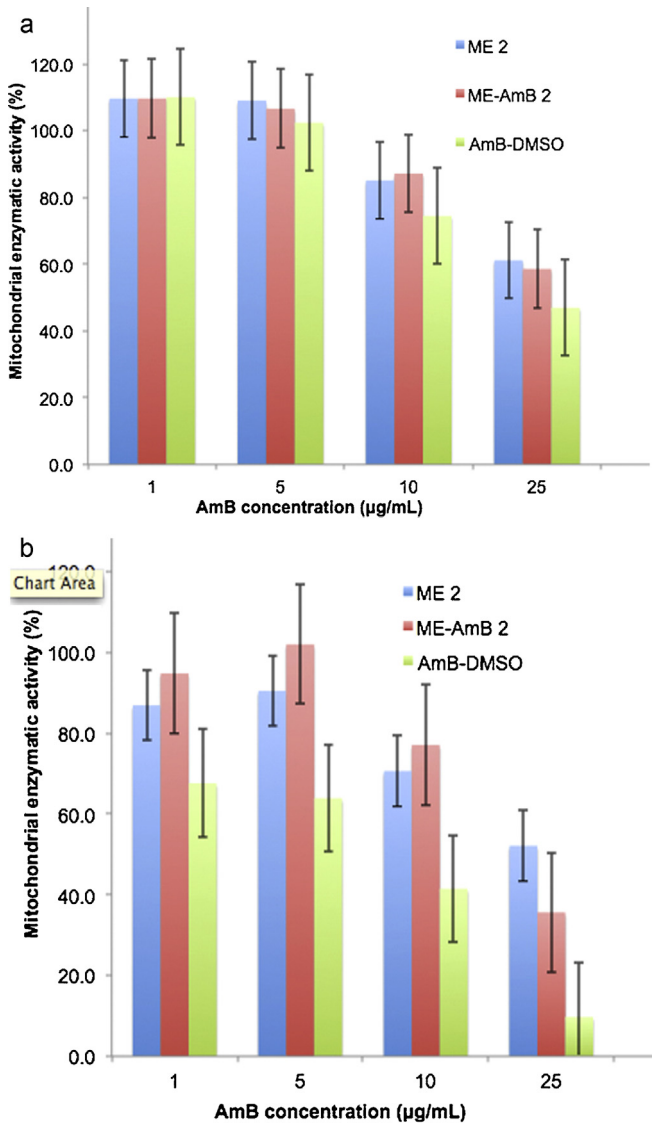

Fig. 7. Dose–response effects of AmB-unloaded and AmB-loaded MEs containing C90 as the oil phase in the weight ratio of 2:8 (oil:surfactant) (ME 2 and ME-AmB 2, respectively) and a solution of AmB in DMSO as a control (AmB-DMSO) on the cytotoxicity on mouse macrophages (J774 cell line) after (a) 1 h and (b) 3 h of incubation, in the MTS conversion assay. Results are presented as a percentage of the activity of untreated cells.

As can be seen in Fig. 7a, the cell viability exceeded 80% after 1 h for the concentrations 1, 5 and 10 μg/mL, but fell to about 60% when the AmB concentration increased to 25 μg/mL, or when the equivalent amount of unloaded MEs were added. This suggests that both blank and drug-loaded ME may be considered as having low acute toxicity probably induced by the volume of ME added to the medium, 50 μL, which contains a large amount of Tween® 80. On the other hand, after 3 h incubation the cell viability remained high at approximately 85% for the AmB concentrations of 1 and 5 μg/mL while the blank ME and drug-loaded ME exhibited slightly higher toxic effects at higher concentrations of AmB. However, the formulations appeared to affect cell viability less than a solution of AmB in DMSO at the same concentration (Fig. 7b). Similar results were obtained for the other three ME formulations (not shown).

The MTS assay revealed that the cytotoxicity of the MEs was time-dependent. There is an indication that the MEs reduce the cytotoxicity at low concentrations, although at AmB concentrations higher than 25 μg/mL no advantage of the MEs could be observed. Regardless of the time of contact between the formulations and the

macrophages, statistical analysis showed no significant differences for the low concentrations of AmB (1 and 5 μg/mL) and the equivalent unloaded MEs, but they were significant for the concentrations of 10 and 25 μg/mL and their corresponding unloaded MEs.

4. Conclusions

Our results demonstrate that it is important to select surfactants and mixtures of surfactants with not only a suitable HLB value, but also with structural similarity with the chosen oil phase for successful ME formulation. The best surfactant mixture to provide O/W ME with medium chain triglycerides based on caprylic esters as the oil phase was shown to have HLB value around 13. More precisely, Tween® 80:Span® 80 in the weight ratio of 9:1 was the surfactant providing greater solubilization capacity for C90 and CPGMC. They also improved the solubility of AmB up to 1000-fold and showed suitable rheological behavior for the oral administration. However, studies of electron absorption and CD spectra revealed the presence of aggregates of AmB. They are likely to be at the origin of the time-dependent cytotoxicity of our formulations, which were slightly less toxic than a solution of AmB in DMSO, although all formulations were well supported by J774 cells at concentrations up to 25 μg/mL of AmB.

Acknowledgments

The authors wish to thank Dr. Claire Gueutin for the support with HPLC analysis and Dr. Nicolas Huang and Sarah Mosbah for the rheological assays. This work was financially supported by CAPES/COFECUB.

References

Acharya, D.P., Hartley, P.G., 2012. Progress in microemulsion characterization. Curr. Opin. Colloid Interface Sci. 17, 274–280.

Barwicz, J., Beauregard, M., Tancrède, P., 2002. Circular dichroism study of interactions of Fungizone or AmBisome forms of amphotericin B with human low density lipoproteins. Biopolymers 67, 49–55.

Bolard, J., Vertut-Croquin, A., Cybulska, B.E., Gary-Bobo, C.M., 1981. Transfer of the polyene antibiotic amphotericin B between single-walled vesicles of dipalmitoylphosphatidylcholine and egg-yolk phosphatidylcholine. Biochim. Biophys. Acta Biomembr. 647, 241–248.

Boudet, G., Bolard, J., 1979. Interaction of the polyene antibiotic amphotericin B with phospholipid bilayer membranes: a circular dichroism study. Biochem. Biophys. Res. Commun. 88, 998–1002.

Dahan, A., Hoffman, A., 2008. Rationalizing the selection of oral lipid based drug delivery systems by an in vitro dynamic lipolysis model for improved oral bioavailability of poorly water soluble drugs. J. Control. Release 129, 1–10.

Damasceno, B.P.G.L., Dominici, V.A., Urbano, I.A., Silva, J.A., Araújo, I.B., Santos-Magalhães, N.S., Silva, A.K.A., Medeiros, A.C., Oliveira, A.G., Egito, E.S.T., 2012. Amphotericin B microemulsion reduces toxicity and maintains the efficacy as an antifungal product. J. Biomed. Nanotechnol. 8, 290–300.

Djekic, L., Primorac, M., Jockovic, J., 2011. Phase behaviour, microstructure and ibuprofen solubilization capacity of pseudo-ternary nonionic microemulsions. J. Mol. Liq. 160, 81–87.

Espuelas, M.S., Legrand, P., Cheron, M., Barratt, G., Puisieux, F., Devissaguet, J.P., Irache, J.M., 1998. Interaction of amphotericin B with polymeric colloids: a spectroscopic study. Colloids Surf. B Biointerfaces 11, 141–151.

Fanun, M., 2012. Microemulsions as delivery systems. Curr. Opin. Colloid Interface Sci. 17, 306–313.

Feng, J.-L., Wang, Z.-W., Zhang, J., Wang, Z.-N., Liu, F., 2009. Study on food-grade vitamin E microemulsions based on nonionic emulsifiers. Colloids Surf. A Physicochem. Eng. Aspects 339, 1–6.

Formariz, T.P., Chiavacci, L.A., Scarpa, M.V., Silva-Junior, A.A., Egito, E.S.T., Terrugi, C.H.B., Franzini, C.M., Sarmento, V.H.V., Oliveira, A.G., 2010. Structure and viscoelastic behavior of pharmaceutical biocompatible anionic microemulsions containing the antitumoral drug compound doxorubicin. Colloids Surf. B Biointerfaces 77, 47–53.

Franzini, C.M., Pestana, K.C., Molina, E.F., Scarpa, M.V., do Egito, E.S.T., de Oliveira, A.G., 2012. Structural properties induced by the composition of biocompatible phospholipid-based microemulsion and amphotericin B association. J. Biomed. Nanotechnol. 8, 350–359.

Gaboriau, F., Chéron, M., Leroy, L., Bolard, J., 1997. Physico-chemical properties of the heat-induced superaggregates of amphotericin B. Biophys. Chem. 66, 1–12.

Han, S.-f., Yao, T.-t., Zhang, X.-x., Gan, L., Zhu, C., Yu, H.-z., Gan, Y., 2009. Lipid-based formulations to enhance oral bioavailability of the poorly water-soluble drug anethol trithione: effects of lipid composition and formulation. Int. J. Nanomed. 379, 18–24.

Hartsel, S., Bolard, J., 1996. Amphotericin B: new life for an old drug. Trends Pharmacol. Sci. 17, 445–449.

Ibrahim, F., Gershkovich, P., Sivak, O., Wasan, E.K., Bartlett, K., Wasan, K.M., 2012. Efficacy and toxicity of a tropically stable lipid-based formulation of amphotericin B (iCo-010) in a rat model of invasive candidiasis. Int. J. Pharm. 436, 318–323.

Lawrence, M.J., Rees, G.D., 2012. Microemulsion-based media as novel drug delivery systems. Adv. Drug Deliv. Rev. 64, 175–193 (supplement).

Mahdi, E.S., Sakeena, M.H.F., Abdulkarim, M.F., Abdullah, G.Z., Sattar, M.A., Noor, A.M., 2011. Effect of surfactant and surfactant blends on pseudoternary phase diagram behavior of newly synthesized palm kernel oil esters. Drug Des. Dev. Ther. 5, 311–323.

Mosca, M., Cuomo, F., Lopez, F., Ceglie, A., 2013. Role of emulsifier layer, antioxidants and radical initiators in the oxidation of olive oil-in-water emulsions. Food Res. Int. 50, 377–383.

Pal, R., 2011. Rheology of simple and multiple emulsions. Curr. Opin. Colloid Interface Sci. 16, 41–60.

Pestana, K.C., Formariz, T.P., Franzini, C.M., Sarmento, V.H.V., Chiavacci, L.A., Scarpa, M.V., Egito, E.S.T., Oliveira, A.G., 2008. Oil-in-water lecithin-based microemulsions as a potential delivery system for amphotericin B. Colloids Surf. B Biointerfaces 66, 253–259.

Pouton, C.W., 2006. Formulation of poorly water-soluble drugs for oral administration: physicochemical and physiological issues and the lipid formulation classification system. Eur. J. Pharm. Sci. 29, 278–287.

Pouton, C.W., Porter, C.J.H., 2008. Formulation of lipid-based delivery systems for oral administration: materials, methods and strategies. Adv. Drug Deliv. Rev. 60, 625–637.

Santos, C.M., Oliveira, R.B., Arantes, V.T., Caldeira, L.R., Oliveira, M.C., Egito, E.S.T., Ferreira, L.A.M., 2012. Amphotericin B-loaded nanocarriers for topical treatment of cutaneous leishmaniasis: development, characterization, and in vitro skin permeation studies. J. Biomed. Nanotechnol. 8, 322–329.

Schmidts, T., Dobler, D., Nissing, C., Runkel, F., 2009. Influence of hydrophilic surfactants on the properties of multiple W/O/W emulsions. J. Colloid Interface Sci. 338, 184–192.

Silva-Filho, M.A., Siqueira, S.D., Freire, L.B., Araújo, I.B., Silva, K.G.H., Medeiros, A.C., Araújo-Filho, I., Oliveira, A.G., Egito, E.S.T., 2012. How can micelle systems be rebuilt by a heating process? Int. J. Nanomed. 7, 141–150.

Torrado, J.J., Espada, R., Ballesteros, M.P., Torrado-Santiago, S., 2008. Amphotericin B formulations and drug targeting. J. Pharm. Sci. 97, 2405–2425.

Varka, E.M., Karapantsios, T.D., 2011. Global versus local dynamics during destabilization of eco-friendly cosmetic emulsions. Colloids Surf. A Physicochem. Eng. Aspects 391, 195–200.

Wasan, E.K., Bartlett, K., Gershkovich, P., Sivak, O., Banno, B., Wong, Z., Gagnon, J., Gates, B., Leon, C.G., Wasan, K.M., 2009. Development and characterization of oral lipid-based amphotericin B formulations with enhanced drug solubility, stability and antifungal activity in rats infected with *Aspergillus fumigatus* or *Candida albicans*. Int. J. Pharm. 372, 76–84.