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The Effects of Lyophilization on the Physico-Chemical Stability of Sirolimus Liposomes

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ABSTRACT

Purpose: The major limitation in the widespread use of liposome drug delivery system is its instability. Lyophilization is a promising approach to ensure the long-term stability of liposomes. The aim of this study was to prepare sirolimus-loaded liposomes, study their stability and investigate the effect of lyophilization either in the presence or in the absence of lyoprotectant on liposome properties. ***Methods:*** Two types of multi-lamellar liposomes, conventional and fusogenic, containing sirolimus were prepared by modified thin film hydration method with different ratio of dipalmitoylphosphatidylcholine (DPPC), cholesterol and dioleoylphosphoethanolamine (DOPE), and were lyophilized with or without dextrose as lyoprotectant. Chemical stability investigation was performed at 4°C and 25°C until 6 months using a validated HPLC method. Physical stability was studied with determination of particle size (PS) and encapsulation efficiency (EE %) of formulations through 6 months. ***Results:*** Chemical stability test at 4°C and 25°C until 6 months showed that drug content of liposomes decreased 8.4% and 20.2% respectively. Initial mean EE % and PS were 72.8 % and 582 nm respectively. After 6 months mean EE % for suspended form, lyophilized without lyoprotectant and lyophilized with lyoprotectant were 54.8 %, 62.3% and 67.1 % at 4°C and 48.2%, 60.4 % and 66.8 % at 25°C respectively. Corresponding data for mean PS were 8229 nm, 2397 nm and 688nm at 4°C and 9362 nm, 1944 nm and 737 nm at 25°C respectively. ***Conclusion:*** It is concluded that lyophilization with and without dextrose could increase shelf life of liposome and dextrose has lyoprotectant effect that stabilized liposomes in the lyophilization process.

Introduction

Sirolimus (Rapamycin, SRL, Rapamune, $C_{51}H_{79}NO_{13}$, CAS No: 53123-88-9), a carbocyclic lactone-lactam macrolide antibiotic, is anatural fermentation product of the streptomyces hygroscopicus discovered in Rapa Nui (Easter Island). Although initially isolated as an antifungal agent with potent anticandida activity, subsequent studies revealed impressive antitumor and immunosuppressive activities. It binds to the immunophilin FKBP12 and interferes with the function of mTOR, thus blocking the progression from G1 to the S phase of the cell cycle and blockage of the response of T and B cells to cytokines and consequently cell proliferation. Although SRL and Tacrolimus are structural analogs, they have different mechanisms of action. Sirolimus is available in oral solution and tablet form. It is rapidly but poorly absorbed after oral administration with an estimated bioavailability of 15%.[1-8]

Liposomes are artificial vesicles, composed of lipidic amphiphiles, usually phospholipids, which organize themselves in water to form an aqueous core surrounded by a lipidic bilayer. This structure allows liposomes to transport both hydrophilic and lipophilic compounds and have led to their clinical use as drug carriers of several drug classes including antibiotics, antifungals and anticancer agents. Liposomes have a 50-year history of use with numerous applications. Their utility has included toxicity buffering of drugs, targeting to specific tissue sites, enhancement of drug efficacy or potency, dissolution of insoluble drugs and sustained release of drugs.[9-16]

However, its potential application as therapeutic agent is still challenged by its physical and chemical instabilities in aqueous dispersions (e.g., hydrolysis and oxidation of phospholipids, encapsulated solute leakage and liposome aggregation) for long-term storage.

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Liposome dispersions prepared from commercially available lipids do not meet the required standards for long-term stability of pharmaceutical preparations. If they are stored as aqueous dispersions the encapsulated drugs tend to leak out of the bilayer structureand the liposomes might aggregate or fuse on storage and it is generally necessary to use them within the first few months of preparation. Accordingly, many methods available for stabilization of liposome have been investigated, such as lyophilization, freezing, spray-drying and supercritical fluid technology. Among these, lyophilization is the main approach used toextend the shelf-life of liposomes, especially for liposome containing thermo-sensitive drugs. Some liposomal products in the market or in clinical trials are provided as a lyophilized powder.[17-21]

A variety of sugars, including sucrose, glucose, fructose, maltose, arabinose and trehalose have been shown to act as lyoprotectant during dehydration/rehydration of liposomes.[22-26]

In the present study, SRL-entrapped multilamellar liposomes were prepared using the thin film hydration method. Physical stability tests of liposomal formulations of SRL were performed at 4°C and 25°C. Moreover the prepared liposomes were lyophilized to study the effect of lyophilization with and without lyoprotectant and also to investigate the lyoprotectant effect to protect liposomes against fusion and leakage during storage at the same temperatures.

Materials and Methods

Materials

Sirolimus was obtained from Poli Company (Lazio, Italy). Dipalmitoylphosphatidylcholine (DPPC) and dioleoylphosphoethanolamine (DOPE) were purchased from Lipoid GMBH (Ludwigshafen, Germany). Cholesterol (Chol) was obtained from Merck Company (Darmstadt, Germany). All solvents were HPLC grade and all reagents were of analytical grade and were purchased from Merck Company (Darmstadt, Germany).

Liposome preparation

SRL multilamellar liposomal formulations with different molar ratios were prepared using the thin film hydration technique. The lipid components (DPPC and Chol) either alone or mixed with DOPE, in the case of fusogenic liposome, with different molar ratios were dissolved in chloroform:methanol mixture (3:1, % v/v) in a round-bottomed flask. The organic solvents were slowly removed under reduced pressure, using a rotary evaporator (Buchi, Zurich, Switzerland), at 45°C, above gel-liquid crystal transition temperature (Tc) of phospholipids, such that a very thin film of dry lipids was formed on the inner surface of the flask. The dry lipid film was slowly hydrated with 20 ml of phosphate buffered saline (PBS) (pH 7.4) containing SRL 500 µg/ml for 3 hrs in rotary leading to the formation of multi-lamellar liposomes. The resulting suspension was sonicated for 10 min to reduce liposome size.

Chemical stability of SRL

Samples of liposomal formulations in suspension and lyophilized form in 4°C and 25°C were investigated initially and monthly until 6 months using previously developed HPLC method.[27]

Lyophilization of liposomes and subsequent reconstitution

The lyoprotectant (dextrose) was dissolved in phosphate buffered saline at concentration of 10%. Liposomal suspensions in buffer with or without lyoprotectant were freeze-dried (ZIRBUS sublimator 400, ZIRBUS Technology, Bad Grund, Germany) where the liquid was frozen at-195°C. Dehydration step lasted for 2 days at temperature of -40°C until dried powder formed. The resulting lyophilized powderswere rehydrated to its original volume at room temperature with PBS, and following the addition of PBS the samples were equilibrated at room temperature for 30 min. Then the samples were subjected to the following tests.

Physical stability of liposomal formulations

Storage stability of all suspensions and lyophilized formulations was tested at 4°C and 25°C for six months.

Determination of drug content and encapsulation efficiency (EE %) of liposomes

Unentrapped drug was separated using dialysis method at temperature below gel-liquid-crystalline transition temperature (Tc) in sink condition after 24 hrs. Drug content in the liposome dispersion and unentrapped drug were analyzed with RP-HPLC system (Beckman, USA). The as follow: Knauer C18 column (4.6×150mm, 5µm) (Berlin, Germany) was used at 54°C, mobile phase consisting of acetonitril and ammonium acetate buffer (pH5.8)(70:30, % v/v) with flow rate of 1.5 ml/min, detection wavelength was set at 278 nm and injection volume was150µl. A linear response was observed over a concentration range of 125–2000 ng/ml (r^2> 0.991). For all quality control (QC) standards in intraday and interday assay, accuracy and precision ranges were 0.96 to 6.30 and 0.86 to 13.74 respectively, demonstrating the acceptable precision and accuracy over the analytical range.

EE %was calculated by the following equation:[28-32]

$$EE\ (\%) = [(C_{total} - C_{free})/C_{total}] \times 100$$

Where C_{total} is total drug concentration which was added and C_{free} is the concentration of unentrapped drug.

Measurement of particle size distribution of liposome

Mean vesicle size and size distribution profile of liposome was determined using particle size analyzer

(Shimadzu, Japan) which uses laser diffraction method. All sample measurements were conducted in triplicate.

Results and Discussion

Chemical stability of SRL

After six months storage at 4°C and 25°C, drug content of liposomes following disruption of liposome in methanol and after enough dilution was analyzed with RP-HPLC method. Results revealed that SRL content of liposomes after 6 months was 91.6 ± 2.3% at 4°C and 79.8 ±3.6% at 25°C for suspended form. Respective results for lyophilized form at the end of 6 months storage were 92.3 ±1.6% and 81.6±2.7% (Figure 1). Therefore it can be concluded that hydrolysis has small effect in SRL degradation.

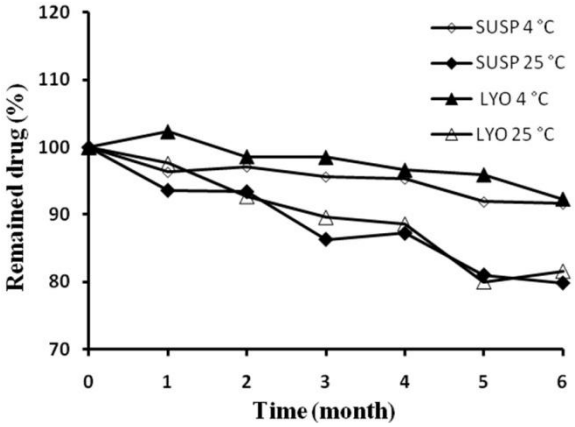

Figure 1. Chemical stability of SRL during 6 months in the suspension and lyophilized form

Physical stability studies

Physical stability study of SRL liposomes was conducted at refrigeration temperature (4°C) and at room temperature (25°C) for a period of 6 months. Drug entrapment of liposomes in suspension and reconstituted liposome was evaluated monthly. The results are demonstrated in Figure 2 in terms of percentage of SRL retained in the liposomes.

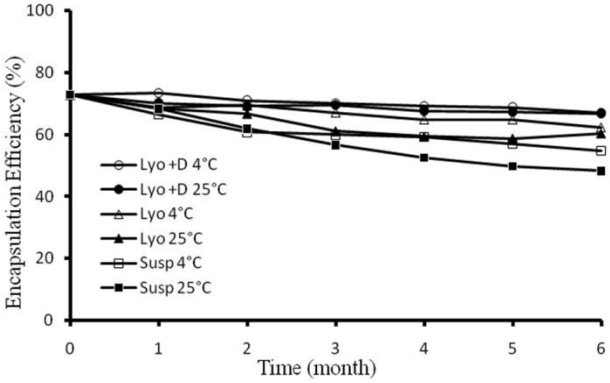

Figure 2. Mean EE % of different forms of multilamellar SRL liposomes stored at 4°C and 25°C

Mean initial EE % for liposomes was 72.8 % and average retained SRL percent in liposomal formulations after 6 months were 54.8 %, 62.3 % and 67.1 % for suspended form, lyophilized without lyoprotectant and lyophilized with dextrose (Lyo + D) respectively. The retained drug in formulations after 6 months storage at 25°C was 48.2 %, 60.4 % and 66.8 % respectively (Figure 2).

Lyophilization increases the shelf-life of liposomal formulation and preserves it in dried form as a lyophilized cake to be reconstituted with water prior to administration. To maintain the same particle size distribution after lyophilization- rehydration cycle, a lyoprotectant needs to be added. Since the process of lyophilization is harmful for the liposome integrity, an obvious decrease in the encapsulation efficiency is seen for lyophilized formulations. In fact freeze-drying leads to destroy the membrane function of the phospholipid bilayer. In the present study we have used dextrose as protecting agent. It is well-known that sugars can be applied to prevent aggregation of nanoparticles during drying and storage. A literature review reveals that in previous studies various sugars were investigated for their ability to protect liposomes against fusion and leakage during lyophilization process. Glavas-Dodov et al, showed that particle size, EE % and release profile did not differ significantly after lyophilization with saccharose.[33] However, in this study dextrose showed protective effect for SRL liposomes. This protective ability can extend both prevention of vesicle fusion and retention of encapsulated drug within the liposome. The protective effect during liposome lyophilization is mainly determined by the formulation factors, such as the nature of the drug, the lipid bilayer composition, and the choice of lyoprotectants. The stabilization property of sugars has been explained by the particle isolation theory, water replacement hypothesis and vitrification theory.[34-38] Liposomes with lyoprotectant showed better stability, as indicated by higher drug retention. Suspended form liposomes had the less stability. Initial mean particle size of liposomes was 582 nm, which after 6 months storage at 4°C were increased up to 8229 nm, 2397 nm and 688 nm for suspended form, lyophilized without lyoprotectant and lyophilized with dextrose respectively. However in storage at 25°C particle sizes were increased more significantly up to 9362 nm, 1944 nm and 737 nm respectively (Figure 3). Considering 0.01 as significance level, only difference in particle sizefor formulation lyophilized with dextrose and stored at 4°C wasn't significant ($P=0.026$) and in all other formulations differences were significant ($P<0.005$).

As mentioned before more SRL retained in liposomes in lyophilized formulations compared to suspension form, but with using dextrose for lyophilization of multi-lamellar liposomes, EE % was higher. The leakage of entrapped SRL could be explained by the fact that in the suspension form, lyophilization and rehydration of a liposomal suspension can result in leakage of internal aqueous contents. Whereas, with using dextrose as a lyoprotectant and in the absence of

protective agent, the amount of entrapped SRL didn't decrease significantly (P> 0.15).

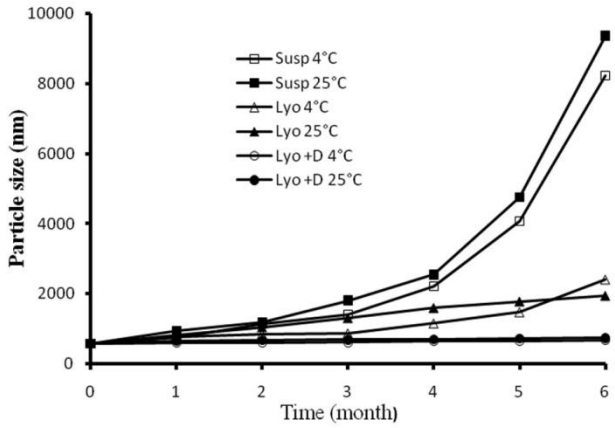

Figure 3. Mean particle size of different forms of multilamellar SRL liposomes stored at 4°C and 25°C.

Conclusion

Instabilities of liposomes during storage are a serious limiting factor for their applicability as drug delivery systems. Lyophilization is a commonly used drying technique for thermolabile pharmaceuticals and also various studies have demonstrated that lyophilization is an effective way to overcome the instability problems of liposomes in the aqueous state. The present work focuses on physicochemical characterization of lyophilized SRL loaded multi-lamellar liposomes and short-term storage stability studies of formulation. The process of lyophilization is often used to prepare pharmaceutical formulations to achieve commercially practicable shelf life and easy handling (shipping and storage). It is important to have a product of a desirable quality and maintain physicochemical characteristics of formulation during storage. It was observed that there was no significant change in drug content at 4°C and 25°C storage conditions for 6 months in lyophilized liposomes with and without dextrose as lyoprotectant (P>015) but decreasing drug content in suspension forms was significant at both 4℃ and 25℃ (P=0.009 and P=0.005 respectively). Whereas significant increase (P<0.005) in size were observed at the same temperatures over 6 months of storage in all formulations except lyophilized form with lyoprotectant (P=0.026). Taken together, studies showed superior stability of the lyophilized product after reconstitution in comparison with those of the suspension product, and physico-chemical stability of products which have dextrose was most superior. Then we could conclude that lyophilization with and without dextrose could increase shelf life of liposome and glucose has lyoprotectant effect that stabilized liposomes in the lyophilization process. Overall, studies on the optimization of formulation and technological parameters to improve the lyoprotective effect are still required for improving liposome lyophilization.

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Conflict of interest

The authors report no conflicts of interest.

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