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Note

Liposomal amphotericin B eye drops to treat fungal keratitis: Physico-chemical and formulation stability

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Abstract

Local fungal infections with *Candida*, *Fusarium*, *Curvularia* and *Aspergillus* can lead to serious ulceration of the cornea and must be treated rapidly. The current treatment consists of 0.15% (w/v) amphotericin B eye drops prepared from Fungizone®, containing deoxycholate, irritant for the cornea, which reduces patient compliance. Eye drops based on liposomal amphotericin B (AmBisome®) would be a convenient alternative; however, according to the manufacturer's instructions, AmBisome® can only be kept refrigerated for 1 week after reconstitution. A longer shelf-life at ambient temperature would be preferable for a preparation made in a hospital pharmacy and delivered to patients. Thus, the possibility of storing an ophthalmic preparation of 0.5% (w/v) liposomal amphotericin B after reconstitution was investigated. After 6 months at room temperature or at +2–8 °C, the hydrodynamic diameter measured by quasi-elastic light scattering remained constant at 108 ± 30 nm with a polydispersity index lower than 0.15. Amphotericin B content, checked by a validated HPLC method, was maintained between 94 and 107%. Amphotericin B and soy phosphatidylcholine proportions remained constant, indicating that the liposomes remained intact and retained the drug. These results show the feasibility of an ophthalmic preparation based on liposomal amphotericin B developed in hospital pharmacies.

Keywords: Liposomes; Amphotericin B; Fungal infections; Eye drops

Amphotericin B is considered the treatment of choice for systemic infections caused by *Candida*, *Aspergillus* and *Fusarium* and is also given intravenously for local infections such as ophthalmic ones. However, it has serious side-effects, particularly dose-limiting nephrotoxicity (Sabra and Branch, 1990; Deray, 2002). Less toxic lipid formulations of amphotericin B can be used in patients with renal diseases or unresponsive to conventional amphotericin B (Sharkey et al., 1996; Walsch et al., 1998). Thus, Goldblum et al. (2000) reported a case of contact-lens related keratitis due to *Fusarium solani* completely resolved under systemic amphotericin B lipid complex (Abelcet®) treatment, while topical and systemic non-liposomal amphotericin B were ineffective. However, in similar cases, the advantage of systemic liposomal or classical amphotericin B was not demonstrated (Goldblum et al., 2000; Schelenz and Goldsmith, 2003).

Topical amphotericin B (0.1–0.3%) is the standard treatment for ocular infections due to *Candida* and related fungi while natamycin (5%) is the usual treatment of filamentous fungi such as *Fusarium* (Thomas, 2003). The current formulation of amphotericin B eye drops (Fungizone®) contains deoxycholate, necessary to solubilise the highly hydrophobic amphotericin B (Cohen et al., 1996), which renders their instillation painful and leads to poor compliance and aggravation of symptoms, especially when direct intravitreal injection of amphotericin B deoxycholate is used to treat fungal endophtalmitis. This toxicity, in particular retinal damage and vitreal opacity, is reduced when lipid formulations, either "in-house" or commercially available such as AmBisome®, are used instead of amphotericin B deoxycholate in rabbit models (Barza et al., 1985; Tremblay et al., 1985; Cannon et al., 2003). Furthermore, the ocular bioavailability of some liposomal drugs has been proved to be equal to or better than that of non-liposomal ones (Kaur et al., 2004; Ebrahim et al., 2005), including amphotericin B bioavailability in the cornea of rabbits (Pleyer et al., 1995). Although amphotericin B levels were higher with classical amphotericin B 15 min

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after instillation, its levels were similar at later time points. In addition, amphotericin B concentrations within the eye were remarkably constant with the liposomal form, which would slow down the clearance of the drug from the eye and act as a reservoir for the molecule.

The general aim of this work was to develop a formulation that would be better tolerated in the eye, with higher amphotericin B content, without using an irritating surfactant. Therefore, we developed a liposomal amphotericin B eye drop formulation using the commercially available speciality AmBisome®.

The eye drops (0.5%, w/v, liposomal amphotericin B) were prepared in the Hôtel-Dieu hospital pharmacy in aseptic conditions from the commercial AmBisome® purchased from Gilead (Foster City, CA, USA), which consists of small unilamellar liposomes. The liposome composition in the original vial is as follows: 50 mg amphotericin B, 213 mg hydrogenated soy phosphatidylcholine, 84 mg distearoylphosphatidylglycerol, 52 mg cholesterol and 0.64 mg alpha-tocopherol. AmBisome® powder was reconstituted with 10 mL of sterile water and filtered through a 0.22 μm syringe filter.

Since patients with ocular infections require several months of treatment, the stability of both the amphotericin B content and the liposomal structure has to be taken into account. Economical and practical constraints in hospital pharmacies and the time necessary to produce the eye drops and dispatch them to patients necessitate an adequate shelf-life. For this reason, we conducted a stability study of several batches of 0.5% (w/v) liposomal amphotericin B packaged in 1 mL portions in glass vials closed with PVC droppers. Samples were stored in sealed tubes for periods up to 6 months (0, 7, 15, 30, 60, 90, 180 days) at both 2–8 °C and room temperature, in both cases in the dark to avoid photo-degradation of amphotericin B.

A previously described HPLC method (Alak et al., 1996; Eldem and Arican-Cellat, 2000) was used with some modifications as a reliable method of quantifying amphotericin B both within and outside liposomes. A stock solution of AmBisome® was prepared by adding 10 mL of sterile water to reach a final amphotericin B concentration of 5 mg/mL. Calibrations samples were prepared by first diluting this stock solution in a mixture of methanol/dimethylsulfoxide (DMSO) 50/50 (v/v) able to solubilise phospholipids and to release all amphotericin B from the liposomes (Alak et al., 1996; Eldem and Arican-Cellat, 2000). A second dilution was performed in the mobile phase: Na_2EDTA 0.02 M/acetonitrile 45/55 (v/v) at pH 5.0 run isocratically. The flow rate was 1 mL/min, detection was performed at 407 nm and the injection volume was 50 μL. At each day of validation, five samples of the same standard concentration were analyzed to calculate the relative standard deviation (R.S.D., $n = 5$) and thus were used for intra-day validation. Inter-day validation was studied over 6 days for each concentration by calculating the R.S.D. ($n = 11$). Accuracy was calculated as the percentage of the nominal concentration of each standard concentration. The limit of quantification was considered as the lowest concentration that could be measured with a R.S.D. lower than 20%.

This method showed good intra-day, inter-day precision and accuracy except for the lowest concentration (0.1 mg/L) with R.S.D. equal to 10.7 and 21.8% and a high inaccuracy (about 156% of the nominal concentration). This concentration was defined as the detection limit. The method proved to be accurate with average percentages ranging between 92.1 and 107.6%. The linearity was evaluated between 0.5 and 10 mg/L. The mean linear regression equations were $y = 304{,}806x\ (\pm 21{,}515) - 15{,}111\ (\pm 8836)$ with a mean correlation coefficient of 0.9997. The limit of quantification was 0.5 mg/L.

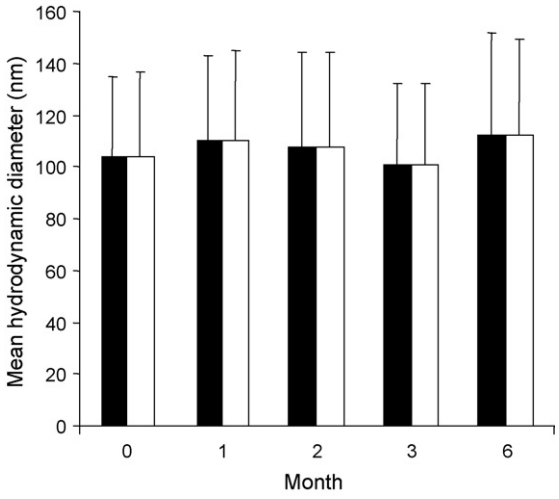

Fig. 1. Mean hydrodynamic diameter of liposomes with the eye drops, measured by quasi-elastic light scattering, after various periods of storage at +2–8 °C (black bars) or room temperature (white bars). Three measurements were made on the same sample. The error bars show the mean of the standard deviations of the distribution calculated by the instrument for each measurement.

Non-liposomal amphotericin B was analyzed daily to provide quality control. A stock solution was prepared in methanol (10 mg/mL) and diluted to 5 mg/mL using the same solvent. The mean concentration obtained was 5.2 ± 0.4 mg/L ($n = 7$) and the accuracy was 103.9 ± 8.7 mg/L ($n = 7$) with R.S.D. = 7.5%.

There was no significant difference between the retention time of the liposomal product and the non-liposomal amphotericin B used as control (about 13.4 min). The peak areas and shapes were similar.

In conclusion, the HPLC method, equally effective for the determination of both liposomal and non-liposomal amphotericin B, is rapid and simple and could be used routinely to control eye drop production.

The amphotericin B content was 5.4 ± 0.2 mg/mL, in agreement with the manufacturer's information. The pH (5.6) and osmolality (350 mosmol/kg) values of all the preparations were compatible with ocular administration. The mean hydrodynamic diameter of the vesicles measured by quasi-elastic light scattering with a Nanosizer N4 Plus (Beckman Coulter, Margency, France) was 107 ± 32 nm with a polydispersity index lower than 0.15, indicating a monodisperse population. The mean diameter of the liposomes (Fig. 1) and the polydispersity index (data not shown) remained unchanged over 6 months, whatever the storage conditions. The amphotericin B content was maintained between 94 and 107% of the initial amphotericin concentration. No precipitation or change in the macroscopic properties (colour, aspect) was observed. Microbiological tests have shown that all samples were sterile (data not shown).

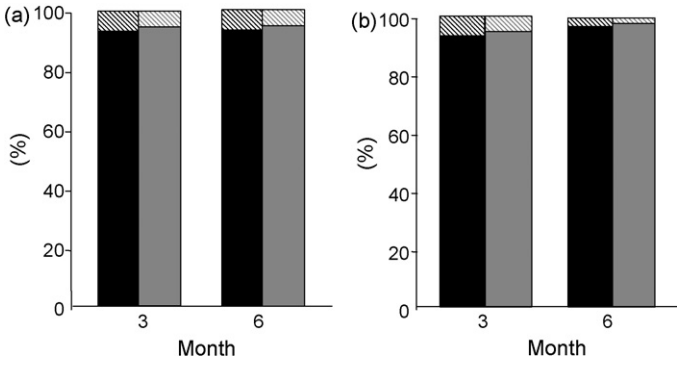

Fig. 2. Distribution of amphotericin B and soy phosphatidylcholine after ultracentrifugation of liposomes after 3 and 6 months of storage at +2–8 °C (a) or room temperature (b). The amounts of soy phosphatidylcholine (grey bars) and amphotericin B (black bars) in the pellet (solid part) and supernatant (cross-hatched part) are expressed as percentages of the total amount recovered.

It was also important to determine whether the amphotericin B in the formulation remained embedded within the liposomal bilayer, so that it would continue to show the improved bioavailability conferred by this formulation. The amount of recovered drug and soy phosphatidylcholine (SPC) was determined at days 90 and 180 after successive purification steps. Centrifugation was chosen as the technique to separate liposomal amphotericin B from other forms of the drug. Low-speed centrifugation (10 min at 9000 × *g* and 4 °C) after dilution of 350 μL of the formulation in 2.15 mL of water showed no precipitation of released amphotericin B within the preparation. Ultracentrifugation (17 h at 150,000 × *g* and 4 °C using a Beckman L7-55, rotor 70.1.TI, Palo Alto, USA) was preferred to other methods (dialysis, ultrafiltration, gel exclusion chromatography) to separate the small unilamellar vesicles from any dissolved amphotericin B in the continuous phase, since the concentration of free drug would be expected to be very low and hydrophobic molecules are known to adsorb to membranes and chromatographic supports. Supernatants were collected and the pellets were resuspended in water to obtain approximately 1.9 g of final suspension. For each step, the samples were weighed to calculate exact dilutions. Amphotericin B was analyzed by HPLC as described above. As SPC represents the main lipid in the formulation, we quantified this compound by an enzymatic method sensitive to phospatidylcholine (Phospholipides enzymatiques PAP 150, Biomerieux, France) (Takayama et al., 1977) in the supernatant and pellet as a marker of the liposomes. Light scattering analysis of undiluted supernatant revealed particles which did not sediment under these ultracentrifugation conditions, with a hydrodynamic diameter of around 70 nm. However, only a small proportion (inferior to 6%) of amphotericin B was detected in these supernatants and was correlated with similar SPC proportions (inferior to 7%, Fig. 2). In contrast, more than 93% of both amphotericin B and SPC was recovered in the pellets whatever the storage temperature (2–8 °C and room temperature) and the time (3 and 6 months) (Fig. 2). These results confirm that the integrity of amphotericin B-loaded liposomes was preserved for at least 6 months even at room temperature. This analysis revealed that the portion found in the supernatants represented less than 7% of the total formulation. The fact that the same percentages of the total recovered amphotericin B and soy phosphatidylcholine were found in the pellet and the supernatant strongly suggests that the liposomes retained their amphotericin B content throughout the 6 months of the study.

This study show the feasibility of an ophthalmic preparation based on liposomal amphotericin B developed in hospital pharmacies. As well as its stability even when stored at room temperature, which would facilitate its use by patients, this formulation contains three times as much amphotericin B (0.5%) as Fungizone® (0.15%). It would be expected to have reduced ocular toxicity, as already shown for intravitreal injection in rabbits and rhesus monkeys (Barza et al., 1985; Tremblay et al., 1985; Cannon et al., 2003), due to the localization of the drug inside the phospholipid bilayer limiting contact with epithelial cells, and the absence of deoxycholate. Thus, a combination of reduced toxicity, a longer persistence at the site of action and a higher amphotericin B concentration would considerably increase the therapeutic index for the antifungal in the treatment of fungal keratitis.

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