International Journal of Pharmaceutics 447 (2013) 38–46

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Hemolytic and pharmacokinetic studies of liposomal and particulate amphotericin B formulations

Dolores R. Serrano[a], Leticia Hernández[b], Laura Fleire[a], Iban González-Alvarez[c], Ana Montoya[b], María P. Ballesteros[a], María A. Dea-Ayuela[c,d], Guadalupe Miró[b], Francisco Bolás-Fernández[d], Juan J. Torrado[a,*]

[a] Farmacia y Tecnologia Farmaceutica, Facultad de Farmacia, Universidad Complutense de Madrid, Plaza Ramon y Cajal, 28040 Madrid, Spain
[b] Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense, Madrid, 28040 Madrid, Spain
[c] Departamento de Farmacia, Facultad de Ciencias de la Salud, Universidad Cardenal Herrera-CEU, Moncada, 46113 Valencia, Spain
[d] Departamento de Parasitología, Facultad de Farmacia, Universidad Complutense, Plaza Ramon y Cajal, 28040 Madrid, Spain

ARTICLE INFO

Article history:
Received 2 January 2013
Received in revised form 13 February 2013
Accepted 14 February 2013
Available online 21 February 2013

Keywords:
Amphotericin B
Pharmacokinetics
Hemolysis
Aggregation state
Albumin microparticles
Passive targeting

ABSTRACT

Amphotericin B (AmB) is a very effective antifungal and antiparasitic drug with a narrow therapeutic window. To improve its efficacy/toxicity balance, new controlled release formulations have been developed based on different encapsulation systems, aggregation states and particle sizes modifications. The kinetics of the hemolytic process was studied not only to characterize the toxicity of different formulations but also as an indicator of drug release. Pharmacokinetic studies in beagle dogs were carried out with those formulations that exhibited the least hemolytic toxicity: liposomal formulation (AmBisome®), poly-aggregated AmB and encapsulated particulate AmB formulation. A novel poly-aggregated AmB formulation proved to be comparable in terms of low hemolytic activity with the marketed gold standard formulation: AmBisome®. Its pharmacokinetic profile, characterized by a smaller area under the curve and larger volume of distribution, was markedly different from AmBisome®, resulting in a cost-effective alternative for the treatment of leishmaniasis which can enhance the AmB passive target by the uptake by the cells of the reticulo-endothelial system. Effects of different variables such as type of formulation, dose, microencapsulation, anesthesia and dog's healthy state on AmB pharmacokinetics were studied.

1. Introduction

AmB is a potent antifungal and antiparasitic drug acting by binding preferentially and selectively to ergosterol present in fungal and bacterial membranes compared to cholesterol localized in mammal membranes and by disrupting them. The molecular interaction of AmB with the natural components of the membranes and the role of different proteins and lipids in this action is complex and has important consequences in its activity and toxicity (Brajtburg and Bolard, 1996; Cheron et al., 2003; Hartsel et al., 2001; Torrado et al., 2008). After AmB formulations intravenous administration, interaction of AmB with RBCs causes their lysis leading to anaemia. Thus, safety of novel AmB delivery strategies such as micelles, microspheres, microemulsions, liposomes, nanoparticles and others can be studied by comparing their hemolysis potential. However, hemolysis assays used by various research groups vary significantly (see Table 1 in supplementary data). Additionally using human to animal origin blood leads to differences, as human red blood cells are more resistant compared to rat making it difficult to compare the safety of novel formulations (Fukui et al., 2003; Knopik-Skrocka et al., 2003). Additionally, studying the hemolysis over various time points is more meaningful when controlled release formulations are under investigation as release from various formulations differs over time and this is not accounted if only one time point is studied (Adams et al., 2003; Adams and Kwon, 2003). These studies are vital to predict the safety of the in vitro formulations that can progress to preclinical studies. Furthermore, due to the low solubility of AmB

Abbreviations: AmB, Amphotericin B; RBCs, red blood cells; RES, reticulo-endothelial system; FD, non-microencapsulated free dimeric AmB formulation; F, Fungizone®; HF, heated-Fungizone; MD, microencapsulated dimer AmB; FP, non-microencapsulated free poly-aggregated AmB formulation; MP, microencapsulated poly-aggregated AmB; DLS, dynamic light scattering; TEM, transmission electron microscopy; SEM, scanning electron microscopy; PBS, phosphate buffer solution; Abs, absorbance; K_d, degradation constant; R, correlation coefficient; NNN medium, Novy-MacNeal-Nicolle medium; AUC, area under the plasma concentration versus time curve; AUMC, area under the first moment curve; C_{max}, concentration at time zero; λ, terminal phase elimination rate constant; $t_{1/2}$, terminal elimination half-life; Cl, body clearance; V_{ss}, volume of distribution at steady state; V_{area}, volume of distribution or volume area; MRT, mean residence time; t_s, mean residence time in systemic circulation; t_p, mean residence time in peripheral tissues.

* Corresponding author at: Departamento de Farmacia y Tecnología Farmacéutica, Facultad de Farmacia, Universidad Complutense, Plaza Ramón y Cajal S/N, 28040, Madrid, Spain. Tel.: +34 913941620; fax: +34 913941736.
E-mail address: torrado1@farm.ucm.es (J.J. Torrado).

0378-5173/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ijpharm.2013.02.038

it is not easy to characterize these delivery systems by conventional drug release tests. For this reason, one of the aims in this work is to propose the hemolysis studies at different times as an ex vivo tool to characterize not only the hemolytic toxicity but also the drug release. We have compared the hemolysis of novel AmB formulations to reference formulations such as Fungizone® and the liposomal formulation AmBisome®. The effect on hemolysis of microencapsulation, aggregation state, particle size and human RBCs concentration has also been explored.

Another parameter that is critical for intravenous formulations is their biodistribution. When particles are administered intravenously, they are quickly coated by the components of the circulation (Davis, 1997). This process renders the injected particles easily recognizable by the RES and the macrophages cells of the liver, spleen, lung and circulating macrophages will remove the opsonised drug from the circulation by phagocytosis (Blunk, 1996). Surface hydrophobicity of particles is directly correlated to the kinetics of plasma clearance by the RES (Berry, 2003). On the other hand, smaller particle size avoids RES elimination and allows for a longer circulation plasma half-life (Gref et al., 1994). The aggregation state, particle size and surface properties can be modified to target AmB to the tissues of interest and to enhance its activity while minimising its toxicity and increasing its therapeutic window (Espada et al., 2008a). In infective diseases such as leishmaniasis, the parasites are accumulated preferentially in the macrophages which are not easily accessible to AmB. In our case, we have developed a novel poly-aggregated formulation that can be used as a cost-effective alternative for the treatment of leishmaniasis which can enhance the AmB passive target by the uptake by the cells of the RES. In this work, we have studied its pharmacokinetic profile after intravenous administration and compared it to commercially available and albumin microparticulate formulations in order to explore the effect of formulation, dose administered, anesthesia and disease state on AmB pharmacokinetics.

2. Materials and methods

2.1. Preparation of AmB experimental formulations

AmB formulations were prepared as it is described by Espada et al. (Espada et al., 2008b). Free-Dimeric AmB formulation (FD) at 5 mg/ml was prepared as a colloidal dispersion similar to the marketed reference formulation Fungizone®. Briefly, 50 mg of AmB (supplied by Azelis, Spain) was dispersed in 5 ml of an aqueous solution formed by 41 mg of sodium deoxycholate (Fluka Chemie A.G., Buchs, Switzerland), 10 mg of dibasic sodium phosphate and 0.9 mg of monobasic sodium phosphate (Panreac S.A., Barcelona, Spain) previously adjusted to pH 12.0 with 2 N sodium hydroxide (Panreac S.A., Barcelona, Spain). Once the drug was homogeneously dispersed, it was acidified to pH 7.4 adding 2 N orthophosphoric acid (Panreac S.A., Barcelona, Spain). Water was added to the resulting mixture up to a final concentration of 5 mg/ml.

Free-Poly-aggregated AmB formulation (FP) was prepared as a suspension at 5 mg/ml. FP was prepared similarly to FD, but without adjusting the initial pH to 12. FP (FP cycle 0) was also centrifuged for six cycles (Hettich Universal 32 centrifuge). The five initial cycles were of 5 min at 700 × *g* and the last one was of 5 min at 4500 × *g*. FP cycle 2, 4 and 6 formulations were obtained from the supernatants collected after the 2nd, 4th, and 6th cycle.

Heated-Fungizone (HF) was obtained after heating Fungizone® (Bristol Myers Squibb, Madrid, Spain) for 1 h at 70 °C. AmBisome® was purchased from Gilead Sciences S.L., Madrid, Spain.

MP (microencapsulated poly-aggregated AmB) and MD (microencapsulated dimer AmB) were prepared from FP or FD respectively as it is described by Sanchez-Brunete et al. (2004). Briefly, each formulation (10 ml) was mixed with 4.49 ml of a 20% serum albumin solution (Instituto Grifols SA, Barcelona, Spain) under moderate stirring. The final mixture was spray-dried (inlet temperature, 125 °C; feed rate, 2.5 ml/min; 100% aspiration) using a Büchi B 191 (Flawil, Switzerland).

2.2. Determination of aggregation state and particle size

Formulations were reconstituted, if required, and diluted as necessary with deionized water. The resulting dilutions were scanned between 300–450 nm (Shimadzu UV-1700 spectrophotometer) as it is described by Sanchez-Brunete et al. (2004). Particle size was assayed by DLS with a Zetatrac Ultra (Microtrac Inc, USA). Mean size (nm) was determined based on size distribution in number. Five runs of 60 s per sample were carried out. Particle size of the microspheres was assayed by laser diffraction (Microtrac S3500, Microtrac Inc, USA) in the same conditions.

2.3. Electron microscopy characterization

The morphological examination of the formulations used for the pharmacokinetic studies (FP, MP and AmBisome®) was studied by electron microscopy. AmBisome® and FP were characterized by TEM (JEM 1010 JEOL) at an accelerating voltage of 80 kV. One drop of the unfiltered AmBisome® and FP formulations diluted to 1 mg/ml with distilled water was placed on Formvar©/Carbon Coated Grid (F196/100 3.05 mm, Mesh 300). Excess sample was filtered off with cellulose filter paper and negatively stained with 2% aqueous uranyl acetate for 20 s with excess filtered off as before. MP were characterized by SEM (JEOL JSM 6335F) at an accelerating voltage of 5.0 kV. A thin film of Au was sputtered onto the substrate prior to imaging.

2.4. Hemolysis test from human erytrocytes (RBCs)

Venous blood obtained from a healthy volunteer was treated by apheresis and RBCs stored at 6 ± 2 °C (Bloodbank of the Spanish Army Hospital Gomez Ulla, Madrid). Whole blood was centrifuged (10 min at 1,600 × *g*) and the supernatant and buffy coat were pipetted off and discarded. RBCs were then washed twice with isotonic PBS of pH 7.4 and were finely dispersed in PBS at different cell densities of 10^8, 2×10^8, 5×10^9, 7×10^9 and 10^{10} cells/ml. Subsequently, 2 ml of the RCBs suspension were mixed with 2 ml of buffer containing several AmB formulations in triplicate. The final AmB concentration was 200 μg/ml in all formulations except for those obtained from centrifugation of FP with 300 μg/ml. Each sample was then incubated at 37 °C in a shaking water bath (100 strokes/min). After 1, 4, 8 and 24 h incubation, hemolysis was stopped by reducing the temperature (0 °C) and unlyzed RBCs were removed by centrifugation (10 min at 2,900 × *g*). The supernatants were collected for analysis of the extent of hemolysis by reading the absorption of the hemoglobin at 575 nm. Results from triplicate experiments were expressed as a percentage of hemolysis with respect to the amount of hemoglobin released in the presence of water, which was taken as measure of complete (100%) lysis.

$$\text{Hemolysis}\ \ (\%) = \frac{100(\text{Abs}_s - \text{Abs}_b)}{\text{Abs}_1 - \text{Abs}_b} \quad (1)$$

where Abs_s is the absorbance of the sample, Abs_b is the average absorbance of the buffer, and Abs_1 is the average absorbance of the lysed samples. In our experimental conditions it was observed a loss of absorbance with time according to a first order process with a degradation constant of 0.043 h^{-1} which was used to correct the absorbances. Differences in hemolysis were studied statistically using a *t*-Student paired test (Excel, Office 2003) and ANOVA test (Statgraphics 5.1, StatPoint Technologies USA). Significance was set at a p value <0.05. The possible protection effect of albumin