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Freeze-Drying of Itraconazole-Loaded Nanosphere Suspensions: A Feasibility Study

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ABSTRACT The present study concerns the stabilization of the association of the new hydrophobic triazole derivative itraconazole within poly-ε-caprolactone-nanospheres by means of freeze-drying. We have investigated the freeze-drying of nanospheres, and especially the cryopreservation conditions, with the help of differential scanning calorimetry and zeta potential measurements. Five commonly used cryoprotective agents were evaluated (glucose, sucrose, trehalose, dextran, mannitol at 0, 5, 10, 20, and 30% [w/v]) after freeze-thawing and freeze-drying. The addition of carbohydrates led to a partial protection of the colloidal suspension, with leakage of 30% of itraconazole under the best cryopreservation conditions (10% of glucose or sucrose). Zeta potential measurements revealed that the main destabilization mechanism during freeze-drying was surface modifications of the nanospheres, and particularly drug desorption. Therefore, the hydrophilic surfactant adsorbed at the surface of the nanospheres played an important role in the cryopreservation. Replacing the commonly used non ionic surfactant PLURONIC®PE F68 by the anionic surfactant sodium deoxycholate resulted in a complete stabilization of itraconazole-loaded nanospheres after freeze-drying, with no drug desorption, in the presence of 10% sucrose, but not in the presence of glucose. As shown by thermal analysis, PLURONIC®PE F68 may crystallize during freezing, which could lead to surface modifications and drug desorption, whereas sodium deoxycholate may not. Moreover, the Tg′ of glucose-containing suspensions is 10°C lower than Tg′ of sucrose-containing suspensions, which may explain the shrinkage of the cake observed in the case of glucose and the homogeneous appearance of the dried product in the case of sucrose.

Key Words: nanospheres, poly-ε-caprolactone, freeze-drying, cryoprotection, itraconazole

INTRODUCTION

Since the rise in the number of immunocompromized patients (AIDS, anticancer therapy) over the last 15 years, systemic infectious diseases have become much more frequent. To overcome the failures of conventional antifungal therapy caused by a lack of activity against deep fungal infections or problems of the toxicity of the active substance, extensive studies have been carried out in the field of colloidal drug carriers, especially liposomes [for reviews see: Bakker-Woudenberg and Roerdink, 1986; Bakker-Woudenberg et al., 1993, 1994; Lopez-Berestein, 1987; Vasan and Lopez-Berestein, 1995]. At the same time,

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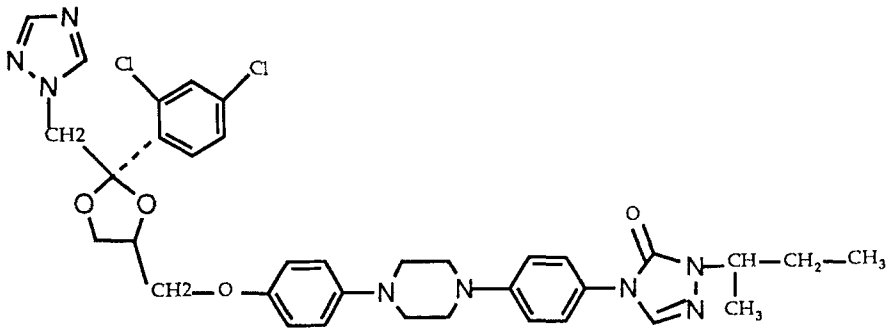

Figure 1. Structural formula of itraconazole (MW = 705.64).

newer azole antifungal compounds have been synthesized and evaluated in vivo, alone [Clemons et al., 1995; Como and Dismukes, 1994; Dismukes, 1988; Fromtling, 1988; Georgiev, 1993; Hay, 1994; Kauffman, 1994; Peng and Galgiani, 1993] or associated with liposomes [Brasseur et al., 1991; Le Conte et al., 1991; Singh et al., 1993] or cyclodextrins [Hostetler et al., 1992; Van Doorne et al., 1988]. Particular interest has been focused on the new triazole derivative itraconazole (Fig. 1), in the light of its specific advantages: broad spectrum of activity including aspergillosis and lower toxicity than amphotericin B, implying a better therapeutic index. However, itraconazole is weakly basic (pKa = 3.7) and highly hydrophobic (octanol/water partition coefficient at pH = 8.1, logP = 5.66). Since it is insoluble in water (Table 1), itraconazole can only be administered by the oral route. We have proposed an intravenously compatible itraconazole-loaded preparation consisting of a poly-ε-caprolactone-based nanosphere (PεC-NS) suspensions [de Chasteigner et al., 1996]. However, these suspensions are not stable with time, because continuous itraconazole desorption from the nanospheres followed by its precipitation in the aqueous phase due to its hydrophobicity occurs.

The improvement of particle suspension stability to reach a shelf-life of several years has already been investigated. Freeze-drying appears to be one of the most attractive methods. Only a few authors have studied freeze-drying of nanoparticles [Auvillain et al., 1989; de Chasteigner et al., 1995; Nemati et al., 1992]. It seems that the matricial network of nanospheres is more resistant to the water crystallization stress of the freezing step than the thin wall of nanocapsules. The literature is much more detailed concerning liposomes [Crowe et al., 1987a; Engel et al., 1994; Talsma et al., 1991a,b, 1992; Tanaka et al., 1992]. Freeze-drying of liposomes leads to several sorts of damages, including drug leakage, fusion, and lateral phase separations. Carbohydrates have proved to be particularly effective at stabilizing phospholipid bilayers, far more so than polyalcohols. Disaccharides are more active than monosaccharides. Among them, trehalose is very often considered as the best cryoprotective agent [Crowe et al., 1984a]. Different techniques have to be used to elucidate the mechanism of cryopreservation of liposomes by carbohydrates, including differential scanning calorimetry, infrared spectroscopy, electron microscopy [Crowe et al., 1984b, 1985a,b, 1987b; Szucs and Tilcock, 1995]. It has been suggested that carbohydrates interact with phospholipids by means of hydrogen bonding between OH groups on the carbohydrate and the phosphate head group of the phospholipid. This direct interaction between the carbohydrate and the phospholipid mimics that between water and the phospholipid. Hence, no residual water retention is necessary for stabilizing dry liposome preparations.

TABLE 1. Solubility of Itraconazole in Various Solvents

Solvents	Solubility (mg/mL)
Water (pH = 7)	0.001
Ethanol	0.30
Methanol	0.71
Acetone	2.0
Polyethyleneglycol 400	2.7
Dimethylsulfoxide	16.0
Tetrahydrofuran	27.3
Dichloromethane	239
Chloroform	363

The aim of this work was to stabilize itraconazole-loaded PεC-NS suspensions by means of freeze-drying and to try to understand the freeze-drying of nanospheres.

MATERIALS AND METHODS

Materials

Egg phosphatidylcholine (E100, non hydrogenated) was purchased from Lipoid, Ludwigshafen, Germany. Sodium deoxycholate (DOC-Na), sucrose

(Suc), trehalose (Tre), and bovine serum albumin (A-8022, 96–99% purity) were supplied by Sigma, l'Isle d'Abeau, Chesnes, France. Poly-ε-caprolactone (PεC) (MW = 40 000) was obtained from Aldrich, Strasbourg, France; PLURONIC®PE F68 (PE F68, an ethyleneoxide propyleneoxide copolymer) from ICI, Clamart, France; MONTANOX®85DF (M85DF, ethoxylated sorbitan trioleate) from Seppic, Paris, France; CREMOPHOR®EL (CrEL, polyoxyethyleneglycerol-triricinoleate), SOLUTOL®HS15 (HS15, polyethyleneglycol 660 hydroxystearate), and KOLLIDON®17PF (K17PF, polyvinylpyrrolidone, MW ≐ 9 000) from BASF, Levallois-Perret, France; and Glucose (Glc), Dextran (Dex) and Mannitol (Man) from Prolabo, Paris, France. Itraconazole (ITZ) was a gift from Janssen Research Foundation, Beerse, Belgium. All other chemicals and solvents were of an appropriate grade (Prolabo, Paris, France).

Preparation of the Nanospheres

Nanospheres were prepared using the process described by Fessi [Fessi et al., 1992]. The organic solution consisted of PεC, itraconazole, E100, acetone, and ethanol. This organic solution (40 mL) was added to an aqueous phase containing PE F68 (80 mL) under moderate magnetic stirring. The preparation was evaporated under reduced pressure and the final volume adjusted to 16 mL (1.25% of polymer). Just after preparation, the suspensions were filtered on a sintered glass filter (porosity 4, mesh size 5–15 μm).

Freeze-Drying Process

The freeze-dryer used for these experiments was an SMH 15 model from Usifroid, Maurepas, France. Firstly, the cryopreserving ability of five commonly used carbohydrates was assessed after a freeze-thawing cycle: 3 h at T = −60°C followed by thawing at room temperature. The cryoprotective agent (glucose, sucrose, trehalose, dextran, or mannitol) was added to 5 mL of itraconazole-loaded suspension at concentrations of 0, 5, 10, 20, or 30% (w/v). Secondly, the cryoprotective action of glucose, sucrose, or trehalose at concentrations of 0, 5, 10, 20, or 30% (w/v) was assessed after a freeze-drying cycle: 3 h freezing at T = −60°C and 10–12 h drying at T = +30°C (RH<5%). The samples were resuspended using 5 mL of water, 0.9% NaCl solution, 5 or 10% glucose solution. When two cryoprotective agents were introduced to 5 mL of itraconazole-loaded nanosphere suspensions, the first one was glucose, sucrose, or trehalose at a concentration of 0, 5, or 10% (w/v) and the second one was dextran, mannitol, or albumin at the respective concentrations of 0.5–1, 0.1–0.2, 0.25–0.5–1% (w/v). All studies were performed in triplicate.

Nanosphere Size Measurement

The particle mean size before and after freeze-thawing and freeze-drying was measured by laser light scattering (Nanosizer®N4MD, Coulter, Harpenden, United Kingdom). For the freeze-thawing study, the experiments were carried out in triplicate and the results were expressed as a ratio:particle mean size after freezing/particle mean size before freezing. A ratio of 1 indicates no modification after freezing of mean particle size.

Determination of the Association of Itraconazole Within the Nanospheres

Itraconazole associated within the nanospheres before and after freeze-drying was measured by HPLC in the suspension after filtration on a sintered glass filter (porosity 4, mesh size 5–15 μm). The filtration step retained unassociated itraconazole which had precipitated instantaneously in the aqueous phase because of its hydrophobicity. The chromatographic analysis was performed using a Waters system (Saint-Quentin-en-Yvelines, France) equipped with a reversed-phase Nova-Pak®C18; 60Å; 4 μm; 3.9 × 150 mm column, and a reversed-phase Nova-Pak®C18 precolumn, after appropriate dilution of the sample in methanol. The samples were eluted with acetonitrile/methanol/ammonium acetate M/40 (600/200/200) adjusted to pH = 9.1 with ammonia, at a constant flow rate of 0.6 mL/min, and detected by UV absorbance at 263 nm. The results are expressed as the percentage of itraconazole remaining associated to the nanospheres, as a ratio itraconazole:polymer (w/w).

Zeta Potential Measurement

The results presented here are all normalized to a value of $\zeta = -55$ mV for the standard solution (carboxylated polystyrene latex supplied by Malvern, Orsay, France). The zeta potentials were measured using the Zetasizer®4, with a Series 7032 Multi-8 Correlator (Malvern) after appropriate dilution in 20 mM phosphate buffer, pH = 7. Since dilution of itraconazole-loaded suspensions was followed by precipitation of part of the drug in the aqueous phase, itraconazole-loaded suspensions were prepared in 20 mM phosphate buffer, and evaporated to 65 mL instead of 16 mL. This permitted direct zeta potential measurements on the suspensions without further dilution, and with no itraconazole desorption and no itraconazole precipitation. When freeze-dried, itraconazole-loaded nanosphere suspensions were prepared in 20 mM phosphate buffer, pH = 7, and the lyophilizate

resuspended using the same phosphate buffer solution.

Transmission Electron Microscopy Observation

Nanospheres were observed by transmission electron microscopy before and after freeze-drying (microscope Philips CM100, Eindhoven, the Netherlands) after appropriate dilution in a phosphotungstate solution.

Thermal Analysis

Thermal analysis was carried out using a differential scanning calorimeter (DSC4, Perkin Elmer, Saint-Quentin-en-Yvelines, France) with samples weighing 13 mg. Programmed cooling of the samples at a rate of 5°C/min from +15°C to −50°C and at a rate of 5°C/min from −50°C to +15°C was carried out.

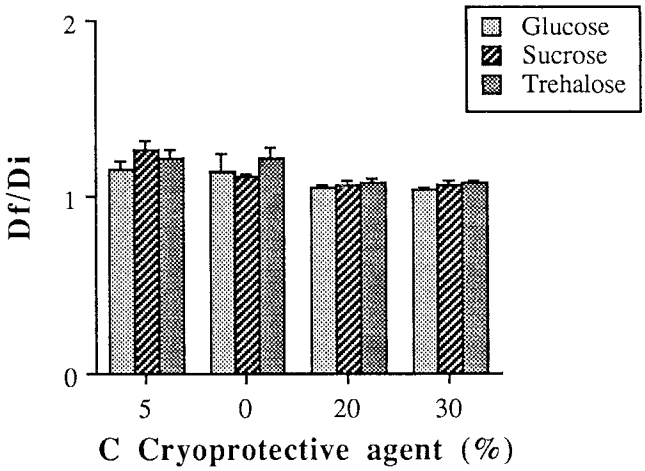

Figure 2. Influence of cryoprotective agents on freezing of itraconazole-loaded PE F68-PεC-NS (Df = mean final particle size; Di = mean initial particle size).

RESULTS

Cryopreservation Conditions

Studies were first carried out on PεC-NS suspensions stabilized by the commonly used PE F68 surfactant. After freeze-thawing, it appeared that cryoprotective agents were necessary to maintain the integrity of the suspension. In the presence of dextran and mannitol the nanospheres were completely destroyed. With the addition of either glucose, sucrose, or trehalose, preservation of the homogeneity of the colloidal system was obtained with only 5% of additive (Fig. 2). Therefore, these three cryoprotective agents were tested during the freeze-drying study. Freeze-drying of PεC-NS suspension led to a partial destabilization of the system: (1) the suspension was partly destroyed and an obvious precipitate was observed. The particle size became polydisperse; (2) part of the associated itraconazole leaked out (Fig. 3), followed by its precipitation in the aqueous phase. Under the best cryopreservation conditions, i.e., at a concentration of 10% of glucose or sucrose, itraconazole leakage was about 30% of the initial drug content. No further improvement was obtained with increasing cryoprotective agent concentrations. Changing the resuspending medium (water, 0.9% NaCl solution, 5 or 10% glucose solution) did not reduce itraconazole leakage. After addition of a second cryoprotective agent (dextran, mannitol, or albumin) itraconazole loss still occurred to a similar or higher extent. Sucrose was retained in subsequent experiments, because the freeze-dried products appeared homogenous, whereas shrinkage of the cake was observed with glucose. As depicted in Figure 4, in the presence of 10% sucrose the influence of the hydrophilic surfactant could not be neglected. Without hydrophilic surfactant, the integrity of the colloidal suspension was not maintained. Thus, after a freeze-thawing cycle in the presence of either glucose, sucrose, or trehalose at concentrations of 0, 5, 10, 20, or 30% (w/v), the surfactant-free nanosphere suspensions were all damaged, whereas both PE F68- and DOC-Na-PεC-NS suspensions were preserved with only 5% of any of the cryoprotective agents studied. When the non ionic surfactant was varied, no improvement, compared with PE F68, was seen after freeze-drying. In contrast, in the presence of the anionic surfactant DOC-Na no precipitate was observed, the nanosphere mean size remained constant, and no itraconazole leakage occurred.

Physico-Chemical Characterization

As shown in the differential scanning calorimetry analysis (Fig. 5), the Tg′ of glucose-containing suspensions was 10°C lower than the Tg′ of sucrose-containing suspensions, which was 2 to 4°C lower than that of trehalose-containing suspensions, whatever the type of nanospheres, and particularly the nature of the hydrophilic surfactant. Moreover, in the presence of PE F68, a second crystallization process during freezing occurred, whereas no crystallization occurred with DOC-Na.

As shown in Table 2, the introduction of 10% sucrose to the suspension was followed by a decrease in the negative surface charge of the nanospheres. This decrease was more pronounced in the presence of itraconazole, and was accentuated after freeze-drying. DOC-Na-PεC-NS were the most negatively charged. As observed in Figure 6a–d, itraconazole-loaded DOC-Na-PεC-NS were less modified than

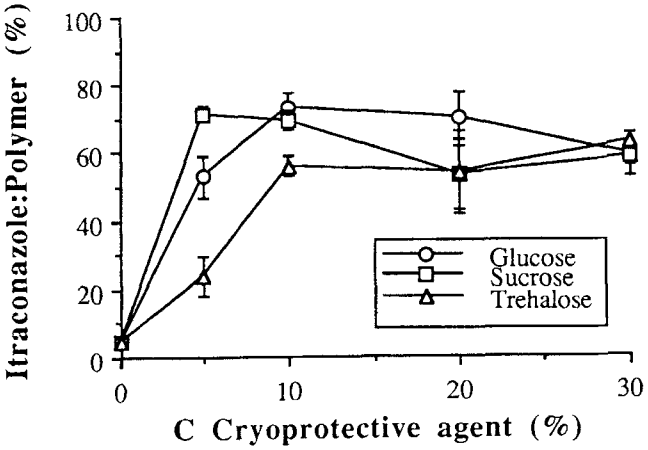

Figure 3. Influence of cryoprotective agents on itraconazole retention after freeze-drying of itraconazole-loaded PE F68-PεC-NS (mean ± SD, N = 3).

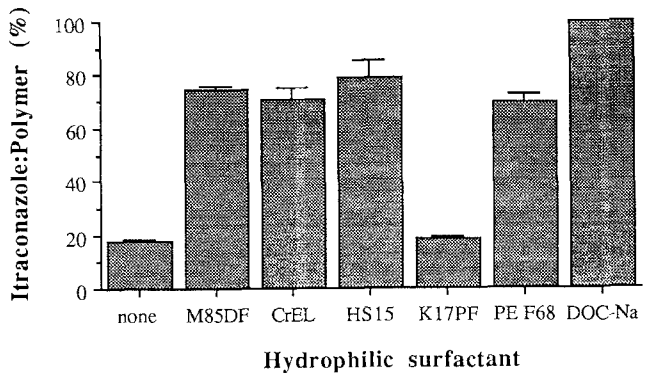

Figure 4. Influence of the hydrophilic surfactant on itraconazole retention after freeze-drying of itraconazole-loaded PεC-NS in the presence of 10% sucrose (w/v) (mean ± SD, N = 3).

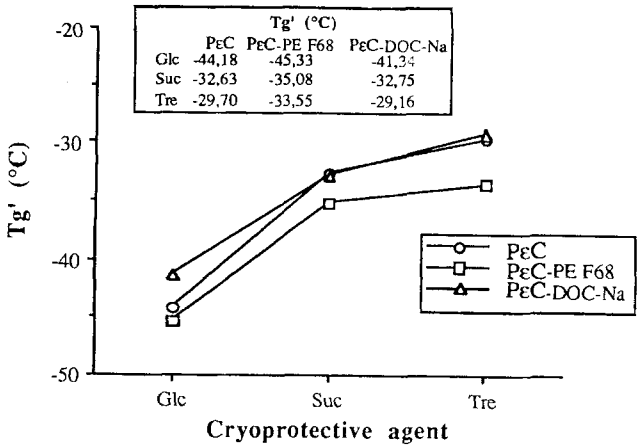

Figure 5. Influence of the cryoprotective agent (10%, w/v) on Tg' of different types of itraconazole-loaded PεC-NS.

itraconazole-loaded PE F68-PεC-NS after freeze-drying compared with the initial itraconazole-loaded PεC-NS: no increase in size, few shape modifications, little if any fusion or particle aggregation, and no reorganization of phospholipids into bilayers were seen.

DISCUSSION

Freezing is the most aggressive step of the freeze-drying operation for colloidal suspensions. Therefore, it is important to improve nanosphere stability by adding cryoprotective agents in order to avoid alteration of the suspension. Although trehalose is usually presented as the cryoprotective agent of choice, we found here that both glucose and sucrose were as effective as trehalose in maintaining the integrity of the nanospheres after freeze-thawing. The three cryoprotective agents gave the same results: no modification of nanosphere mean size, hence no nanosphere aggregation or fusion and no itraconazole desorption from the nanosphere surface, which would lead to its precipitation in the aqueous phase.

After freeze-drying, the nanosphere suspensions containing cryoprotective agents were only partly altered, compared with the cryoprotective agent-free nanosphere suspensions, which were totally destroyed. The addition of carbohydrates thus led to partial cryopreservation. Since freeze-drying of free PεC-NS in the presence of the same cryoprotective agents was not accompanied by any modification of the mean size of the nanospheres and of the appearance of the suspension, the alteration of freeze-dried itraconazole-loaded PεC-NS was mainly due to itraconazole desorption from the surface of the nanospheres followed by its rapid precipitation in the aqueous phase. This may explain the heterogeneity in the particle size observed after freeze-drying. The best results in terms of nanosphere size preservation, itraconazole leakage, and aspects of their freeze-dried product were obtained with 10% sucrose. Whatever the type of nanospheres, the Tg' value of the freeze-concentrated solution in the presence of glucose was 10°C lower than that with sucrose. This could have led to a different rearrangement of the freeze-concentrated solution in an amorphous state during the freeze-drying process, and to a different content of unfrozen water, which is known to act as a plasticizer and decrease Tg'. The lower the Tg', the more the lyophilizates tend to collapse. Finally, the macroscopic aspect of freeze-dried products differed as a function of the cryoprotective agent, since sucrose-containing lyophilizates were homogeneous and glucose-containing lyophilizates were collapsed. When cryoprotective agents were added at higher concentrations, the freeze-drying process was disturbed. The

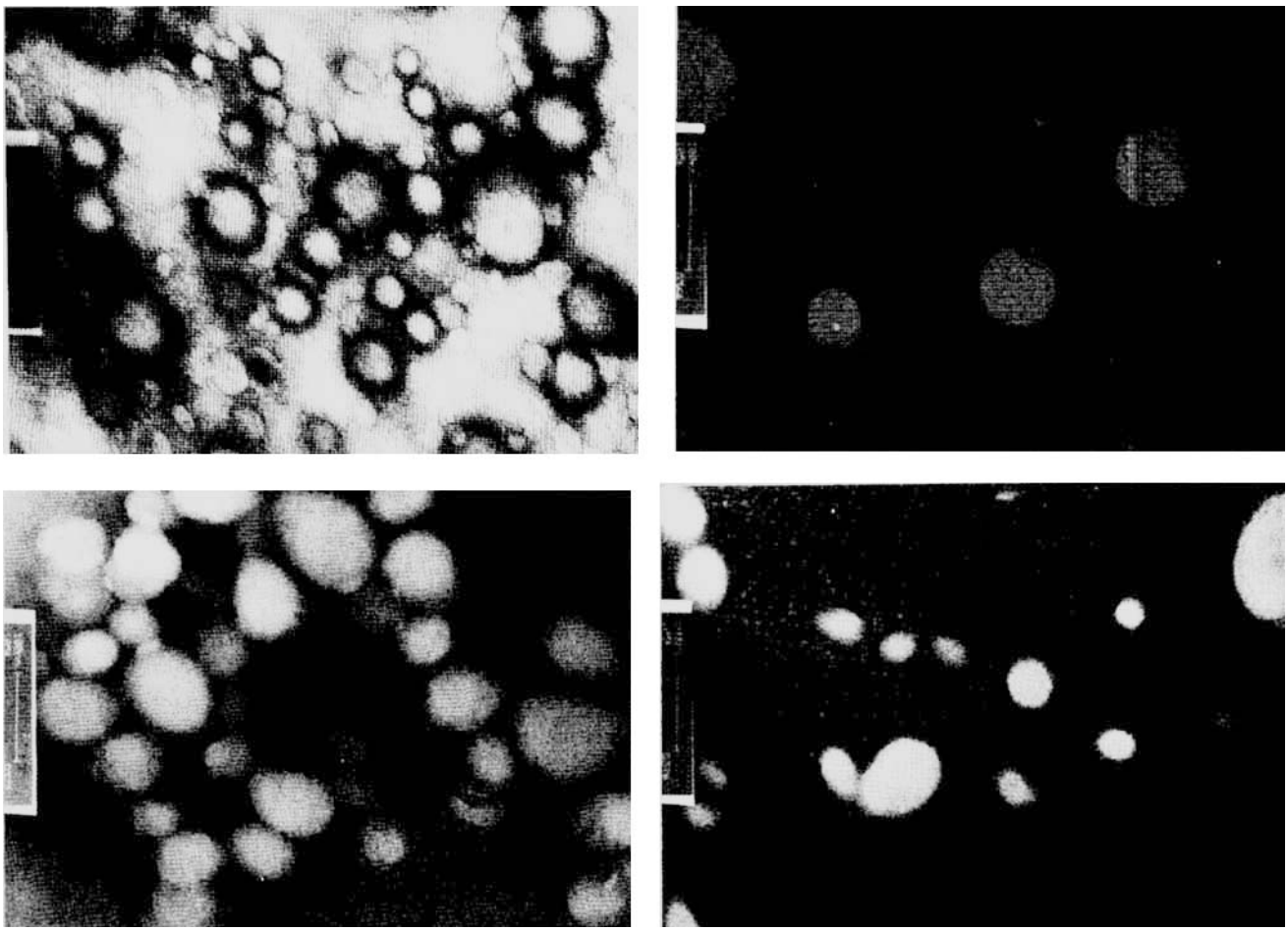

Figure 6. Transmission electron microscopy observations. **(above left)** Itraconazole-loaded PE F68-PεC-NS; **(above right)** Itraconazole-loaded DOC-Na-PεC-NS; **(below left)** Freeze-dried intraconazole-loaded PE F68-PεC-NS; **(below right)** Freeze-dried itraconazole-loaded DOC-Na-PεC-NS.

resuspending medium may also play an important role, as has been shown for resuspension of freeze-dried proteins [Zhang et al., 1995]. Since resuspension of nanospheres may occur more rapidly than resolubilization of cryoprotective agents, an osmotic stress may be created, leading to compensating itraconazole desorption from the surface of the nanospheres. However, itraconazole leakage seemed to occur before rather than during the resuspending step, since resuspending in iso- or hyper-osmotic solutions instead of water did not reduce itraconazole desorption. The modifications that take place during freeze-drying, i.e., solute concentration, ice growth, glass transition of the cryoprotective agent (Tg') [Her and Nail, 1994], may interfere with the stability of the system [te Booy et al., 1992]. This could be particularly true for the surface of the nanospheres which may be very susceptible to the different destabilization processes. To overcome these phenomena, it is possible to introduce a second cryoprotective agent into the preparation: dextran or mannitol, in order to prolong the amorphous state, i.e., to increase Tg' of the main cryoprotective agent and hence to diminish possible destabilization due to water crystallization. However, itraconazole desorption from the surface of the nanospheres after freeze-drying was not avoided when dextran or mannitol were added. Water crystallization and ice growth could still displace the itraconazole located at the surface of the nanospheres leading to its desorption when the lyopholizate was resuspended. Moreover, itraconazole desorption was enhanced: dextran or mannitol seemed to prevent sucrose from expressing its full cryopreservation efficacy, maybe because they competed for covering the surface of the nanospheres. Since itraconazole possesses a high affinity for albumin, we postulated

TABLE 2. Influence of Freeze-Drying (f-d) on Surface Charge of Free and Itraconazole-Loaded PεC-NS (Mean ± SD of 3 Measurements)

	ζ (mV, no itraconazole)			ζ (mV, with itraconazole)		
	Before f-d		After f-d	Before f-d		After f-d
	Suc = 0%	Suc = 10%	Suc = 10%	Suc = 0%	Suc = 10%	Suc = 10%
PE F68-PεC-NS	−43.4 ± 0.1	−40.9 ± 0.2	−20.4 ± 0.6	−24.2 ± 0.3	−19.7 ± 0.1	−17.0 ± 0.6
DOC-Na-PεC-NS	−56.0 ± 1.6	−44.0 ± 1.3	−46.3 ± 1.1	−52.2 ± 1.8	−44.7 ± 1.2	−35.1 ± 1.6

that desorbed itraconazole molecules would preferably bind to albumin rather than precipitating in the aqueous phase. However, the addition of albumin to the suspension was not sufficient to either improve itraconazole stabilization at the surface of the nanospheres, hence limiting its desorption, or avoid its precipitation after freeze-drying. From these results, we can conclude that total protection of PεC-NS after freeze-drying could not be obtained by adding a simple cryoprotective agent. Moreover, the maximum itraconazole leakage (30% of the total association yield) observed under the best cryopreservation conditions, i.e., with 10% sucrose, did not exceed the initial adsorbed itraconazole amount (40% of the total association yield). This supports the hypothesis that in the presence of 10% sucrose no nanosphere fusion or destruction occurs and that itraconazole desorption is the main alteration of itraconazole-loaded PεC-NS suspension after freeze-drying. The freeze-dried suspension polydispersity is only due to itraconazole desorption and precipitation.

The results suggest that the hydrophilic surfactant participates in the cryopreservation of the colloidal suspension, as well as the cryoprotective agent. In the absence of any hydrophilic surfactant, the nanosphere suspension is partly altered, even after a single freeze-thawing cycle. Moreover, it has been shown here that replacing the commonly used non ionic surfactant PE F68 by the anionic surfactant DOC-Na resulted in a complete stabilization of itraconazole-loaded PεC-NS after freeze-drying in the presence of 10% sucrose: Tyndall effect still present, nanosphere mean size unchanged, and no itraconazole leakage. The nanosphere mean size and the drug content remain constant for 24 h after resuspending in water. These results were confirmed by transmission electron microscopy observations, showing that itraconazole-loaded DOC-Na-PεC-NS were far less altered than itraconazole-loaded PE F68-PεC-NS. As reported previously [Mehl, 1995; Murase et al., 1991], salt composition and concentration play an important role in the cryopreservation or freeze-drying of biological materials. We can assume here that PE F68 crystallization led to destabilization of the weakly adsorbed drug at the surface of the nanospheres leading to its desorption when resuspending the lyophilizate, without further nanosphere alterations. On the other hand, DOC-Na, which did not crystallize, stabilized itraconazole association. The formulation is particularly sensitive to this effect because both the hydrophilic surfactant and the adsorbed drug are present at the surface of the nanospheres. To explain the greater stability of the DOC-Na-PεC-NS, the stronger hydrophobic interactions between DOC-Na and itraconazole than between PE F68 and itraconazole should be taken into account. Furthermore, the negatively charged DOC-Na-PεC-NS are more stable than uncharged PE F68-PεC-NS because of electrostatic repulsion. This contributes to the better stabilization of adsorbed itraconazole molecules at the surface of DOC-Na-PεC-NS after freeze-drying. These results support the hypothesis that the main destabilization mechanism acting on PεC-NS during freeze-drying consists of surface modifications, and particularly drug desorption.

This hypothesis was investigated by measuring the zeta potential of particles before and after freeze-drying. The addition of 10% sucrose to the suspension before freeze-drying decreased the negative surface charge of the nanospheres. This could be due to part of the nanosphere surface being masked as a result of hydrogen bonding between OH groups of the cryoprotective agent and the surface of the nanosphere, as has been proposed between OH groups of the cryoprotective agent and the phosphate head groups of the phospholipid for liposomes [Crowe et al., 1984b, 1985a,b, 1987b; Szucs and Tilcock, 1995]. After freeze-drying, the decrease in the negative surface charge is accentuated, showing a rearrangement of the surfactants at the surface of the nanosphere, leading to a possible desorption of itraconazole molecules in the case of itraconazole-loaded nanospheres. Finally, DOC-Na-PεC-NS remain the most negatively charged after freeze-drying, and hence the most stable, as discussed above [Elimelech and O'Melia, 1990].

CONCLUSION

The main drawback of itraconazole-loaded nanosphere suspensions appeared to be their instability with time because of continuous desorption of itraconazole. To overcome this problem freeze-drying of the suspension was carried out. The study indicated that the nature and the electric charge of the hydrophilic surfactant, as well as the nature and the concentration of the cryoprotective agent, were of great importance in the cryopreservation of the colloidal suspension. We found that itraconazole-loaded DOC-Na-PεC-NS was the only preparation which remained unchanged after freeze-drying with 10% sucrose. Work is in progress to determine the pharmacokinetics and efficacy of itraconazole after intravenous administration of itraconazole-loaded DOC-Na-PεC-NS.

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