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Preparation and evaluation of paclitaxel-containing liposomes

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Received May 24, 2006, accepted June 9, 2006

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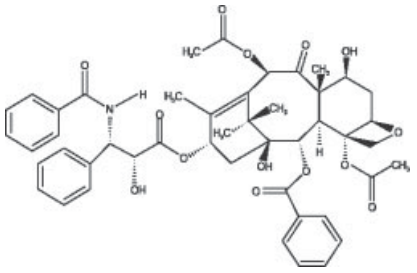
Pharmazie 62: 126–132 (2007) *doi: 10.1691/ph.2007.2.6093*

Paclitaxel, an antitumoral drug, is poorly soluble in aqueous media. Therefore, in a commercialised formulation (Taxol®), paclitaxel (30 mg active compound) is dissolved in polyethoxylated castor oil (Cremophor® EL) and ethanol. After dilution of Taxol® in aqueous media paclitaxel tends to precipitate. Several side effects, attributed to the surfactant Cremophor® EL, occur, e.g. bronchospasm, hypotension, neuro- and nephrotoxicity, and anaphylactic reactions. To eliminate these side effects, the solubility of paclitaxel was enhanced using liposomes instead of Cremophor® EL. The amount of entrapped paclitaxel in crystal-free liposomes was 0.5 mg/ml liposome suspension, i.e. almost 85 times the native solubility. Thus, 30 mg paclitaxel had to be dissolved in 60 ml liposome suspension, of either multi-lamellar vesicles (MLV's) or of small unilamellar vesicles (SUV's) with 5% sucrose as cryoprotector. No precipitation was observed after dilution of the MLV-formulation with (physiological) water or with 5% aqueous dextrose solution, which proves their suitability for administration with perfusions. The chemical stability of paclitaxel in the prepared MLV's stored at 4 °C was demonstrated during a period of 5 months. The chemical degradation to conjugated dienes and hydroperoxides, two oxidative degradation products of EPC, was negligible (less than 1%).

1. Introduction

In 1967, paclitaxel has been isolated from the bark of *Taxus brevifolia* (Taxaceae) (Cserhati and Hollo 1994). Paclitaxel, an antitumor product, is a natural complex diterpene with on C_{13}, an extented side chain required for activity (Kearns 1997; Straubinger et al. 1995a).



Paclitaxel

Paclitaxel is almost insoluble in water (AVGI 2001) and is susceptible to hydrolysis. The chemical stability is highest at pH range 3–5 (Dordunoo and Burt 1996). Clinical studies proved the antitumor effect against breast and ovarian cancer. There is also activity against head and neck cancers, non-small-cell lung carcinoma, prostate cancer, melanoma, Kaposi's carcoma and tumors in the gastro-intestinal tract (AVGI 2001; Balasubramanian and Straubinger 1994; KNMP 1999; Martindale 1999; Monroe and Wani 1995; Needham and Sarpal 1998; Sharma et al. 1997; Walle et al. 1995).

The problem of paclitaxel in formulation development is its poor water solubility, which is a challenging limitation. Surfactants, polymers, cyclodextrins, emulsions and liposomes have been used in the literature to improve its solubility (Balasubramanian and Straubinger 1994; Merisko-Liversidge et al. 1996; Needham and Sarpal 1998; Sharma et al. 1993, 1995a, 1997; Straubinger et al. 1995b; Tarr et al. 1987b).

The commercial formulation Taxol® (Bristol Myers Squibb) contains 6 mg paclitaxel, dissolved in 527 mg of the surfactant Cremophor® EL (polyethoxylated castor oil) and water-free ethanol added till 1 ml (AHFS 2002; Sharma et al. 1997). Both excipients are used to improve the solubility of paclitaxel. Before administration, this formulation must be diluted with either physiological solution (0.9% NaCl solution) or 5% dextrose solution, up to a concentration of 0.3 to 1.2 mg/ml (AVGI 2001; Pfeifer et al. 1993; Straubinger et al. 1995a; Trissel and Bready 1992). Often, needle shaped precipitation is noticed (Balasubramanian and Straubinger 1994). To avoid that these particles enter the blood stream, a 0.22 μm filter is placed at the end of the infusion set (AVGI 2001; Leikin and Paloucek 1996; Lubejko and Sartorius 1993).

The Cremophor® EL excipient has been observed to cause serious anaphylactic reactions with some patients (Balasubramanian and Straubinger 1994; Lubejko and Sartorius 1993; Sharma et al. 1993, 1995a, b, 1997; Straubinger et al. 1995a, b), as it causes the release of histamine in the body (Leikin and Paloucek 1996). Therefore, Taxol® treatment includes a prophylactic regimen of corticosteroids (dexamethasone), H_1- (diphenhydramine) and H_2-anti-histamines (ranitidine, cimetidine) combined with prolonged infusion times, which is aggravating for the patient (KNMP 1999; Lubejko and Sartorius 1993; Sharma et al.

1993, 1995a, 1997; Straubinger et al. 1995a, b). With premedication, the severity and incidence of serious hypersensitivity reactions is reduced, but not eliminated (Sharma et al. 1993, 1995a, 1997; Straubinger et al. 1995a, b). Cremophor® EL also has been associated with bronchospasm, hypotension, neuro- and nephrotoxicity (Arbuck et al. 1993; Ramaswamy et al. 1997). It is physically incompatible with the compounds of some intravenous (I.V.) infusion sets, as evidenced by the extraction of plasticizers, such as diethylhexylphalate (DEHP), which is hepatotoxic (Lubejko and Sartorius 1993; Pearson and Trissel 1993). Therefore, Taxol® solution must be stored in glass or polyoleofin containers and administrated by polyethylene I.V. tubings (AVGI 2001; Lubejko and Sartorius 1993; Pearson and Trissel 1993). The doses of paclitaxel vary from 2 to 20 mg/kg (Sparreboom et al. 1996) with infusion times of 3 to 24 h (Gianni et al. 1995; Huizing et al. 1995).

To avoid these disadvantages, due to Cremophor® EL, new formulation possibilities were searched for. Tarr and Yalkowsky (1987a) used Pluronic® as solubility enhancer. Nevertheless, paclitaxel cristallized. Preparation of emulsions (Tarr et al. 1987b) gave no improvement regarding precipitation, while nanocrystalline suspensions (Merisko-Liversidge et al. 1996) resulted in no improvement in the tumor model compared to preparations with Cremophor® EL (Sharma et al. 1995b). Another application was the oral administration of paclitaxel (Paxene®), also dissolved in Cremophor® EL and ethanol. Cremophor® EL is not absorbed after oral administration, but the absorption and bioavailability of paclitaxel is also poor (Malingré et al. 1999; Meerum-Terwoght et al. 1998).

The use of liposomes as alternative for Cremophor® EL has been tested by different researchers. In one study, precipitation was noticed at a concentration of 3 mol% (= 0.425 mg/ml) paclitaxel (Balasubramanian and Straubinger 1994). Needham and Sarpal (1998) were able to incorporate 12 mol% paclitaxel, but the used lysolipid is toxic and not suitable for clinical research. Liposomes are prepared either with a single lipid, often phospholipids, or with a combination of lipids. Cholesterol is often incorporated in the bilayers of the liposomes to enhance their structural stability (New 1990). The literature about liposomes is exhaustive and the requirements for their I.V.-use are well documented (Lasic and Papahadjopoulos 1998). Some formulations of paclitaxel liposomes are proposed (Balasubramanian and Straubinger 1994; Needham and Sarpal 1998; Sharma et al. 1993, 1995b, 1997; Straubinger et al. 1995b), but often it is not clear which type of liposome has been used, how much paclitaxel can be incorporated, whether crystals occur or how the incorporated amount is verified. Because of these lacks of clarity in the literature, in this work liposomes were prepared and their physical and chemical stability was investigated. The lack of physical and chemical stability, especially of long-term stability, is a problem for the industrial preparation of those formulations.

The potential stability problems in liposome preparations are various. Oxidation of the fatty acids, especially of the polyunsaturated, represented by linoleic acid and arachidonic acid in egg phosphatidylcholine (EPC), is the most common degradation of the phospholipid. A free-radical chain reaction results in excessive formation of conjugated dienes and trienes, while further oxidation processes of the conjugated double bounds form two types of peroxides: hydro- and endoperoxides (New 1990). Paclitaxel can undergo solvolysis (hydrolysis in aqueous environment).

The aim of this paper was to develop and to test liposome formulations containing 30 mg paclitaxel. Such formulations are expected to have fewer side effects than the marketed formulations containing Cremophor® EL as solubility enhancer.

2. Investigations, results and discussion

2.1. Preliminary experiments

The HPLC method for paclitaxel was based on the literature (Ringel and Horwitz 1987; Sharma et al. 1995a). The retention time of paclitaxel was about 9 min and the retention factor was 2.58. Calibration lines were found to be linear ($r = 0.9994$) in the concentration range of 0.01–1 mg paclitaxel/ml. The repeatability of the injection of two paclitaxel standards was 0.86% and 0.80% RSD, which is in agreement with the Pharmacopoeia criteria (European Pharmacopoeia 2002).

The HPLC-method was checked on its ability to investigate possible degradation of paclitaxel during the experiments. Therefore paclitaxel was submitted to forced degradation conditions. Samples of paclitaxel, exposed to 1N HCl or to 1 N NaOH, resulted in the formation of degradation products, well separated from the paclitaxel peak. Paclitaxel is also sensible to oxidation; a chromatogram of a diluted solution of hydrogen peroxide (3% H_2O_2) demonstrated oxidation products, which again were well separated from the paclitaxel peak. The HPLC system (MeOH-water: 70/30 (v/v)) thus seemed suitable for stability-indicating measurements. No peak deformations or shoulders, which could indicate the presence of a degradation product under the paclitaxel peak, were observed.

2.2. Liposomes

2.2.1. Preparation of paclitaxel-loaded liposomes

2.2.1.1. Selection of crystal-free paclitaxel liposomes

The composition of the liposomes was based on earlier investigations (Gabriëls et al. 2002).

In a study to prepare crystal-free paclitaxel liposomes with highest entrapment efficiency, several MLV-liposome samples with paclitaxel concentrations of 1, 1.5 and 2 mg paclitaxel per ml liposome suspension, were prepared. In all samples fine, needle-shaped crystals, often lying in groups forming stars, immediately were seen. This phenomenon is also described in the literature (Balasubramanian and Straubinger 1994; Sharma et al. 1995a). Therefore, the amount of paclitaxel was decreased and liposomes with 0.4, 0.5, 0.6, 0.75 and 1 mg paclitaxel per ml liposome suspension were prepared. Only in the samples with $\leq$0.5 mg paclitaxel/ml liposome suspension, no crystals were found, even not after days of storage. Therefore, the crystal-free liposome formulation with the highest amount of paclitaxel, i.e. 0.5 mg/ml, was selected for further investigations.

2.2.1.2. Physical stability of crystal-free paclitaxel liposomes: visual evaluation and morphology

A first possibility to evaluate whether the MLV- and SUV-formulations were well prepared is visually (see further). Afterwards, some selected were made visible after a 'freeze-fraction' procedure (Mayer et al. 1985; Schurtenberger and Hauser 1993), of which the results can be seen in Fig. 1.

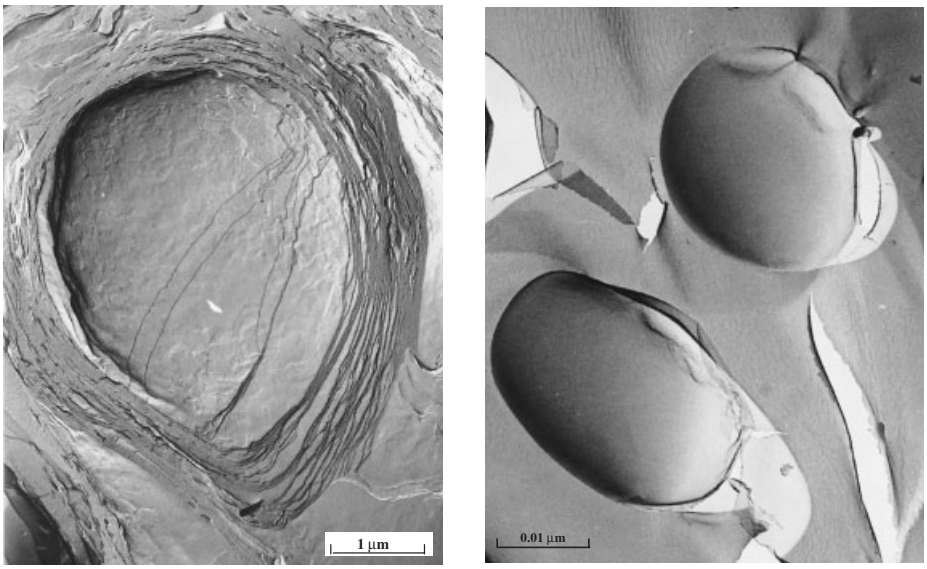


Fig. 1:
(a): Freeze-fraction-image of loaded MLV's, in which paclitaxel is incorporated
(b): Reconstituted SUV solution

The prepared MLV's (with 0.5 mg paclitaxel/ml liposome suspension) remained visibly stable during the examined period of 1 week: the white colour remained. This preparation was also morphologically investigated. Fig. 1a clearly shows the lamellar structure of the MLV. No crystals were observed.

The prepared SUV's (transparent blue-grey colour) were only stable during approximately 24 h; they slowly transformed again to MLV's (white colour). This colour change was also noticed by New (1990). Therefore, different SUV's, i.e. without cryoprotector, with 1 and 5% of sorbitol, and with 1, 3 and 5% of sucrose as cryoprotector, were prepared and lyophilised to obtain a more stable formulation. The lyophilised SUV's were reconstituted with water before sampling, vortexed and visually compared with those not lyophilised.

After reconstitution of the SUV's with sorbitol as cryoprotector, the white colour of the MLV's was observed. There was no visual difference between the 1 and 5% sorbitol preparations. This indicated that the SUV's did not withstand the lyophilisation. Ausborn et al. (1992) confirm that sorbitol is not a good cryoprotector since in fact it promotes the fusion of lipids. According to Ausborn et al. (1992) and Macdonald and Macdonald (1993) sucrose should be better. Indeed, after reconstitution of these SUV's, a blue-grey colour was perceived. The formulation with 5% sucrose was more transparent (thus less white colour of reforming MLV's) than with 3% and 1%, and this transparency was similar to that of the SUV's before lyophilisation. Therefore, the SUV-formulation with 5% sucrose became the selected and the unilamellar structure is clearly visible in Fig. 1b.

Finally, two formulations were selected: 60 ml of MLV's containing 30 mg paclitaxel, and 60 ml of SUV's containing 30 mg paclitaxel and 5% sucrose as cryoprotector.

2.2.2. Evaluation of the paclitaxel liposomes

2.2.2.1. Dose of paclitaxel in the liposomes

Determination of free paclitaxel. Non-capsulated paclitaxel was found in the supernatant: 6 µg/ml paclitaxel, which was similar to our determination of the solubility of paclitaxel in water.

This is 1000 times below the targeted concentration of 6 mg/ml, occurring in the currently marketed Taxol® formulation. In the literature, water-solubility values between 0.35–30 µg/ml are reported (Balasubramian et al. 1994; Dordunoo and Burt 1996; Liggings et al. 1997; Needham and Sarpal 1998; Sharma et al. 1995b; Suffness 1993; Straubinger et al. 1995a; Szente et al. 1999).

Determination of total paclitaxel. Methanol and chloroform were employed to dissolve the liposomes, which was necessary to determine the total paclitaxel concentration. No interference of the liposomes components with the paclitaxel peak in the chromatograms was observed. The incorporated paclitaxel was recovered (100.4%, $n = 3$, $RSD = 1.09\%$).

For SUV liposomes, 97.6% ($n = 2$, $RSD = 0.89\%$) and after lyophilisation, 97.5% ($n = 2$, $RSD = 0.95\%$) of paclitaxel was recovered. No degradation products of paclitaxel were noticed in the chromatograms, immediately after preparation (nor after lyophilisation). The loss of 2.4% were MLV's which were situated in the precipitate eliminated after sonification (see 3.3.1.): sonification did not convert all MLV's into SUV's. Partly because of this and also because of the short shelf life of the SUV's (24 h) (see above), we decided to abandon the study for a SUV-formulation.

The following experiments were continued with only the MLV-formulation, containing 30 mg paclitaxel/60 ml MLV liposome suspension.

2.2.2.2. Diluting paclitaxel liposome formulations

Before evaluating the stability of paclitaxel in the liposomes and of the liposomes themselves, crystal formation was checked by diluting the formulations. Taxol®, the commercial paclitaxel formulation, was administered intravenously. By diluting, precipitation occurs in the perfusion (Lubejko and Sartorius 1993). To avoid the particles entering the blood stream, a 0.22 µm filter is placed at the end of the infusion set (AVGI 2001; Leikin and Paloucek 1996; Lubejko and Sartorius 1993). The amount of paclitaxel lost in this approach is unknown.

No crystals were found in the dilutions of the liposome formulation during a 12 h control, the maximum therapeutic time of a Taxol® perfusion. This indicated the suitability of the selected formulation for administration with perfusions.

2.2.2.3. Chemical stability of paclitaxel in MLV liposomes

No degradation products of paclitaxel were noticed after 0, 30, 40 and 52 days, both when stored at 4 and at 25 °C. The initial concentration of paclitaxel was recovered after 52 days at 25 °C (100.4%, n = 3, RSD = 0.98%), but 20% was lost after 100 days (79.9%, n = 3, RSD = 0.94%). The samples, stored at 4 °C, remained stable, even after 140 days (99.8%, n = 3, RSD = 0.95%). Therefore, it is to be recommended to store the liposome formulation in the refrigerator.

2.2.3. Chemical stability of the liposomes

The preparation of MLV's with paclitaxel was adequate, but we still did not know whether the liposomes themselves were stable during a given disposal time. Therefore the chemical stability of the liposome components was examined.
Despite the great need for quality control of liposomal preparations, because most of the degradation products are precursors of severe diseases, as atherosclerosis, rheumatism and cancer (Jiang et al. 1992), the chemical stability of liposomal compounds has received very little attention in the literature. Therefore, it is important that the prepared liposomes contain as less as possible oxidation products.

2.2.3.1. Separation of phospholipids from the liposomes

Before examining the degradation of the polyunsaturated fatty acids in the phospholipids (EPC), it was necessary to separate them from the other liposome compounds: α-tocopherol and CH in case of empty liposomes, and α-tocopherol, CH and paclitaxel in case of loaded liposomes. The solid phase extraction was based on the method of New (1990) (Fig. 2). Fraction 2, the chloroform fraction, contained the phospholipids, CH and the anti-oxidant. This fraction was further separated on a silica SPE column. Separation between CH, α-tocopherol (fraction 3) and EPC (fraction 4) took place. Before examining the latter fraction on degradation, it was necessary to evaluate whether paclitaxel does not disturb the determination of the conjugated dienes or the hydroperoxides. So, in case of loaded liposomes, it was necessary to know that paclitaxel would not eluted in fraction 4, the phospholipid fraction. Paclitaxel was only found in the first fraction, the acidic methanol. In the other, neither paclitaxel nor any degradation product was detected. We could conclude that paclitaxel will not disturb the determination of the conjugated dienes or the hydroperoxides. The other liposome components: α-tocopherol and CH will not disturb the determination of the EPC oxidation products as they were separated from EPC.

2.2.3.2. Determination of conjugated dienes

It is worth noting that a free radical chain reaction can result in an extensive formation of conjugated dienes and trienes without the appearance of peroxides. Consequently, no single test is sufficient to determine the extent of oxidation of phospholipids (New 1990).
To determine conjugated dienes, the method described by New (1990) was applied. The samples were diluted until a concentration of approximately 0.05 mg lipids/ml ethanol. The molar absorbance coefficient (ε) for dienes at 233 nm is 30,000 (New 1990).
The concentration of the dienes was calculated from the measured absorbance and the theoretical ε value. Directly after preparation and after 1 month of storage at 25 °C and 4 °C, no dienes were detected, neither in the empty nor in the loaded liposomes. After 3 months, the different liposomes were re-analysed. The percentages conjugated dienes were 0.87% and 0.80% at 4 °C, and 0.89% and 0.85% at 25 °C for the paclitaxel (n = 2) and for the empty liposomes (n = 2), respectively. The percentages of the conjugated dienes thus were small. No difference between the liposomes with and without paclitaxel at the different temperatures was seen. No FDA guidelines exist for the presence of such unwanted degradation products. Less than 1% of oxidation products, which is negligible, were formed. Longer time stability will reveal whether the amount of conjugated dienes will increase considerably. Oxidation is minimised using inert atmosphere (nitrogen) and an antioxidant (α-tocopherol).

2.2.3.3. Oxidation of liposomes: determination of hydroperoxides

The oxidation products of lipids are hydroperoxides (−ROOH). To quantify them, the modified International

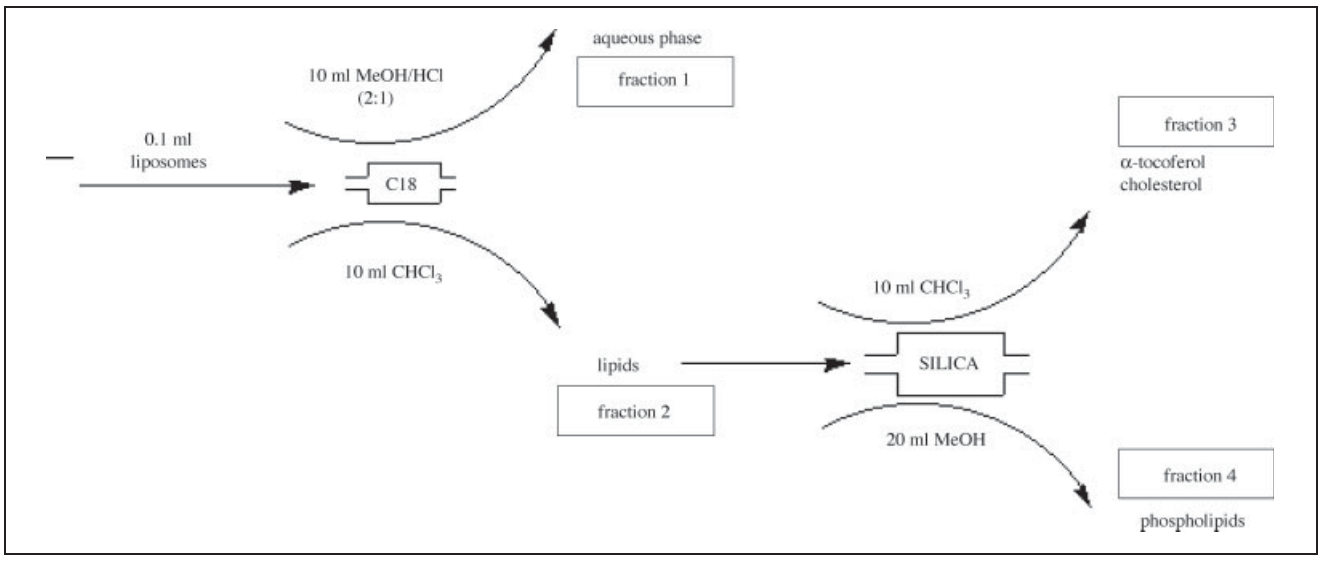
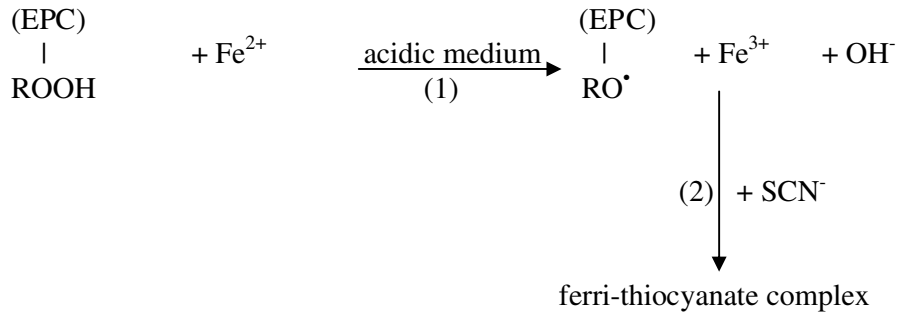


Fig. 2: Schedule of the solid phase extraction to extract phospholipids from the liposomes

Scheme: Modified International Diary Federation method (Gabriëls et al. 2000) applied for the determination of hydroperoxides



Diary Federation method, developed by Gabriëls et al. (2000), was used. The reaction applied is given in the Scheme and the method described in 3.3.4.2. Of course, EPC must be present during the ferro-ferri-oxidation (1), but it should be removed from the reaction medium before the coloured complex of ferri (Fe^{3+}) with thiocyanate (SCN^-) (2) develops. Removal was necessary as a secondary complex between ferri-thiocyanate complex and the organic phosphor of EPC can be formed. Therefore, an extraction method, based on the triangle of Bligh and Dyer (1959), was developed to separate the ferri-salt and EPC. Bligh and Dyer described a phase diagram of three components: water, chloroform and methanol. Depending on the proportions of these three solvents, either a mono- or a bi-phasic system is obtained. After development of the ferri-salt in the mono-phase (containing water, methanol and chloroform), EPC was extracted to the chloroform phase by creating a bi-phasic system. The cumene hydroperoxides (and EPC, in case of liposomes) are extracted in the chloroform phase, not interfering in the reaction of the formed ferri-ions with thiocyanate. The ferri-thiocyanate-complex was then spectrometrically determined at 478 nm in the supernatants (water-MeOH). Cumene hydroperoxide, containing 80% of cumene, was used as standard. An excess of the Fe^{2+} solution is needed. The concentrations of the cumene hydroperoxide standards with their corresponding absorbances are given in the Table. No hydroperoxides were formed in the liposomes ($A \leq 0.033$). The absorbances for the stored empty or loaded and the fresh prepared liposomes all were similar. After 3 months, still no hydroperoxides were noticed which is an important indication of the stability of the liposomes.
This later stage of the oxidation process takes place with the consumption of dienes, so that for longer stored formulations, it is possible to notice an increase in peroxides.

Table: Preparation and absorbances of the cumene hydroperoxide standards

Number standard	Working solution (ml) in standard	Standard conc. (μM) in mono-phase	End conc. of standard (μM) in bi-phase	Absorbance
blank	0	0	0	0
1	1	22.39	14.72	0.185
2	2	44.77	29.45	0.371
3	3	67.16	44.18	0.490
4	4	89.54	58.90	0.569
5	5	111.93	73.63	0.751

Mono-phase: volume proportion: $MeOH/CHCl_3/H_2O$: 2/1/0.8
Bi-phase: volume proportion: $MeOH/CHCl_3/H_2O$: 2/2/1.8

2.3. Conclusion

Paclitaxel is a potent antitumor agent, of which the formulation Taxol® has several side effects, due to the presence of the surfactant Cremophor® EL, included to improve solubility of paclitaxel. Much investigation is done to improve the solubility and to reduce the side effects correlated to Cremophor® EL. The solubility of paclitaxel in water is reported to be 0.35 to 30 μg/ml, while we found 6 μg/ml. This is about 1000 times below the targeted concentration of 6 mg/ml, occurring in Taxol® (30 mg/5 ml). The amount of encapsulated paclitaxel in crystal-free liposomes was only 0.5 mg/ml in a liposome suspension, composed of 144 mg egg phosphatidyl choline, 56 mg cholesterol and 1.5 mg α-tocopherol per ml. Therefore, 60 ml liposomes were needed to incorporate the required paclitaxel.
First, two formulations were selected: 30 mg paclitaxel/60 ml MLV's and 30 mg/60 ml lyophilised SUV's with 5% sucrose as cryoprotector. As after the SUV preparation only 97.5% of paclitaxel remained and because of the short shelf life of the SUV's, only the MLV-formulation was maintained. No crystal formation after dilution in perfusion liquids was noticed, which indicated its suitability for administration with perfusions. Liposomal formulations often have chemical stability problems. Paclitaxel remained stable in the prepared MLV's at 4 °C during the tested period of almost 5 months (140 days). The MLV's, empty or loaded with 0.5 mg paclitaxel/ml liposome suspension, remained stable during the tested period of 3 months.
The long-term stability (after 3 months) of the MLV's remains to be studied. In case, they are not long enough stable to be of industrial interest, their lyophilisation is possible.

3. Experimental

3.1. Materials

3.1.1. Chemicals

Paclitaxel (Mr = 854) was purchased from Acros Organics (Geel, Belgium), egg phosphatidylcholine (EPC) from Lipoid (Ludwigshafen, Germany), cumene hydroperoxide from Fluka (Buchs, Switzerland), hydrogen peroxide (H_2O_2, 30%) and sorbitol both from Federa (Brussels, Belgium), sucrose from Acros Organics and dextrose from UCB (Brussels, Belgium). The following products were all from Merck (Darmstadt, Germany): cholesterol (CH), α-tocopherol, ferrous chloride quadrihydrate, ammonium thiocyanate (NH_4SCN), disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12\,H_2O$), citric acid (mono hydrate), hydrochloric acid (37% w/w) (HCl), sodium hydroxide (NaOH), sodium chloride (NaCl), methanol (MeOH), chloroform ($CHCl_3$), dichloromethane (CH_2Cl_2) and absolute ethanol.
In all experiments mQ water was used, prepared in-house with a mQ water purification system (Millipore, Molsheim, France).

3.1.2. Apparatus

The HPLC system consisted of an L-6000 pump (Merck-Hitachi, Tokyo, Japan), equipped with a Rheodyne 7125-075 injector (Cotati, California) with a 20 µl loop, a variable wavelength Perkin Elmer LC 90 UV spectrophotometric detector (Shelton, Connecticut) and a D-2500 Chromato-integrator (Merck-Hitachi). An analytical reversed-phase (RP) C_{18} Lichrospher® 100, 250 × 4 mm, 5 µm column was used in connection with a C_{18} guard column Lichrospher® 100, 4 × 4 mm; 5 µm, both from Merck. An Uvikon 860 spectrophotometer (Kontron Instruments, Zürich, Switzerland) was used for measurements in the UV and visible range in 1 cm quartz cells Suprasil® 110-QS (Hellma, Aartselaar, Belgium). Solids were weighed either on a Sartorius basic analytical balance (Sartorius, Göttingen, Germany) or on a Mettler-Toledo M5SA microbalance (Mettler-Toledo, Zürich, Switzerland). The vortex mixer was from VWR International (Amsterdam, The Netherlands), the Certa centrifuge from International Equipment Company (Bedfordshire, England) and the water bath shaker from GFL (Gesellschaft für Labortechnik, Burgwedel, Germany). The pH measurements were performed using a Radiometer Copenhagen PHM 26 pH meter (Copenhagen, Denmark) calibrated daily using pH 4.00, 7.00 and 10.00 standard buffers (Merck). The pH of small volumes was measured by a WTW Multical pH meter (Metro Parkway, Florida). During forced degradation, an oven (Memmert, Swabach, Germany) was used. Solid phase extraction (SPE) columns, C_{18}, 3 ml, were obtained from Bond-Elut Analytichem International (Germany) and Seppak 3 ml silica gel columns from Baker (Philipsburg, NJ).

3.2. Methods

3.2.1. High-performance liquid chromatography (HPLC) analysis of paclitaxel

The mobile phase consisted of methanol-water 70/30 (v/v). The flow rate was 1 ml/min and UV absorbance detection was accomplished at 227 nm. Analyses were performed at ambient temperature (approximately 22 °C).
Standard solution. An accurately, on the microbalance weighed, quantity of 20 mg paclitaxel was dissolved in 100.0 ml methanol.
Linearity. The calibration line was prepared from a stock solution containing of 1.0 mg paclitaxel/ml mobile phase. From the stock solution working solutions were prepared in a concentration range of 0.01–1 mg/ml mobile phase.
Precision. The repeatability of injection was determined by six replicate injections of two paclitaxel standard solutions. The relative standard deviation (% RSD) of the peak area was calculated.
Degradation of paclitaxel. Forced degradation of paclitaxel was done with 240 µg/ml solutions. Samples were exposed to 1 N HCl and to 1 N NaOH at room temperature. Paclitaxel was also stored in 3% H_2O_2 at 45 °C during several days.

3.3. Liposomes

3.3.1. Preparation of liposomes: multi-lamellar vesicles (MLV's) and small unilamellar vesicles (SUV's)

Empty liposomes were MLV's or SUV's without activum, loaded liposomes contained paclitaxel as activum further in this paper.
Liposomes with 200 mg lipids per ml buffer were prepared. They contained 144 mg EPC, 56 mg CH and 1.5 mg α-tocopherol per ml liposome suspension. All compounds were dissolved in dichloromethane (CH_2Cl_2). This organic solvent then was evaporated under a nitrogen stream rotating on a Rock-'n-Roller (Snijders, Tilburg, The Netherlands) to inhibit oxidation of the EPC, CH (and paclitaxel, in case of loaded liposomes). Evaporation was done for 18 h (Lyolab Secfroid, Aclens-Lausanne, Switzerland). The next step was hydratation of the lipid dry film with an isotonic aqueous buffer at 4 °C during 24 h under slow rotation, resulting in an MLV suspension. The isotonic buffer consisted of $Na_2HPO_4 \cdot 12\ H_2O$/citric acid: 50/50, brought to pH 5 with 1 N NaOH and made isotonic with NaCl.
To obtain a SUV suspension, the MLV suspension with addition of 5% sucrose as cryoprotector was sonificated during 30 min with a titanium probe (Branson sonifier 250, Heinemann, Schwäbisch Gmünd, Germany) under N_2-stream and centrifuged (9760 × g) during 15 min at 4 °C. The precipitate (the remaining MLV's) on the bottom was eliminated. The SUV's were frozen during 30 min at −45 °C and then lyophilised for at least 18 h.

3.3.2. Preparation of paclitaxel-loaded liposomes

A series of 20 ml MLV-liposomes was prepared (as described in 3.3.1.) but with paclitaxel concentrations of 0.4, 0.5, 0.6, 0.75, 1.0, 1.5 and 2.0 mg per ml liposome suspension. The presence of paclitaxel crystals in these liposome formulations was evaluated using an optic microscope (Carl Zeiss, Oberkochen, Germany, enlargement 10 × 100).
SUV's, containing 0.5 mg paclitaxel/ml liposome suspension, were also made with 1 and 5% sorbitol and with 1, 3 and 5% sucrose as cryoprotector.

3.3.3. Evaluation of the paclitaxel liposomes

3.3.3.1. Investigation of the liposomes morphology

The microscopy 'freeze-fraction' method (Mayer et al. 1985; Schurtenberger and Hauser 1993) was applied to make the liposomes (empty and loaded) visible. Liposomal droplets were frozen by direct immersion in liquid Freon 22 and maintained in liquid nitrogen (−196 °C). The frozen blocks were admitted at −150 °C into the vacuum chamber of a Balzers BAF 400 freeze-etching unit (Liechtenstein) and cracked with a knife blade. Sublimation of water (freeze-drying) was achieved for 1 min at −100 °C. The etched faces were converted into replicates after platinum shadowing (45°, 8 s) and carbon coating. The specimens were rinsed away with water, sulphuric acid and sodium hypochlorite. Replicates were observed by a JEOL 100 SX transmission electron microscope (JEOL, Japan) at 80 kV accelerating voltage.

3.3.3.2. Total paclitaxel content and trapping efficiency of paclitaxel in the liposomes

To determine the total paclitaxel content in the liposome formulation, a sample of 0.5 g crystal-free liposome suspension was accurately weighed into a 5.0 ml volumetric flask and dissolved in a mixture of methanol/chloroform (2/1, v/v). A ten time diluted sample with methanol was injected into the HPLC system.
To measure the free (not entrapped) paclitaxel, approximately 1 ml of liposome suspension (MLV's or SUV's) was centrifuged at 2500 × g for 15 min at 4 °C. The supernatant was then analysed with the HPLC method.
The trapping efficiency (TE) was calculated, using the following formula:

$$\%\ \mathrm{TE} = (\text{total paclitaxel} - \text{free paclitaxel})/\text{total paclitaxel}^{*}\ 100 \quad (1)$$

in which total paclitaxel is the total concentration of paclitaxel measured in the crystal-free liposome formulation, and free paclitaxel is the total concentration paclitaxel in the upper aqueous phase after centrifugation of the liposomal suspension.

3.3.3.3. Diluting paclitaxel liposome formulations

MLV formulations loaded with 0.5 mg paclitaxel/ml liposome suspension were prepared. The dilution fluids were H_2O, NaCl 0.9% and dextrose 5%, respectively. The dilution was similar to the therapeutic one, i.e. to reach a concentration of 0.3 mg/ml perfusion solution. Every 30 min, the solution was visually evaluated for crystal formation under a Carl Zeiss microscope (Oberkochen, Germany, enlargement 10 × 100) during 12 h.

3.3.3.4. Chemical stability of paclitaxel in liposomes

Empty MLV's and MLV's loaded with 0.5 mg paclitaxel/ml liposome suspension were stored at 4 °C and at 25 °C. The chemical stability of paclitaxel was examined after 0, 30, 40, 52, 100 days storage both at 4 °C and at 25 °C and also after 120 and 140 days storage at 4 °C.

3.3.4. Chemical stability of the liposomes

3.3.4.1. Solid phase extraction of phospholipids from the liposomes (Fig. 2)

A C_{18} SPE column was first conditioned with 3 ml MeOH, then 0.1 ml liposome suspension was added. The column was eluted with 10 ml MeOH/HCl 0.1 N (2/1, v/v) (fraction 1) and 10 ml chloroform (fraction 2). The chloroform fraction was brought on a silica SPE column. Elution with 10 ml chloroform (fraction 3) and 20 ml MeOH (fraction 4) was performed. The fractions 1 and 4 were analysed with HPLC.

3.3.4.2. Determination of hydroperoxides and conjugated dienes

Calibration line of cumene hydroperoxide standard. A stock solution of 8 mM cumene hydroperoxide was prepared in MeOH/chloroform (2/1, v/v) (= solvent). A working solution of 0.56 mM was obtained from this stock by a dilution of 7 ml stock solution to 100 ml solvent.
Different volumes of the cumene hydroperoxide working solution were diluted with MeOH/ chloroform (2/1, v/v) to 25 ml (Table). To 3.0 ml of each of the obtained standards, 0.8 ml of a 1.14 mM ferrous chloride solution was added and vortexed. A mono-phasic system was obtained: the reaction of the hydroperoxides with the Fe(II)-ions could take place with the formation of Fe(III)-salts. To create a biphasic system, 1.0 ml chloroform and 1.0 ml water were added. This solution was vortexed and centrifuged. To 2.0 ml of the supernatant (water-MeOH), 0.4 ml of an aqueous colour agent 60 mM ammonium thiocyanate solution, was added. The *in situ* formed ferri chloride complexated with the ammonium thiocyanate. The complex was spectrophotometrically measured at 478 nm.
Determination of hydroperoxides and conjugated dienes in liposomes. The empty and loaded liposomes, stored at 25 °C and 4 °C, were investigated for the possible presence of conjugated dienes and hydroperoxides. A volume of 0.2 ml liposome suspension was added to the C_{18} SPE column and subsequently to the silica SPE column (see 3.3.4.1.). The last elution fraction (fraction 4 = 20 ml MeOH on the silica column) was divided into

15 ml and 5 ml. Both fractions were evaporated to dryness. The first was redissolved in 3 ml MeOH/chloroform (2/1, v/v) for the detection of hydroperoxides. The latter was dissolved in ethanol up to a concentration of 0.05 mg EPC/ml (New 1990) for the determination of the conjugated dienes. This solution was spectrophotometrically measured at 233 nm against ethanol as blank and the molar absorbance coefficient (ε) for dienes of 30,000 at 233 nm was used (New 1990).

Acknowledgements: Prof. G. Goffinet, Université de Liège, Dept. biologie générale et morphologique ultrastructural is gratefully thanked for the investigation on the morphology of the liposomes.

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