SCIENTIFIC PAPERS
OF THE UNIVERSITY OF PARDUBICE
Series A
Faculty of Chemical Technology
13 (2007)

PREPARATION AND CHARACTERIZATION OF A NEW LIPOSOMAL PACLITAXEL FORMULATION

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Received September 24, 2007

Stable lyophilised liposomal preparation of paclitaxel (7 mol %) encapsulated in the liposomes composed of SOPC/POPG/MOPC (60:20:20 molar ratio) and doped with vitamin E (5 mol %) was prepared by various procedures: hydration of lipid film, the proliposome-liposome method and lyophilisation from 2-methyl-butan-2-ol. Extrusion through 0.2 μm polycarbonate membranes was applied as a secondary processing technique to reduce the size distribution of liposomes to about 180 - 190 nm (PDI 0.1). A negative charge (zeta-potential of –30 mV in saline phosphate buffer) was sufficient to prevent flocculation and fusion of liposomes in suspension. An isocratic reverse-phase high performance liquid chromatography (HPLC) method with ultraviolet (UV) detection was used for determination of paclitaxel in liposomal preparations. The value of paclitaxel

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encapsulation efficiency (EE_{PTX}) was determined to be approximately 95 %. Mannitol, lactose, sucrose and trehalose were tested as cryoprotective agents. Except for mannitol, all the tested saccharides were equally cryoprotective at lipid:saccharide molar ratios of 1:5 and 1:10.

Introduction

The taxanes represent one of the most important new classes of oncology drugs approved in the past two decades. Paclitaxel (Fig. 1), the prototype of this class, is a diterpenoid pseudoalkaloid compound isolated from the bark of Western Pacific Yew, *Taxus Brevifolia* [1]. This drug has a unique mode of action and was found to be effective in treating various kinds of cancer [2,3]. The commercially available injectable preparation is a sterile solution of paclitaxel in Cremophor EL® with dehydrated alcohol. Presently, cancer chemotherapy with paclitaxel is associated with hypersensitivity reactions in spite of suitable premedication with corticosteroids and anti-histamines [4]. Paclitaxel in Cremophor EL® shows also an incompatibility with the components of the administration sets. Cremophor EL®ethanol leaches the plasticizer bis(ethylhexyl) phthalate from the poly(vinyl chloride) infusion bags [5]. A suitable drug formulation remains still a problem, because paclitaxel has a low therapeutic index owing to its high lipophilic character. Hence, the development of an improved delivery system for paclitaxel is of high importance. Current approaches focus mainly on the development of formulations that are devoid of Cremophor EL®, investigation of the possibility of large-scale preparation and seeking for longer-term stability. These different approaches have shown some promising possibilities to substitute Cremophor EL®, they are as follows: (a) micelle, liposome and niosome formulations [6-9], (b) water-soluble prodrug preparations [10], (c) enzyme-activatable prodrug preparations conjugated with antibodies or albumin [11,12], (d) parenteral emulsions [13], (e) microspheres [14], (f) cyclodextrins [15] and (g) nanocrystals [16]. However, none of these alternatives is ready to replace Cremophor EL® in the clinic now.

Liposomes, lipid membrane vesicles, represent powerful and versatile drug carriers for a wide range of drugs [17]. Recent advances in this area have led to the development of some products for human medicine (e.g. liposomal doxorubicin and amphotericin) [18]. Some liposomal paclitaxel formulations have already been reported and tested in mouse cancer models [7,8]. Lower toxicity, higher efficiency and increased maximum tolerated dose of paclitaxel were demonstrated in comparison with Cremophor EL®. The maximum entrapment capacity for paclitaxel in these liposomal formulations was only about 3 mol %. There have been reports suggesting that the paclitaxel encapsulation can be further increased by using lysophospholipids in the liposome formulation. These lipids increase the

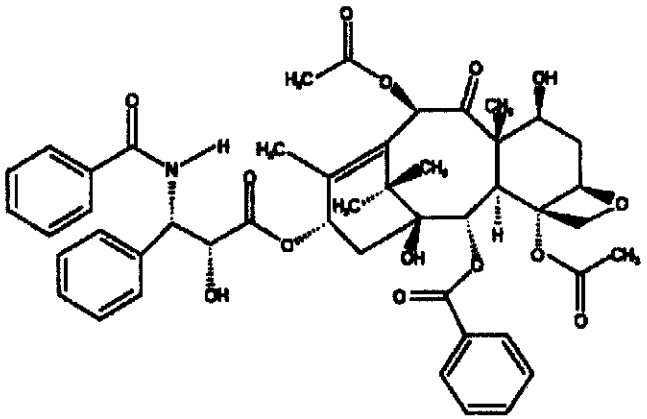

Fig. 1 Chemical structure of paclitaxel

membrane fluidity and create bilayer "pockets" in which bulky and hydrophobic molecules such as paclitaxel can be encapsulated [19].

The analysis of paclitaxel and related taxane agents is generally performed by high performance liquid chromatography (HPLC), although other techniques have been reported. The reversed phase separations on various stationary phases are preferred, but normal phase chromatographic analysis has also been used [20]. Mobile phases typically consist of mixtures of methanol, acetonitrile and water or buffer. The available HPLC methods have ordinarily employed mass spectrometric and ultraviolet detection, mostly in a narrow region of 225-230 nm [21]. Generally, the paclitaxel analysis is carried out for the purposes of separation and identification of taxanes from the natural sources (e.g. needles, twigs and bark) [22], quantification in biological samples [23] especially pharmacokinetic monitoring in plasma and blood [24] and determination of drug content in liposome-based paclitaxel formulations [25]. Characterization of paclitaxel content and its stability in the prepared liposomal formulations is important information for next active investigation.

Now, we report improvements in this approach leading to a new optimal stabilized formulation of liposomal paclitaxel (up to 7 mol %) that is stable during storage.

Experimental

Chemicals and Reagents

Lipids comprising SOPC (1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine), MOPC (1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine) and POPG (1-

palmitoyl-2-oleoyl-*sn*-glycero-phosphatidylglycerol) were purchased from Avanti Polar Lipids (USA). All the organic solvents used (reagent or HPLC grade) and vitamin E were obtained from Sigma-Aldrich (the Czech Republic). Paclitaxel (purity of 97 %) was purchased from Houser Chemical Research Inc. (Boulder, CO, USA).

Liposome Preparation

Hydration of Lipid Film, Proliposome – Liposome method

Liposomes containing vitamin E and paclitaxel were prepared by the proliposome-liposome method and hydration of lipid film method followed by the extrusion through 0.2 μm polycarbonate membrane in an analogous way to that described previously by Turánek *et al.* [26,27]. The hand-operated device Mini-Extruder (Avanti Polar Lipids, USA) was used for the extrusion of small liposome volumes (up to 1 ml).

Lyophilisation from 2-methylbutan-2-ol

Lipids, vitamin E and paclitaxel were solubilised in 2-methylbutan-2-ol instead of *tert*-butyl alcohol reported in [8]. This mixture (12 mg lipids ml^{-1}, 1 ml total) was frozen at –70 °C and lyophilised for 24 h in a Lyovac GT2 instrument (Finaqua, Finland). Stepwise hydration of lipids in PBS buffer (0.2 μm filtered, pH = 7.2) was accomplished via step-by-step addition of an aqueous phase to the lyophilate (20 μl additions during 20 min, total volume 200 μl) under continuous magnetic stirring. After lipid hydration, the volume was adjusted to 1 ml, and optional extrusion through 0.2-μm polycarbonate membrane was performed.

The Cryoprotective Effect of Various Saccharides

Liposomal paclitaxel samples were extruded through a polycarbonate membrane of 0.2 μm pore size. Various saccharides (lactose, mannitol, sucrose and trehalose) were added to the liposomal preparation at different lipid:saccharide molar ratio (1:3, 1:5, 1:7, 1:10). These mixtures were frozen at –70 °C and lyophilised for 24 h. The parameters chosen to judge the saccharide cryoprotective efficiency were as follows: the protection of liposome size and the absence of paclitaxel crystals after rehydration.

Sample Preparation and Standards

The liposomal paclitaxel preparations (0.944 mg paclitaxel ml^{-1}), volume of 200 µl, were diluted with PBS buffer up to the final volume of 3 ml in a glass tube and gently stirred. The samples were prepared for the chromatographic analysis by ultracentrifugation at 100.000 g for 30 min. The supernatant (containing possibly unencapsuled drug) was separated from the sediment. The liposome sediment was then redispersed in the same volume of PBS buffer. The supernatant, the redispersed sediment and the non-ultracentrifuged liposomes were frozen at –70 °C and lyophilised for 24 h. All samples were redissolved in the same volume of methanol (3 ml) and vortexed. A part of the content of the tubes was transferred into a glass vial and 20 µl aliquots were injected into the HPLC system.

The standard stock solution (100 µg ml^{-1}) of paclitaxel in methanol was always freshly prepared prior to use. The calibration standards were obtained by sequential dilution of the methanolic stock solution; the concentrations were as follows: 1 µg ml^{-1}, 10 µg ml^{-1}, 25 µg ml^{-1}, 50 µg ml^{-1} and 100 µg ml^{-1}. The chromatographic run of each standard was made in triplicate. The calibration curves were obtained by linear regression analysis.

Chromatographic Instrumentation and Conditions

The chromatography was carried out using a Waters system (Milford, MA, USA) composed of a 717 plus auto-injector, a 600 gradient pump and a 996 diode array detector. A Nova-Pak (C_{18}, 150 × 3.9 mm I.D., 4-µm particle size) stainless-steel analytical column was attached. The mobile phase consisting of acetonitrile and water (45:55, v/v) was degassed by sonication prior to use. The separation was carried out isocratically, the temperature was set on 35 °C and the flow rate was 1.1 ml min^{-1}. The detector wave-length was 229 nm. Millennium 2010 programme was applied for the data collection and integration.

Determination of Paclitaxel Levels

The encapsulation efficiency (EE_{PTX}) of paclitaxel loading into the liposomal preparation was calculated according to the following equation

$$EE_{PTX}(\%) = \frac{A_{LIP\text{-}PTX}}{A_{TOTAL\text{-}PTX}} \times 100 \qquad (1)$$

where $A_{LIP\text{-}PTX}$ represents the amount of paclitaxel that remains associated in liposomes and $A_{TOTAL\text{-}PTX}$ is the total amount of paclitaxel.

Size Distribution and Zeta-potential of Liposomes

The size distribution and zeta-potential of the liposomal preparations were determined by dynamic light scattering and micro-electrophoresis using a Nano Sizer SZ (Malvern, UK).

Paclitaxel Crystallization

The prepared and stored liposomal samples were tested for the presence of paclitaxel crystals by phase contrast microscopy. These crystals were detected in the optical field of Eclipse 600 microscope (Nikon, Japan).

Results and Discussion

Preparation of Liposomal Paclitaxel

The first method evaluated was the hydration of a lipid film. The lipid films were prepared with an initial paclitaxel content of 7 mol % and hydrated with 10 mM HEPES buffer (pH = 7.2) at 25 °C for 60 min. No paclitaxel crystals were seen and the achieved encapsulation efficiency (EE_{PTX}) values were about 91 %. When the proliposome-liposome method was used, paclitaxel was dissolved in methanol at 60 °C for 10 min. No thermal decomposition of paclitaxel was detected by HPLC, however the final EE_{PTX} values were approximately 80 % and crystallization was observed. In these experiments, the best results were obtained by lyophilisation of a powderized mixture of lipids-paclitaxel from 2-methylbutan-2-ol followed by hydration in PBS at 25 °C for 60 min. The values of EE_{PTX} were found to be at least 94 % and paclitaxel crystallisation was not registered over several days.

Liposomes were prepared from the mixture of SOPC, POPG and MOPC according to the general composition SOPC/POPG/MOPC (80-X:X:20 molar ratio), where X value is 0, 10, 20 or 30 %. Different liposomal paclitaxel formulations were prepared separately by lyophilisation from 2-methylbutan-2-ol of a powderized lipids-paclitaxel. It was followed by the hydration step and the extrusion through 0.2-μm polycarbonate membrane using a hand-operated Mini-Extruder. The resulting liposome particles were of the size range of 175-195 nm and the polydispersity index was about 0.1, as measured by dynamic light scattering (DLS). The presence of POPG increased the values of negative zeta-potential of liposomal paclitaxel particles and helped to optimize EE_{PTX} (Fig. 2). SOPC/POPG/MOPC (60:20:20 molar ratio) with 7 mol % of paclitaxel was chosen

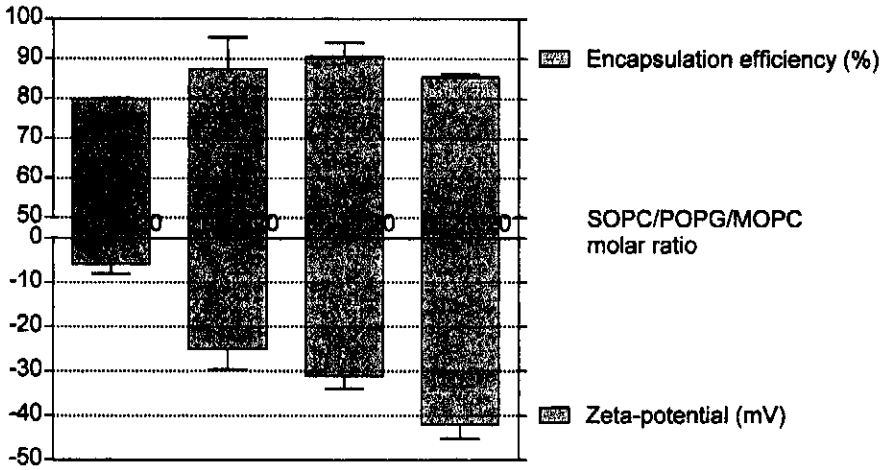

Fig. 2 Effect of negative charged POPG on the encapsulation efficiency and zeta-potential of liposomal paclitaxel formulations. Zeta-potential was measured in the PBS buffer

to be the optimum liposomal paclitaxel formulation. Then, the influence of storage temperature (4 °C or 37 °C) was investigated. The size of liposomal particles and the crystalline state of paclitaxel were monitored daily by DLS and phase contrast microscopy. Based on these measurements, SOPC/POPG/MOPC (60:20:20 molar ratio) with 7 mol % of paclitaxel was confirmed to be the optimum liposomal paclitaxel formulation once again (Table I).

Table I Influence of lipid composition on the paclitaxel crystallization from liposomal paclitaxel formulations. The amount of paclitaxel needles in the optical field was scored by phase contrast microscopy

Storage		SOPC/POPG/MOPC molar ratio			
Time	Temperature	80:00:20	70:10:20	60:20:20	50:30:20
a.p.	4 °C / 37 °C	–	–	–	–
1st day	4 °C / 37 °C	–/–	–/–	–/–	–/–
2nd day	4 °C / 37 °C	++/++	–/–	–/–	–/–
4th day	4 °C / 37 °C	+++/+++	+/+	–/+	+/+
7th day	4 °C / 37 °C	+++/+++	++/++	+/+	++/++

a.p. – immediately after preparation
– no crystals, + sporadic crystals, ++ several crystals, +++ plenty of crystals

Lyophilisation from 2-methylbutan-2-ol has proven to be a simple and robust method for the preparation of optimal liposomal paclitaxel samples, for rapid screening of various lipid compositions and long-term storage. This is gratifying since a preparation of long-term stable liposomal paclitaxel formulations by a combination of hydration of lipid film, lyophilisation from 2-methylbutan-2-ol, sonication and second lyophilisation [28] has recently been described as too complicated and expensive for further clinical applications.

Vitamin E and Saccharide Cryoprotectants

Vitamin E (0, 5, 10 and 15 mol %) was added to this optimal liposomal paclitaxel (7 mol %) formulation. These formulations were prepared by lyophilisation from 2-methylbutan-2-ol with subsequent hydration in PBS and extrusion through 0.2 μm polycarbonate membrane, as it was previously mentioned. The size of the optimal liposomal paclitaxel particles, containing 15 mol % of vitamin E, tended to be smaller in comparison to the formulations with less amount of vitamin E. Zeta-potential of these liposomes was also decreased (Table II). Paclitaxel crystals were not observed after hydration during storage within 26 days at 4 °C and 3 days at 37 °C and no important changes in EE_{PTX} were detected.

Table II Influence of different molar percentages of vitamin E on the size and zeta-potential of liposomal paclitaxel formulations. The liposomal composition was SOPC/POPG/MOPC (60:20:20 molar ratio) with 7 mol % paclitaxel. The size distribution and zeta-potential were measured in the PBS buffer. The polydispersity index was approximately 0.1 for all the liposomal preparations

Parameter	Molar % of vitamin E			
	0	5	10	15
Encapsulation efficiency, %	79.8 ± 0.4	94.7 ± 3.8	93.4 ± 0.8	94.4 ± 0.9
Zeta-potential, mV	–31.2 ± 3.2	–28.4 ± 4.5	–33.5 ± 3.8	–24.9 ± 3.1
Size, nm	190 ± 2	176 ± 6	194 ± 6	129 ± 5

The lyophilised optimal formulations of liposomal paclitaxel containing different amounts of vitamin E (5, 10 and 15 mol %) were stable for at least 6 months during long-term storage at 4 °C. Liposomal particles were physically stable in the presence of sucrose as a cryoprotectant after rehydration. Paclitaxel did not crystallize out of these liposomal formulations for at least 40 days of the storage at 4 °C. These experimental results were obtained after rehydration by the measurements of size distribution, zeta-potential and EE_{PTX} indicating the release of paclitaxel out of liposomes.

The optimum level of vitamin E was stated to be 5 mol % in the presence of various cryoprotective agents. In this case, lactose, sucrose and trehalose were found to be almost equal with respect to their cryoprotective effectivity (Fig. 3). The minimum lipid:saccharide molar ratio necessary for the size stabilization of the lyophilised and rehydrated liposomal paclitaxel particles was found to be 1:5. The composition of SOPC/POPG/MOPC (60:20:20 molar ratio) with 7 mol % of paclitaxel and 5 mol % of vitamin E was established to be the optimum for the stabilization of the liposomal paclitaxel formulation.

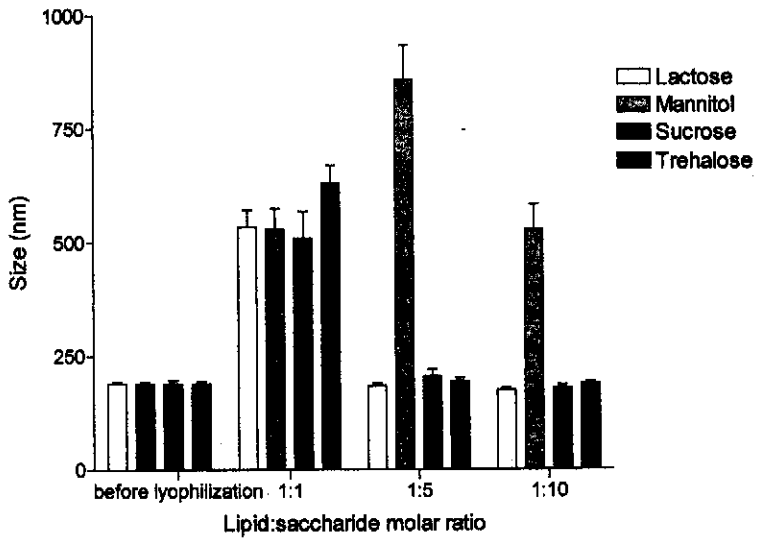

Fig. 3 Effect of various saccharide cryoprotectants on the physical stability of lyophilised liposomal paclitaxel formulation after rehydration. Lactose, mannitol, sucrose and trehalose were used as the cryoprotective agents for the lyophilisation of the paclitaxel containing liposomes. Lipid:saccharide molar ratios were as follows: 1:1, 1:5 and 1:10. The liposomes extruded through the membrane of the pore size of 0.2 μm in the solution of the tested saccharides were used as the control of the size prior to lyophilisation

Liposomal paclitaxel formulations are known to be problematic because of the low encapsulation capacities. Maximal achievable content of paclitaxel is 3 mol % [29]. The use of lysophosphatidylcholine significantly increased encapsulation capacity of liposomes for paclitaxel [19]. The inclusion of vitamin E (5-10 mol %) in this optimum formulation contributes to the stabilization of the lipid bilayers and prevents the paclitaxel crystallization in lyophilised and rehydrated stages during storage. We developed liposomal paclitaxel systems that were able to encapsulate up to 10 mol % of paclitaxel. These liposomal systems were physically unstable for long-term storage at high levels of paclitaxel (9-10 mol %). Disintegration and aggregation of liposomal particles frequently appeared

and paclitaxel tended to crystallize spontaneously. Seven molar percent of paclitaxel was found to be the optimum amount with respect to the development of stable formulations appropriate for long-term storage.

Chromatographic Analysis

An isocratic reverse-phase HPLC method with UV detection was used for paclitaxel determination in our liposomal formulations. The composition of the mobile phase was optimized in order to prevent interfering peaks co-eluted with the compound of interest. This assay was found to be linear over the range of 1-100 μg ml^{-1} of paclitaxel, with regression correlation coefficient > 0.999. Paclitaxel quantities were determined in total preparation, supernatant and sediment using calibration curves (data not shown). The EE_{PTX} values of liposomal samples were calculated according to Eq. (1). The highest observed values of EE_{PTX} were approximately 95 % in optimal paclitaxel formulation. They represent a successful paclitaxel loading into the liposomal membrane bilayers.

We developed an ultracentrifugation technique with a lyophilisation step followed by fast and simple HPLC assay. This procedure allows to determine total, bound and free fractions of drug in the liposome formulation instead of measuring only free drug content [25]. Thus, we invented powerful tool for the monitoring of the level of the encapsulated drug. The measuring of EE_{PTX} has shown no dramatic changes during storage. Contrary, a great decrease in encapsulation efficiency due to the massive paclitaxel crystallization has been reported in sterically stabilized liposomes within several hours [30]. Mass detection is by far superior to UV detection [31], however, taking into account the impact of both availability and cost, we decided for UV detection of the paclitaxel in liposomal samples.

We suppose that our optimal stabilized formulation, comprising SOPC/POPG/MOPC (60:20:20 molar ratio) with 7 mol % paclitaxel and 5 mol % vitamin E should be more acceptable for clinical trials.

Our optimal stabilized liposomal paclitaxel formulation has already been tested *in vivo*. It was well tolerated at high doses and the anticancer has been proven in B16F10 melanoma-bearing mice. Significant reductions in lung metastases were observed when high doses of liposomal paclitaxel (50, 100 mg kg^{-1}) were administered (publication in preparation).

Acknowledgements

This work was supported by grants from the Ministry of Agriculture of the Czech Republic (grant No. MZE 0002716201) and the Ministry of Education, Youth and Sport of the Czech Republic (project MSM 0021627502).

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