Efficacy Evaluation of a Novel Submicron Miconazole Emulsion in a Murine Cryptococcosis Model

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Submicron emulsions of miconazole were stabilized by using a combination of three emulsifiers comprising phospholipids, poloxamer, and deoxycholic acid (DCA). The presence of DCA was vital for prolonged emulsion stability owing to its contribution to the elevated zeta potential of the emulsion. Further, the results by the phospholipid surface labelling colorimetric technique clearly suggested that poloxamer molecules interacted with phospholipid polar-head groups of the mixed DCA-phospholipid interfacial film, resulting in the stabilization of the emulsion by a steric enthalpic entropic mechanism. The plain emulsion vehicle was well tolerated up to a dose of 0.6 ml injected i.v. to BALB/c mice. The maximum tolerated dose of miconazole was 80 and 250 mg/kg in Daktarin® i.v. (a marketed product) and emulsion, respectively, showing an improved safety ratio of 1 to 3 in favor of the emulsion. These results tended to confirm that the adverse effects associated with Daktarin® i.v. injection should be associated with the vehicle rather than with the miconazole itself. In a murine cryptococcosis model, only one mouse out of ten remained alive by day 15 in the infected group treated with Daktarin® i.v., while in the miconazole emulsion treated group, mice began to die from day 16 up to day 25 post inoculation. Thus, the multiple-dose treatment with the miconazole emulsions improved the protection offered to the infected mice. However, the therapeutic levels of miconazole that were reached in the target organ (brain) were lower than those required for complete eradication of *Cryptococcus neoformans*, which is known to multiply preferentially in the brain.

KEY WORDS: submicron; emulsion; miconazole; stability; toxicity; cryptococcosis.

INTRODUCTION

Miconazole is a synthetic imidazole with a broad spectrum of antifungal activity (1,2). It is poorly absorbed by the oral route of administration and is used primarily as a topical agent for cutaneous mycoses and as an alternative systemic antifungal agent when amphotericin B is either ineffective or contraindicated (3,4).

The clinical use of systemic antifungal agents is continually growing as a result of the increasing incidence of life-threatening fungal infections, particularly in immunocompromised hosts such as cancer patients (5), patients after transplantation (6), and HIV-infected patients (7). According to a 1980 review (4), the therapeutic use of miconazole yielded encouraging results. However, due to the toxicity of miconazole injections, attributed to pharmaceutical excipients rather than to the drug molecule itself, the place of this imidazole in the therapy of fungal disease remains uncertain since its role has never been defined properly (8).

The marketed formulations, Monistat® i.v. or Daktarin® i.v. (Janssen Pharmaceutical Inc., Titusville, NJ) contain Cremophor EL®, a polyethoxylated castor oil surfactant needed for micellar solubilization of this lipophilic drug. The parenteral use of miconazole is frequently associated with severe side effects, mainly anaphylaxis, phlebitis, and thrombocytosis, which are believed to be related to the Cremophor EL® (9). Anaphylactoid reactions were also reported in the literature following i.v. injection of other products containing Cremophor El® as a solubilizing agent (10,11).

The main objective of this study was, therefore, to develop and evaluate an alternative miconazole formulation. This study includes a description of a miconazole emulsion formulation and a comparative evaluation of safety and efficacy of the emulsion and Daktarin® i.v. in BALB/c mice.

MATERIALS AND METHODS

Materials

Miconazole base was kindly supplied by Janssen Pharmaceutical, Belgium, via Abic Ltd., Netanya, Israel. Lecithin used was intravenous grade (Lipoid E-80 from Lipoid KG, Ludwigshafen, Germany, containing according to manufacturer specifications, 81.5% phosphatidylcholine, 8.5% phosphatidylethanolamine, 2.3% lysophosphatidylcholine, and 2.7% sphingomyelin). Deoxycholic acid (DCA) was purchased from Sigma Chemical Co. (St. Louis, MO). Medium-chain triglycerides (MCT) of fatty acids ranging from eight to ten carbons (minimum 95% purity) were obtained from the Societe des Oleagineux, Saint Blangy, France. Poloxamer (Pluronic F-68) was furnished by BASF (Ludwigshafen, Germany) and conformed with USP specifications. All other ingredients used were of pharmaceutical grade.

Emulsion Preparation

The submicron emulsion of miconazole was prepared using the manufacturing process previously described for the preparation of a submicron emulsion of diazepam (12,13), with few modifications. The phospholipids were first dissolved in alcohol and immediately dispersed in the aqueous phase. Alcohol was removed by evaporation prior to mixing the aqueous phase with the oil phase in which DCA and miconazole were previously dissolved. The emulsion was then prepared by emulsifying the oil phase with the aqueous phase according to the procedure already reported. The formulation (%, w/w) consisted of miconazole 1.0, MCT 20.0, fractionated egg yolk phospholipids 1.0, DCA 0.5, glycerin 2.25, α-tocopherol 0.02, and water for injection to 100 g. The pH was adjusted to 7.4 with 1N NaOH. The emulsions were

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packed in 10-ml polybutylene vials in a nitrogen atmosphere and sterilized by a standard autoclaving cycle (steam sterilization at 121.1°C over 15 min).

Emulsion Property Evaluations

1. Particle size evaluation

The droplet size distribution of the emulsions was determined by photon correlation spectroscopy (14), which is considered the most appropriate method for studying the droplet size below 1 μm (Malvern System 4700, Malvern, England)

2. Zeta potential

The zeta potential of the charge on emulsion droplets was determined using the moving boundary electrophoresis technique, as previously described (12). Briefly, an emulsion sample diluted with water (1:10) was eluted, under an appropriate potential gradient, into an aqueous electrolyte solution which consisted of 1% glycerol, 0.5% poloxamer and NaOH to adjust the pH to 7.4. Zeta potential value was calculated from the electrophoretic mobility using the Helmholtz–Smoluchowski equation.

3. Sterility and test evaluations

The sterility of the emulsions was assessed using a technique based on the Bactec 46 apparatus (Johnson Laboratories, Towson, MD) (15). One milliliter of the emulsion was placed into aerobic and anaerobic radioactive soybean-casein digested broth in blood culture bottles 6B and 7D, respectively, which were incubated at 37°C for one week.

4. Miconazole quantitative analysis

Miconazole content was analyzed by using an HPLC system consisting of a Milton Roy HPLC (Model Constametric 3000, Riviera Beach, FL) equipped with a variable wavelength ultraviolet detector (Spectro Monitor 3100, Milton Roy) and a Milton Roy Integrator. A Lichrosphere C-18, 5 μm (Merck Co., Darmstadt, Germany), 25 cm × 4.6 mm I.D. reverse-phase column, was used. The column was eluted with a solvent system containing methanol-aqueous solution (9:1, v/v). The aqueous solution was comprised of 0.05 M $NH_4H_2PO_4$. The eluent was run at a rate of 1.5 ml/min and monitored at 230 nm following injected volumes of 20 μl of miconazole standard solutions and samples. The calibration curve was found to be linear in the range of 1 to 40 μg/ml. In order to determine the total content of miconazole in the emulsion, the emulsion was first dissolved in isopropyl alcohol (1:10), and the resulting solution was then diluted with methanol (1:10). Each sample was analyzed in triplicate.

5. Determination of phosphatidylethanolamine exposed to TNBS labelling

The extent of exposure of the aminophospholipids (APL) at the emulsified oil droplet interface was determined by chemical labelling of the APL with 2,4,6-trinitrobenzene sulfonic acid (TNBS, Sigma, St. Louis, MO), a membrane-impermeable hydrophilic reagent (16). It should be noted that phosphatidylethanolamine contributes more than 95% of the total egg APL.

In the case of bilayer vesicles, the mole percent of exposed APL should reflect the lipid mass distribution between the various vesicle leaflets as a function of vesicle type and size (16). For large unilammelar bilayer vesicles, the ratio of intact to total labelled bilayer vesicle should approach the minimal value of 0.50, indicating that half of the APL molecules are oriented towards the internal aqueous core where no contact with TNBS could occur.

In the emulsion not contaminated by liposomes, all the APL reacting groups are exposed towards the external aqueous phase (monolayer formation). Hence, the ratio of total to intact labelled emulsion should be equal to unity (17).

The exposure extent of APL is estimated from the mole percent of APL reacting with TNBS resulting in the trinitrophenyl-APL (TNP-APL) compound. The calculation is based on the following equation:

$$\% \text{ Exposure} = \frac{\text{TNP -APL in intact emulsion} \times 100}{\text{Total TNP-APL in dissolved emulsion}}$$

The intact emulsion and total dissolved emulsion were subjected separately to TNBS labelling as described below.

a. Intact emulsion. Twenty microliters of the emulsion was diluted with 125 ml water and 0.8 ml of 0.8N $NaHCO_3$. To this solution, 80 μl of 5% TNBS was added, the solution was mixed and allowed to incubate in the dark for 1h. After the incubation, 0.5 ml water, 1.5 ml chloroform, and 1.5 ml methanol were added to the sample, which was then mixed and centrifuged. All centrifugations were carried out at 2000 × g for 5 min at room temperature. The upper, aqueous phase was removed, and 1.5 ml of the synthetic upper phase composed of chloroform: methanol: water (6:96:98, v/v) was then added to the chloroformic phase. The upper, aqueous methanolic phase was separated from the organic phase following centrifugation.

The choloroformic lower phase was then washed several times with the upper synthetic phase until unreacted TNBS was completely removed, as indicated by the disappearance of the yellow color in the upper aqueous phase.

b. Total emulsion labelling. Twenty microliters of emulsion was solubilized in 1 ml of isopropanol: heptane: 0.8 M $NaHCO_3$ (40:10:1, v/v). Then, 120 μl of 0.8 N $NaHCO_3$ and 80 μl of 5% TNBS were added, and the solution was mixed and allowed to incubate in the dark for 1h. After the incubation, 0.5 ml heptane and 0.5 ml water were added, followed by mixing and centrifugation. The upper, heptanic phase was removed, and 1.5 ml chloroform, 0.8 ml water, and 0.7 ml isopropanol were then added, followed by mixing and centrifugation. Two phases were formed. The aqueous, upper phase was again discarded and the chloroformic, lower phase was washed several times with the synthetic upper phase composed of chloroform: methanol: water (6:96:98, v/v) until removal of unreacted TNBS as described above.

The final washed chloroformic phases of the intact and total dissolved emulsion containing the TNP-APL were quantified spectrophotometrically at 410 nm against a calibration curve. This approach has already been validated in

the case of liposome and lipid mixture studies (16-18) and was recently applied successfully to the study of an injectable submicron emulsion formulation (19).

It should be noted that APL-TNBS reactions were not affected by the presence of the various emulsion excipients, since the same O.D. was obtained in the totally dissolved emulsion and ethanol standard solution when the same range of APL concentrations was used.

Four concentrations of phospholipids, ranging from 5 to 20 μg/ml (the concentration of the emulsion can range from 10 to 17 μg/ml) in an alcoholic solution, were used for calibration purposes. Various calibration curves were constructed and found linear over the range of phospholipid concentrations tested (r^2 ranging from 0.99 to 0.995, with maximum linear slope deviation of ±5%).

Animal Studies

Acute Toxicity Study

Single dose. Male albino BALB/c mice weighing 20 g were injected through the tail vein with various doses of miconazole either as Daktarin® i.v. or as various emulsion formulations. Each miconazole dosage form was administered i.v. by single-bolus injections (varying in volume from 0.04 to 0.2 ml) to groups of 10 mice. Since this is a novel emulsion formulation, the toxicity of the plain emulsion was tested by i.v. injection of various doses to groups of 10 mice. Survival was followed up to 30 days.

Multiple dose. The safety of 5 consecutive daily doses of miconazole was examined by injection into the tail vein of mice (groups of 10), either as Daktarin® i.v., or as an emulsion (10, 20, 40, 60, 80, 100 mg/kg, daily). The plain emulsion (0.1–0.2 ml) was also injected over 5 consecutive days in a control group of 10 mice. This study was needed as a control for the efficacy study of the multiple-dose treatment.

Systemic Murine Cryptococcosis

In all the experiments, 3×10^5–3×10^6 cells of *Cryptococcus neoformans* B-3501 (serotype D), from a 48-hr culture on Sabouraud dextrose agar (SDA) at 30°C, was injected through the tail vein of male albino BALB/c mice (20 ± 3 g) by a single bolus of 0.2 ml PBS suspension. With these inocula, systemic infections were regularly produced in mice causing total killing within 10–20 days. The appropriate inoculum for each experiment was experimentally determined prior to each efficacy experiment. Yeast concentration was determined by hematocytometer count. Viable count was measured as colony forming units (CFU) on SDA after 2 days of incubation at 30°C. In some experiments, in addition to the mortality, multiplication in the brain, as measured by CFU of homogenized organs, was also monitored.

Efficacy Evaluation of Miconazole Dosage Forms in Systemic Murine Cryptococcosis

Mice infected with 3×10^5 cells (CFU) of *Cr. neoformans* B-3501 (serotype D) as described above, were treated with either Daktarin® i.v. or miconazole emulsion at an identical dose of 40 mg/kg. Ten infected mice were used for each treatment. Each group was maintained in a separate cage. Treatment began 48 hr after the infection by injection of a single bolus (0.08 ml) of the miconazole dosage forms for five consecutive days (40 mg/kg). A control group of 10 infected mice treated with saline instead of miconazole was included. The number of surviving animals in each group was recorded daily over a period of 30 days.

Statistical Analysis

The results of the survival data were analyzed using the Kolmogorov–Smirnov goodness of fit procedure (20), originally developed to test differences in distributions of two samples of continuous observations. In the present case, the statistical comparison was carried out first between each group vs. control and then between miconazole-treated animal groups in an attempt to identify a significant difference between the two survival curves obtained with the miconazole emulsion and Daktarin® i.v. treated animal groups.

RESULTS

Formulation Design

The concentration of miconazole in the marketed product Daktarin® i.v. is 10 mg/ml.. To reach the desired identical drug concentration in the emulsion, containing 20% oil phase, a solubility of at least 50 mg/ml should be reached in the appropriate oil solvent. Such a concentration was attained with MCT which was able to dissolve up to 70 mg/ml. Therefore, MCT was selected for the emulsion oil phase.

In an attempt to predict the potential behavior of miconazole in the emulsion in vivo, the *n*-octanol : water and MCT water partition coefficients were determined by dissolving a desired amount of miconazole in 0.5 ml *n*-octanol and MCT respectively. Ten milliliters of water was added and the biphasic systems were gently shaken at 37° C over 48 hr. The concentration of miconazole in the various phases was determined using the HPLC method previously described. The K_p value was 1.1×10^6 and 3.2×10^5 for *n*-octanol:water and MCT:water respectively.

The combination of the emulsifiers was based on the results of a previous study on an amphotericin B emulsion which was stabilized using phospholipids, poloxamer, and sodium deoxycholate (21). The appropriate actual combination consisted of phospholipids, poloxamer, a nonionic emulsifier, and DCA as an anionic emulsifier, the presence of which was vital to prolonged emulsion stability owing to its contribution to the elevated zeta potential of the emulsion.

Physical Emulsion Evaluation

The well-established combined technique for sizing to the submicron range, utilizing a high-shear mixer for emulsification, followed by a two-stage high-pressure homogenizer, yielded fine emulsions with a mean droplet size of approximately 125 nm (Table I). Zeta potential values were -41 ± 6.5 mV (Table I) and the content of miconazole was 10 mg/ml, as confirmed by HPLC.

The incorporation of miconazole in the emulsion did not alter the physical stability over a 17-month storage period at 4°C, as indicated in Table I. No change was noted either in mean droplet size or in drug content. However, a moderate

Table I. Stability follow-up of the miconazole emulsion

Emulsion properties*	t_0	17 months at 4° C
Zeta potential, mV	−41 ± 6.5	−49.4 ± 6.9
Mean droplet size, nm	125 ± 35	137 ± 46
Drug content, % of original	100.0	97.8 ± 1.5

* The mean droplet size and zeta potential values represent the mean ± SD of three different batches manufactured, stored and analyzed under identical experimental conditions.

increase in zeta potential value was noted. Various inter- and intra-formulation modifications were performed on the emulsion; ten different formulations were prepared and evaluated (data not shown). The formulation chosen was based on the results of the physicochemical and stability evaluation over the first 6 months of storage.

Phosphatidylethanolamine Surface Labelling

The percent exposure of phosphatidylethanolamine in miconazole emulsion prepared according to the manufacturing procedure previously described, but without poloxamer, was 98%, whereas the percent exposure of phosphatidylethanolamine in the miconazole decreased with increasing poloxamer concentration, as depicted in Fig. 1.

Animal Studies

Acute Toxicity

Single dose. The acute toxicity results (Table II) were obtained from at least two independent single-dose experiments: Daktarin® i.v. at a concentration of 100 mg/kg caused immediate death, whereas no deaths occurred at 80 mg/kg. However, at a concentration of 50 mg/kg, severe adverse CNS effects such as tremors and convulsions were observed immediately after the i.v. injection. These effects lasted at least 1 hr.

The miconazole emulsion was tolerated at higher doses of up to 200 mg/kg, and no side effects were noticed with these doses. A dose of 250 mg/kg caused convulsions but was not lethal, while a dose of 300 mg/kg caused immediate death.

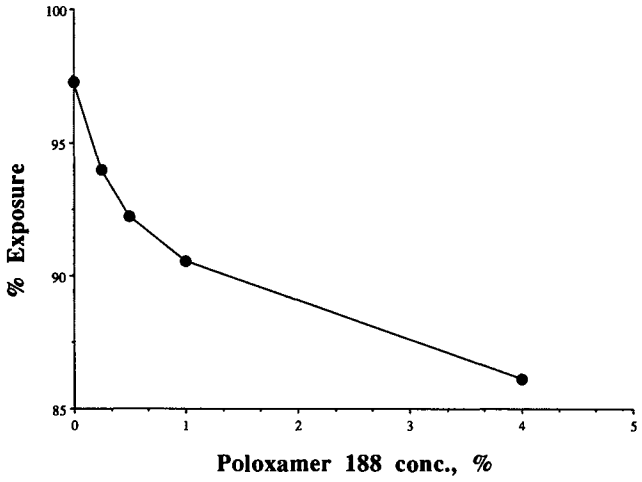

Fig. 1. Effect of poloxamer concentration on the extent of surface exposure of aminophospholipids in the miconazole emulsion.

Table II. Acute toxicity of emulsion vehicles and miconazole dosage forms in single-dose experiments

Treatment[a] (0.1 or 0.2 ml i.v.)	Tremor, severe CNS adverse effects[b]	% Survival after 30 days
PBS	-	100
Emulsion control		
0.1 ml	-	100
0.2 ml	-	100
0.6 ml[c]	-	100
0.8 ml[c]	-	death within 1h
Daktarin® i.v.		
20 mg/kg	-	100
50 mg/kg	+ +	100
80 mg/kg	+ + +	100
100 mg/kg		immediate death
Miconazole emulsion		
50 mg/kg	-	100
100 mg/kg	-	100
150 mg/kg	-	100
200mg/kg[c]	-	100
250 mg/ kg[c]	+ +	100
300 mg/kg[c]		immediate death

[a] A single bolus of the drug (0.1 or 0.2 ml) was injected i.v. to a group of 10 BALB/c mice.
[b] The effects lasted more than one hour after injection.
[c] When the volume of one single injection exceeded 0.2 ml, appropriate consecutive injections of either 0.1 or 0.2 ml at 10 min intervals were performed.

Multiple doses. No death was observed over a period of 30 days in mice administered with 5 consecutive daily doses of 100 mg/kg of the miconazole emulsion (Table III), while a dose of 70 mg/kg of the marketed drug, Daktarin® i.v. was lethal. Therefore, it was decided to proceed in the efficacy experiments with the relatively well-tolerated dose of Daktarin® i.v. (40 mg/kg) and an equidose of the miconazole emulsion. Any death in the infected mice should be attributed to the lack of treatment efficacy rather than toxicity due to multiple dose administration.

Efficacy Evaluation

The death rate and median survival time (the time required for death of 50% of the infected mice) were related to inoculum concentrations ranging from 1×10^4 to 1×10^7 yeast cells per mouse. Two different experiments were carried out using at least one identical inoculum of 3×10^5 yeast cells per mouse. The results of the survival curves (data not shown) showed that all 10 untreated mice died from the injection 10–18 and 8–14 days after the i.v. injection of an inoculum of 3×10^5 and 1×10^6 yeasts, respectively.

The results of a representative efficacy experiment of Daktarin® i.v. as compared to a miconazole emulsion at an equal dose of 40 mg/kg following an infection with an inoculum of 3×10^5 yeast are depicted in Fig. 2.

Treatment was initiated two days after inoculum. It was found that by this time colonization of internal target organs

Table III. Toxicity of miconazole emulsion and Daktarin® i.v. in a multiple-dose experiment[a]

Group	% Survival (30 days)	Comments
Emulsion controls		--
0.1 ml	100	--
0.2 ml	100	--
Daktarin® i.v.	(10 mg/ml)	--
10 mg/kg	100	--
20 mg/kg	100	--
40 mg/kg	100	--
60 mg/kg	100	--
70 mg/kg	0	--
Miconazole emulsion (10 mg/ml)		--
20 mg/kg	100	--
40 mg/kg	100	--
50 mg/kg	100	--
60 mg/kg	100	--
70 mg/kg	100	--
80 mg/kg	100	--
90 mg/Kg	100	--
100 mg/Kg	100	Neurotoxic shock[b]

[a] Five consecutive daily doses of the drug (0.1 ml) were injected i.v. to a group of 10 BALB/c mice.

[b] At this dose, neurotoxic shock was observed with the 25 mg/ml emulsion but not with the 10 mg/ml emulsion.

such as the brain had already taken place (Table IV). All the infected untreated control animals died 15 days after the injection of *Cr. neoformans*, confirming the results of the previous calibration experiments which had been carried out to identify the appropriate inoculum. All of the infected mice treated with Daktarin® i.v. died from the injection 13–16 days after the injection, while a prolonged survival rate was observed with the miconazole emulsion (deaths occurring from 16 to 25 days) (Fig. 2). The survival curve of the mice treated with the miconazole emulsion was significantly different from both the control and Daktarin® i.v. survival curves ($p < 0.01$). No statistically significant difference was observed between the control survival curve and the survival curve of the mice treated with Daktarin® i.v. (Fig. 2).

In the brains of all animals tested, viable *Cr. neoformans* were found 10 and 17 days after inoculation, indicating

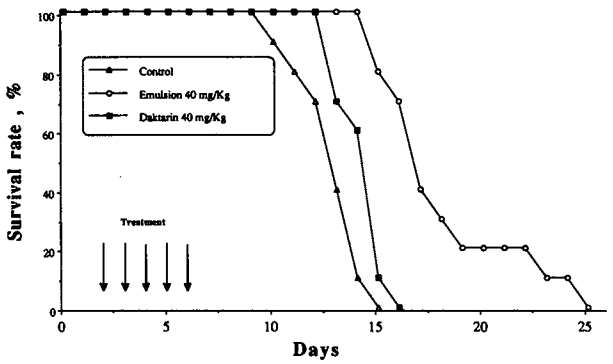

Fig. 2. Efficacy of miconazole dosage form in murine cryptococcosis. Ten mice were infected with 5×10^5 yeast cells (CFU) per mouse. Antifungal treatment was initiated 48 hr after infection by 5 consecutive daily i.v. injections.

Table IV. Replication of *Cr. neoformans* in brain during treatment

Days of infection	CFU/g brain 2	10	17
Controls	3×10^4	1×10^7	4×10^6
	3×10^4	7×10^6	1×10^7
Emulsion (control)		7×10^6	1×10^6
		1×10^6	2×10^7
Miconazole 40 mg/kg		1×10^7	2×10^7
		1×10^7	1×10^7
Miconazole 60 mg/kg		7×10^6	1×10^7
		9×10^6	8×10^6

the persistence of the infection in spite of the drug treatment, irrespective of the dosage form used. It was deduced that all the animals in these groups died from infection and not from a toxic reaction.

DISCUSSION

Formulation Design

The presence of DCA together with the combination of phospholipids and poloxamer led to stabilization of the submicron formulation since oil phase separation was noted only in the emulsion formulations prepared without DCA (data not shown). It should be emphasized that the physical stability of submicron emulsions may be reduced by the incorporation of drugs in the formulation as a result of surfactant solubility alteration in the oil phase, resulting in an impoverishment of surfactant molecules at the oil-water interface (22). This was again confirmed in the present study where the combination of phospholipids and poloxamer as a co-emulgator complex in the emulsion was unable to prevent oil separation and emulsion breaking following incorporation of miconazole. Stability was markedly improved (Table I) only after the addition of a third surfactant, DCA, to the emulsion.

Un-ionized free cholic acids are known to be poor surface active agents. Nevertheless, it was preferred to prepare emulsions with DCA instead of sodium deoxycholate as a result of better localization of the free acid in the interface of the oil/water emulsion owing to its high lipid solubility. The pKa of DCA is 6.5. Thus adjusting the final pH of the emulsion to 7.4 resulted in conversion of DCA molecules from the un-ionized to the ionized state in the oil/water interface of the emulsions without being excluded from the surface regions of the oil droplets. This is also supported by the increase in negative zeta potential values with increasing concentration of DCA in the emulsion, while all other parameters, especially the anionic components of the phospholipid mixtures, were kept constant (data not shown). Furthermore, identical experiments were carried out with sodium deoxycholate. The concentration of the corresponding salt needed to ensure maximum emulsion stability was twice that of DCA, owing probably to the poor localization of the ionized form at the oil/water interface of the emulsion.

Phosphatidylethanolamine Surface Labelling

The TNBS results reflect the actual "surface exposure" of APL since the reagent is membrane impermeable and can-

not penetrate the oil droplet or any lipid structure, as has already been shown (16–19). Therefore, any interaction between APL and TNBS should occur in the outer interface, towards the water phase or in the bulk aqueous phase where some individual APL molecules could be solubilized. We made several repeated studies of standard solutions, and of intact and totally dissolved emulsions.

The high percentage (98%) of APL exposure (Fig. 1), as determined by TNBS-APL, revealed the lack of phospholipid liquid crystal or large multilamellar bilayer formation in the emulsion without poloxamer. That large multilamellar liposomes are absent in the emulsion is further supported by results obtained from independent TEM freeze-fracturing experiments, which involved a similar amphotericin B emulsion formulation (21). It should be emphasized that the possible occurrence of small unilamellar liposomes at low concentration cannot be ruled out by the techniques used. Increasing the poloxamer concentration to 4% resulted in an increased coverage of the aminophospholipid which reacted to a lesser extent with TNBS. The gradual decrease in surface exposure from 98% to 86% (Fig. 1) is highly significant and does not lie in the range of experimental error (±5%). It should be mentioned that the minimal surface exposure volume that can be reached is 50% in the case of total unilamellar liposome formation. Since the phospholipid concentration was kept constant, these results indicate that poloxamer molecules interacted with phospholipid polar-head groups which were oriented towards the aqueous phase at the oil/water emulsion interface, confirming the molecular arrangement model of film-forming surfactants previously suggested by Levy *et al.* (23) when investigating a combination of emulsifiers comprising phospholipids, poloxamer, and oleic acid (23). The model involved partial penetration of the poloxamer hydrophobic moieties into the oleic acid phospholipid film, while the hydrophilic components of poloxamer were immersed in the aqueous subphase interacting with the hydrophilic moieties of phospholipids and oleic acid. A similar model can be visualized in the present case with deoxycholic acid instead of oleic acid.

Such an arrangement can stabilize an emulsion by a steric enthalpic-entropic mechanism where the hydrophobic polyoxypropylene groups of the poloxamer molecules act as anchoring moieties and the hydrophilic polyoxyethylene segments of poloxamer molecules provide stability in the surrounding aqueous medium by a repulsion effect (24). The overall chemo-physical results presented in this study provided evidence that a monolayer co-emulgator interfacial film was formed at the oil/water interface of the emulsion. This film efficiently prevented coalescence of adjacent droplets upon collisions through a combined stabilizing mechanism. This involves steric stabilization accompanied by an electrostatic repulsive surface charge resulting from molecular interactions of the various film-forming components (phospholipids, DCA, and poloxamer).

Animal Studies

Toxicity

The present, modified, submicron emulsion formulation contains a high content of DCA. Therefore, the toxicity of the emulsion vehicle was addressed. It should be mentioned that in a previous study, the toxicity of a similar emulsion vehicle prepared with sodium deoxycholate was investigated (21). In the present study, it was preferred to incorporate DCA, which is oil soluble, to allow a better localization of this surface active agent at the oil/water interface of the dispersed oil droplets in the aqueous medium.

The plain emulsion was well tolerated at the doses of 0.1 and 0.2 ml (25 and 50 mg/kg DCA and corresponding sodium salt, respectively), using either one single dose, or five consecutive daily doses. The deaths observed after the cumulative injections of 0.8 ml of emulsion vehicle (Table II) could be attributed to the hemodynamic changes or to the presence of the cholate surfactants. Considering that the entire plasma volume of a 20-g mouse is estimated to be less than 1 ml, the cumulative volume of the plain emulsion injected (0.8 ml), which almost equals the plasma volume, may induce severe toxic effects, probably due to changes in hemodynamics, resulting in death. In previous toxicity studies of fat emulsions it was noted that small animals, such as rats, were much more susceptible than dogs to the same dose injected, indicating that lipid emulsion toxicity probably depends on the ratio of lipid injected to plasma volume (26). Recently, it has been reported that the LD_{50} of sodium deoxycholate injected i.v. is 150 mg/kg in normal mice (25). This LD_{50} is smaller than the maximum concentration of sodium deoxycholate injected in the present study (200 mg/kg, corresponding to the cumulative injections of 0.8 ml of emulsion vehicle).

The emulsion formulations and Daktarin® i.v. were injected into groups of ten mice in doses of 20–300 mg/kg in two independent studies (Table II). It was noted that the maximum tolerated dose of Daktarin® i.v. (80 mg/kg) remained unchanged, indicating that the results were reproducible. Furthermore, the maximum tolerated dose of the miconazole emulsions was again 250 mg/kg, maintaining the initial improved safety ratio of 1 to 3.

It has already been reported that the *in vitro* release of miconazole from the submicron emulsion is rapid and similar to that of the micellar solution, Daktarin® i.v. under perfect sink conditions (27). Therefore, the improvement in this safety ratio should be attributed to the change in the dosage form (i.e., replacement of the micellar form by an o/w submicron emulsion). These results reveal that miconazole release from the emulsion is governed by a partition process of miconazole in favor of plasma. Furthermore, based on pharmacokinetic data reported by Kakutani *et al.* (28), only in drugs which exhibit an n-octanol to water partition coefficient higher than 10^8, will the carrier modify the pharmacokinetic profile by increasing the area under the curve. The miconazole n-octanol to water partition coefficient was found to be much smaller than the above-mentioned value, indicating that the incorporation of miconazole in the emulsion should not alter the pharmacokinetic profile. Thus, it is expected that the drug will be released rapidly from the carrier upon i.v. injection, due to the large dilution, as was found *in vitro* in the cited study. Therefore, the immediate death observed upon cumulative injection of 300 mg/kg miconazole emulsion should be attributed to the intrinsic toxicity of the drug molecule and not to the emulsion excipient vehicle, the safety of which was previously assessed, since

the same volume (3 × 0.2 ml) did not induce any deaths (Table II). The actual results confirmed the previous hypothesis that the adverse effect associated with Daktarin® i.v. injection should be associated with the vehicle rather than with the miconazole itself.

Efficacy

Cr. neoformans is a neurotropic pathogenic yeast-like fungus which preferentialy multiplies in the brain. Therefore, its multiplication was only tested in this organ (Table 4). Systemic drug treatment of this life-threatening fungal infection is practically ineffective as a result of the low permeability of the blood-brain barrier to current systemic antifungal drugs. Unless the drug is lipophilic, this barrier prevents any drug from diffusing into the brain, reaching therapeutic concentrations, as in the case of miconazole. Thus, it was anticipated that incorporation of miconazole in a negatively-charged submicron lipid emulsion might promote the passage of miconazole through the blood-brain barrier, resulting in increasing drug concentration in the brain. Such a plausible effect may lead to new potential therapeutic applications of a miconazole emulsion.

The multiple-dose treatment with the miconazole emulsion improved the protection offered to the infected mice. All treated mice survived up to 15 days, while only one mouse remained alive by day 15 in the infected group treated with Daktarin® i.v. and no survivor remained in the control group (Fig. 2). In the miconazole emulsion-treated group, mice began to die from day 16 up to day 25 post-inoculation. Our data suggest that the brain was more accessible to miconazole from the emulsion than from the micellar solution but the emulsion dosage form of miconazole at 40 mg/kg was unable to eradicate the yeast cells from the brain of the treated mice (Fig. 2, Table IV).

It was therefore decided to inject multiple doses much above 40 mg/kg of miconazole (i.e., 60 mg/kg) which is the largest tolerated dose of miconazole in the Daktarin®-tested group (Table III). Increasing the dose from 40 to 60 mg/kg, both the micellar solution and in the lipid emulsion, did not improve the survival rate of the infected mice in the murine cryptococcosis model, as confirmed by the multiplication of *Cr. neoformans* in the brain (Table IV). No dose-response relationship could be extracted from such results. In future research we will attempt to improve the emulsion formulation in order to increase brain penetration without significantly increasing the injected miconazole dose.

This study demonstrates that the submicron miconazole emulsion was more effective than Daktarin® i.v. in treatment of murine cryptococcosis. It can be deduced that a negatively-charged submicron emulsion did enhance drug permeation through the blood-brain barrier compared to the corresponding micellar solution of miconazole. However, the levels of miconazole that were reached in the target organ (brain) were lower than those required for complete eradication of the infection.

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