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Preparation, Characterization, and Stability of Liposome-Based Formulations of Mitoxantrone

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ABSTRACT The preparation, characterization, and stability of lyophilized liposome-based formulation of mitoxantrone was investigated. Mitoxantrone was entrapped inside small, unilamellar liposomes composed of dioleoylphosphocholine (DOPC), cholesterol, and cardiolipin. The mean vesicle size and drug entrapment efficiency of the liposomes were ~150 nm and ~99%, respectively. Less than 1% of drug was lost and mean vesicle size remained unchanged after sterile filtration. The pre-lyophilized (pre-lyo) formulations were characterized by a differential scanning calorimetric (DSC) method. Results showed that the glass transition temperatures (Tg′) increased as the molar ratios of sucrose:lipid and trehalose:lipid in the formulations were increased. The maximum Tg′ of the pre-lyo formulations containing 10:1 sucrose:lipid and trehalose:lipid molar ratios were −37C and −41C, respectively. After reconstitution of the lyophilized cake of the sucrose-containing formulation, the mean vesicle size was comparable to pre-lyo liposome size. *In vitro* release studies showed that less than 2% of mitoxantrone was released after an extensive dialysis against phosphate buffered saline (PBS) at 37C, indicating that the mitoxantrone was highly associated and retained inside the liposomes. Short-term stability studies of the sucrose-containing formulations revealed that the reconstituted and eight-fold diluted formulations were stable for up to 8 hours at room temperature. Long-term stability studies of lyophilized liposomal mitoxantrone showed that the lyophilized formulation was stable for up to 13 months after storage at refrigerated condition.

KEYWORDS Mitoxantrone stability, Liposomes, Injectable formulation, Lyophilization

INTRODUCTION

Liposomes are drug delivery vehicles that have been used to enhance therapeutic efficacy, reduce toxicity, and modify the pharmacokinetics of anticancer drugs (Gregoriadis et al., 1988). Since the endothelial membrane of tumors are more "leaky" than normal membranes, liposomes with particle sizes <200 nm easily pass through the fenestrated blood vessels and

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accumulate at tumor and disease sites (Bally et al., 1994; Gabizon, 1992). Hence, by encapsulating anticancer agents inside the liposomes, distribution and exposure to healthy cells and tissues are minimized while the drug payload to the tumor is enhanced, thus resulting in reduced toxicity and enhanced efficacy (Bally et al., 1994; Gabizon, 1992). Liposome encapsulated drugs are also known to accumulate in organs of the reticuloendothelial system (Caride et al., 1976; Hinkle et al., 1978; Rahman et al., 1982).

Mitoxantrone (1, 4-dihydroxy-5, 8-bis[[2-[(2-hydroxyethel)amino] ethylamino]-9,10-anthracenedione) is an antracenedione derivative and, like doxorubicin, has been used to treat various forms of leukemia, breast, stomach, liver, and ovarian cancers; however, mitoxantrone is less cardiotoxic than doxorubicin (Neidhart et al., 1986). The mechanism by which mitoxantrone exerts its antitumor activity may be due to the inhibition of DNA and RNA synthesis via intercalation into DNA and RNA base pairs (Reska et al., 1997) and inhibition of topoisomerase II, an enzyme involved in DNA repair (Ehninger et al., 1990). Some of the common side effects associated with the mitoxantrone therapy are phlebitis, alopecia, nausea, vomiting, diarrhea, and myelosuppression (Novantrone Immunex Co., Seattle, WA). The dose-limiting toxicity is myelosuppression. Coincidentally, the occurrence of myelosuppression has lead to its use in the treatment of multiple sclerosis (Minow et al., 1977).

Significant efforts have been made to develop liposomal formulations of mitoxantrone (Gokhale et al., 2001; Lim et al., 2000; Poujol et al., 1999). Some of these formulations have been demonstrated to improve safety, therapeutic efficacy, and pharmacokinetics in preclinical and clinical studies (Gokhale et al., 2001; Lim et al., 2000; Poujol et al., 1999). Gokhale et al. (2001) recently developed a cardiolipin-based liposomal formulation of mitoxantrone. The entrapment of mitoxantrone inside the liposomes was achieved by formation of electrostatic complex between the cationic drug and cardiolipin (Novantrone Immunex Co., Seattle, WA). This formulation is currently provided in a two-vial system, with one vial containing the lyophilized lipids (dioleoyl-sn-glycero-3-phosphocholine, cholesterol, and cardiolipin) and the other vial containing the commercial product Novatrone® (2 mg/mL mitoxantrone hydrochloride for injection). Prior to administration, the liposomal mitoxantrone was prepared by comixing the lyophilized lipids and 2 mg/mL mitoxantrone base (Novatrone), hydrating for 60 minutes, and then sonicating for 30 minutes. However, this formulation has several drawbacks: it is difficult to use, has a nonuniform particle size distribution profile, and has a potential for microbial contamination.

In the present study, we developed a more efficient formulation process for the preparation of sterile and stable liposomal mitoxantrone. The new process involves the entrapment of mitoxantrone inside the cardiolipin-containing liposomes, size-reduction, sterilization, and lyophilization. The final lyophilized formulation only requires reconstitution with sterile water for injection just prior to administration. Other advantages of the new formulation include high entrapment efficiency and mono-modal particle size distribution profile. The specific objectives of our study were 1) to prepare an easy-to-use lyophilized liposomal mitoxantrone (LEM-ETU) formulation, 2) characterize the formulation, and 3) evaluate short- and long-term stability of the formulation.

MATERIALS AND METHODS

Materials

Mitoxantrone HCl was obtained from GensiaSicor, Irvine,-CA. Lipids: dioleoly-sn-glycero-3-phosphocholine (DOPC), cholesterol, and 1,1′,2,2′ tetramyristoyl cardiolipin were obtained from Avanti Polar Lipids (Alabaster, AL). Alpha-tocopheryl acid succinate was obtained from Sigma (St. Louis, MO). Ethyl alcohol, USP, was purchased from Aaper (Shelbyville, KY). Sodium chloride, USP, and potassium hydrogen phosphate, USP, were obtained from EM Science (Gibbstown, NJ). Sucrose, NF, was purchased from Mallinkrodt Baker Inc. (Paris, KY). Trehalose was obtained from ICN Biomedical (Aurora, OH). Nitrogen gas, NF, was procured from BOC Gases (Carol Stream, IL). Spectra Por® Dialysis membrane tubing, molecular weight cutoff ~12–14,000 daltons, was obtained from Spectrum Labs (Los Angeles, CA). Water for Injection (WFI) was obtained from Baxter (Round Lake, IL). All chemicals were used as received.

Mitoxantrone and Lipid Assays

The high-performance liquid chromatography (HPLC) system used for drug and lipid content analysis consisted of Agilent 1100 modules (Wilmington, DE), a quaternary pump, mobile phase degasser, autosampler with thermostat, and a column heater compartment. ChemStation software (Agilent Technologies, Wilmington, DE) was used for data acquisition and analysis. For mitoxantrone drug content assay, an ultraviolet (UV) variable detector at wavelength of 608 nm and a Hypersil BDS (C8) column (4.6 mm × 150 mm, 5 μm) were utilized. The mobile phase consisted of a mixture of 0.1% trifluoroacetic acid and acetonitrile in gradient proportions. During the analysis, 20-μL samples were injected in duplicate into the HPLC system at flow rate of 1 mL/min. The mitoxantrone was quantitatively determined using external standards (Miller et al., 2004). For lipid analysis, an evaporative light scattering detector (Polymer Laboratories, Amherst, MA) and a diol column 250 mm × 4.6 mm, ASTEC, Inc., Whippany, NJ), equilibrated at 40°C were used. The mobile phase consisted of chloroform:methanol:ammonium acetate buffer pH 9.9, 71:26:3 %v/v at a flow rate of 1 mL/min. Lipids were quantitatively determined using a standard curve comprised of a minimum of six working standard concentrations (Simonzadeh et al., 2004).

Liposome Preparation, Sterilization, and Lyophilization

Preparation and Sterilization

Liposomes were prepared by the ethanol injection method (Campbell, 1995). Briefly, lipid components (DOPC, cholesterol, cardiolipin, and alphatocopheryl acid succinate) were solubilized in ethanol. Once dissolved, the ethanol/lipid mixture was transferred and diluted into an aqueous solution of lyoprotectant (either sucrose or trehalose in varying concentrations) and mitoxantrone, resulting in the formation of multilamellar mitoxantrone liposomes. The multilamellar vesicles were then extruded (Northern Lipids, Inc., Vancouver, British Columbia, Canada) under a nitrogen atmosphere (pressure ~300–400 psi) through two stacks of 0.2 and 0.1 μm polycarbonate membrane filters (Whatman, Inc., Clifton, NJ) at room temperature until the size was reduced to approximately 150 nm. Ethanol was removed by evaporation under vacuum. The resulting mitoxantrone liposomes were filtered through 0.2 μm PVDF membrane sterilizing filters (Millipore, Billerica, MA) and filled into 10 mL Type I serum vials.

Tg′ Determination

The Tg′ of the pre-lyophilized mitoxantrone liposomes were determined using differential scanning calorimeter (DSC) (Model TA Q100 series, TA Instruments, New Castle, Delaware). Briefly, 20-μL samples were hermetically sealed in aluminum pans and placed in the heating block of the instrument under continuous streams of nitrogen. The cycle consisted of a ramp from to −80°C to 25°C at 5°C/min., isothermal hold at −80°C for 30 minutes, and a ramp to 25°C at 5°C/min. Tg′ was determined from the heating scan and calculated as the temperature at the half height of the glass transition region.

Lyophilization Procedure

The filled formulation vials were placed in Virtis lyophilizer (Advantage, The Virtis Company, Gardiner, NY). The cycle consisted of freezing of the formulation at −45°C for 2 h, primary drying at −35°C for at least 24 h, and secondary drying at 25°C for at least 8 h.

Characterization of Mitoxantrone Liposomes

Vesicle Size Measurement

Mean vesicle size and distribution were measured by dynamic light scattering technique using a submicron particle sizer (Nicomp 380, Particle Sizing Systems, Santa Barbara, CA). Prior to sample measurement, polystyrene beads of standard size were used to verify the performance of the instrument. Samples were measured in duplicate. Data collected was analyzed by CW388 software (Particle Sizing Systems, Santa Barbara, CA), assuming that vesicles are spherical and scatter light according to Mie theory. The data is reported as volume-weighted mean diameter size distribution.

Drug Entrapment Efficiency

An aliquot of reconstituted mitoxantrone liposomes was diluted eight-fold in 0.9% sodium chloride, mixed, and 4 mL were transferred into a centrifuge tube. The sample was centrifuged at 200,000 × g for 2 hours at 4°C using a Beckman L-90k ultracentrifuge (Palo Alto, CA). The total mitoxantrone concentration in the formulation and supernatant after centrifugation was determined by a HPLC method. The percent entrapment efficiency (%EE) was calculated as follows:

$$\%EE = \frac{D_t - D_s}{D_t} \times 100$$

where D_t and D_s are total drug concentration in LEM-ETU and drug concentration in supernatant, respectively.

In Vitro Drug Release

The *in vitro* release of mitoxantrone from the formulation was performed by membrane dialysis at 37°C against phosphate-buffered saline (PBS) at pH 7.4. In a regular dialysis mode, 2 mL aliquot of reconstituted mitoxantrone liposomes were transferred into dialysis tubing and placed in temperature-controlled, jacketed beaker containing 100 mL of PBS at 37°C. Sink condition was maintained during the release experiments. At various time intervals, samples were withdrawn from the release medium and assayed for mitoxantrone. In a reverse dialysis mode, several dialysis bags containing 2 mL of PBS were placed into 98 mL of PBS contained in jacketed beakers maintained at 37°C. Two-mL aliquot of the reconstituted mitoxantrone liposomes were added directly into the 98 mL of PBS. At various time intervals, one dialysis bag was withdrawn and the contents of the bag were analyzed for mitoxantrone by HPLC.

Stability

The stability of the reconstituted and diluted samples was assessed after storage at room temperature (20–25°C) for 8 hours. The stability of the lyophilized cake was evaluated after storage at 2–8°C for up to 13 months. The pH, percent entrapment efficiency, mean vesicle size, mitoxantrone, DOPC, cholesterol, and cardiolipin concentrations of the samples were determined as a function of the storage time and temperature. The lipid and drug concentrations were determined by HPLC. The mean vesicle size was measured using the submicron particle sizer as described previously.

RESULTS AND DISCUSSION

Formulation Composition

The liposomal formulation was composed of 2 mg/mL mitoxantrone, DOPC, cholesterol, and cardiolipin. The drug:lipids ratio and DOPC:cholesterol:-cardiolipin mole percent ratio were 1:18 and 50:30:20, respectively. The rationale for using DOPC was to maximize mitoxantrone loading into the liposomes. Unsaturated phospholipids such as DOPC form flexible bilayers that can accommodate greater amounts of drug. It was necessary to include cholesterol in the formulation in order to enhance both in vitro and in vivo stability of the liposomes (Palatini, 1992). Inclusion of cardiolipin was also essential, since it has been reported that this phospholipid plays a significant role in modulating multidrug resistance (Oudard et al., 1991; Thierry et al., 1993), and also provides a negative charge for interaction with positively charged mitoxantrone.

Sterilization

Table 1 summarizes the effect of sterile filtration on liposome size and mitoxantrone assay. As the results show, the formulation was sterile filtered without significant loss in mitoxantrone or change in vesicle size. Since this formulation was intended to be freeze-dried, aseptic filtration was the only viable method, provided that the liposomes can pass through a 0.22-μm filter. Mitoxantrone liposomes were size reduced to less than 150 nm (99 percentile size distribution less than 220 nm), hence sterile filtration was successfully achieved.

TABLE 1 Effect of Sterile Filtration on Liposome Size and Mitoxantrone Assay[a]

Sample	Mitoxantrone (% of initial concentration)	Mean vesicle size (nm)
Pre-sterilization	100	126
Post-sterilization	99.1	129

[a]Values represent mean of two determinations.

TABLE 2 The Dependence of Major Glass Transition Temperature (Tg′) of Pre-lyophilized (Pre-lyo) LEM-ETU Formulations on Sucrose:Lipid and Trehalose:Lipid Ratios[a]

Disaccharide:lipid molar ratios		Tg′ ± SD (C)
Sucrose-lipid ratios	5.0	−47.7 ± 0.8
	7.5	−40.7 ± 0.7
	10	−37.6 ± 0.4
Trehalose:lipid ratios	5.0	−43.2 ± 0.2
	7.5	−42.0 ± 0.1
	10	−41.8 ± 0.5

[a]Values represent mean (±standard deviation) of three determinations.

Lyophilization

Lyophilization of liposomal products can minimize lipid hydrolysis and enhance long-term storage stability as well as obviate the need to freeze the liquid formulation; however, a major challenge is to lyophilize liposomes such that upon reconstitution the pre-lyophilization vesicle size is restored, and the drug is retained inside the liposomes. In the studies outlined above, the feasibility of freeze-drying liposomal mitoxantrone was examined. The dependence of glass transition temperatures (Tg′) of pre-lyophilized formulations on sucrose:lipid or trehalose:lipid ratios are presented in Tables 2 and 3. Results show that the glass transition temperatures (Tg′) increased as the molar ratios of sucrose:lipid and trehalose:lipid in the formulations were increased (Table 2). The maximum Tg′ of the pre-lyo formulations containing 10:1 sucrose:lipid and trehalose:lipid molar ratios were −37°C and −41°C, respectively. In a preliminary experiment, we successfully lyophilized formulations containing 5:1 lyoprotectant:lipid ratios at the primary drying temperature set of −35°C. We found that product temperature probe reading was below the set shelf temperature by approximately 7−8°C (data not shown); therefore, the product temperature safety margin is wide enough to prevent cake collapse during lyophilization when the shelf temperature is set at −35°C.

Vesicle Size

The mean vesicle diameters (nm) of pre-lyo and post-lyo LEM-ETU as a function of lyoprotectant:lipid ratios are summarized in Table 3. The sucrose-containing formulations retained their size after lyophilization and rehydration at sucrose:lipid ratios of 7.5 or greater. The mean vesicle size of trehalose-containing liposomes at all ratios studied were significantly higher after lyophilization and rehydration. Previous studies have shown that the freeze dry of liposomal products in the absence of lyoprotectants resulted in large and unstable liposomes upon rehydration, due to aggregation and fusion of liposomes (Bridges & Taylor, 2001; Crowe et al., 1985); however, the addition of disaccharides such as trehalose and sucrose to the formulation inhibited aggregation and fusion (Bridges & Taylor, 2001; Crowe et al., 1985). Since at the same sugar:lipid ratio, the formulation containing trehalose showed lower Tg′, it is likely that the product temperature may have exceeded the Tg′ during lyophilization. This may have resulted in micro-collapse of the cake and subsequent fusion of the liposomes.

Drug Entrapment Efficiency

Mitoxantrone entrapment efficiency was found to be greater than 99% (data not shown). The ionization constants of mitoxantrone have been previously reported to be 5.99 and 8.13 (Beijnen et al., 1988); therefore, at formulation pH 5.5, mitoxantrone will exist primarily as posivitely charged ions and thus will

TABLE 3 The Dependence of Mean Vesicle Diameter (nm) of Pre-lyo and Post-lyo LEM-ETU on Lyoprotectant-to-Lipid Ratios[a]

	Mean vesicle diameter (nm)			
	Sucrose		Trehalose	
Lyoprotectant:lipid molar ratios	Pre-lyo	Post-lyo	Pre-lyo	Post-lyo
5.0	151	1474	158	974
7.5	141	170	169	1165
10	140	140	155	867

[a]Values represent mean of two determinations.

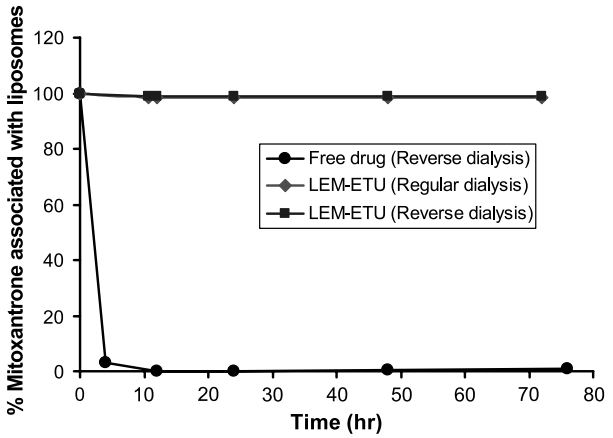

FIGURE 1 ***In vitro*** **release of mitoxantrone from LEM-ETU at 37°C bt dialysis method.**

be expected to interact avidly with negatively charged cardiolipin within the liposomes.

In Vitro Release

The release of mitoxantrone was studied by regular and reverse dialysis methods. As shown in Fig. 1, free mitoxantrone in control samples diffused freely across the membrane, and in fewer than 8 hours of dialysis, nearly 100% of the free drug had crossed the membrane. However, less than 2% of drug was released from the liposomal formulation after 72 hours of dialysis for reconstituted samples. These results suggest that mitoxantrone is strongly associated with liposomes in the formulation. This result also corroborates with high entrapment efficiency data previously reported. The release profiles of free drug obtained by reverse and regular dialysis methods were comparable.

Short-Term and Long-Term Stability

Lyophilized LEM-ETU product was stored under refrigerated conditions and analyzed at specified time points. As shown in Table 4, the lyophilized formulation was stable for up to 13 months at 2–8°C storage condition. The mitoxantrone, DOPC, cardiolipin assay values, entrapment efficiency, pH, and mean vesicle size were comparable to initial values. The stability data for reconstituted and diluted LEM-ETU formulations is summarized in Table 5. Based on the data, the lyophilized formulation reconstituted in sterile water and diluted eight-fold in normal saline is physically and chemically stable for 8 hours at room temperature (20–25°C).

CONCLUSIONS

We have developed a one-vial, easy-to-use, liposomal formulation of mitoxantrone with high entrapment

TABLE 4 **Stability Data of Lyophilized LEM-ETU at Refrigerator Condition (2–8°C)**[a]

Time (months)	Mitoxantrone (% of initial)	Cardiolipin (% of initial)	Cholesterol (% of initial)	DOPC (% of initial)	Entrapment efficiency (%)	Mean vesicle size (nm)
Initial	100	100	100	100	>99	121
1	96	104	100	98	>99	127
3	110	104	104	100	>99	127
6	106	104	106	98	>99	120
13	103	99	101	107	>99	122

[a]Values represent mean of two determinations.

TABLE 5 **Stability Data for Reconstituted and Eight-Fold Diluted LEM-ETU Samples Stored at Room Temperature (20–25°C)**[a]

	Time (h)	Mitoxantrone % of initial	Cardiolipin % of initial	Cholesterol % of initial	DOPC % of initial	pH	Entrapment efficiency (%)	Mean vesicle size (nm)
Reconstituted	Initial	100	100	100	100	4.75	>99	140
	8	97	95	94	96	4.77	>99	145
Eight-fold diluted	Initial	100	100	100	100	5.43	>99	146
	8	101	95	98	100	5.35	>99	148

[a]Values represent mean of two determinations.

efficiency and uniform size distribution profile. This formulation can be reconstituted with suitable injectable diluent prior to administration. The lyophilized formulation is physically and chemically stable upon storage at 2–8°C for up to 13 months. When reconstituted with sterile water for injection and/or further diluted in normal saline, the formulation is stable for up to 8 hours at room temperature (20–25°C) storage condition.

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