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Research paper

Paclitaxel/β-cyclodextrin complexes for hyperthermic peritoneal perfusion – Formulation and stability

Wim Bouquet [a], Wim Ceelen [b], Bernd Fritzinger [c], Piet Pattyn [b], Marc Peeters [d], Jean Paul Remon [a], Chris Vervaet [a,*]

[a] *Laboratory of Pharmaceutical Technology, Ghent University, Ghent, Belgium*
[b] *Department of Abdominal Surgery 0K12C, Ghent University Hospital, Ghent, Belgium*
[c] *Department of Organic Chemistry, Ghent University, Ghent, Belgium*
[d] *Department of Gastro-enterology 1K12E, Ghent University Hospital, Ghent, Belgium*

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Abstract

Due to its low aqueous solubility paclitaxel is currently formulated in a Cremophor EL®/ethanol mixture. However, the vehicle of this formulation causes several side-effects. Our objective was to formulate a tensioactive-free and solvent-free paclitaxel solution, which can be used for a hyperthermic intraperitoneal chemoperfusion procedure (HIPEC). The potential of chemically modified β-cyclodextrins to form complexes with paclitaxel was investigated as a means to increase the aqueous solubility of paclitaxel. Methylated β-CDs (randomly methylated and 2,6-dimethylated) showed the best ability to solubilise paclitaxel compared to sulfobutyl-ether- and hydroxypropyl-β-CD. The minimal ratio of paclitaxel versus randomly methylated-β-cyclodextrin (RAME-β-CD) yielding 100% inclusion efficiency was 1/20 (mol/mol). Paclitaxel/RAME-β-CD inclusion complexes prepared via freeze drying were stable for at least 6 months when stored at 4 °C. A 5 mg/ml paclitaxel solution was formulated using paclitaxel/RAME-β-CD-complexes. Upon dilution of these solutions, no precipitation was seen. After 24 h storage at room temperature or 2 h at HIPEC conditions (41.5 °C) the 1/40 (mol/mol) ratio showed the highest stability at paclitaxel concentrations of 0.1 and 0.5 mg/ml. When hydroxypropyl methylcellulose (HPMC) was added to the reconstitution medium, the stability significantly increased, offering the opportunity to reduce the amount of RAME-β-CDs in the formulation.

Keywords: Paclitaxel; β-Cyclodextrins; Hyperthermic intraperitoneal chemoperfusion (HIPEC); HPMC

1. Introduction

Taxol®, containing paclitaxel as a microtubule stabilising agent, was approved by the FDA for the treatment of breast and ovarian cancer in 1992 and 1994, respectively [1]. Unlike most antineoplastic agents, paclitaxel does not depolymerize the microtubules, but it promotes the polymerization of tubulin. The formed microtubules are extremely stable and inhibit the normal microtubule dynamics, which are essential for the cell cycle, thus causing cell death [2].

Since paclitaxel has a low aqueous solubility (0.4 μM or 0.34 μg/ml [3]), the drug is currently formulated in a 1:1 (v/v) Cremophor EL®/ethanol mixture, available as Taxol®. Amongst multiple problems caused by this vehicle, in particular by Cremophor EL®, the most important side effects include hypersensitivity reactions, nephrotoxicity and neurotoxicity [4]. Furthermore, the leaching of the plasticizer diethylhexylphthalate (DEHP) from polyvinylchloride (PVC) infusion sets into the Cremophor EL®/ethanol formulation should be considered as another

* Corresponding author. Laboratory of Pharmaceutical Technology, Harelbekestraat 72, 9000 Ghent, Belgium. Tel.: +32 9 264 80 69; fax: +32 9 222 82 36.
E-mail address: Chris.Vervaet@UGent.be (C. Vervaet).

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severe problem. Therefore, a lot of research is performed to reformulate paclitaxel using the following approaches: co-solvency, emulsification, micellisation, liposome formation, non-liposomal lipid carriers (microspheres, nanocapsules), cyclodextrins and local drug delivery devices [4].

β-Cyclodextrins (β-CDs) are cyclic oligosaccharides which consist of 7 covalently linked glucopyranose units. β-CDs have a cone-like structure, combining a hydrophilic exterior with a hydrophobic interior which can encapsulate hydrophobic drug molecules or parts of these molecules. The resulting inclusion complexes increase the aqueous solubility of the drug. β-CDs are chemically modified to enhance their solubility and their complexing activity.

Hyperthermic intraperitoneal chemoperfusion (HIPEC) after debulking surgery is a novel strategy in the treatment or prevention of peritoneal carcinomatosis. Using this technique the peritoneum is perfused with a solution (40–43 °C) containing a cytostatic agent. The rationale for this treatment lies in the combination of the tumoricidal properties of intraperitoneal hyperthermia [5] and various chemotherapeutic drugs. In addition, hyperthermia is known to enhance the antitumoral effect of several cytostatic drugs (doxorubicin [6], gemcitabine [7], cisplatin [8], tumor necrosis factor [8], mitomycin C [9]). By applying the drug locally, there is a pharmacokinetic advantage as the clearance of the drug is delayed and also higher concentrations of the cytotoxic drug can be applied. Randomized clinical trials are available to support HIPEC in the treatment and prevention of peritoneal carcinomatosis following resection of pathological gastric cancer [10]. Phase I data showed a $\geqslant$1000-fold exposure to paclitaxel in the peritoneal cavity compared with the systemic compartment [11]. The dose-limiting toxicity of intraperitoneal administered paclitaxel was found to be abdominal pain which could be due to the drug, the vehicle (ethanol/Cremophor EL®-mixture in case of Taxol®) or both. The latter seems the most plausible, as in recent years it has become clear that Cremophor EL® is not an inert vehicle, but exerts a range of biological effects, some of which have important clinical implications [12].

The purpose of this study was to formulate a solvent- and tensioactive-free paclitaxel solution, based on chemically modified β-CDs, which can be used for a HIPEC procedure.

2. Materials and methods

2.1. Materials

Randomly methylated-β-cyclodextrin (RAME-β-CD) with a total degree of substitution (TDS) of 13 and 2, 6-dimethyl-β-cyclodextrin (DIME-β-CD) with a TDS of 14 were purchased from Cyclolab (Budapest, Hungary). The following cyclodextrins were donated: sulfobutylether-β-cyclodextrin (SBE-β-CD), TDS 6.5 (Captisol®, Cydex, Overland Park, USA); hydroxypropyl-β-cyclodextrin (HP-β-CD), TDS 3 (Encapsin®, Johnson & Johnson, Beerse, Belgium); hydroxypropyl-β-cyclodextrin (HP-β-CD), TDS 4.5 (Kleptose HPB®, Roquette, Lestrem, France); hydroxypropyl-β-cyclodextrin (HP-β-CD), TDS 5.2 (Cavitron 82005®, Cerestar, Vilvoorde, Belgium); hydroxypropyl-β-cyclodextrin (HP-β-CD), TDS 6.1 (Cavitron 82006®, Cerestar, Vilvoorde, Belgium). Hydroxypropyl methylcellulose (HPMC) (Metolose® 60SH-4000, Shin-Etsu, Tokyo, Japan) was also donated. Paclitaxel was purchased from Acros Organics (Geel, Belgium).

2.2. Preparation of the inclusion complexes

First the required amount of paclitaxel was dissolved in absolute ethanol (Merck, Overijse, Belgium), in a ratio of 1–60 (w/w). β-Cyclodextrins were added to the solution to obtain paclitaxel/β-cyclodextrin ratios varying from 1/60 to 1/20 (mol/mol) and the solution was placed in an ultrasonic bath (Sonis 3 GT, Iskra-Pio, Šentjernej, Slovenia) for 5 min. Next, phosphate-buffered saline (PBS) (10 PBS tablets (Sigma, Bornem, Belgium) in 2 l demineralised water) was added in a ratio of 1/2 (w/w) versus the CD-fraction. Next, the solution was placed in an ultrasonic bath for 1 min and afterwards stirred with a magnetic stirrer for 5 min. After evaporating most of the solvent from the solution under reduced pressure by a rotavap, the solution was freeze dried for 24 h at −50 °C and at 1 mbar, after being frozen at −70 °C using solid carbon dioxide. After freeze drying, a white amorphous powder [13] was obtained.

2.3. Paclitaxel determination

The HPLC-system consisted of a pump (L-6000, Merck-Hitachi, Tokyo, Japan), an integrator (D-2000, Merck-Hitachi, Tokyo, Japan), an injector (Vici, Valco Instruments, Houston, USA) with a loop of 25 μL and a UV/vis detector (UV 2000, Spectra-systems, Darmstadt, Germany). Detection was performed at a wavelength of 227 nm. Chromatographic separation was achieved with a guard column (Lichrospher® 100-RP-18, 4 × 4 mm (5 μm), Merck, Darmstadt, Germany) and an analytical column (Lichrospher® 100-RP-18, 125 × 4 mm (5 μm), Merck, Darmstadt, Germany). Before use, the mobile phase consisting of acetonitrile (Biosolve, Valkenswaard, The Netherlands) and 0.1% (v/v) phosphoric acid in water (Acros Organics, Geel, Belgium) (42:58, v/v) was degassed by ultrasonication under vacuum. A calibration curve was validated for a concentration ranging from 1 to 100 μg paclitaxel/ml.

2.4. Determination of inclusion efficiency

In order to evaluate the inclusion procedure and to measure the amount of paclitaxel that was complexed with β-cyclodextrins, an amount of freeze dried material, equivalent to 1 mg paclitaxel, was dispersed either in 10 ml PBS or in 10 ml mobile phase used for HPLC-analysis. The concentration of dissolved paclitaxel in both samples was

determined with the HPLC-method. The mobile phase dissolved free as well as complexed paclitaxel, whereas only complexed paclitaxel dissolved in PBS (aqueous solubility of paclitaxel: 0.34 μg/ml). Therefore, the concentration in the mobile phase was regarded as the 100% value and the concentration in PBS was expressed relative to the concentration in the mobile phase to calculate the inclusion efficiency. Thus, any increase in aqueous solubility was considered to be due to the interaction and formation of complexes between paclitaxel and β-CDs.

2.5. Characterisation of paclitaxel/RAME-β-CD complex

(1) Thermal analysis of the samples was performed via differential scanning calorimetry (2920 Modulated DSC, TA Instruments, Leatherhead, UK). Samples (approximately 10 mg) were heated in sealed aluminum pans at 10 °C/min to 400 °C under N_2 purge. Both complexes and physical mixtures of paclitaxel and RAME-β-CDs (molar ratios ranging from 1/20 to 1/60) were analysed.
(2) ^{1}H NMR spectra of complexes in 1/20 and 1/40 (mol/mol) ratios were obtained in D_2O solution at 25 °C at 700 MHz using a Bruker AVANCE II spectrometer equipped with a 5 mm TXI Z-gradient probe head.

2.6. Stability

The stability of paclitaxel in PBS was tested at room temperature (25 °C, during 24 h) and at HIPEC temperature (41.5 °C, during 2 h) for 0.1 and 0.5 mg/ml paclitaxel solutions with or without HPMC and at 4 °C for a 5 mg/ml paclitaxel solution (during 2 weeks). In the experiments where HPMC was used, the polymer was added to the reconstitution medium in a concentration of 0.1% (w/v). The solutions were stored in sealed vials exposed to daylight, except for the 5 mg/ml solution which was stored in the dark. Samples, taken at set intervals, were filtered through a 0.22 μm filter (Spartan 30/0.2 RC) (Schleicher & Schuell, Dassel, Germany) and analysed by HPLC (following appropriate dilution using PBS). When precipitation was observed in the 5 mg/ml paclitaxel solution, the samples were centrifuged at 450*g* for 10 min and aliquots of the supernatans were analysed by HPLC after filtration using a 0.2 μm filter (Spartan 30/0.2 RC). To determine the stability of the freeze dried product, the paclitaxel concentration was determined by HPLC after storing the freeze dried material for 3 and 6 months in sealed vials at 4 °C.

2.7. Analysis of the freeze dried product

The water and ethanol content of the starting material and the freeze dried products was determined. The products (200 mg) were analysed for their water content using a Karl Fisher titrator (Mettler Toledo DL 35, Beerse, Belgium), while their ethanol concentration was determined using a head-space gas chromatography flame ionization detection (GC-FID) method. About 20 mg material was dissolved in 0.5 ml H_2O, next 0.5 ml H_2O (HPLC grade) and 0.5 ml of an internal standard solution (*tert*-butanol in H_2O, 40 mg/100 ml) were added before injection into the GC-FID system.

2.8. Statistical analysis

Results were analysed with SPSS 14.0 statistical software. For the 24 h stability experiment the effect of the ratio was evaluated using a one way ANOVA and a Scheffé post hoc test. The stability data at HIPEC conditions were evaluated using a two way ANOVA with pairwise comparisons within each factor, using a Bonferroni correction. For all results the homogenity of variances was analysed with the Levene's test and the normality of the residuals was checked for by a Kolmogorov–Smirnov test.

3. Results and discussion

3.1. Solubilisation of paclitaxel

3.1.1. Selection of the chemically modified β-cyclodextrins

In this research project, different chemically modified β-cyclodextrins were evaluated in order to obtain a tensioactive- and solvent-free paclitaxel formulation based on these β-cyclodextrins. By reformulating the commercially available Taxol® solution, it was the intention to reduce the dose-limiting toxicity of Cremophor EL® by replacing it with β-cyclodextrins, being fully aware that these compounds could also exert toxic effects, especially renal toxicity. This will be closely monitored during future in vitro and in vivo experiments. RAME-β-CD, DIME-β-CD, SBE-β-CD and the different HP-β-CDs were investigated for their paclitaxel solubilising effect. In a first screening experiment at a fixed cyclodextrin concentration (1/80 ratio (w/w)) the highest amount of paclitaxel in PBS was recovered using methylated-β-cyclodextrins (approximately 90% inclusion efficiency). SBE-β-CD and HP-β-CD had a very low affinity for paclitaxel and induced only a small increase of paclitaxel solubility (about 2% inclusion efficiency). The degree of substitution of HP-β-CD had a minor effect on paclitaxel inclusion. These results confirmed previous literature reports: methylated-β-cyclodextrins provided a better enhancement of the aqueous solubility of paclitaxel compared to HP-β-CD [3,14]. As both methylated-β-cyclodextrins produced similar results, RAME-β-CD were chosen for futher research because of economical reasons (lower cost). Using the inclusion procedure via freeze drying the amount of RAME-β-CD could be reduced to a molar ratio of 1/20 and still complete inclusion of paclitaxel was obtained. After reconstitution in PBS, all ratios (1/20 to 1/60 (mol/mol)) produced visually clear solutions at a

concentration of 1 mg/ml paclitaxel. At a lower ratio (<1/20), not all paclitaxel molecules (about 60% inclusion efficiency) were complexed by RAME-β-CD and no visually clear solutions could be obtained. The water content of the freeze dried powder varied between 1.4% and 2.2%, whereas the original RAME-β-CD material had an average water content of 5.4%. Due to the large volumes perfused through the peritoneal cavity during HIPEC and because ethanol is known to enhance the sedative effects of anaesthetics it is imperative that the ethanol content (used as a co-solvent during production of the freeze dried material) be kept to a minimum. The ethanol content of the freeze dried material varied between 2.5% and 3.7%. At these low concentrations, no problems are expected during HIPEC.

3.1.2. Characterisation of paclitaxel/RAME-β-CD complex

Differential Scanning Calorimetry (DSC) was used to identify the complexes in the freeze dried powder. In contrast to a physical mixture of paclitaxel and RAME-β-CD, no endothermal melting peak of paclitaxel (at 225 °C) was observed in the freeze dried material, indicating that inclusion complexes have been formed during processing (Fig. 1). This was most obvious at the lowest molecular ratio (1/20) as at higher ratios (>1/40 molar ratio) the sensitivity for the paclitaxel signal was rather low due to the large amount of cyclodextrins in the formulation.

^{1}H NMR spectroscopy was used to further support complex formation. Given the very low solubility of paclitaxel in D_2O, the fact that a set of clearly visible resonances, distinctive for paclitaxel (between 7.5 and 8.5 ppm), is observed can only be explained from complexation as uncomplexed paclitaxel (pure and in a physical mixture) was unable to produce a spectrum (Fig. 2).

3.2. Stability

To test the stability of paclitaxel solutions, the freeze dried material was reconstituted with PBS. Stability is expressed as the concentration of paclitaxel dissolved in the solution at the time of sampling compared to the concentration obtained immediately after reconstitution. As it is the objective of this study to formulate an alternative for Taxol®, which is available in a concentrated solution of 6 mg paclitaxel/ml, a maximum concentration of 5 mg/ml was chosen for the paclitaxel/RAME-β-CD-formulation. This concentration could be obtained at all ratios, but using lower molar ratios (<1/40) precipitation occurred within 48 h. The higher ratios (⩾1/40) remained stable for 2 weeks: after storage at 4 °C and out of the light, they contained ⩾95% of paclitaxel. The improved stability of solutions containing higher amounts of RAME-β-CD is in accordance with data reported in the literature [14]: cyclodextrins not only enhanced solubility, but also the stability of the solutions, containing paclitaxel.

Precipitation upon dilution can be a major problem when drugs with a poor aqueous solubility are given intravenously via an infusion set. Throughout all experiments precipitation upon dilution did not occur with this paclitaxel/RAME-β-CD formulation at all molar ratios. These observations are in good agreement with Sharma et al. [3], who reported no precipitation upon dilution of paclitaxel solutions which have a concentration below 12 mg/ml in 50% DIME-β-CD, whereas higher paclitaxel concentrations precipitated when diluted. Concentrations above 12 mg/ml are of no interest for a HIPEC procedure as lower concentrations are administered due to the large volume used during intraperitoneal perfusion. At all molar ratios the freeze dried material remained stable for at least 6 months when stored at 4 °C.

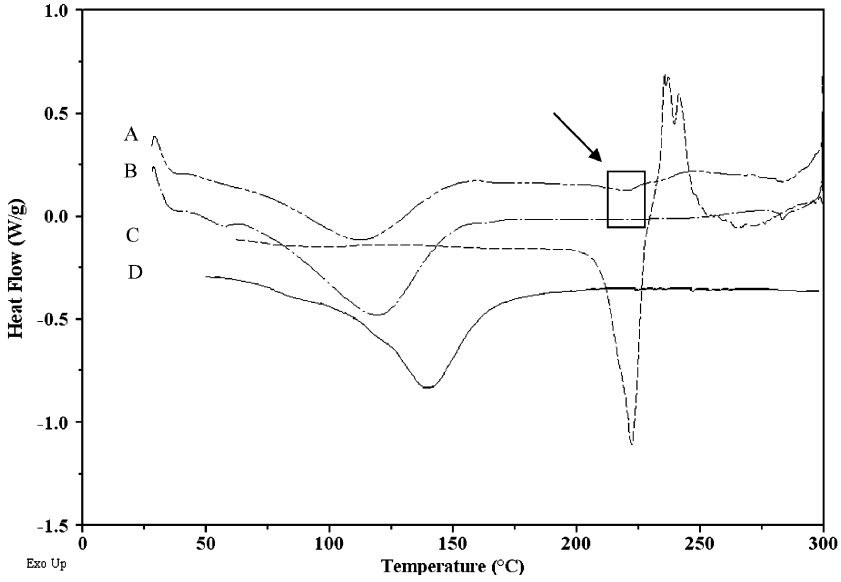

Fig. 1. DSC thermograms of a physical mixture (A) and an inclusion complex (B) of paclitaxel and RAME-β-CD (1/20 molar ratio), paclitaxel (C) and RAME-β-CD (D). The frame indicates the zone of interest for evaluation of complex formation between paclitaxel and RAME-β-CD.

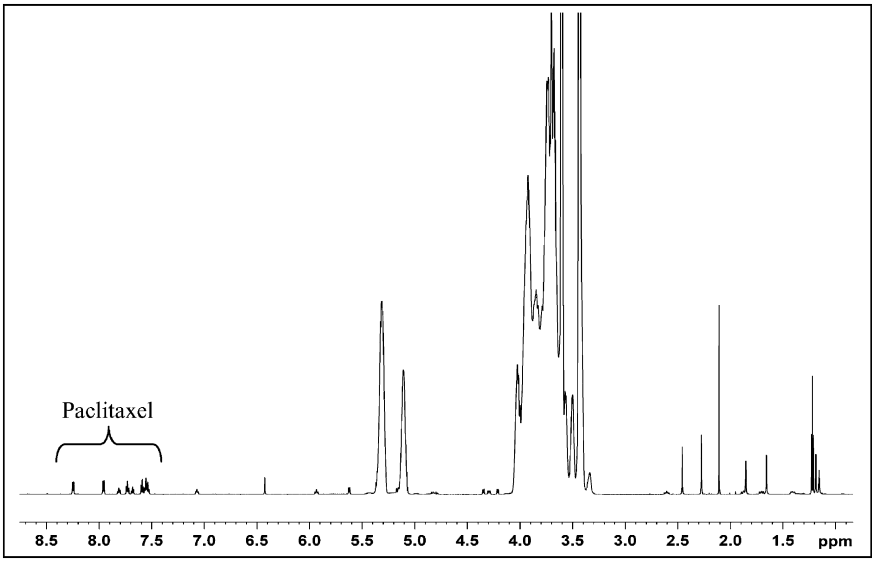

Fig. 2. Seven hundred megahertz ^{1}H NMR spectrum of the 1/20 (mol/mol) inclusion complex. Clearly distinguishable paclitaxel resonances, from the phenylgroups, appear between 7.5 and 8.5 ppm.

As it is the objective to use this RAME-β-CD/Pac formulation for an experimental HIPEC procedure in rats, the stability of these solutions was evaluated at room temperature for 24 h and at HIPEC conditions (41.5 °C, 2 h). The stability results at room temperature will be an indication whether the solutions should be prepared immediately before administration or can be prepared in advance. Two paclitaxel concentrations (0.1 and 0.5 mg/ml) were chosen, taking into consideration the body surface area of a rat (0.03–0.04 m^2), the intraperitoneal dose (±170 mg/m^2) and the volume used for the HIPEC procedure (±80 ml).

The stability of a 0.1 mg/ml paclitaxel solution after 24 h storage at room temperature was poor (Table 1). Only the formulation with a 1/40 molar ratio had a stability above 50% (69.5 ± 20.4%). A lower ratio significantly reduced the stability of the complex, whereas a higher ratio did not result in a significant improvement of stability. At a paclitaxel concentration of 0.5 mg/ml the stability was higher and ranged from 34.4% to 97.0% (Table 1). However, due to the large variation on these data, it was impossible to perform any statistical analysis, even after transformation. Taking both concentrations into consideration the 1/40 molar ratio was the most stable. The reason for this optimum in drug/RAME-β-CD ratio remains unclear, but might be due to a molecular interaction of paclitaxel [15]. Depending on the drug concentration and the polarity of the solvent paclitaxel interacts via intermolecular hydrogen bonds, forming a stack of drug molecules in solutions. These stacks influence the physical stability because they act as nuclei which promote aggregation and finally precipitation of paclitaxel. Since precipitation was the primary cause of instability of these paclitaxel/RAME-β-CD solutions, we assume that this self-association may be influenced by the β-cyclodextrin concentration present in the solutions. A variable degree of self-association could explain the high variability in paclitaxel stability.

Stability data at HIPEC conditions showed no interaction between molar ratio and time, hence both variables were evaluated individually (Fig. 3). At both drug concentrations, the stability at a 1/20 molar ratio was significantly lower compared to the other evaluated ratios (1/40 and 1/60). For all ratios a significant decrease of stability in function of time was measured, but – similar to the stability data at room temperature – the highest drug

Table 1
Stability ($n = 3$) of paclitaxel/RAME-β-CD solutions, with or without HPMC[a] added, stored for 24 h in sealed vials at room temperature (25 °C)

Pac/RAME-β-CD ratio (mol/mol)	Without HPMC	With HPMC
	0.1 mg/ml Stability (%)	0.1 mg/ml Stability (%)
1/60	29.2 ± 21.9	97.0 ± 1.1
1/52	13.7 ± 5.1	96.9 ± 1.6
1/46	43.4 ± 22.0	96.8 ± 0.9
1/40	69.6 ± 20.4	96.4 ± 1.0
1/33	14.0 ± 8.7	95.5 ± 0.1
1/26	5.2 ± 3.6	95.1 ± 0.8
1/20	2.9 ± 1.3	94.3 ± 1.1
	0.5 mg/ml Stability[a] (%)	0.5 mg/ml Stability[a] (%)
1/60	95.4 ± 3.5	96.8 ± 14.2
1/52	78.8 ± 31.5	99.0 ± 2.3
1/46	97.0 ± 1.6	99.0 ± 6.2
1/40	94.8 ± 3.6	90.4 ± 10.2
1/33	66.3 ± 53.6	93.9 ± 4.7
1/26	35.1 ± 55.9	93.7 ± 2.9
1/20	34.5 ± 43.5	98.5 ± 4.9

Pac, paclitaxel; RAME-β-CD, randomly methylated-β-cyclodextrins; HPMC, hydroxypropylmethylcellulose.
[a] Metolose® 60SH-4000.

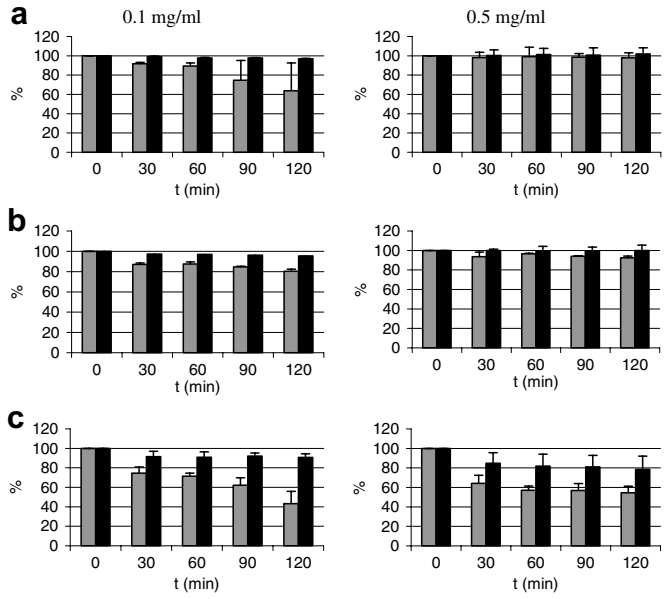

Fig. 3. Stability ($n = 3$) at hyperthermic conditions (41.5 °C) of aqueous paclitaxel solutions (▒ without and ■ with HPMC added to the reconstitution medium) formulated using different paclitaxel/RAME-β-CD ratios (mol/mol) (a) 1/60; (b) 1/40; (c) 1/20.

concentrations after 2 h at HIPEC conditions were detected for the 1/40 molar ratio.

As it has been reported that HPMC significantly increased the complexation behaviour of cylcodextrins (even at a HPMC concentration as low as 0.1%, w/v) [16,17], it was evaluated if HPMC might improve the stability of the complex and reduce the variability of the stability data. Adding 0.1% (w/v) HPMC to the medium used to dissolve the freeze dried paclitaxel/RAME-β-CD powder resulted after 24 h at room temperature in paclitaxel concentrations higher than 90% of the initial values (Table 1). Even after 10 days no precipitation was seen and more than 70% of the drug was recovered (data not presented). In contrast to the solutions without HPMC, there were no significant differences in stability between the different paclitaxel/RAME-β-CD ratios, offering the opportunity to lower the amount of cyclodextrins in the formulation. Adding HPMC also reduced the variability of the stability data (Table 1).

Similar to the stability testing without HPMC, an effect of the drug/CD ratio was seen after 2 h at HIPEC conditions (Fig. 3), independent of the paclitaxel concentration: 1/20 performed significantly worse than 1/40 and 1/60 (although its stability at a concentration of 0.1 mg/ml was also higher than 90%). Stability of the HPMC-containing formulations was time-dependent in case of 0.1 mg/ml solutions: drug concentrations at each time point were significantly lower compared to initial concentration, but the concentrations between 30 and 120 min were not significantly different. Adding HPMC to the formulation significantly improved stability at 41.5 °C (except for 0.5 mg/ml solution at a 1/60 ratio), although HPMC could not enterily prevent the precipitation of paclitaxel at a 1/20 molar ratio. These experiments showed that it was not necessary to add the hydrophilic polymers during the complexation process, but that adding the polymer to the reconstitution medium also had a beneficial effect.

The exact mechanism behind the stabilising effect of HPMC is not known. However, Ribeiro et al. [18] proposed a mechanism in which the polymer was able to stabilise the binary drug/CD complex by hydrogen bonds and van der Waals interactions, thus forming a stable ternary complex. Although the preparation method in this study (manufacturing binary drug/CD complexes which were dissolved in reconstitution medium containing HPMC) was different from the method used by Ribeiro et al. (immediate preparation of ternary complexes) interaction between the drug/CD complexes and HPMC is very likely since the Pac/CD complexes completely dissolved in the dissolution medium. In addition to the stabilising effect, we believe that the binding of the polymer to the binary complex also prevented precipitation since solutions without HPMC formed a precipitate and HPMC-containing solutions remained clear (even after 10 days). The limited loss of paclitaxel in solutions with HPMC was due to chemical degradation, evidenced by the appearance of additional peaks in the HPLC chromatogram. Based on these observations we assume that the interactions between the binary complex and the polymer not only stabilised but also prevented the binary complexes to aggregate and precipitate because of the steric hindrance the polymer caused.

4. Conclusion

Complexation of paclitaxel and RAME-β-CDs using a freeze-drying procedure allowed one to formulate tensioactive-free paclitaxel solutions suitable for HIPEC. Both the freeze dried product and paclitaxel solutions (0.1, 0.5 and 5 mg/ml) were stable during a sufficient period of time. Addition of HPMC to the reconstitution medium significantly improved the stability of the paclitaxel/RAME-β-CD formulation, allowing one to reduce the amount of RAME-β-CD in the formulation. This formulation of paclitaxel/RAME-β-CD will be used for further in vitro and in vivo experiments to evaluate its efficiency and toxicity.

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