Pharmaceutical development of investigational anticancer agents: focus on EO-9, AP5346, and GMP implications

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Farmaceutische ontwikkeling van nieuwe geneesmiddelen tegen kanker toegespitst op EO-9, AP5346 en GMP implicaties

(met een samenvatting in het Nederlands)

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“Als we wisten wat we deden,
heette het geen onderzoek.”

Albert Einstein

Contents

General introduction

Cancer is a major public health problem in western countries. In the United States currently, one out of four deaths is due to cancer. Incidence rates stabilized among men from 1995 through 2000, but continued to increase among females by 0.4% per year from 1987 through 2000 [1]. In the Netherlands, an increase in contribution of cancer to total mortality was seen in 2004 [2]. This indicates the necessity of more cancer research and the development of innovative anticancer agents. Especially for patients with advanced disease, chemotherapy is the best option.

The development of anticancer agents for chemotherapy is a complicated process and starts with the acquisition of the active pharmaceutical ingredient (API). Nature is the primary source of APIs with compounds isolated from plants (e.g. taxanes, camptothecins, and vinca alkaloids) and microbial sources (e.g. dactinomycin, doxorubicin). A relatively new source is the marine ecosystem. This source provides a growing number of promising agents [3-6]. Subsequently, the cytotoxic activity is tested in both *in vivo* and *in vitro* test models. Once this screening process is successfully completed, the selected compounds are manufactured in sufficient quantities according to the GMP-guidelines [7] and should be appropriately pharmaceutically characterized and formulated before animal toxicity studies are performed [8]. Formulation can be defined as the process in which the raw material of a compound is transformed into a pharmaceutical product, which can be administered safely, from the pharmaceutical point of view, to humans. In most cases, APIs can not be administered to humans as pure compound, because this is often hampered by poor aqueous solubility and stability characteristics of the API. Therefore, formulations are often complex mixtures which contain the API together with excipients like diluents, stabilizers, or preservatives. Since any component of the formulation may contribute to or modify the toxicity, it is imperative to evaluate the formulated pharmaceutical product as such in an early stage of the development process.

Anticancer drug formulations for experimental use are generally intended for intravenous use to obtain absolute bio-availability, circumvent possible disturbance of or degradation in the GI-tract and to be able to adjust or stop administration of the drug in case of acute toxicity. Consequently, the development of a pharmaceutical formulation of a novel anticancer agent is focussed on issues associated with the design of sterile and stable injectable products. Table I summarizes the steps of pharmaceutical development.

Table I. Steps of pharmaceutical development
Characterization of the API (structural and analytical)
Solubility- and stability studies
Design of the formulation
Manufacturing
Quality control analysis
(Bio)compatibility studies

The novel anticancer agents EO-9 and AP5346 described in this thesis are examples of potentially active anticancer agents. EO-9 is a mitomycin derivative and AP5346 is platinum-containing polymer. Both compounds are currently used in phase I and II clinical trials.

Characterization of API

For each recently developed, novel API a set of analytical methods has to be developed to enable characterization and quality control of this compound. The requirements for analysis of APIs can be found in regulatory guidelines of the FDA and the European Commission [7,9]. Characterization of the API has to be performed before solubility- and stability studies are initiated and is often comprised of a set of techniques (e.g. ultraviolet/visible spectrophotometry, nuclear magnetic resonance, mass spectrometry, infrared spectroscopy, chromatography), necessary to fully elucidate the structure. Furthermore, the purity, residual moisture content and/or residual solvent content are often determined. Subsequently, a reference standard must be selected (to compare other batches with) and specifications defined [10].

Solubility- and stability studies

Most anticancer agents possess poor solubility- and stability characteristics. Therefore, special formulations have to be developed before the APIs can be administered to the patients.

In this thesis, the formulation development of two different APIs, EO-9 and AP5346, is described. For EO-9 both alkaline and acid degradation is of importance as was seen for its precursor Mitomycin C [11,12]. Because of this instability, freeze drying was selected as manufacturing process. Furthermore, the use of an organic solvent and complexation with a cyclic sugar were shown to improve the solubility of EO-9.

For AP5346 solubility was not an issue. AP5346 is metal containing polymer and normally, metal complexes obtain low aqueous solubility and high susceptibility to ligand exchange, resulting in low stability, especially in solution. However, in AP5346 these problems were solved by binding of the metal complex to a biocompatible water

soluble macromolecule. Because of stability reasons, freeze drying was selected as manufacturing process. Furthermore, due to stability problems of both EO-9 and AP5346, heat sterilization in the final container was not possible. Therefore, aseptic manufacture was selected for both products.

Design of the formulation

During this phase a pharmaceutical formulation is developed taking into account the stability- and solubility characteristics of the API. Furthermore, in case of anticancer agents, minimizing the damage of healthy tissues by the API is also a point of concern. This can be obtained by local administration or drug targeting.
In this thesis, local administration of EO-9 is described for the treatment of superficial bladder cancer. Therefore, a formulation for a bladder instillation of this API was developed. The low solubility and instability of EO-9 in both aqueous solutions and physiological environment were the main items to be improved. These items were improved by dissolving EO-9 in 40% v/v tert-butyl alcohol containing sodium bicarbonate to keep EO-9 stable. After filling of the vials tert-butyl alcohol was subsequently removed during freeze drying, resulting in freeze-dried cake as final product. Prior to administration into the bladder, this cake was reconstituted with a special reconstitution solution to be able to dissolve EO-9 rapidly and to keep it stable in the bladder for an acceptable period of time.
Drug targeting was applied for the macromolecule AP5364, which was targeted to tumor tissue in a passive way. Due to an increased permeability of the tumor vasculature and decreased lymphatic drainage, macromolecules extravasate and accumulate mainly at the tumor side.
During these formulation studies, further development of the analytical methods is performed to enable characterization of the pharmaceutical products.

Manufacturing

After selection of a pharmaceutical product for further development, manufacture of batches for the clinic is the next step. Therefore, a manufacturing process is developed and optimized for each product. During the optimization, compatibility of the pharmaceutical formulation with the materials used during manufacture should be tested, especially when excipients are used (e.g. complexation agents, organic solvents). One of the problems which could occur is extraction of lipophile compounds from silicone tubing and is therefore discussed in this thesis.
In our formulation unit sterile products are manufactured which requires specific requirements. Although the production is generally performed on a small scale for phase I and II clinical trials, the manufacture has to comply with the guidelines for

Good Manufacturing Practice [13-16]. The two compounds described in this thesis, EO-9 and AP5346, are manufactured under aseptic conditions. Therefore, high standards for personnel and production facilities are required. According to the GMP-guidelines, risk assessment of the manufacturing process should be performed for each product [17-19]. In this thesis it is described how this can be performed using mixed effects analysis.

Quality control analysis

After manufacture, quality control analysis of each product is performed. As soon as a product enters phase I clinical trials, requirements and specifications must be selected in order to obtain and maintain a product of good quality. This is beneficial to the patient, but also very important for the outcome of the clinical trials (i.e. the results of a clinical trial with a pharmaceutical product of inhomogeneous quality could be negative even when the API is promising). However, setting of specifications of new experimental products is quite difficult, due to the availability of only a limited data-set of manufactured batches [20]. Furthermore, the manufacturing process and analytical methods are often not fully validated at this stage of development, resulting in larger deviations than found for established pharmaceutical products. The FDA and European commission have made regulations concerning the manufacture of experimental agents. However exact specification limits are not given [14,16]. Therefore, in this thesis suggestions are made how to set specifications and how to control product quality of experimental agents.

(Bio) compatibility

The last step in pharmaceutical development is the compatibility study. During this study, the stability of the API in the developed pharmaceutical product is tested in primary packaging materials and administration devices (e.g. infusion containers, tubing). This was performed for EO-9 as well as AP5346. Furthermore, biocompatibility studies can be performed depending on the route of administration. Biocompatibility studies are performed to simulate the *in vivo* situation to estimate the effect of the physiological environment on the product and vice versa. In case of EO-9 the stability of the formulation mixed with urine at 37ºC was tested, because EO-9 is administered and retained in the bladder for one hour, where it mixes with urine.

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CHAPTER 1

Pharmaceutical development, analysis & manufacture of EO-9

Chapter 1.1

Development of a bladder instillation of the indoloquinone anticancer agent EO-9 using tert-butyl alcohol as lyophilization vehicle

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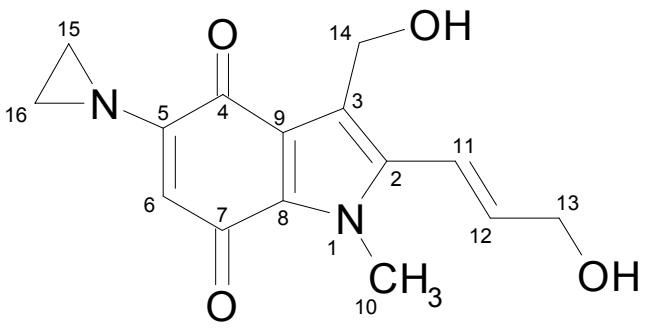
Abstract

EO-9 is a bioreductive alkylating indoloquinone and an analogue of the antitumor antibiotic mitomycin C. EO-9 is an inactive prodrug, which is activated by reduction of the quinone moiety to semiquinone or hydroquinone, generating an intermediate with an electrophilic aziridine ring system, which serves as a target for nucleophilic DNA. For the treatment of superficial bladder cancer a pharmaceutical formulation for a bladder instillation with EO-9 was developed. To improve solubility and stability of EO-9, tert-butyl alcohol was chosen as co-solvent for the solution vehicle in the freeze-drying process. Because EO-9 is most stable at alkaline pH sodium bicarbonate was used as alkalizer. Stability and dissolution studies revealed an optimal formulation solution for freeze-drying composed of 4 mg/ml EO-9, 10 mg/ml sodium bicarbonate, and 25 mg/ml mannitol in 40% v/v tert-butyl alcohol in water for injection. Optimization of the freeze drying process was performed by determination of the freeze drying characteristics of tert-butyl alcohol/water systems and differential scanning calorimetry analysis of the formulation solution. Furthermore, the influence of the freeze drying process on crystallinity and morphology of the freeze dried product was determined with X-ray diffraction analysis and scanning electron microscopy, respectively. Subsequently, a reconstitution solution was developed. A stable bladder instillation was obtained after reconstitution of freeze dried product containing 8 mg of EO-9 per vial to 20 ml with a reconstitution solution composed of propylene glycol/water for injection/sodium bicarbonate/sodium edetate 60/40/2/0.02% v/v/w/w, followed by dilution with water for injection to a final volume of 40ml. This pharmaceutical product of EO-9, named EOquin^{TM}, is currently used in Phase II clinical trials.

Introduction

EO-9, 3-hydroxymethyl-5-aziridinyl-1-methyl-2-(1H-indole-4,7-dione)-prop-ß-en-α-ol, is a bioreductive alkylating indoloquinone and a synthetic analogue of the antitumor antibiotic mitomycin C (MMC) (Figure I). The use of MMC is limited due to its dose-limiting toxicities. To improve the applicability of MMC, analogues such as EO-9 are developed. EO-9 is an inactive prodrug, like MMC, which is activated by reduction of the quinone moiety to semiquinone or hydroquinone, generating an

Figure I. Molecular structure of EO-9 (Mw = 288Da)



intermediate with an electrophilic aziridine ring system. This electrophilic ring system serves as a target for nucleophilic DNA. Reduction occurs under conditions of either low oxygen tension (hypoxic areas) or in presence of high levels of specific reducing enzymes. Preferential cytotoxicity of EO-9 towards hypoxic versus aerobic tumor cells and lack of bone marrow toxicity were seen [1]. Because solid tumors contain hypoxic areas, due to a discrepancy between capillary growth and oxygen need of the growing tumor [2], EO-9 is considered a promising treatment option for those tumors. Furthermore, EO9 is a good substrate for several reducing enzymes, e.g. NADPH:cytochrome P450 [3] and DT-diaphorase [4]. Therefore, EO-9 may be a promising alkylating indoloquinone for the treatment of cancer.

A freeze dried product for intravenous administration of EO-9 was developed by Jonkman-de Vries et al [5]. They dissolved EO-9 and lactose in water for injection. Due to its low solubility rate, EO-9 drug substance had to be grinded prior to dissolution. Furthermore, the aziridine moiety of EO-9 is easily protonated in acidic and neutral environment, followed by nucleophilic attack of water and formation of EO-5a. Therefore, Jonkman-de Vries et al. added sodium hydroxide to the formulation solution to pH 9-9.5 and freeze dried aliquots of 40 ml in 50 ml vials, resulting in a product composed of 8 mg EO-9 and 200 mg lactose per vial. However, after intravenous administration, no tumor response of EO-9 was seen [6,7]. This is likely due to instability of EO-9 in physiological environment of pH 7.4 (i.e. EO-9 is rapidly converted into the inactive EO-5a before it reaches the tumor)[8]. To administer EO-9 more directly to the tumor, the route of administration was changed to intravesical administration for the treatment of superficial bladder cancer and a formulation for a bladder instillation had to be developed. A complete new formulation was developed with the aim to resolve the disadvantages of the formulation for intravenous administration (i.e. instability of EO-9 after administration, grinding of a hazardous drug substance and the large filling volume per vial).

This paper describes the development of a freeze dried intravesical formulation of EO-9 with use of the organic solvent tert-butyl alcohol in the manufacturing process to improve solubility and stability of EO-9, resulting in much smaller filling volumes and making grinding of EO-9 redundant. Furthermore, the freeze drying program was optimized by determination of the freeze drying characteristics of tert-butyl alcohol/water systems and by differential scanning calorimetry analysis of the formulation solution. Subsequently, the influence of the freeze drying process on crystallinity of the freeze dried product was determined with X-ray diffraction analysis. Furthermore, a reconstitution solution was designed to obtain a stable bladder instillation.

Materials and methods

Chemicals

EO-9 drug substance was supplied by Spectrum Pharmaceuticals, Inc. (Irvine, CA, United States). Mannitol and sodium bicarbonate (Ph.Eur. grade) were purchased from BUFA (Uitgeest, The Netherlands). Tert-butyl alcohol was obtained from Merck (Darmstadt, Germany). Sterile water for injection was purchased from B. Braun (Melsungen, Germany). Phosphate buffer (5mM) was prepared in-house at the Department of Pharmacy & Pharmacology of the Slotervaart Hospital (Amsterdam, The Netherlands). Methanol was purchased from Biosolve B.V. (Amsterdam, The Netherlands). All chemicals obtained were of analytical grade and used without further purification.

High Pressure Liquid Chromatography (HPLC)

HPLC analysis was performed using an isocratic P1000 pump, AS 3000 autosampler and an UV 1000 UV/VIS detector, all from Thermo Separation Products (Breda, The Netherlands). The mobile phase consisted of 5mM phosphate buffer pH 7/methanol 70/30% v/v. A Zorbax SB-C18 analytical column (750 x 4.6mm ID, particle size 3.5 µm, Agilent Technologies, Palo Alto, California, USA) preceded by a guard column (reversed phase 10 x 3mm, Varian, Palo Alto, California, USA) was used. Detection was performed at 270 nm. An injection volume of 10 µL, flow rate of 0.7 ml/min and a run time of 10 minutes were used. The calibration curve showed a linear relationship with a correlation coefficient > 0.999. The retention time of EO-9 was approximately 8.2 min. The method showed to be stability indicating with EO-5a, the main degradation product of EO-9, at a relative retention time to EO-9 of 0.68. Therefore,

the purity (expressed as the peak area of EO-9 as percentage of the total area of all peaks present in the chromatogram) was taken as stability parameter in this study.

Gas chromatography (GC)

Traces of residual tert-butyl alcohol (TBA) in freeze dried products were determined by gas chromatographic analysis. The GC system was composed of a Model 5890 gas chromatograph equipped with a flame ionisation detector (FID), a split-splitless injector and a Model 6890 series autosampler (Hewlett Packard). Separation was achieved with a crossbond® 6% cyanopropylphenyl - 94% dimethylpolysiloxane (30m x 0.53mm ID x 3.0µm film thickness) column. Calibration solutions of 30-500µg/ml TBA in dimethylsulfoxide (DMSO) were used. A sharp signal of TBA appeared at 2 minutes. A large signal of DMSO was seen at 9.3 minutes. The calibration curve was linear with a correlation coefficient of 0.9999. No internal standard was required.

Pharmaceutical formulation development

Solubility and stability in tert-butyl alcohol (TBA)

The maximum solubility and stability of EO-9 in TBA solutions were assessed. Solutions of 0, 10, 20, 30, 40, and 50% v/v TBA in water for injection (WfI) containing 1% w/v sodium bicarbonate were saturated with EO-9. The pH of the solutions increased with increasing TBA content and varied from pH 8.4 to 9.5 for solutions containing 0% to 50% v/v TBA, respectively. The solutions were shaken for 24 hours at room temperature and ambient light. After 6 and 24 hours samples were filtered using Millex® HV filters (0.45µm x 4mm, Millipore, Etten-Leur, The Netherlands) and the content and purity of the filtrates were determined with HPLC-UV.

Furthermore, the stability of EO-9 in freeze dried products containing a bulking agent and an alkalizing agent was determined. The bulking agents mannitol (crystalline bulking agent) and polyvinylpyrrolidone (an amorphous bulking agent) and the alkalizing agents sodium bicarbonate, sodium hydroxide and meglumine were tested. Sodium bicarbonate has also been used for bladder instillations of MMC. It was shown that addition of sodium bicarbonate to MMC bladder instillations increased time to recurrence of patients with superficial bladder cancer [9]. For each solution the bulking agent, alkalizing agent and EO-9 were dissolved in 40% v/v TBA to concentrations of 25mg/ml, 10mg/ml and 4mg/ml, respectively. Sodium hydroxide was added to pH 8.5. Vials (8 ml hydrolytic class I glass type Fiolax-clear, Aluglas, Uithoorn, The Netherlands) were filled with aliquots of 2 ml of each solution and subsequently freeze dried. Vials were frozen to -43ºC at ambient chamber pressure in 1 hour. This

condition was maintained for 2 hours. Subsequently, primary drying was performed at -43ºC and a chamber pressure of 0.20 mbar. After 32.5 hours of primary drying, secondary drying was performed at +25ºC. The chamber pressure of 0.20 mbar was maintained. Vials were closed under vacuum after 8 hours of secondary drying and stored at 5±3°C/ambient relative humidity (RH), 25±2°C/60±5%RH and 40±2°C/75±5%RH, all in the dark. The content and purity were determined with HPLC-UV analysis after 1, 2, 4 and 8 weeks of storage. The formulation in which EO-9 was most stable was selected for further development.

Differential scanning calorimetry (DSC)

DSC was used to characterize the formulation solution composed of EO-9 4 mg/ml, mannitol 25 mg/ml, and sodium bicarbonate 10mg/ml in 40% v/v TBA and to optimize the freeze-drying process. DSC was performed with use of a Q1000 V2.5 DSC equipped with a refrigerated cooling accessory (RCS) for low temperature in the T4P mode (TA Instruments, New Castle, DE, USA). Temperature scale and heat flow were calibrated with indium. Samples of approximately 10 mg were transferred into aluminium pans (TA Instruments), hermetically closed, cooled to -40ºC and heated to –20°C. After an isothermal step for 5 minutes at -20ºC, samples were cooled to -40ºC and finally heated to +10ºC. Cooling and heating were performed with 1°C/min and 5ºC/min, respectively. An empty pan was used as reference.

Manufacture and stability

The selected formulation solution for pharmaceutical product manufacture, composed of EO-9 (4 mg/ml), mannitol (25 mg/ml) and sodium bicarbonate (10 mg/ml) in 40% v/v TBA was filtered through a 0.2µm filter (Midisart 2000, Sartorius, Goettingen, Germany). Aliquots of 2.0 ml were filled into 8 ml colorless glass vials (hydrolytic class 1 type Fiolax-clear, Münnerstadter Glaswarenfabrik, Münnerstadt, Germany), partly closed with grey butyl rubber lyophilization stoppers (Type FM157/1, Helvoet Pharma N.V., Alken, Belgium) and loaded into the freeze dryer (Model Lyovac GT4, GEA Lyophil GmbH, Hürth, Germany). Several test batches were prepared. Vials of the first batch were frozen to -43ºC in one hour at ambient pressure. This temperature and pressure were maintained for two hours. Subsequently, the pressure was decreased to 0.20 mbar for primary drying at a shelf temperature of -43ºC. Primary drying was performed for 45 hours and was followed by an increase in temperature to +25ºC in 15 hours at a chamber pressure of 0.20 mbar for secondary drying. The total freeze drying time was 66 hours. Due to a high moisture content of this batch, the freeze drying program was adapted for the following 2 batches: the shelf temperature of the secondary drying phase was raised from +25ºC to +30ºC and the chamber pressure was lowered from 0.20 to 0.05 mbar. Vials of the three batches were closed

pneumatically under vacuum, stored at 5±3ºC and 25±2ºC/60±5%RH, in the dark, and analyzed after 3, 6, 12, and 18 months of storage with HPLC-UV analysis to determine the stability of the freeze dried finished product.

Reconstitution and dilution

Solutions containing various amounts of propylene glycol in water for injection were tested as reconstitution solutions for the freeze dried product. Sodium bicarbonate (2% w/v) was added as alkalizing agent and sodium edetate (0.02% w/v) as chelating agent. Subsequently, the solubility and stability of EO-9 after reconstitution and dilution of the freeze dried product were tested.

Optimization of the freeze drying program

The maximum product temperature allowed during primary drying was determined using DSC analysis. Subsequently, the influence of shelf temperature on product temperature was determined. Vials filled with 2 ml EO-9 formulation solution composed of EO-9 (4 mg/ml), mannitol (25 mg/ml) and sodium bicarbonate (10 mg/ml) in 40% v/v TBA were freeze dried (Model Lyovac GT4, STERIS, Hürth, Germany) at different shelf temperatures. The product temperature during freeze drying was continuously recorded using platinum electrodes.

Furthermore, the freeze drying characteristics of TBA/WfI solutions were studied and the primary drying time for the solution composed of 40% v/v TBA was determined. Mixtures of 0, 5, 7.5, 10, 15, 20, 30 and 40% v/v of TBA in WfI were prepared. Glass vials (8 mL, hydrolytic class 1 glass type Fiolax-clear, Aluglas, Uithoorn, The Netherlands) were filled with aliquots of 2 mL and partly closed with grey butyl rubber lyophilization stoppers (Type FM157/1, Helvoet Pharma N.V., Alken, Belgium). Per time point, 10 vials of each solution were loaded at random on one shelf of the freeze drier at ambient temperature (Model Lyovac GT4, STERIS, Hürth, Germany). The vials were frozen to -35ºC in one hour, followed by an annealing step at –20°C. Primary drying was performed at a shelf temperature of -10ºC and a chamber pressure of 0.20 mbar. Vials were closed 1, 1.5, 2, 4, 5 and 6 hours after starting of the primary drying phase. The amount sublimed during primary drying was calculated by weighing the vials before and after freeze drying. Subsequently, the TBA/WfI ratios of the remaining solutions were determined with refractive index analysis using a calibration curve of 1%, 5%, 10%, 20%, 30%, and 40% v/v TBA in WfI. Refractive index analysis was performed using an Abbe refractometer 302 (Atago, Tokyo, Japan).

Subsequently, the secondary drying phase was optimized. Several batches composed of 25 vials containing 2 mL of EO-9 formulation solution were freeze dried. The freeze drying process of all batches started with freezing to -35ºC, followed by an

annealing step at –20°C. Primary drying of 7 hours was performed at a shelf temperature of -10ºC and a chamber pressure of 0.20 mbar. Optimization of the secondary drying phase was performed by varying chamber pressure and shelf temperature. Per batch, vials were closed at two different time points during secondary drying. The maximum secondary drying time tested, was 24 hours. At each time point, residual moisture content and residual TBA content were determined in duplicate using Karl Fisher titration and gas chromatography analysis, respectively. The purity of the EO-9 batches freeze dried with a secondary drying phase of 24 hours and a shelf temperature of more than +25°C was determined with HPLC-UV analysis.

Scanning electron microscopy (SEM)

SEM was performed on a XL30FEG scanning electron microscope (FEI, Eindhoven, The Netherlands) at 5.0 kV. Samples were mounted on stubs with double-sided cohesive tape and subsequently coated with 4 nm platinum/palladium mixture. Freeze dried products before and after optimization of the freeze drying program were analyzed. Furthermore, a sample of untreated EO-9 drug substance was observed as reference.

X-ray diffraction (XRD)

XRD of the freeze dried product, EO-9 drug substance and the excipients was performed using a model PW 3710 PC-APD diffractometer (Philips, Eindhoven, The Netherlands) at atmospheric humidity in the angular range 5-40*(2 theta). The CuKalpha radiation from the anode operating at 40kV and 50mA was monochromized using a 15µm Ni foil. Scan step size was x*(2theta) and steptime 0.05-5.0s.

Results and discussion

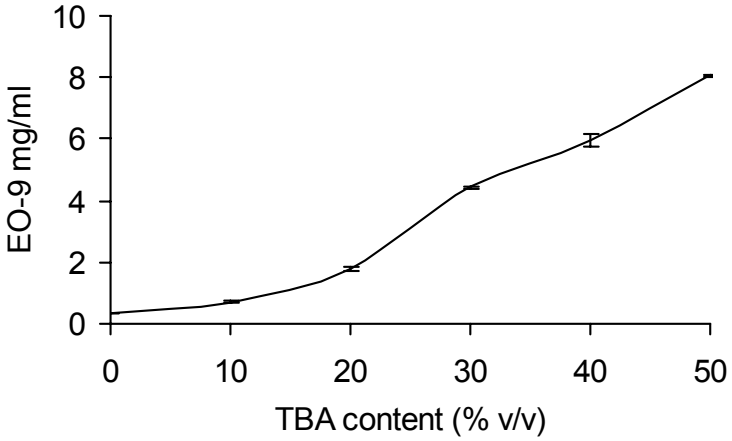
Pharmaceutical formulation development

Solubility and stability of EO-9 in aqueous TBA solutions

The maximum solubility, solubility rate, and stability of EO-9 in aqueous solutions were the main items to be improved with respect to the formulation for intravenous administration developed previously [5]. In literature, the use of organic solvents in formulation development for freeze dried products, has been described earlier [10]. Besides positive effects on stability and solubility of some drugs in the bulk solution, it has been shown that organic solvents increased sublimation rate and decreased drying time during freeze drying. The use of the organic solvent TBA in freeze drying experiments has been described earlier [10-16]. Due to the higher vapor pressure of TBA

(26.8 mmHg compared to 20 mmHg of water, at 20ºC), the mass transfer during the freeze drying process is accelerated, resulting in shorter freeze drying times. Furthermore, it has been shown that lyophilization from TBA resulted in significantly higher specific surface areas of freeze dried products which may promote rapid reconstitution [15]. Because of these advantages and due to our experience with freeze drying of TBA solutions [16], we decided to use TBA for the formulation development of EO-9. The results of the solubility study show that the maximum solubility of EO-9 increased with increasing TBA content (Figure II).

Figure II. Solubility of EO-9 in aqueous TBA solutions containing 1% w/v sodium bicarbonate.



At all TBA concentration levels measured, the maximum solubility of EO-9 was reached within 6 hours (data not shown). Jonkman-de Vries et al. showed that EO-9 is most stable at pH 8.75 [17] and therefore, sodium bicarbonate was added to all solutions. Increasing the TBA content up to 20% v/v improved the stability further (Figure III). However, for solutions containing 30, 40 and 50% v/v TBA no further improvement in stability of EO-9 was found within 24 hours. The maximum solubility of EO-9 in these solutions was 4.4, 6.0 and 8.1 mg/ml respectively. The dosing range for Phase I clinical study was expected to be 0.5–32 mg EO-9 per intravesical instillation with a volume of 40 ml. A target content of 8 mg per vial was selected for the pharmaceutical product designated for phase I clinical trial.

As a 4 mg/ml EO-9 solution was readily obtained using a 40% v/v TBA vehicle without the necessity of grinding, this concentration was selected and resulted in a 20-fold reduction of filling volume compared to the original formulation [5].

To obtain a homogeneous, visually acceptable and stable freeze dried product of EO-9, bulking- and alkalizing agents are required. As described earlier, at acidic and neutral pH the nitrogen of the aziridine ring is protonated, followed by water attachment and opening of the ring (Figure IV).

Figure III. Stability of EO-9 in aqueous solutions containing 1% w/v sodium bicarbonate and 0% (◆), 10% (□), 20% (▲), 30% (x), 40% (∗), and 50% (•) v/v TBA.

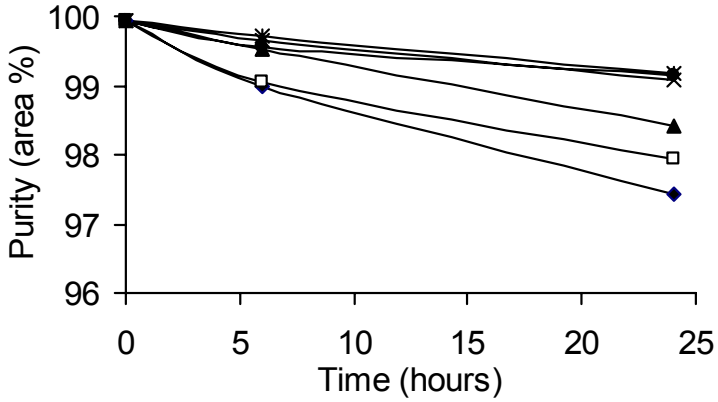
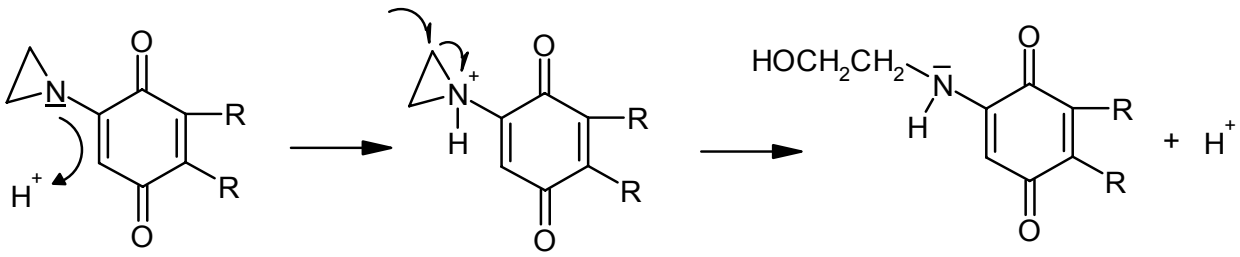


Figure IV. Acid degradation pathway of EO-9



EO-9 EO-5a

To prevent this ring-opening and thus inactivation of EO-9, several alkalizing agents (sodium hydroxide, meglumine, and sodium bicarbonate) were tested to obtain a pH of approximately 9. Mannitol and PVP were selected as bulking agents. As disaccharides are not stable at high pH, these were not considered. During the stability study of the freeze dried products rapid degradation of EO-9 was seen in both formulations containing meglumine after storage for one week at 40ºC/75%RH (Table I). The EO-9 content decreased in the formulation with meglumine and mannitol to 81% and with PVP to 25%. The purities (calculated as percentage of the total peak area) were 98% and 90%, respectively. Jonkman-de Vries et al. [17] proposed a degradation mechanism of EO-9 in alkaline environment in which the aziridine ring is substituted by an hydroxyl ion. However, this is apparently not the mechanism of degradation caused by meglumine, because then EO-9 would degrade more rapidly in presence of sodium hydroxide (a stronger base) and this was not seen. The precise interaction between meglumine and EO-9 remains to be elucidated. Due to the poor stability, the stability of meglumine-containing formulations was not explored further. The two formulations containing sodium bicarbonate were most stable and therefore, additional stability experiments at 40ºC/75%RH with these two formulations were performed. In these

experiments the amounts of bulking agent were varied and the combination of mannitol and PVP was tested. The results showed that the freeze dried product composed of 8 mg EO-9, 50 mg mannitol, and 20 mg sodium bicarbonate per vial was

Table I. Stability of EO-9 in 6 freeze dried products *

Product **	Storage Condition ***	1 week		2 weeks		4 weeks		8 weeks	
		Content (%)	Purity (%)	Content (%)	Purity (%)	Content (%)	Purity (%)	Content (%)	Purity (%)
1	40°C/75%RH	91.66	97.03	87.27	96.54	86.87	93.62	85.54	93.37
	25°C/60% RH			89.30	97.51	86.99	93.18	91.30	94.86
	5°C					98.70	99.80	103.2	99.04
2	40°C/75%RH	95.59	98.52	93.18	98.10	90.64	96.61	87.79	94.69
	25°C/60% RH			96.43	99.30	94.02	96.35	96.79	98.56
	5°C					96.94	99.71	101.7	99.68
3	40°C/75%RH	97.12	99.60	97.79	99.65	97.27	98.92	98.56	98.61
	25°C/60% RH			100.3	100.1	100.1	98.98	96.50	99.99
	5°C					99.12	100.2	102.4	100.2
4	40°C/75%RH	99.25	99.33	95.67	98.44	94.10	97.67	94.95	96.00
	25°C/60% RH			97.79	99.42	98.07	98.13	99.50	98.33
	5°C					101.4	99.85	101.8	99.73
5	40°C/75%RH	80.94	98.30						
	25°C/60% RH								
	5°C					Not analysed			
6	40°C/75%RH	24.93	90.28						
	25°C/60% RH								
	5°C								

* The content and purity are calculated as percentage of the initial values

** Composition of the freeze dried products per vial:

1: EO-9 8 mg, mannitol 50 mg, NaOH qs.
2: EO-9 8 mg, PVP 50 mg, NaOH qs.
3: EO-9 8 mg, mannitol 50 mg, $NaHCO_3$ 20 mg
4: EO-9 8 mg, PVP 50 mg, $NaHCO_3$ 20 mg
5: EO-9 8 mg, mannitol 50 mg, meglumine 20 mg
6: EO-9 8 mg, PVP 50 mg, meglumine 20 mg

*** All samples were stored in the dark, RH = relative humidity

Table II. Stability of EO-9/mannitol/$NaHCO_3$ freeze dried product with and without PVP at 40ºC/75%RH.

Product **	1 week		4 weeks		8 weeks		12 weeks	
	Content (%)	Purity (%)	Content (%)	Purity (%)	Content (%)	Purity (%)	Content (%)	Purity (%)
I	98.29	99.7	93.15	99.1	95.74	98.7	92.62	98.4
II	93.38	98.9	86.53	97.9	90.94	97.2	86.56	96.8
III	93.22	98.9	87.27	96.6	90.30	94.0	85.55	91.8

* The content and purity are calculated as percentage of the initial values

** Composition of the freeze dried products:

I: EO-9 8 mg, mannitol 50 mg, NaHCO3 20 mg

II: EO-9 8 mg, mannitol 100 mg, NaHCO3 20 mg

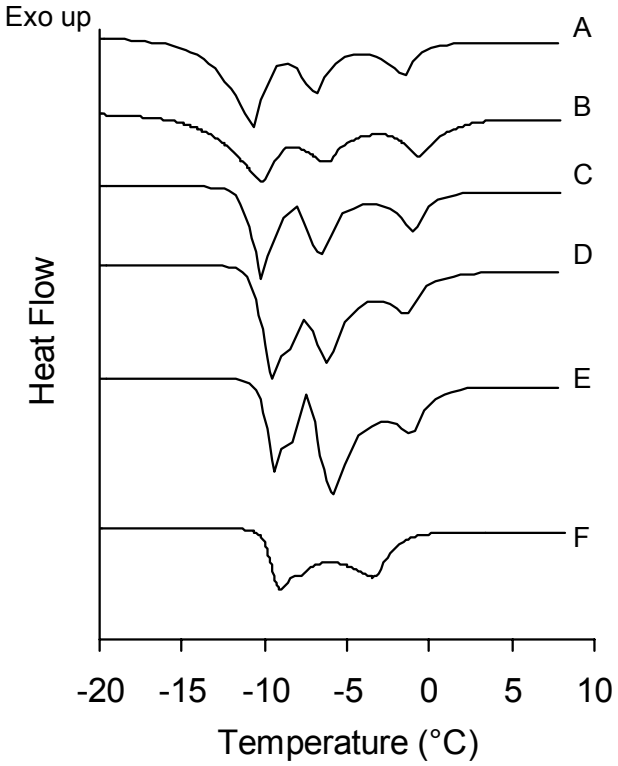
III: EO-9 8 mg, mannitol 50 mg, PVP 50 mg, NaHCO3 20mg

most stable (Table II). The purity of EO-9 was still 98% after 12 weeks of storage at 40ºC/75%RH. Therefore, this formulation was chosen for further development.

Differential scanning calorimetry

DSC analysis was performed to indicate at what temperatures thermal events occur during freezing and reheating of the formulation solution. During primary drying, the product temperature must be held below the temperature at which the first thermal event is seen to obtain a homogeneous pharmaceutical product. For the formulation solution the first thermal event was seen to commence at approximately -18ºC (Figure V, curve A). Furthermore, during warming upon cooling of 40% v/v TBA, a re-crystallization exotherm was seen at –20°C (data not shown), indicating that not all TBA was crystallized. Recrystallization of TBA was reported earlier at -20ºC by Telang et al [18] and at -30ºC in presence of 5% sucrose by Wittaya-Areekul et al [13]. Crystallization of TBA during the freeze drying process reveals a porous cake structure, resulting in a decreased cake resistance and thus increasing sublimation rate. Furthermore, a porous cake structure facilitates reconstitution of the pharmaceutical product. Therefore, complete crystallization of TBA was desired. No recrystallization exotherm was seen after annealing at -20ºC, indicating that this thermal treatment resulted in complete crystallization of TBA (Figure V). No change in formation of the TBA crystals was seen upon addition of mannitol (25 mg/ml) and/or sodium bicarbonate (10 mg/ml) and/or EO-9 (4 mg/ml). This was concluded from the DSC thermograms of all solutions containing the same endothermic signals as the thermogram of 40% v/v TBA (Figure V).

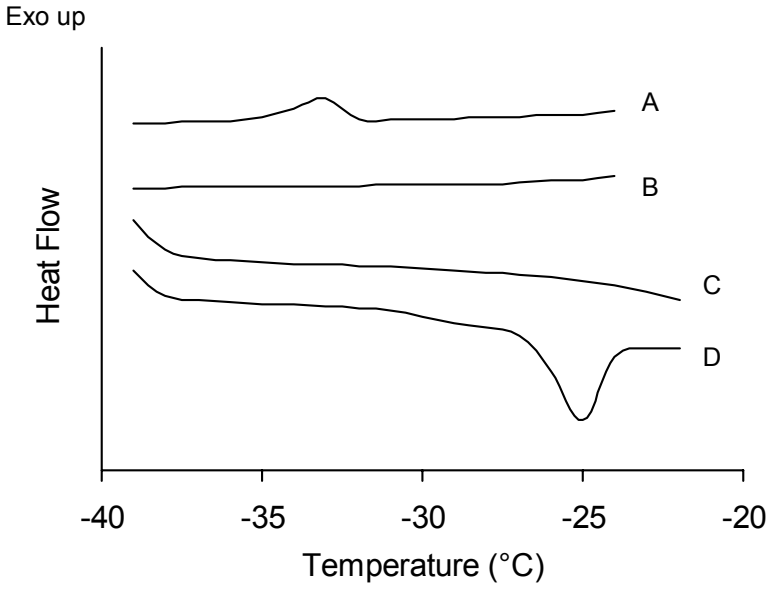
Figure V. DSC thermal analysis with an annealing step at -20ºC of the formulation solution composed of 4 mg/ml EO-9, 10 mg/ml sodium bicarbonate, and 25 mg/ml mannitol (A); 10 mg/ml sodium bicarbonate and 25 mg/ml mannitol (B); 10 mg/ml sodium bicarbonate (C); 25 mg/ml mannitol (D) (A-D all dissolved in 40% v/v TBA); 40% v/v TBA (E) and 20% v/v TBA (F).



However, one small change was seen: the onset of the endotherm at approximately -10ºC of the solutions containing both sodium bicarbonate and mannitol (curves A and B) was less sharp than the endotherms in the other solutions. This is probably due to a less complete crystallization of TBA [14]. Earlier studies showed that TBA crystallization can be inhibited by the presence of an amorphous cosolute (for example a sugar) [13,18]. In our formulation solution, TBA crystallization is probably inhibited by the presence of amorphous mannitol. Normally, mannitol crystallizes easily. This was also seen in the thermogram of 40% v/v TBA with mannitol (2.5% w/v) during cooling from ambient temperature to -40ºC. This thermogram showed an exotherm with an onset at -32ºC (Curve A, Figure VI). Subsequently heating to -20ºC revealed an endothermic signal with an onset at -27ºC (Curve D). Both thermal signals were not seen in presence of sodium bicarbonate (Curves B and C) and are probably due to inhibition of mannitol crystallization by sodium bicarbonate. Previously, this was also reported by Telang et al [19]. They showed that the presence of 5% w/v sodium chloride, sodium citrate and sodium acetate completely inhibits mannitol crystallization in

aqueous solutions. During crystallization of mannitol a hydrate can be formed [20] and the water retained by this hydrate may be released during storage of the freeze dried product, probably decreasing the stability. Therefore, inhibition of mannitol crystallization during freeze drying may be favorable to the pharmaceutical product.

Figure VI. DSC thermograms of solutions containing 40% v/v TBA and 2.5% w/v mannitol with (B and C) and without (A and D) 1% w/v sodium bicarbonate. Thermogram A and B are recorded during the first cooling step, thermogram C and D during warming from -40ºC to -20ºC before annealing.



All thermograms of the solutions containing 40% v/v TBA (corresponding to 34% w/w TBA) show three endothermic signals with onsets at approximately –14°C, –8°C, and -3ºC (Figure V). No meta-stable events or glass transitions were seen, indicating that all TBA was crystallized after annealing at -20ºC. The endotherm with the onset at –14°C is due to melting of the eutectic at 20% w/w TBA of TBA hydrate-ice, the second (with an onset at -8ºC) is due to melting of TBA hydrate. This TBA hydrate contains approximately 70% w/w TBA. The endotherm of this TBA hydrate is not present in the thermogram of 20% v/v TBA. For 20% v/v TBA an endotherm of the eutectic (onset of -11ºC) and an endotherm due to melting of ice (onset of -5ºC) were seen. This is all conform the phase diagram of Kasraian et al [12]. However, the third endotherm of 40% v/v TBA (with an onset at -3ºC) can not be explained with this phase diagram. Visually, no particles were seen in 40% v/v TBA at room temperature, but with cryoscopy small crystals were visible (data not shown). It is likely that these crystals are formed of pure TBA. Perhaps the presence of TBA crystals induced the formation of this third TBA hydrate crystal during freezing. According to the phase

diagram of Kasraian et al [12] this hydrate, with an onset at -3ºC, should contain approximately 90-95% w/w TBA.
Based on the results of this DSC analysis an annealing step at -20ºC was added to the freeze drying program to obtain complete crystallization of TBA. Furthermore, we decided to maintain the product temperature below -20ºC during primary drying to obtain a homogeneous freeze dried product.

Manufacture and stability

We found that addition of TBA increases the maximum solubility and stability of EO-9 in aqueous solutions. Because administration of TBA into the bladder will probably induce scarring and inflammation of bladder tissue [21] and to enhance shelf life of the product, freeze drying was chosen for the removal of TBA and WfI, respectively. Since another freeze dried product developed at Slotervaart Hospital (Amsterdam, The Netherlands) contained the same solution vehicle (40% v/v TBA) with mannitol as bulking agent, the freeze drying program of this product was initially chosen for the manufacture of the freeze dried product of EO-9 [16].

Table III: Results of the quality control of the first three batches of EOquin™ freeze dried product (8 mg EO-9 per vial)

Test Item	Batch 1*	Batch 2**	Batch 3**
Reconstitution	Clear, purple solutions***	Clear, purple solutions***	Clear, purple solutions***
Content	94.9%	102.3%	105.0%
Purity	99.8%	99.9%	99.8%
pH after reconstitution	9.5	9.4	9.4
Moisture content	6.0%	3.90%	3.52%
Sterility	Sterile	Sterile	Sterile
Bacterial endotoxins	< 12.5 EU/ vial	< 12.5 EU/ vial	< 12.5 EU/ vial

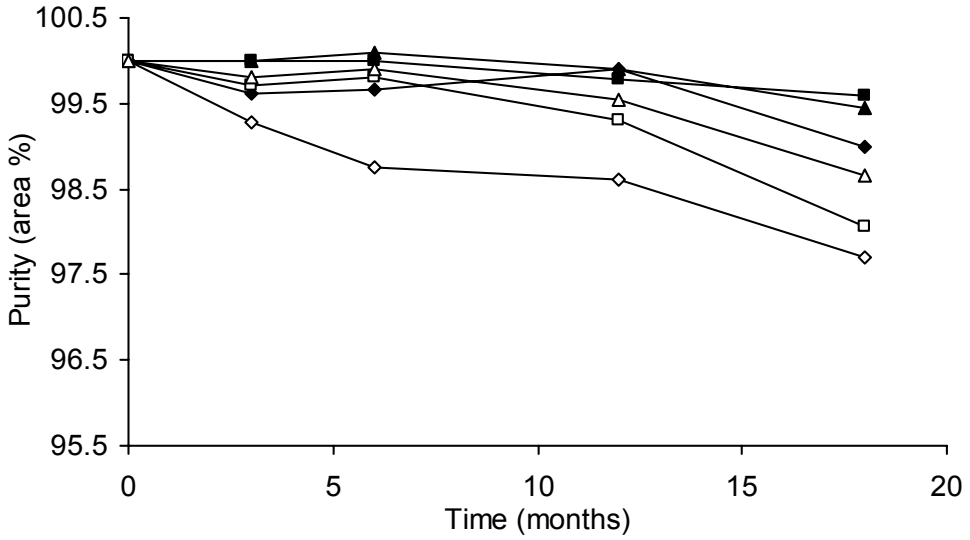
* Secondary drying phase at a shelf temperature of +25ºC and a chamber pressure of 0.20 mbar.

** Secondary drying phase at a shelf temperature of +30ºC and a chamber pressure of 0.05 mbar.

*** After reconstitution with reconstitution solution. No visible particles were seen.

Results of the quality control of the three batches of freeze dried product are depicted in Table III. The moisture content of the first batch is substantially higher than that of the other two batches, indicating that lowering of chamber pressure and increasing of shelf temperature during secondary drying result in decreased moisture levels in the freeze dried product. The purity during storage of the three batches is depicted in Figure VII. The results show that all three batches are stable for at least 18 months at 5ºC. At 25ºC/60%RH however, the purity of the first batch decreases more rapidly than batch 2 and 3, especially in the first 6 months of storage, probably due to its high moisture content. This indicates that EO-9 freeze dried product is susceptible to moisture and therefore, the moisture content was minimized with optimization of the freeze drying cycle.

Figure VII: Stability of EOquin™ freeze dried product batch 1 at 5ºC (◆) and 25ºC/60%RH (◇), batch 2 at 5ºC (■) and 25ºC/60%RH (□), and batch 3 at 5ºC (▲) and 25ºC/60%RH (△).



Reconstitution and dilution

EOquin final product exhibits poor solubility in aqueous solutions (e.g. 0.9% w/v sodium chloride, 5% w/v glucose). Furthermore, a high pH after reconstitution and dilution is important to keep EO-9 stable. Therefore, a reconstitution solution fulfilling these requirements had to be developed. To improve the solubility of EO-9, propylene glycol was used as cosolvent. Propylene glycol is frequently used in parenteral drug products (e.g. Melphalan HCl, phenytoin sodium) [22]. It was shown that both solubility and stability of EO-9 increased with increasing propylene glycol content. The maximum solubility was reached within 6 hours and increased from 0.49 mg/ml in WfI containing 1% w/v sodium bicarbonate to 3.82 mg/ml in 60% v/v propylene

glycol. The purity increased from 97.1% after 6 hours in WfI containing 1% sodium bicarbonate to 98.6% in 60% v/v propylene glycol. Concentrations of more than 60% v/v propylene glycol were not tested because of increasing viscosity. To ensure equal sodium bicarbonate load during phase I dose-escalating study (0.5-32mg EO-9 per bladder instillation), additional sodium bicarbonate was added to the reconstitution solution. Due to the high pH metal ions can be extracted from the walls of the glass vials which facilitate oxidation reactions and therefore sodium edetate (0.02% w/v) was added as chelating agent. The final reconstitution solution was composed of propylene glycol/water for injection/sodium bicarbonate/sodium edetate of 60/40/2/0.02% v/v/w/w. At these concentrations, both sodium edetate and propylene glycol exert an antimicrobial effect. Reconstitution of the freeze dried product composed of 8 mg EO-9, 20 mg sodium bicarbonate and 50 mg mannitol per vial with 10 ml of this reconstitution solution was complete within approximately 5 minutes.
The dosing range in phase I clinical trials was expected to be 0.5-32 mg EO-9 per 40 ml bladder instillation fluid. A special reconstitution and dilution procedure was developed to ensure constant propylene glycol, sodium bicarbonate and sodium edetate levels with varying EO-9 concentration for all doses. The freeze dried product was reconstituted with 10 ml reconstitution solution. The amount of reconstituted product required was then drawn from the vial, diluted (if necessary) with reconstitution solution to make up a total volume of 20 ml, and subsequently diluted 1:1 v/v with water for injection, yielding a final volume of 40 ml. The stability of EO-9 after this reconstitution and dilution procedure was studied for the lowest and highest dose. The results are given in Table IV.

Table IV. Stability of EO-9 after reconstitution and dilution at ambient temperature and light.

Time (h)	0.5mg/40ml			32mg/40ml		
	Conc. (µg/ml)	Purity (%)*	pH	Conc. (µg/ml)	Purity (%)*	pH
0	11.73 ± 0.02	99.3	8.89	771.0 ± 7.80	101.9	9.06
3	11.71 ± 0.06	99.1	8.89	767.9 ± 10.0	101.5	9.06
6	11.64 ± 0.14	98.5	8.89	766.4 ± 3.33	101.3	9.06
24	11.35 ± 0.11	96.0	9.04	756.4 ± 10.2	100.0	9.16
48	10.87 ± 0.13	91.9	9.08	718.7 ± 2.86	95.0	9.20

* Calculated as percentage of the initial purity

EO-9 tended to be less stable in the solution of 0.5 mg / 40 ml compared to the 32 mg / 40 ml solution with purities of 98.5% and 101.3% 6 hours after reconstitution and dilution, respectively. However, a purity of 98.5% was considered acceptable. Therefore, this reconstitution solution and reconstitution procedure were used for the phase I clinical study.

Optimization of the freeze drying program

The aim of the optimization of the freeze drying program was to obtain a homogeneous freeze dried product with minimal residual moisture and residual TBA contents. Furthermore, shortening of the freeze drying cycle was studied to prevent unnecessary long and expensive freeze drying cycles. First, the influence of TBA on the freeze drying characteristics of TBA/WfI solutions and primary drying time were determined, followed by optimization of the secondary drying phase.

With DSC it was shown that the product temperature must be kept below -20ºC during primary drying to prevent thermal events during sublimation, probably resulting in an inhomogeneous product. A product temperature of -30ºC was obtained with a shelf temperature of -10ºC and a chamber pressure of 0.20 mbar. Increasing the shelf temperature to -5ºC resulted in the formulation solution bursting out of the vials, likely due to rapid sublimation of TBA. Therefore, a shelf temperature of -10ºC and chamber pressure of 0.20 mbar were chosen for primary drying. Subsequently, the influence of TBA on the freeze drying characteristics of TBA/WfI solutions was determined during primary drying at these conditions.

A linear correlation ($R^2 = 0.998$) was found between the TBA content (expressed as % v/v) and refractive index (RI) of TBA/WfI solutions and was used for the determination of the TBA content of the remaining solutions in the vials during primary drying. Furthermore, a linear correlation was found between the density (g/ml) and TBA content (% v/v) of TBA/WfI solutions ($R^2 = 0.981$). The density and amount (g) of solution in the vials were used to calculate the total volume present in the vials. Subsequently, the volumes of WfI and TBA remaining in the vials were calculated using the calculated total volume and the determined TBA content (% v/v).

The sublimation of TBA/WfI solutions after 1, 2, and 4 hours of primary drying was calculated as fraction sublimed of the initial content of the vials. An increase in sublimed fraction with increasing primary drying time and increasing TBA content was seen (Figure VIIIa). A positive effect of TBA on the sublimation rate of TBA/WfI solutions was reported earlier by Kasraian et al [15]. Furthermore, they found that in a solution composed of 20% w/w TBA in water, the TBA phase sublimed as fast as the water phase. To compare the sublimation rate of the WfI phase with the sublimation rate of the TBA phase, taking into account the initial volumes of TBA and WfI present in the vials, we calculated the sublimation rates as fractions sublimed of the initial

Figure VIIIa. Sublimation of solutions containing 0 – 40% v/v TBA after 1 (◆), 2 (■), 4 (▲) hours of primary drying. The amount of solution removed during freeze drying is calculated as fraction of the initial content of the vials.

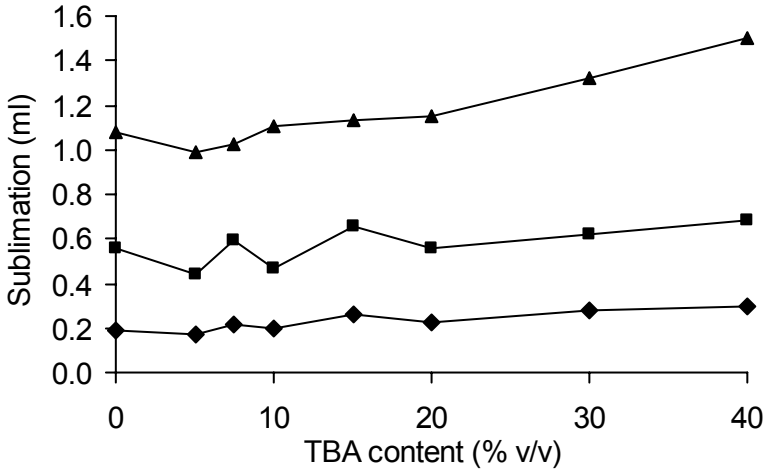
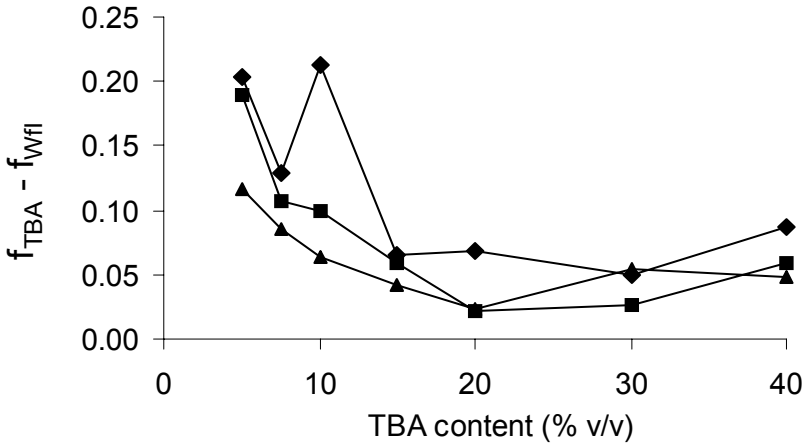


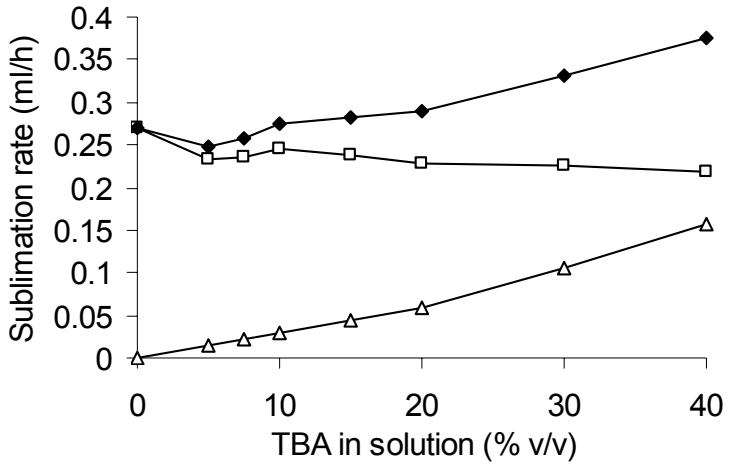
Figure VIIIb. Fraction of TBA sublimed (f_{TBA}) minus fraction of WfI sublimed (f_{WfI}) of solutions containing 0 – 40% v/v TBA after 1 (◆), 2 (■), 4 (▲) hours of primary drying.



contents of WfI and TBA, respectively. Results of the subtraction of the fraction of sublimed WfI from the fraction of sublimed TBA of solutions containing 5-40% v/v TBA after 1, 2 and 4 hours of primary drying are depicted in Figure VIIIb. This figure clearly shows that for all solutions the fraction sublimed of TBA is higher than the fraction sublimed of WfI. This difference in sublimation decreases with increasing primary drying time. For solutions containing less than 20% v/v TBA no correlation was seen between the differences in fractions sublimed and TBA content after one hour of primary drying. However, increasing of primary drying time resulted in a more uniform sublimation.

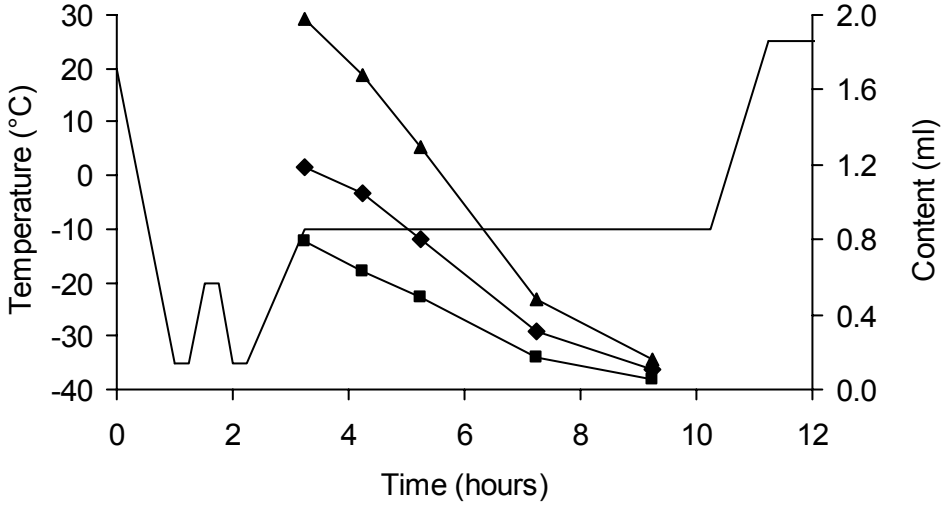
Furthermore, the sublimation rates of the total solution, TBA fraction and WfI fraction were calculated over the first 4 hours of primary drying. A slightly decreasing sublimation rate of the WfI fraction was seen with increasing TBA content (Figure VIIIc). For the TBA fraction a positive linear correlation was found between the TBA content of the solution and the sublimation rate. No correlation between the sublimation rate of the total solution and TBA content was seen for solutions containing 0-10% v/v TBA. However, a slightly positive effect of TBA on the sublimation rate was seen for solutions with a TBA content of 10-20% v/v and a clearly positive effect was seen for solutions containing more than 20% v/v TBA. This indicates that a positive effect of TBA on sublimation during primary drying can only be obtained with TBA concentrations higher than 10% v/v.

Figure VIIIc. Sublimation rate of solutions containing 0 – 40% v/v TBA of the total solution (◆), water fraction (□) and TBA fraction (△).



Because 40% v/v TBA was selected as vehicle for the formulation solution, the sublimation of this solution was studied more thoroughly. The sublimation of the TBA fraction, WfI fraction and total solution during primary drying were determined. The results show that WfI sublimes somewhat faster than TBA (Figure IX). Almost all WfI and TBA sublimed in 6 hours of primary drying. To compensate for a probable minor decrease in sublimation rate due to the addition of EO-9 and excipients, a primary drying phase of 7 hours was selected for freeze drying of the formulation solution containing 40% v/v TBA.

Figure IX: TBA (■), WfI (◆) and total (▲) content (right y-axis) of a 40% v/v TBA solution remaining in the vials as function of the freeze-drying program (left y-axis).



Subsequently, the secondary drying phase was optimized. The effects of chamber pressure and shelf temperature during secondary drying on the residual moisture and residual TBA content were studied. A decrease of chamber pressure from 0.20 mbar to 0.05 mbar did not influence the residual moisture content, but a decrease in moisture content was obtained with increasing shelf temperature. After 24 hours of secondary drying at + 25ºC a moisture content of 7.00 ± 0.29 % w/w was found. Further increase of the shelf temperature to +35ºC and +45ºC resulted in moisture contents of 4.59 ± 0.76% w/w and 3.64 ± 0.38% w/w, respectively. This effect of chamber pressure and temperature on the residual moisture content was described earlier [23]. The TBA content was not affected by either chamber pressure nor shelf temperature and remained approximately 0.6% w/w, corresponding to 0.47 mg TBA per vial containing 8 mg EO-9. TBA belongs to class 3 organic solvent according to the ICH guidelines Q3C [24]. In the ICH guidelines this class of organic solvents is described as "solvents with low toxic potential to man" and a permitted daily exposure (PDE) of 50 mg or more per day is given. The dosing range of phase I clinical trial of EOquin is 0.5-32 mg EO-9 per instillation, corresponding to 0.03-1.87 mg TBA. This is far below the PDE of 50 mg and therefore, no further decrease of the TBA content is required.

The drug purity (determined with HPLC-UV and calculated as percentage of the total peak area) of the final product after 24 hours of secondary drying at +35°C and +45°C was 99.53 ± 0.03% and 99.22 ± 0.20%, respectively. This difference in purity is very small and therefore a shelf temperature of +45°C and a chamber pressure of 0.20 mbar were chosen for secondary drying of 24 hours to minimize moisture content and freeze drying time. The moisture content after optimization of the freeze drying program is

equal to the moisture contents of batch 2 and 3 manufactured before the optimization of the freeze drying time, indicating that a decrease of the moisture content below 3.5 % w/w is difficult. Stability results of freeze dried product showed that batches 2 and 3 containing 3.9 and 3.5% w/w moisture, respectively, are stable for at least 18 months at 5ºC and therefore no further attempts were made to reduce the moisture content. Optimization of the freeze drying cycle was also performed to shorten the freeze drying time and revealed a freeze drying cycle of 35 hours instead of 66 hours.

Scanning electron microscopy

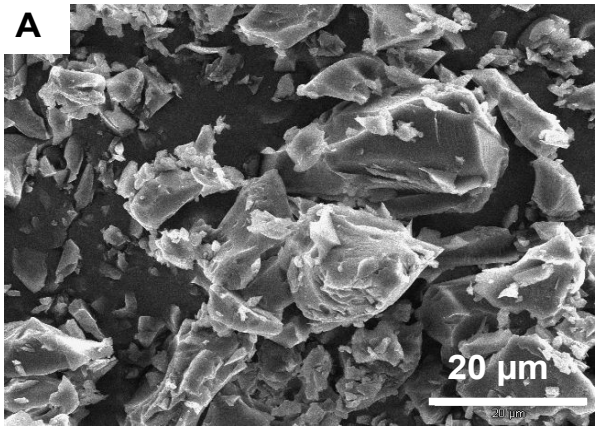
The results of the SEM analysis are depicted in Figure X A-C. EO-9 drug substance appeared as small irregular formed crystals (Figure X A). These EO-9 crystals were not seen in the freeze dried products, indicating that with both freeze drying programs the crystallinity of EO-9 changed or EO-9 became amorphous. Furthermore, different morphologies of the freeze dried product before and after optimization were seen. The freeze dried cake before optimization appeared as a network-like structure with little crystals attached to it. These crystals were not seen in the freeze dried product after optimization. This is a more homogeneous product composed of larger porous particles. The presence of crystals in the product before optimization indicates that during freeze drying probably supersaturation of the solution occurred resulting in precipitation and crystallization of sodium bicarbonate. The formation of sodium bicarbonate crystals was not seen with DSC analysis of the formulation solution, but this is probably due to different conditions in the DSC compared to the freeze dryer (e.g. larger volume and vacuum in the freeze dryer). Furthermore, the crystals were not visible in the product freeze dried with the optimized program, which might indicate that more complete crystallization of TBA prevents crystallization of sodium bicarbonate.

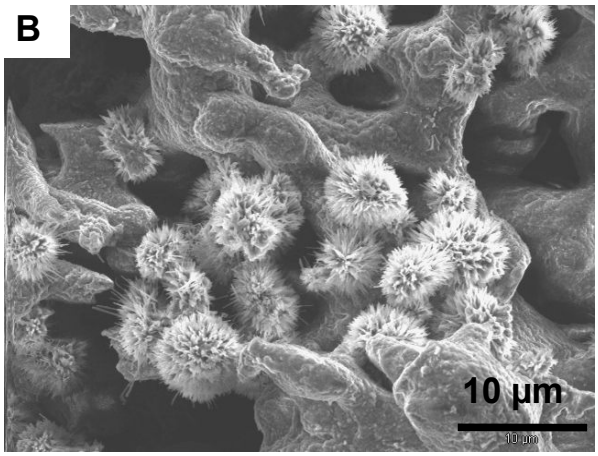
These scanning micrographs show that the product freeze dried with the optimized freeze drying program is a homogeneous and porous product. Therefore, this program was selected for further manufacture of this product.

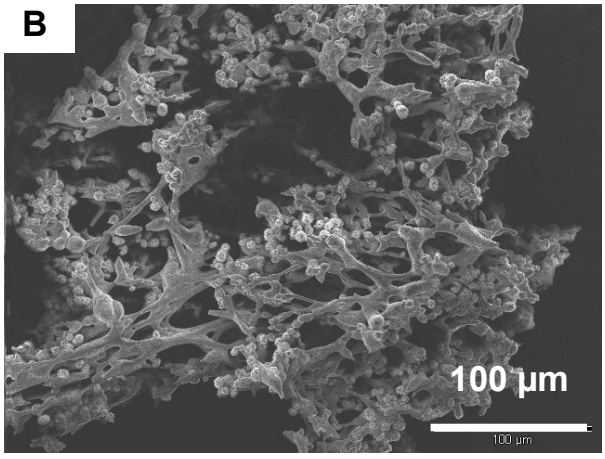
X-ray diffraction

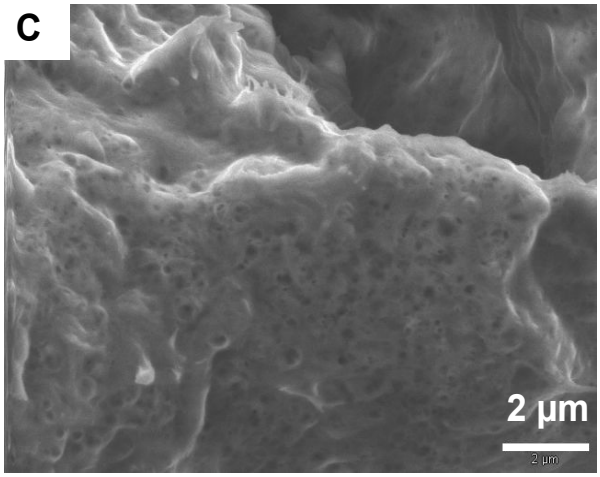
The developed freeze drying process includes an annealing step to induce complete crystallization of TBA, resulting in increased drying rates. Moreover, the reconstitution time of the freeze dried product is decreased, which is favorable to the product, in case EO-9 is amorphous after freeze drying. However, annealing might also induce (undesired) crystallization of excipients and/or EO-9. Therefore, the influence of annealing on the crystallinity of the final product was analyzed using XRD analysis.

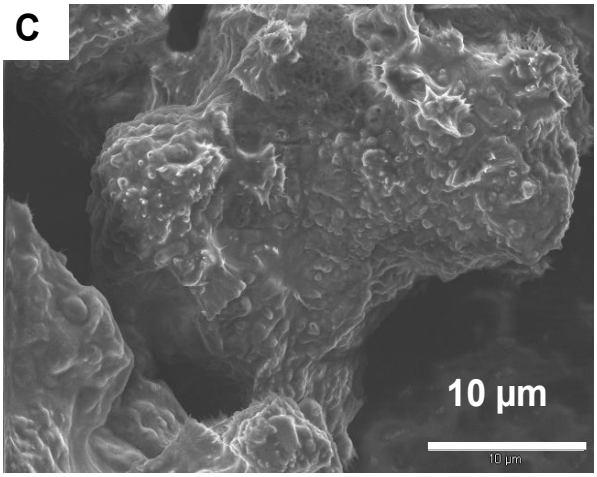
Figure X: Scanning electron micrographs of EO9 drug substance (A) and of EOquin freeze dried product before (B) and after (C) optimization of the freeze drying program.





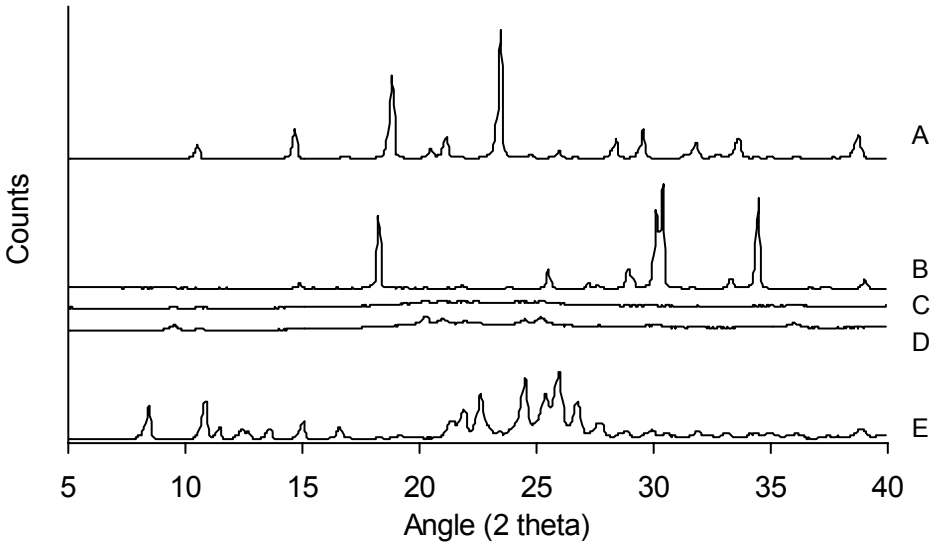






Analysis was performed on EO-9 drug substance, the excipients, and the final product. The final product was analyzed after freeze drying with annealing at -20ºC (the optimized freeze drying program) and without annealing (the old freeze drying program). The XRD spectra (Figure XI) clearly show that mannitol, sodium bicarbonate and EO-9 are crystalline substances. After freeze drying without annealing, a complete amorphous product is formed. With annealing, a few very small signals are seen, indicating that some parts of the freeze dried product are crystalline. One of the signals, at an angle of approximately 9.5, is not present in any other sample. This was also found by Telang et al [25]. They assigned this signal to the δ-polymorph structure of mannitol, formed in presence of sodium chloride. Probably, this polymorph is also formed in presence of sodium bicarbonate when crystallization is more complete due to annealing. Because just a few small signals are present in the XRD spectrum of the final product after annealing, it was not expected that annealing prolongs the reconstitution process. Indeed, reconstitution of the product freeze dried with annealing was complete within 5 minutes after reconstitution with 10 ml of the reconstitution solution (equal to the reconstitution time of the freeze dried product manufactured with the 66-hour freeze drying program).

Figure XI: XRD spectra of mannitol (A), sodium bicarbonate (B), EOquin freeze dried without annealing (C), EOquin freeze dried with annealing at –20°C (D), and EO-9 drug substance (E).



Conclusion

A bladder instillation of the indoloquinone anticancer agent EO-9 for treatment of superficial bladder cancer was developed. The solubility and stability of EO-9 in the formulation solution prior to freeze drying were improved dramatically by addition of the organic solvent TBA. In addition, TBA increased the sublimation rate of the formulation solution during freeze drying. To keep EO-9 stable in the formulation solution, addition of sodium bicarbonate was required. Furthermore, mannitol was added as bulking agent, revealing a formulation solution composed of 4 mg/ml EO-9, 25 mg/ml mannitol and 10 mg/ml sodium bicarbonate in 40% v/v TBA.

DSC thermograms of 40% v/v TBA indicated that during freezing three crystals are formed: TBA hydrate-ice crystals, crystals of TBA hydrate and a third crystal, probably composed of TBA hydrate crystals containing approximately 90-95% TBA. Furthermore, it was shown that crystallization of TBA-hydrate was inhibited in presence of both sodium bicarbonate and mannitol.

Optimization of the freeze drying process resulted in a freeze drying program of 35 hours instead of 66 hours of the initially chosen freeze drying program and an annealing step at -20ºC was added to ensure complete crystallization of TBA. A minor increase in crystallinity of the freeze dried product and formation of the δ-polymorph of mannitol was seen after annealing. However, no effect of this minor increase in crystallinity was seen on the reconstitution time of the freeze dried product.

Due to the low solubility and stability of EO-9, a solution had to be developed for reconstitution of the freeze dried product and resulted in a reconstitution solution composed of propylene glycol/water for injection/sodium bicarbonate/sodium edetate of 60/40/2/0.02% v/v/w/w.

The developed pharmaceutical product is now used in phase II clinical trials with patients suffering from superficial bladder cancer. Formulation related side effects have not been observed hitherto.

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Chapter 1.2

Complexation study of the investigational anticancer agent EO-9 with 2-hydroxypropyl-β-cyclodextrin

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Submitted for publication

Abstract

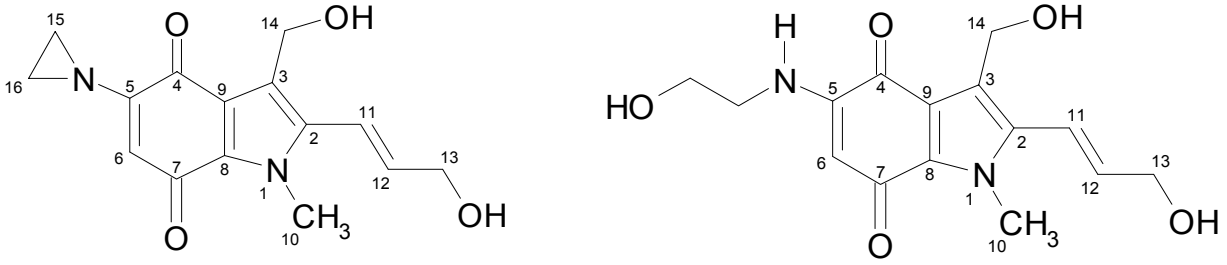
For the development of a bladder instillation of the indoloquinone agent EO-9 use of the complexing agent 2-hydroxypropyl-β-cyclodextrin (HPβCD) was considered. Therefore, a complexation study of EO-9 with HPβCD was performed. Complexation was studied in aqueous solution and in solid freeze dried products. A phase solubility study, UV/VIS analysis and analysis of the effect of HPβD on the stability of EO-9 were performed. With the phase solubility study a $K_{1:1}$ of 32.9, a CE of 0.0457 and an U_{CD} of 38.3 were calculated. These $K_{1:1}$ and CE values indicate a weak complex, but the U_{CD} shows that HPβCD can be very useful as solubilizer in the desired formulation. Furthermore, a positive effect of HPβCD on the chemical stability of EO-9 in solution was seen. Subsequently, complexation in the freeze dried products was studied more thoroughly using FTIR, DSC, XRD, and SEM analysis.
HPβCD was found to be an excellent pharmaceutical complexing agent for application in formulations for EO-9 bladder instillations. Reconstitution prior to use of the developed freeze dried products can simply be accomplished with water for injection.

Introduction

EO-9, 3-hydroxymethyl-5-aziridinyl-1-methyl-2-(1*H*-indole-4,7-dione)-prop-ß-en-α-ol (Figure I), is a bioreductive alkylating indoloquinone and a synthetic analogue of the antitumor antibiotic mitomycin C. For the treatment of superficial bladder cancer a formulation of EO-9 for intravesical instillation was developed. However, this formulation is a freeze dried product which has to be reconstituted with a separate solution. This is less practical in the clinic and induces higher costs. Furthermore, the reconstitution solution contains propylene glycol which is hyperosmotic and may induce local irritation of bladder tissue. Therefore, we attempted to improve the formulation of EO-9 for intravesical administration by making the reconstitution solution redundant.
For several drugs solubility and stability were improved upon complexation with cyclodextrins [1-4]. Cyclodextrins are cyclic oligosaccharides of D-glucopyranose units α-(1,4) linked in a ring formation, which possess a relatively hydrophobic cavity and hydrophilic outer surface. The natural cyclodextrins (α-, β-, γ-cyclodextrins) have a poor solubility in both water and organic solvents, but their most critical drawback is irreversible renal toxicity due to crystallization of cholesterol-cyclodextrin complexes

in the kidneys [5]. Hydroxypropyl derivatization of β-cyclodextrin resulted in the formation of 2-hydroxypropyl-β-cyclodextrin (HPβCD) and improved the solubility and reduced the toxicity of this cyclic sugar [6].

Figure I. Molecular structure of EO-9 and its main degradation product EO-5a



It was shown that HPβCD has a very low toxicity when administered via parenteral route and that any toxic effects on the kidney are reversible [7,8]. This improvement was due to a higher solubility of HPβCD-cholesterol complexes, resulting in a decrease in crystallization of those complexes in the kidneys [5]. HPβCD has already been used in a licensed, parenteral product of the antifungal agent itraconazol (Sporanox®, Janssen-Cilag, Berchem, Belgium). Because of these advantages, HPβCD was chosen to investigate its efficacy on the solubility and stability of EO-9 drug substance.
This article describes the complexation study of EO-9 with HPβCD intended as alternative formulation for intravesical administration of EO-9.

Material and methods

Materials

EO-9 drug substance (Mw = 288 Da) was supplied by Spectrum Pharmaceuticals, Inc. (Irvine, CA, United States). HPβCD (Mw = 1399 Da) was purchased from Roquette Freres (Lestrum, France). Sodium bicarbonate ($NaHCO_3$) was purchased from BUFA (Uitgeest, The Netherlands). Tert-butyl alcohol (TBA) and tri(hydroxymethyl)-aminomethane (Tris) originated from Merck (Darmstadt, Germany). Sterile water for injection (WfI) and normal saline were purchased from B. Braun (Melsungen, Germany). All chemicals obtained were of analytical grade and used without further purification.

High performance liquid chromatography with UV detection (HPLC-UV)

HPLC-UV analysis was performed using an isocratic P1000 pump, AS 3000 autosampler and an UV 1000 UV/VIS detector, all from Thermo Separation Products (Breda, The Netherlands). The mobile phase consisted of 5mM phosphate buffer pH 7/methanol 70/30% w/w. A Zorbax SB-C18 analytical column (750 x 4.6mm ID, particle size 3.5 μm, Agilent Technologies, Palo Alto, California, USA) preceded by a guard column (reversed phase 10 x 3mm, Varian, Palo Alto, California, USA) was used. Detection was performed at 270 nm. An injection volume of 10 μL, flow rate of 0.7 ml/min. and run time of 10 minutes were used. Chromatograms were processed using Chromeleon software (Dionex Corporation, Sunnyvale, CA, USA).

High performance liquid chromatography with photodiode-array detection (HPLC-PDA)

Samples were analyzed with high performance liquid chromatography (HPLC) using a system composed of a HP1100 Series binary HPLC pump and degasser (Agilent Technologies, Palo Alto, CA, USA), a Model SpectraSERIES AS3000 automatic sample injection device equipped with a 100 μl sample loop (Thermo Separation Products, Breda, The Netherlands). Gradient chromatography was performed using a Synergi 4U Fusion-RP 80A column (150 x 2.0 mm ID, particle size of 4.0 μm; Phenomenex, Torrance, CA, USA). The mobile phase consisted of ammonium hydroxide (pH 8.5; 1mM) in water and methanol, pumped at a flow-rate of 0.2 mL/min. The gradient started with 5% methanol and 95% 1mM ammonium hydroxide. This condition was maintained for 15 min. After 15 min. the amount of methanol was linearly increased to 70% in 15 min. and subsequently increased to 80% in 5 min. This condition was maintained for 10 min.. After a run time of 45 min. the gradient was returned to 5% methanol in one minute and the column was stabilized for 4 minutes, resulting in a total run time of 50 minutes. A sample injection volume of 10 μL was used. Detection was performed with a PDA detector Model Waters™ 996 (Waters Chromatography B.V., Etten-Leur, The Netherlands) at 270 nm with PDA detection from 800 to 200 nm. Chromatograms were processed using Chromeleon software (Dionex Corporation, Sunnyvale, CA, USA).

Complexation in liquid environment

Phase solubility

The phase solubility of EO-9 in HPβCD solutions was studied according to the procedures described by Higuchi and Conners [9]. An excess amount of EO-9 was added to solutions containing 100, 150, 200, 250, 300 and 400 mg/ml HPβCD in WfI. All

solutions contained 10 mg/ml $NaHCO_3$ and were prepared in duplicate. Solutions were sonicated for 3 hours and subsequently shaken for 17 hours at room temperature and ambient light. Next, samples were filtered using a hydrophilic Minisart 0.45µm filter (Millipore, Etten Leur, The Netherlands). Subsequently, the EO-9 content of the filtrates were determined with HPLC-UV analysis.

UV/VIS spectrophotometry

UV/VIS analysis was performed on a UV-1650PC UV-VISIBLE spectrophotometer using UV Probe 2.20 software, both from Shimadzu (Kyoto, Japan). Cuvettes split in half by a transparent screen ("tandem mix") were used. Three solutions were prepared: 10 mg/ml $NaHCO_3$ in WfI, 40 µg/ml EO-9 dissolved in 10 mg/ml $NaHCO_3$ solution and 500 mg/ml HPβCD dissolved in 10 mg/ml $NaHCO_3$ solution. Exact 1.0 ml of the EO-9 solution was transferred in the cuvette on one side of the screen and 1.0 ml HPβCD solution on the other side. Subsequently, the UV-spectrum was recorded. Next, the solutions were mixed by gently shaking of the cuvette and a second spectrum was recorded. This procedure was performed in duplicate. Furthermore, UV spectra were recorded of the EO-9 solution together with the $NaHCO_3$ solution (without HPβCD) before and after mixing. These two UV-spectra were used as reference.

Effect of HPβCD on the stability of EO-9

Four solutions composed of EO-9/$NaHCO_3$, EO-9/HPβCD/$NaHCO_3$, EO-9/Tris and EO-9/HPβCD/Tris were prepared by 5-fold dilution of a stock solution of 500 µg/ml EO-9 in methanol with WfI containing HPβCD and/or $NaHCO_3$ and/or Tris. The diluted solutions contained 100 µg/ml EO-9 and/or 15 mg/ml HPβCD and/or 500 µg/ml $NaHCO_3$ and/or 30.3 µg/ml Tris. The weight ratios of EO-9 drug substance and excipients in these solutions were equal to the weight ratios present in the freeze-dried products. The pH of the freshly prepared solutions composed of EO-9/HPβCD/$NaHCO_3$ and EO-9/HPβCD/Tris was measured. Aliquots of 1 ml of each solution were filled in autosampler vials. The EO-9 content and presence of any degradation products in these solutions were determined immediately after preparation and after storage for 15 hours at 70°C, in the dark. Samples were analyzed using HPLC-PDA detection.

Complexation in solid state

Preparation of solid mixtures

To study complexation freeze dried products and physical mixtures composed of EO-9/HPβCD/$NaHCO_3$ and EO-9/HPβCD/Tris with different EO-9/HPβCD weight ratios were prepared.

Freeze dried product

Formulation solutions composed of EO-9/HPβCD/$NaHCO_3$ (2/300/10 mg/ml), EO-9/HPβCD/$NaHCO_3$ (2/100/10 mg/ml), HPβCD/$NaHCO_3$ (100/10 mg/ml), EO-9/HPβCD/Tris (2/300/0.5 mg/ml), EO-9/HPβCD/Tris (2/100/0.5 mg/ml), HPβCD/Tris (100/0.5 mg/ml) in 20% v/v TBA were sonicated for 2 hours. Aliquots of 2 ml were filled in 8 ml glass vials (hydrolytic class I type Fiolax-clear, Münnerstadter Glaswarenfabrik, Münnerstadt, Germany), partially closed with grey butyl rubber lyophilization stoppers (Type FM157/1, Helvoet Pharma N.V., Alken, Belgium) and subsequently freeze dried (Model Lyovac GT4, GEA Lyophil GmbH, Hürth, Germany). The solutions were frozen to –35°C in one hour. The primary drying phase started after 2 hours and was performed at a shelf temperature of –35°C and a chamber pressure of 0.20 mbar for 45 hours. The product temperature during primary drying was -30ºC. For secondary drying the temperature was raised to +25°C in 15 hours. The chamber pressure of 0.20 mbar was maintained. Vials were closed after 3 hours of secondary drying at a chamber pressure of 0.20 mbar.

Physical mixture

Physical mixtures were prepared by grinding EO-9, HPβCD and one of the alkalizers $NaHCO_3$ or Tris, with mortar and pestle to form a homogeneous powder. The excipients and EO-9 were mixed in the same amount ratios as present in the freeze dried products.

Fourier Transform Infrared analysis

The freeze dried products composed of EO-9/HPβCD/$NaHCO_3$ (4/200/20 mg/vial), EO-9/HPβCD/Tris (4/200/1 mg/vial) and the corresponding physical mixtures were analyzed using Fourier Transform Infrared (FTIR) analysis. Freeze dried blanks (HPβCD/$NaHCO_3$ 200/20 mg/vial and HPβCD/Tris 200/1 mg/vial) were analyzed as reference. FTIR was performed on a FTIR-8400S Fourier Transform Infrared spectrophotometer equipped with a "golden gate device" using IRsolution software, all from Shimadzu (Kyoto, Japan). IR spectra of dry powder samples were recorded from 600 to 4000 cm^{-1} in the slow scan mode, with a step size of 2 cm^{-1}.

Differential scanning calorimetry

Differential scanning calorimetry (DSC) analysis was performed with use of a Q1000 V2.5 DSC equipped with a refrigerated cooling accessory (RCS) for low temperature in the T4P mode (TA Instruments, New Castle, DE, USA). DSC thermograms were recorded in duplicate of the freeze dried products and physical mixtures composed of EO-9/HPβCD/$NaHCO_3$ (4/600/20 mg/vial) and EO-9/HPβCD/Tris (4/600/1 mg/vial). Approximately 10 mg powder of each sample was weighed in aluminium pans (TA

Instruments) and hermetically closed. Samples were cooled to 0ºC and subsequently heated to 210ºC with 10ºC/min. Temperature scale and heat flow were calibrated with indium and an empty pan was used as reference.

X-ray diffraction

X-ray diffraction (XRD) analysis of the freeze dried products and physical mixtures composed of EO-9/HPβCD/$NaHCO_3$ (4/200/20 mg/vial) and EO-9/HPβCD/Tris (4/200/1 mg/vial) was performed using a model PW 3710 PC-APD diffractometer (Philips, Eindhoven, The Netherlands) at atmospheric humidity in the angular range 5-40*(2 theta). The CuKalpha radiation from the anode operating at 40kV and 50mA was monochromized using a 15 µm Ni foil. Scan step size was x*(2theta) and steptime 0.05-5.0s. Furthermore, EO-9 drug substance and the excipients HPβCD, Tris and $NaHCO_3$ were analyzed as reference.

Scanning electron microscopy

Scanning electron microscopy (SEM) was performed on a XL30FEG (FEI, Eindhoven, The Netherlands) at the Electron Microscopy group Utrecht. Samples were mounted on stubs with double-sided cohesive tape and subsequently coated with 4 nm platinum/palladium mixture. Freeze dried products composed of EO-9/HPβCD/$NaHCO_3$ (4/600/20 mg/vial) and EO-9/HPβCD/Tris (4/600/1 mg/vial) were analyzed. Furthermore, samples of untreated EO-9 drug substance and HPβCD were scanned as reference.

Results and discussion

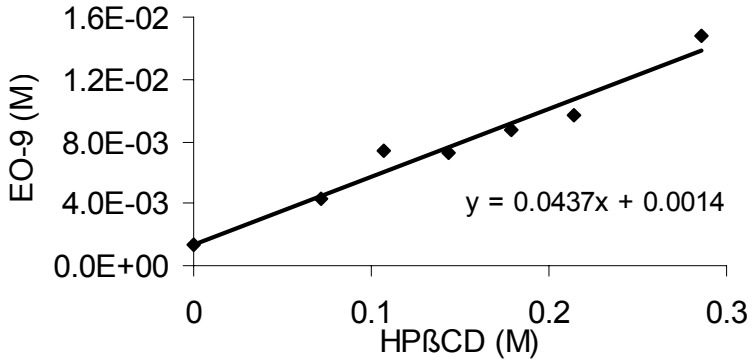
Complexation in liquid environment

Phase solubility

The influence of HPβCD on the solubility of EO-9 in WfI was determined in a phase solubility study. EO-9 raw drug substance appeared as rather large crystalline particles. The phase solubility study was started with sonication for 3 hours to accelerate disintegration of those particles. To prevent degradation of EO-9 10 mg/ml $NaHCO_3$ was added to all solutions [10].

Addition of HPβCD resulted in a linear increase in the solubility of EO-9 (Figure II). A complexation constant ($K_{1:1}$) of 32.9 M^{-1} was calculated using the formula of Higuchi and Conners [9] (Equation 1).

Figure II. Phase solubility diagram of EO-9 with HPβCD in water for injection containing 10 mg/ml $NaHCO_3$.



$K_{1:1}$ = slope / S_0*(1-slope) (1)

Where S_0 is the intrinsic solubility of EO-9 ($1.39*10^{-3}$ M or 0.4 mg/ml) and $K_{1:1}$ is the complexation constant. In this calculation the assumption is made that a complex with a 1:1 stoichiometry is formed (i.e. one molecule EO-9 forms a complex with one molecule HPβCD). Mostly, the value of $K_{1:1}$ is within 50-2000 M^{-1} for all cyclodextrins[11]. The low $K_{1:1}$ value of the EO-9/HPβCD complex indicates that EO-9 will dissociate more rapidly from HPβCD than most other molecules. Calculation from M to mg/ml, revealed maximum solubilities of EO-9 of 2.1 mg/ml, 2.8 mg/ml and 4.3 mg/ml in presence of 200 mg/ml, 300 mg/ml and 400 mg/ml HPβCD, respectively.
To calculate the number of HPβCD molecules required to obtain one EO-9/cyclodextrin complex the Complexation Efficiency (CE) of Lofttson et al [12] was used. The CE is determined by the ratio of free cyclodextrin and drug-cyclodextrin complex. The CE is calculated according to Equation 2.

$$CE = \frac{[DCD]}{[CD]} = \frac{Slope}{1 - Slope} \qquad (2)$$

Where [DCD] is the concentration of dissolved drug-cyclodextrin complex, [CD] is the concentration of dissolved free cyclodextrin and Slope is the slope of the phase-solubility profile. Loftsson et al. [12] found for 38 drug-cyclodextrin complexes an average CE of 0.3, meaning that on average one of every four cyclodextrin molecules in solution forms a complex with a drug. From our phase-solubility profile of EO-9 with HPβCD a CE of 0.0457 was calculated, indicating that approximately one of every 23 cyclodextrin molecules forms a complex with EO-9.
In order to asses the efficacy of HPβCD as complexing agent, the utility number (U_{CD}) was calculated according to equation 3 [13].

$$U_{CD} = \frac{KS_0}{1+KS_0}\frac{m_{CD}}{m_D}\frac{MW_D}{MW_{CD}} \qquad (3)$$

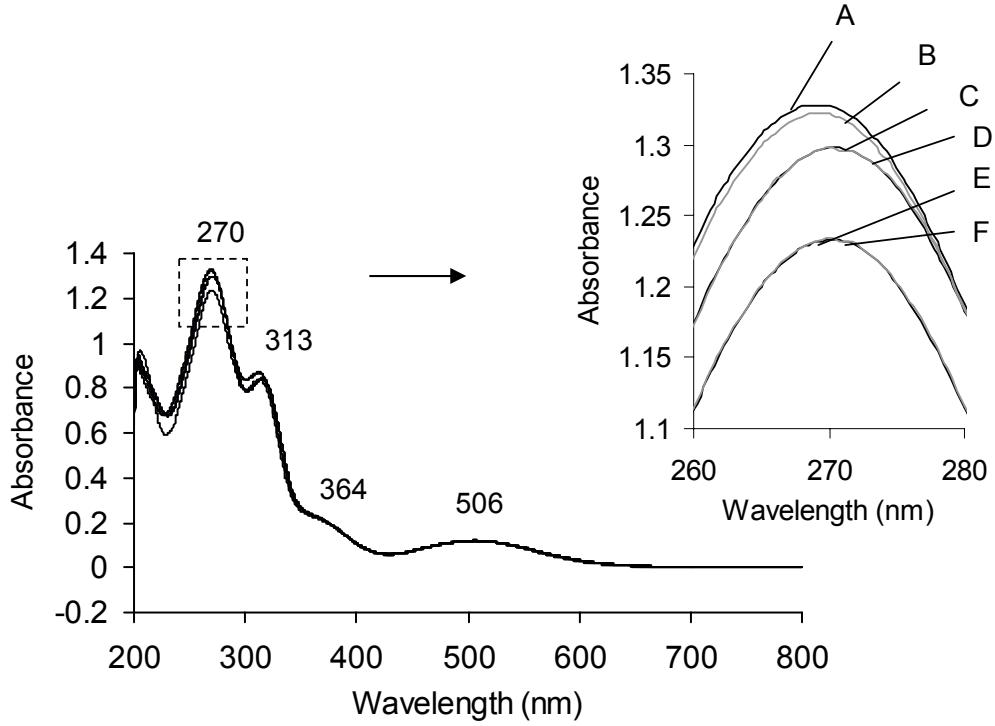
Where K is the complexation constant of a complex with a 1:1 stoichiometry, S_0 the intrinsic solubility of the drug, m_D the drug dose, m_{CD} the workable amount of HPβCD, and MW_{CD} and MW_D are molecular weights of HPβCD and drug, respectively. Phase I and II clinical trials performed with the current formulation of EO-9 revealed a target dose of 4 mg EO-9 per bladder instillation. For HPβCD a maximum concentration of 40% w/v in the formulation solution is workable due to increasing viscosity with increasing HPβCD concentration, resulting in a workable amount of 800 mg HPβCD per vial if a fill volume of 2 ml is used. Typically, a U_{CD} greater or equal to 1 indicates that solubilization is adequately provided by complexation with the cyclodextrin tested. We calculated a U_{CD} of 38.3 for HPβCD. The high value of this parameter is due to the large workable amount of HPβCD compared to the drug dose. Because of the high U_{CD} HPβCD was chosen for further development of an alternative bladder instillation.

UV/VIS spectrophotometry

A change in UV/VIS absorption of a drug in complex with cyclodextrin was reported earlier[14,15]. This indicates interaction of HPβCD with the chromophore of the drug. Because EO-9 has a very characteristic UV/VIS spectrum and changes in absorption might indicate complexation, UV/VIS analysis was also performed to study complexation. Due to the relatively low $K_{1:1}$ and CE values found for EO-9 with HPβCD only minor changes in absorption were expected. Small changes in absorption might also occur due to a change in pH upon mixing of two solutions in "tandem mix" cuvettes. Therefore, EO-9 and HPβCD were both buffered by 10 mg/ml $NaHCO_3$. To affirm further complex formation in solution, we investigated differences in light absorbance of solutions of EO-9 with HPβCD before and after mixing in the cuvette in the molar ratio 1:1. However, no differences were seen which can be explained by the low complexation constant found with the phase solubility study. As shown in this study, just one out of 23 molecules of HPβCD forms a complex with EO-9 indicating that only 4% of the EO-9 molecules in the solution with a 1:1 molar ratio will form a complex with HPβCD. Therefore, we also used a higher HPβCD concentration (500 mg/ml) to study the complexation. The results show that in presence of HPβCD, there is only a very small difference between the duplicates before mixing (Figure III, curves A&B) and no difference between the duplicates after mixing (Figure III, curves C&D). As expected, for the reference no difference in absorption was seen before and

after mixing (Figure III, curves E&F). However, small but significant differences were seen between the separate and mixed EO9/HPβCD solutions. After mixing a small hypochromic shift of the signals with λ_{max} at 270 nm and 313 nm was seen. Furthermore, a tendency towards a bathochromic shift was seen. These results may indicate that a part of the UV/VIS-absorbing light chromophore of EO-9 is included in or interacted with the HPβCD molecule.

Figure III. UV spectra of EO-9 before and after mixing with HPβCD solution. With the duplicates before mixing (A & B), after mixing (C & D), and the reference before and after mixing (E & F). The duplicates are depicted as grey and black curves.



Effect of HPβCD on the stability of EO-9

During complex formation of EO-9 with HPβCD, the stability of EO-9 could increase if for example the aziridin moiety is incorporated in and protected by the cyclodextrin molecule. Therefore, the influence of HPβCD on the stability of EO-9 in solution was tested. Because a high pH (8.5-9) is required for stabilization of EO-9 in the formulation solution, the influence of the alkalizers $NaHCO_3$ and Tris on the stability of EO-9 was also assessed.

The pH values of the solutions composed of EO-9/HPβCD/$NaHCO_3$ and EO-9/HPβCD/Tris were 8.14 and 7.36, respectively. Freshly prepared solutions were pink and had a purity of 98.8 ± 0.01%. After storage at 70ºC all solutions turned purple, indicating the presence of (or analogues of) EO-5a, the main degradation product of

EO-9 (Figure I)[10]. The percentage of degradation of EO-9 in the solutions containing $NaHCO_3$ were 86.7% and 100% for the solutions with and without HPβCD, respectively. Solutions containing Tris instead of $NaHCO_3$ showed degradation percentages of EO-9 of 68.1% and 78.4% (with and without HPβCD, respectively). These results clearly show that EO-9 is more stable in the solutions containing Tris than in the solutions containing $NaHCO_3$. This is not a pH-effect, because according to the pH profile, EO-9 should be less stable in the solution with Tris (pH 7.36) than with $NaHCO_3$ (pH 8.14) [10]. Furthermore, we showed that the addition of HPβCD increased the stability of EO-9. This increase is likely to be higher in absence of methanol, because methanol may have a negative effect on complexation constants of drug-cyclodextrin complexes[16]. The positive effect of HPβCD on the stability of EO-9 is an indication for complexation and/or interaction of EO-9 with HPβCD.

Complexation in solid state

Fourier Transform Infrared

Complexation between drugs and cyclodextrins has been shown earlier using FT-IR analysis[17-20]. The advantage of FT-IR analysis is that changes in absorption bands indicate what part of the drug molecule interacts with the cyclodextrin molecule. For EO-9 however, showing complexation of EO-9 is difficult due to the interference of the relatively large amount of HPβCD present in the freeze dried products. Therefore, freeze dried products and physical mixtures were used with less HPβCD per vial (200 mg instead of 600 mg). Furthermore, the FT-IR spectrum of EO-9 was recorded. No differences in the spectra between the physical mixtures and freeze dried products were seen in the fingerprint area. This is due to the very large signal of HPβCD. However, small differences were seen in the C=O region of EO-9 at approximately 1600 cm^{-1} for the products composed of EO-9/HPβCD/$NaHCO_3$ and EO-9/HPβCD/Tris (Figure IV a and b, respectively). Small signals of EO-9 at approximately 1625 cm^{-1} and 1580 cm^{-1} were seen in both physical mixtures which were absent in the freeze dried products and freeze dried blanks. Disappearance of these signals of EO-9 in both freeze dried products could indicate interaction and/or complexation of the quinone (C=O) part of EO-9.

Figure IVa. Part of the IR spectra of EO-9 (1), of EO-9/HPβCD/NaHCO3 4/200/20 mg physical mixture (2), EO-9/HPβCD/NaHCO3 4/200/20 mg freeze dried product (3), and freeze dried blank composed of HPβCD/NaHCO3 200/20mg (4).

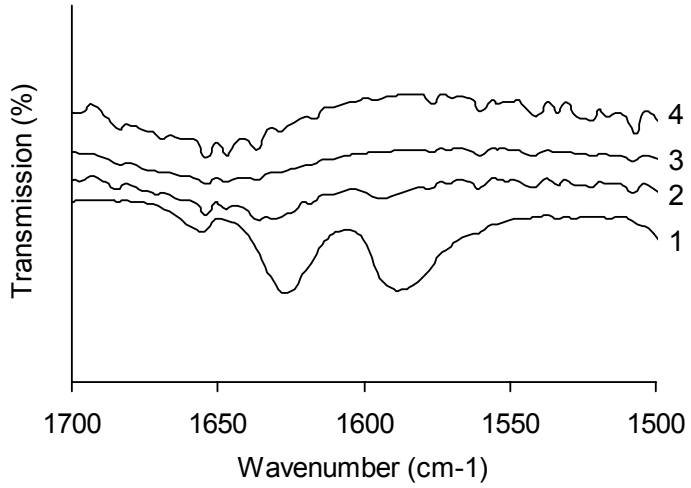
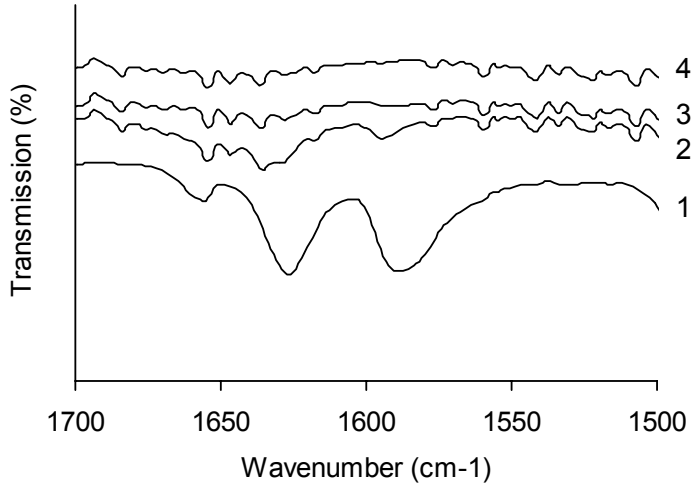


Figure IVb. Part of the IR spectra of EO-9 (1), of EO-9/HPβCD/Tris 4/200/1 mg physical mixture (2), EO-9/HPβCD/Tris 4/200/1 mg freeze dried product (3), and freeze dried blank composed of HPβCD/Tris 200/1 mg (4).



Differential scanning calorimetry

DSC analysis was used to detect thermal events during heating of EO-9 drug substance, physical mixtures and freeze dried products with EO-9. Typically, crystalline solid drug substances show a melting endotherm during heating. For EO-9 drug substance, a large exothermic signal was seen at approximately 170-195°C (Figure V, curve A). The melting point of EO-9 is 187°C and this exothermic signal is probably due to oxidation of EO-9 during and immediately after melting. A similar phenomenon was reported earlier for ampelopsin [21]. Due to this large exotherm, no endotherm from the melting crystal could be seen. The thermogram of HPβCD (Curve

B) only showed a broad endothermic signal at approximately 120-150°C. This signal is due to evaporation of water. Because cyclodextrins are amorphous compounds no melting endotherm was seen nor expected. A broad endotherm due to evaporation of water was also seen in the DSC thermograms of $NaHCO_3$ and Tris (Curve C and D). Furthermore, the thermogram of Tris showed an endothermic signal at 171°C, corresponding to the melting point of Tris. $NaHCO_3$ has a melting point of 270°C. Therefore no melting endotherm of $NaHCO_3$ was seen.
To study complexation between EO-9 and HPβCD, DSC thermograms were recorded of the freeze dried products containing 600 mg HPβCD per vial (and $NaHCO_3$ or Tris) and the physical mixtures with the same EO-9/HPβCD/alkalizer ratios. The DSC thermograms of both physical mixtures did not show a clear endothermic signal, however in both curves a sharp bend at 186-187°C was seen (Curves E and G). This bend was not seen in the thermograms of HPβCD, $NaHCO_3$ or Tris and is probably due to a small endothermic signal of EO-9 superimposed on the endothermal signals of HPβCD and/or one of the alkalizers $NaHCO_3$ or Tris. The endothermic signal of EO-9 is very small. This can be explained by the fact that the amount of EO-9 is very small compared to the amount of HPβCD in the DSC pan (e.g. 10 mg physical mixture contains only 0.07 mg EO-9). The DSC thermogram of EO-9 drug substance was recorded with approximately 10 mg of pure drug substance in the DSC pan. These small endotherms seen in the physical mixtures were not seen in the DSC thermograms of both freeze dried products, indicating drug amorphization and/or inclusion complex formation. This effect of freeze drying on complexation of drugs with cyclodextrins has been reported earlier [1,3,17,18,20,22].

X-ray diffraction

Crystalline compounds, such as EO-9, show a characteristic XRD pattern. If a crystalline compound is partly included in the cavity of an amorphous cyclodextrin molecule, signals of the XRD spectrum will decrease or disappear when a part of the molecule or the whole molecule is encapsulated. Therefore, XRD analysis is often used to study complexation [3,4].
The XRD spectra show that EO-9 (Fig. VIa/b, Curve B), Tris (Fig. VIa, Curve A) and $NaHCO_3$ (Fig. VIb, Curve A) are crystalline compounds. The XRD spectrum of the physical mixture composed of EO-9/HPβCD/ $NaHCO_3$ (Fig. VIb, curve E) showed signals of both EO-9 and $NaHCO_3$. With XRD analysis of the physical mixture composed of EO-9/HPβCD/Tris (Fig. VIIa, Curve E) only signals of EO-9 were seen. This is probably due to the small amount of Tris present in the physical mixture compared to $NaHCO_3$ (1 mg of Tris compared to 20 mg of $NaHCO_3$). The XRD spectra of both freeze dried products resemble the XRD spectrum of HPβCD, an amorphous excipient. These results show that both freeze dried formulations are

amorphous powders, indicating amorphization and/or complexation of EO-9 with HPβCD.

Figure V. DSC thermograms of EO-9 (A), HPβCD (B), NaHCO3 (C), Tris (D), EO-9/ HPβCD/Tris (4/600/1 mg) physical mixture (E), EO-9/HPβCD/Tris (4/600/1 mg/vial) freeze dried product (F), EO-9/HPβCD/NaHCO3 (4/600/20 mg) physical mixture (G), EO-9/HPβCD/NaHCO3 (4/600/20 mg/vial) freeze dried product (H).

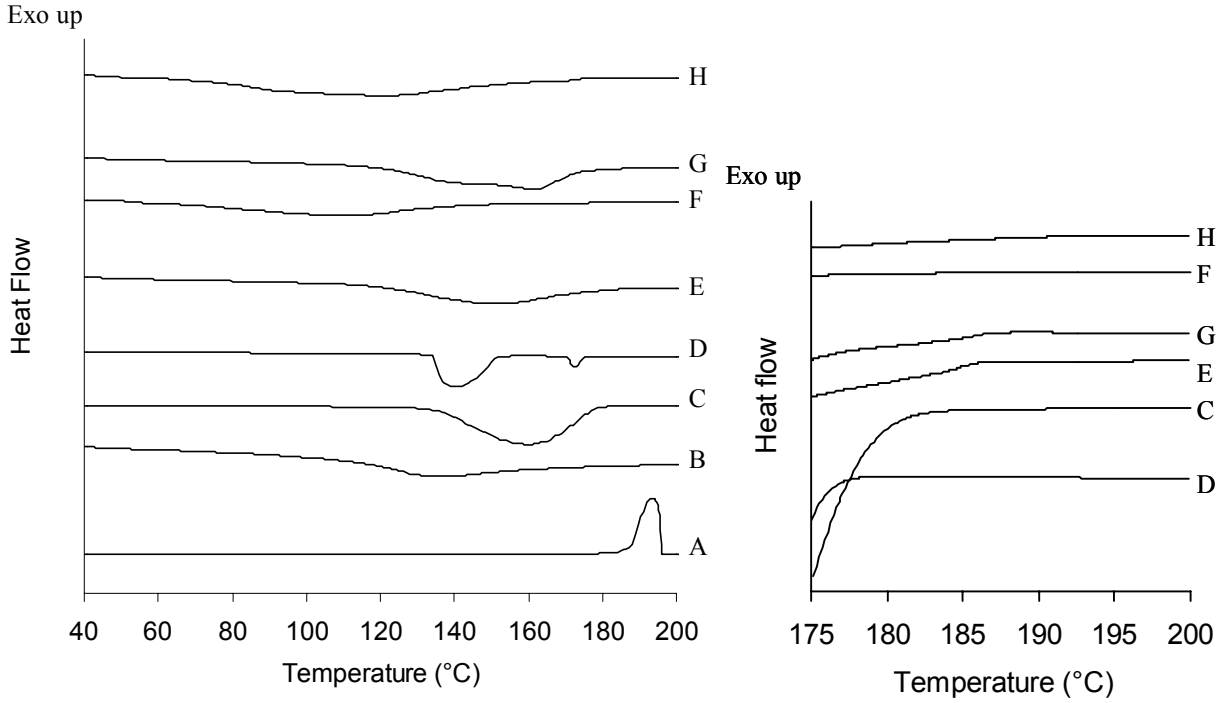


Figure VIa. XRD spectrum of Tris (A), EO-9 (B), HPβCD (C), EO-9/HPβCD/ Tris freeze dried product (4/200/1 mg) (D), EO-9/HPβCD/Tris (4/200/1 mg) physical mixture (E).

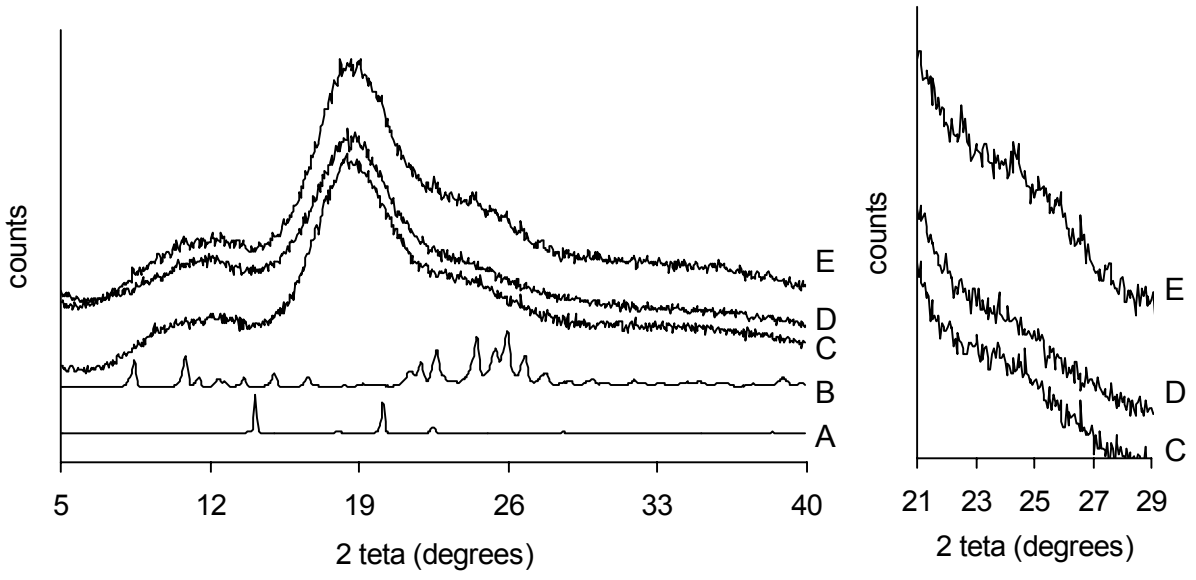
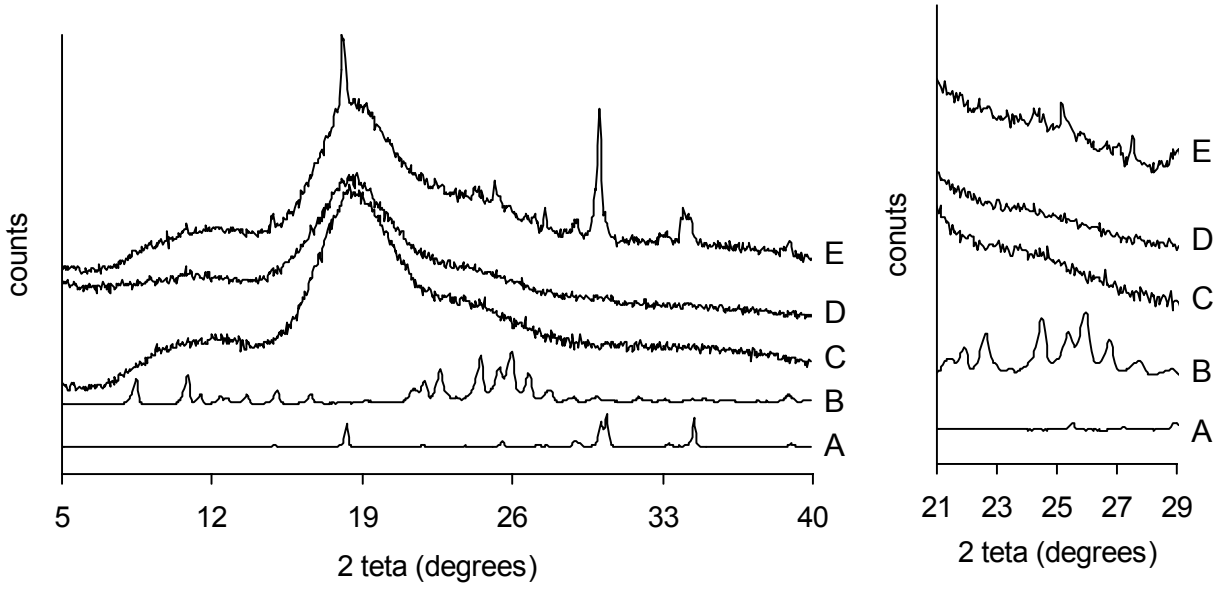


Figure VIb. XRD spectrum of NaHCO3 (A), EO-9 (B), HPβCD (C), EO-9/HPβCD/ NaHCO3 (4/200/20 mg) freeze dried product (D), EO-9/HPβCD/ NaHCO3 (4/200/20 mg) physical mixture (E).

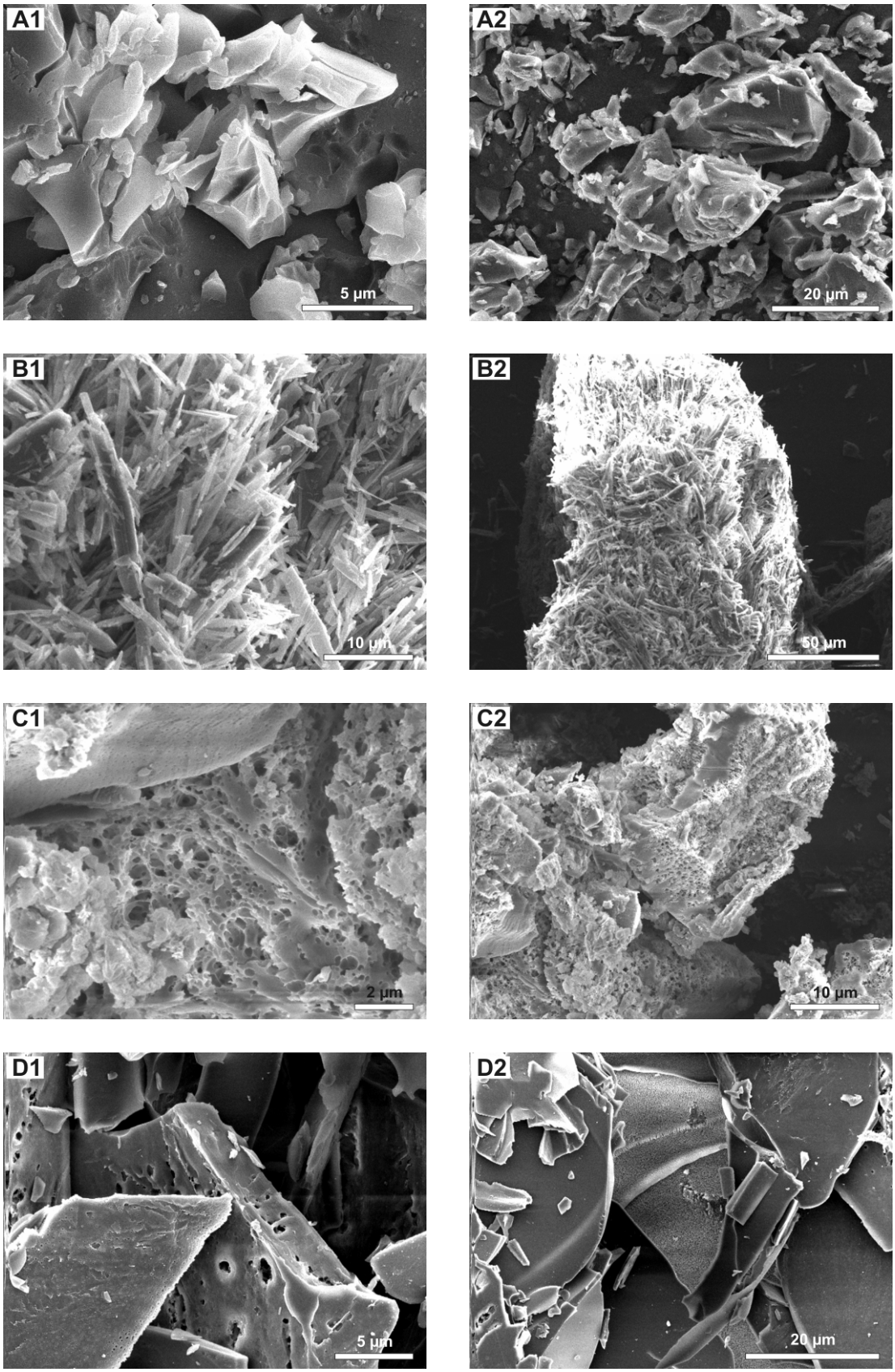


Scanning electron microscopy

SEM is not a suitable technique to detect what part of a drug molecule is included in the HPβCD cavity. However, the morphology of the products can be visualized by this technique. The morphology of EO-9 drug substance and HPβCD are depicted in Figure VII A and B, respectively. EO-9 is composed of irregular formed crystals, HPβCD is composed of large needle-like structures. None of both structures are seen in the freeze dried products containing EO-9/HPβCD/$NaHCO_3$ and EO-9/HPβCD/Tris (Figure VII C and D).

Furthermore, in both freeze dried products just one structure is seen, indicating that EO-9 adhered homogeneously or formed a complex with HPβCD. Remarkable is the different morphology of both freeze dried products. The product with $NaHCO_3$ is composed of sponge-like structures and the product with Tris of larger plate-like structures. This indicates that alkalizing agents can exert a major effect on the morphology of a freeze dried product, probably influencing reconstitution characteristics and/or stability of freeze dried products.

Figure VII. SEM data of EO-9 (A1 and A2), HPβCD (B1 and B2), and freeze dried products composed of EO-9/HPβCD/NaHCO3 (C1 and C2) and EO-9/HPβCD/Tris (D1 and D2).



Besides the analytical methods described above, two other methods were also investigated for their usefulness to detect complexation. One of these methods was liquid chromatography coupled to mass spectrometry (LC-MS). The main problems were the viscosity after dissolution of the freeze dried products in combination with a low complexation constant. The performance of the ion spray decreases with increasing viscosity of the solution. Because HPβCD increases the viscosity, the freeze dried products had to be reconstituted and diluted resulting in EO-9 concentrations of 200 µg/ml. This concentration is below the intrinsic solubility of EO-9 and with the low complexation constant, disintegration of the EO-9/HPβCD complex can be expected (in the solution and/or in the ion spray). Therefore, only masses of EO-9 and HPβCD (and not the complex) were seen and no indication of complexation was found. Furthermore, ^{13}C NMR was performed using high-power proton decoupling (HP-DEC) and cross polarization magic angle spinning (CP-MAS). However, due to the large signals of HPβCD, no signals of EO-9 could be seen in the freeze dried products or physical mixtures. Therefore, these methods could not be used to study complexation.

Based on this complexation study, HPβCD was selected as complexing agent for the development of an alternative pharmaceutical formulation of EO-9. Furthermore, to increase the solubility rate of EO-9, the organic solvent TBA was added to a final concentration of 20% v/v in the formulation solution. TBA was selected because positive effects of TBA on both stability and solubility of EO-9, and on the sublimation rate during primary drying were seen earlier. As alkalizers both $NaHCO_3$ and Tris are used, resulting in prototype formulations composed of EO-9/HPβCD/$NaHCO_3$ 4/600/20 mg/vial, EO-9/HPβCD/Tris 4/600/1 mg/vial and EO-9/HPβCD/Tris 4/600/6 mg/vial. Due to the chemical instability of EO-9[10], freeze drying was selected as manufacturing process for the new formulation.

Conclusion

Efforts were made to obtain a novel formulation for the EO-9 bladder instillation which can be reconstituted with WfI. The results of the phase solubility showed a positive effect of HPβCD on the solubility of EO-9 in aqueous solutions. However, the calculated complexation constant was relatively low, indicating that a relatively high amount of HPβCD is required to achieve complexation of EO-9. Due to the low complexation constant, complexation of EO-9 with HPβCD was difficult to proof, but an indication of complexation was obtained with UV/VIS, DSC, XRD, SEM, and IR analysis. Furthermore, a positive effect of HPβCD on the stability of EO-9 in solution

was also seen, indicating interaction of HPβCD with EO-9. Because of this positive effect of HPβCD on the solubility of EO-9, three alternative bladder instillation formulations with HPβCD were developed. These formulations are freeze dried products composed of EO-9/HPβCD/Tris 4/600/1 mg/vial, EO-9/HPβCD/Tris 4/600/6 mg/vial EO-9, and EO-9/HPβCD/$NaHCO_3$ 4/600/20 mg/vial which can be reconstituted with 1.45 ml WfI and are now further pharmaceutically developed.

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Chapter 1.3

Purity profile of the indoloquinone anticancer agent EO-9 and chemical stability of EO-9 freeze dried with 2-hydroxypropyl-β-cyclodextrin

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Submitted for publication

Abstract

Two new bladder instillations of the investigational anticancer agent EO-9 containing 2-hydroxypropyl-β-cyclodextrin (HPβCD) and the alkalizers sodium bicarbonate ($NaHCO_3$) and tri(hydroxymethyl)aminomethane (Tris) were developed. During the stability study of these freeze-dried products, formation of new degradation products was seen. We have characterized these products by using high performance liquid chromatography in combination with photodiode array detection and mass spectrometry. In total, five new degradation products were identified of which three were detected in both freeze-dried products and two only in the freeze-dried product composed of EO-9/HPβCD/$NaHCO_3$.

Furthermore, the purity profile of two lots of EO-9 drug substance was investigated. Five, probably synthetic intermediates were found. However, the amount of total impurities was very small for both lots of drug substance and below acceptable international limits for pharmaceutical use.

Introduction

EO-9, 3-hydroxymethyl-5-aziridinyl-1-methyl-2-(1*H*-indole-4,7-dione)-prop-ß-en-α-ol, is a bioreductive alkylating indoloquinone and a synthetic analogue of the antitumour antibiotic mitomycin C (MMC). The formulation of EO-9 for intravesical instillation currently used in phase II clinical trials for the treatment of superficial bladder is a freeze-dried product, which has to be reconstituted with a separate solution prior to administration. This bladder instillation fluid contains 30% v/v propylene glycol after reconstitution and dilution. To avoid the need of a special reconstitution solution and use of the hyper-osmotic organic co-solvent propylene glycol, alternative formulations for intravesical administration were developed. These formulations were freeze-dried products containing EO-9, 2-hydroxypropyl-β-cyclodextrin (HPβCD) and the alkalizers sodium bicarbonate ($NaHCO_3$) and tri(hydroxymethyl)aminomethane (Tris).

During the stability study of these freeze-dried products, formation of new degradation products were seen and therefore, characterization of these products was performed.

This article describes the characterization of the degradation products of EO-9 formed in freeze-dried products containing HPβCD and the alkalizers $NaHCO_3$ or Tris using high performance liquid chromatography in combination with photodiode array

detection and mass spectrometry. Furthermore, two lots of EO-9 drug substance and EO-9 drug substance after forced degradation in media of pH 6 and pH 11 were analyzed as reference.

Material and methods

Materials

EO-9 drug substance (Mw = 288 Da) was supplied by Spectrum Pharmaceuticals, Inc. (Irvine, CA, United States). HPβCD (Mw = 1399 Da) was purchased from Roquette Freres (Lestrum, France). $NaHCO_3$ was purchased from BUFA (Uitgeest, The Netherlands). Tris and tert-butyl alcohol (TBA) were obtained from Merck (Darmstadt, Germany). Sterile Water for Injection (WfI) was purchased from B. Braun (Melsungen, Germany). All chemicals obtained were of analytical grade and used without further purification.

High performance liquid chromatography with photodiode array detection (HPLC-PDA)

HPLC was performed using a system composed of a HP1100 Series binary HPLC pump and degasser (Agilent Technologies, Palo Alto, CA, USA), a Model SpectraSERIES AS3000 automatic sample injection device equipped with a 100 µl sample loop (Thermo Separation Products, Breda, The Netherlands). Gradient chromatography was performed using a Synergi 4U Fusion-RP 80A column (150 x 2.0 mm ID, particle size of 4.0 µm; Phenomenex, Torrance, CA, USA). The mobile phase consisted of ammonium hydroxide (pH 8.5; 1mM) in water and methanol, pumped at a flow-rate of 0.2 ml/min. The gradient started with 5% methanol and 95% 1mM ammonium hydroxide. This condition was maintained for 15 min. After 15 min. the amount of methanol was linearly increased to 70% in 15 min. and subsequently increased to 80% in 5 min. This condition was maintained for 10 minutes. After a run time of 45 minutes the gradient was returned to 5% methanol in one minute and the column was stabilized for 4 minutes, resulting in a total run time of 50 minutes. A sample injection volume of 10 µl was used. Detection was performed with a PDA detector Model Waters™ 996 (Waters Chromatography B.V., Etten-Leur, The Netherlands) at 270 nm with PDA detection from 800 to 200 nm. Chromatograms were processed using Chromeleon software (Dionex Corporation, Sunnyvale, CA, USA).

High performance liquid chromatography with mass spectrometry (HPLC-MS)

Analysis was performed using a HPLC system consisting of a HP1100 Series binary HPLC pump, degasser and HP1100 autosampler (Agilent Technologies, Palo Alto, CA, USA). The same analytical column, mobile phase, gradient and flow were used as described for the HPLC-PDA analysis. The HPLC eluate was fed directly into an API 365 triple quadrupole MS equipped with an electrospray ion source and controlled with Analyst™ 1.2 software (Sciex, Thornhill, ON, Canada). The quadrupoles were operated in the positive ion mode. Nebulizer gas (compressed air), turbo gas (compressed air) and curtain gas (N_2) were operated at 8 psi, 7 ml/min and 15 psi, respectively. The ionspray voltage was kept at 5500 V, with a source temperature of 250ºC. Sample injections of 5 µl were carried. Total Ion Current (TIC) and Q1 spectra were used for identification of intermediates and degradation products utilizing Analyst™ software version 1.2 (Sciex). For the Q1 spectra, background subtraction was performed.

Manufacture of freeze-dried products

Two different freeze-dried products were manufactured. One product was composed of EO-9, HPβCD and $NaHCO_3$ and the other was composed of EO-9, HPβCD and Tris. Formulation solutions of EO-9 (2 mg/ml), HPβCD (300 mg/ml) and $NaHCO_3$ (10 mg/ml) or Tris (0.5 mg/ml) in 20% v/v TBA in WfI were sonicated for 2 hours. Subsequently, aliquots of 2 ml were filled in 8 ml glass vials (hydrolytic class I type Fiolax-clear, Münnerstadter Glaswarenfabrik, Münnerstadt, Germany), partially closed with grey butyl rubber lyophilization stoppers (Type FM157/1, Helvoet Pharma N.V., Alken, Belgium) and freeze dried (Model Lyovac GT4, GEA Lyophil GmbH, Hürth, Germany). The solutions were frozen to –35°C in one hour. The primary drying phase started after 2 hours and was performed at a shelf temperature of –35°C and a chamber pressure of 0.20 mbar for 45 hours. The product temperature during primary drying was -30ºC. For secondary drying the temperature was raised to +25°C in 15 hours. The chamber pressure remained 0.20 mbar. Vials were closed under vacuum after 3 hours of secondary drying.

Identification of intermediates and degradation products

Both freeze-dried products were stored at the accelerated storage condition of 40±2ºC and 75±5% relative humidity (RH). Samples of EO9/HPβCD/Tris and EO9/HPβCD/$NaHCO_3$ freeze-dried product were taken after 2 and 3 months of storage, respectively. Furthermore, two lots of EO-9 drug substance were analyzed, Lot A and B (stored at 2-8°C, in the dark). Lot A and B were manufactured by different suppliers and via different pathways. Lot A was synthesized in July 1993 and

was used as reference standard. Lot B was synthesized in November 2003 and used for the manufacture of both freeze-dried products. Of both lots of EO-9 drug substance a stock solution of 100 µg/ml was prepared in methanol and diluted one hundred times with a solution composed of 1mM NH_4OH:methanol 7:3 v:v prior to analysis. Furthermore, the pH of the diluted stock solution of drug substance Lot A was adapted to pH 6 and pH 11 with 1M HCl and 1M NaOH, respectively, to study degradation of EO-9 in absence of excipients (the optimal pH of EO-9 is 8.75[1]). EO-9 was incubated in these solutions for approximately one hour at room temperature. Subsequently, intermediates (i.e. compounds assumed to be formed during synthesis of EO-9 drug substance) and degradation products of EO-9 were characterized with use of HPLC-PDA and LC-MS analysis.

Results and discussion

HPLC-PDA and HPLC-MS analysis

The mobile phase was composed of methanol and 1mM NH_4OH of pH 8.5. This is the pH of the minimum of the log k-pH profile of EO-9 [1] and therefore, no degradation of EO-9 was expected during analysis. Furthermore, a wide range in percentage of methanol in the gradient was chosen to be able to detect both polar and non-polar synthetic intermediates and degradation products of EO-9 in drug substances and freeze-dried products. With PDA detection UV-spectra of detected compounds were recorded. With use of these UV-spectra an indication of the chromophore was obtained. However, this technique alone was insufficient to fully elucidate the molecular structure of intermediates and/or degradation products. Therefore, LC-MS analysis was also performed.

Identification of intermediates and degradation products

Two Lots of EO-9 drug substance (Lot A and B), EO-9 drug substance Lot A in alkaline solution (pH 11), EO-9 drug substance Lot A in mild acidic/neutral solution (pH 6) and both freeze-dried products fresh and after storage at 40±2ºC/75±5%RH were analyzed using HPLC-PDA and HPLC-MS analysis. The m/z values of the Q1 spectra and the λ_{max} values of the UV spectra of the degradation products and putative intermediates found in these products are given in Table I. The TIC spectra are depicted in Figure I.

Table I. Intermediates and degradation products of EO-9 drug substance, EO-9 in alkaline and acidic environment, and EO-9 in freeze-dried products composed of EO-9/HPβCD/$NaHCO_3$ and EO-9/HPβCD/Tris.

Signal	Retention time (min.)	EO-9 Lot A [1] F1 m/z	λ	EO-9 Lot B [2] F2 m/z	λ	EO-9 pH 11 [3] F3 m/z	λ	EO-9 pH 6 [4] F4 m/z	λ	EO-9/CD/Bic [5] F5 m/z	λ	EO-9/CD/Bic [6] 40ºC/75% RH F6 m/z	λ	EO-9/CD/Tris [7] F7 m/z	λ	EO-9/CD/Tris [8] 40ºC/75% RH F8 m/z	λ
A	1.6	- a - b		-	-	246 286 492 550	210 281 311 365 541	-	-	-	209 280 311 359 541	-	209 280 311 360 544	-	-	-	213 281 311 363 552
B	2.3	-	-	-	-	-	-	-	-	-	-	257 287 305	-	-	-	-	-
C	33.0 EO-5a	-	212 277 323 363 541	-	-	-	-	307 329 636	212 277 323 364 545	-	213 278 324 365 546	-	212 277 321 365 547	-	212 277 323 363 547	-	212 277 323 365 546
D	33.4	-	-	229 273 313	-	-	-	-	-	229 273 291 313	204 252 307	-	-	-	-	-	-
E	33.7	-	-	-	-	231 260 272 300 559 578	272 353 487	-	-	-	-	-	207 251 276 322 357 541	-	-	-	-

Signal	Retention time (min.)	EO-9 Lot A [1] F1 m/z	λ	EO-9 Lot B [2] F2 m/z	λ	EO-9 pH 11 [3] F3 m/z	λ	EO-9 pH 6 [4] F4 m/z	λ	EO-9/CD/Bic [5] F5 m/z	λ	EO-9/CD/Bic [6] 40ºC/75% RH F6 m/z	λ	EO-9/CD/Tris [7] F7 m/z	λ	EO-9/CD/Tris [8] 40ºC/75% RH F8 m/z	λ
F	33.9 EO-9	241 271 289 312 542 560 578 595	203 270 313 365 505	241 272 289 311 542 560 578	269 313 363 507	241 260 272 289 312 542 559 578 600	270 312 363 506	241 272 289 312 542 559 600	201 270 313 366 506	241 271 289 312 542 559 578 595	270 313 364 506	241 271 289 311 542 559 578 595	208 271 313 364 512	241 271 289 542 559 578 595	270 313 363 506	272 289 542 559 578 595	201 270 313 366 506
G	34.5	-	-	-	-	-	-	-	-	-	-	164 441 500 720 940 1160	-	163 280 441 720 940 1160	-	-	-
H	34.9	119 179 321	208 278 319 365 544	119 179 321 343 664	253 273 321 369 534	119 179 321 343	203 252 277 324	179 304 321 343 664	206 270 321 363 544	-	252 314	179 270 287 309	227 279 376	119 179	201 252	-	201 252 275 321 369 527
I	35.5	-	-	-	-	-	-	-	-	-	-	291	212 278 322 364 549	-	-	326 347	-
J	36.1	-	270 317 367 544	117 145 163 335 357	226 268 318 364	-	-	145 163 326 347 559 599	201 276 320 361 544	-	-	-	-	145 163 325 335	230 264 318	-	201 276 321 366 540

Signal	Reten-tion time (min.)	EO-9 Lot A [1] F1 m/z	λ	EO-9 Lot B [2] F2 m/z	λ	EO-9 pH 11 [3] F3 m/z	λ	EO-9 pH 6 [4] F4 m/z	λ	EO-9/CD/Bic [5] F5 m/z	λ	EO-9/CD/Bic [6] 40ºC/75% RH F6 m/z	λ	EO-9/CD/Tris [7] F7 m/z	λ	EO-9/CD/Tris [8] 40ºC/75% RH F8 m/z	λ
K	38.1	129 148 171 229	-	129 148 171 230	-	129 148 172 191 229	-	129 148 171 189 229	-	129 148 171 239	-	129 148 171 239 364 385	210 278 322 368	129 147 171 229	-	364 385	210 277 325 369 547
L	38.6	141 183 241	-	129 141 183 241	-	131 183 241	-	155 183 241	-	129 183 241	-	129 183 241 650 672	-	129 141 183 241	-	141 183 241	-
M	44.3	-	-	-	-	-	-	-	-	106 288 311	-	-	-	106 289 311 598	-	-	-
N	45.1	-	-	149 205 280	-	-	-	-	-	149 205 279	-	149 205 279	-	130 149 205 280	-	-	-

[a] m/z values seen in the Q1 spectrum

[b] λ_{max} values seen in the UV/VIS spectrum

[1] EO-9 drug substance manufactured by Kyowa Hakko (Japan) in July 1993

[2] EO-9 drug substance manufactured by Irix Pharmaceuticals, Inc. (USA) in November 2003

[3] EO-9 drug substance Lot A incubated for approximately 1h at room temperature at pH 11

[4] EO-9 drug substance Lot A incubated for approximately 1h at room temperature at pH 6

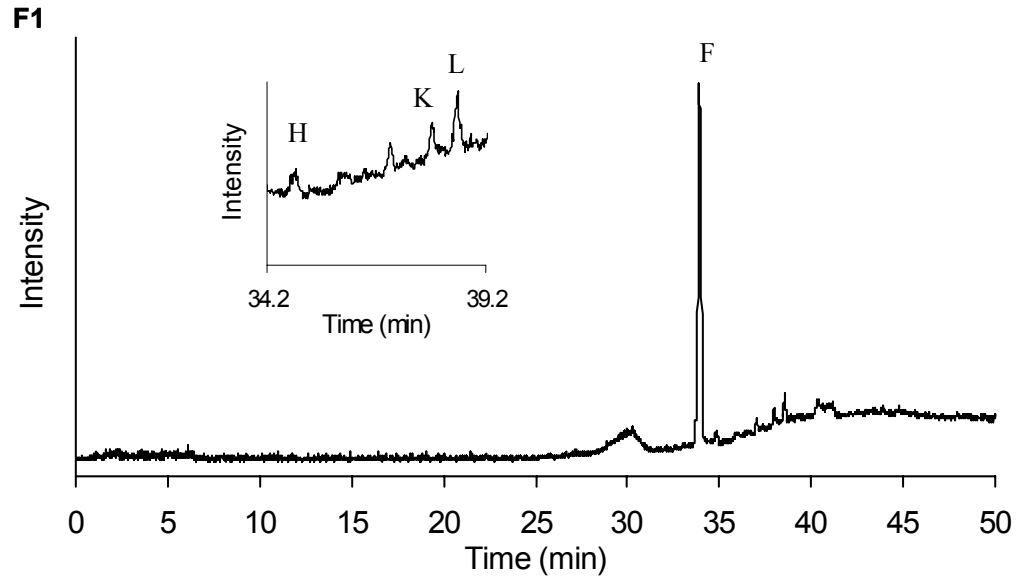
[5] Freeze-dried product prepared from EO-9 drug substance Lot B, HPβCD and $NaHCO_3$ (4/600/20 mg/vial respectively).

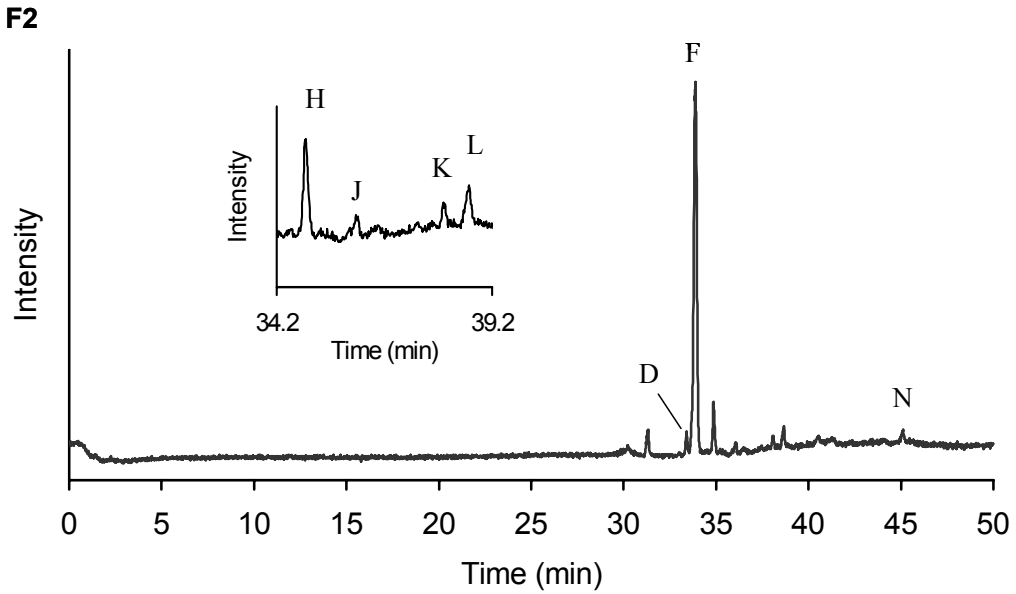
[6] Product 5 stored at 40ºC/75%RH for 3 months

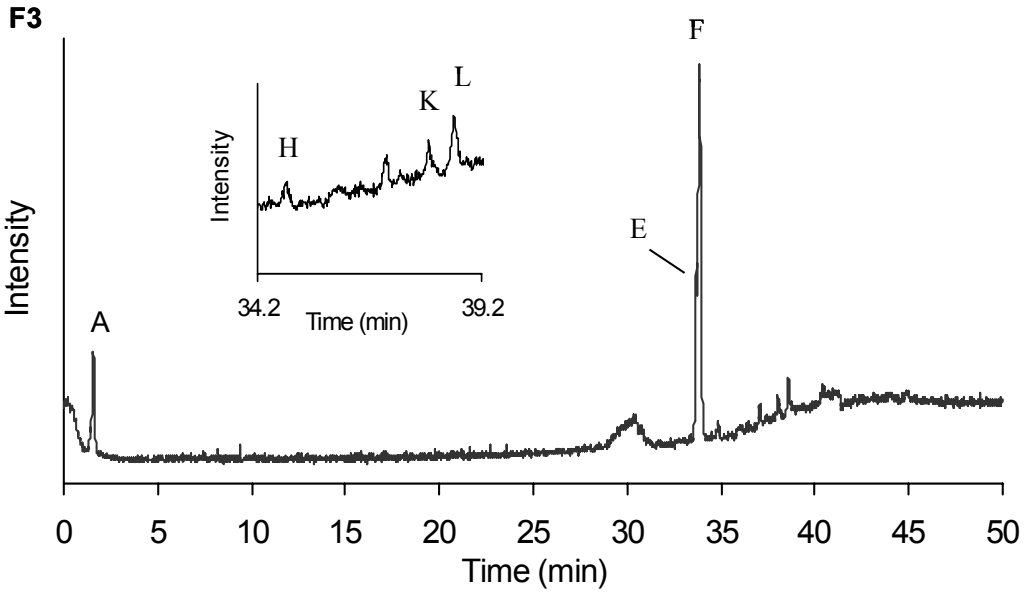
[7] Freeze-dried product prepared from EO-9 drug substance Lot B, HPβCD and Tris (4/600/1 mg/vial respectively).

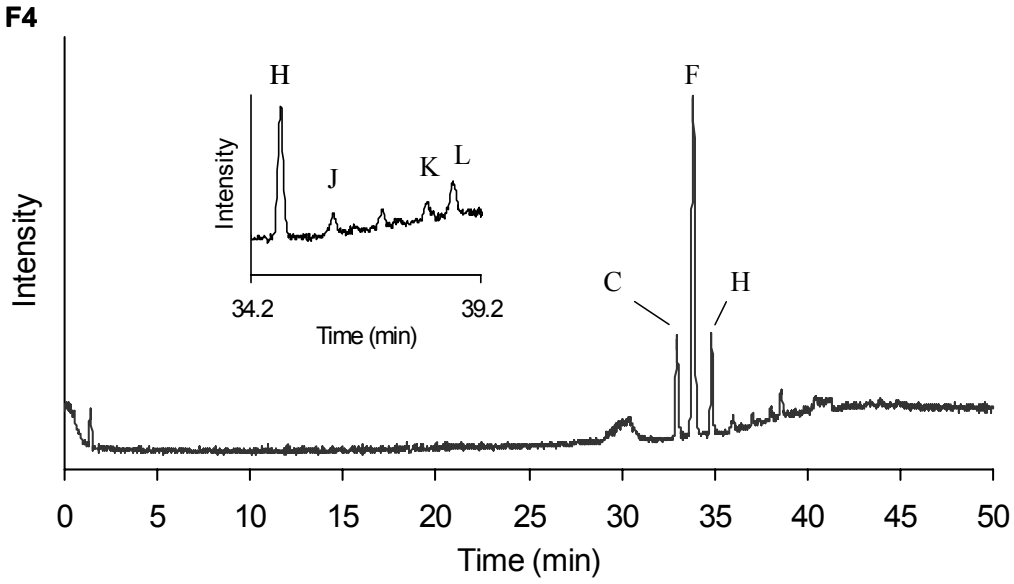
[8] Product 7 stored at 40ºC/75%RH for 2 months

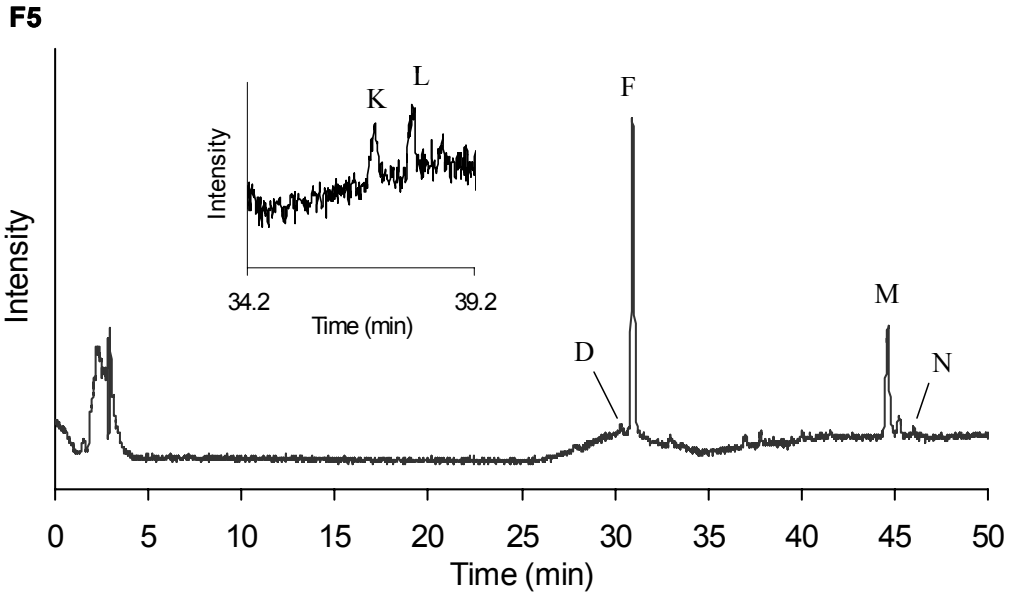
Figure I. TIC of EO-9 drug substance Lot A (Figure 1, F1), EO-9 drug substance Lot B (F2), degradation of EO-9 at pH11 (F3), degradation of EO-9 at pH6 (F4), fresh freeze-dried product composed of EO-9/HPβCD/$NaHCO_3$ (F5), freeze-dried product composed of EO-9/HPβCD/$NaHCO_3$ stored at 40ºC/75%RH for 3 months (F6), fresh freeze-dried product composed of EO-9/HPβCD/Tris (F7), freeze-dried product composed of EO-9/HPβCD/Tris stored at 40ºC/75%RH for 2 months (F8).

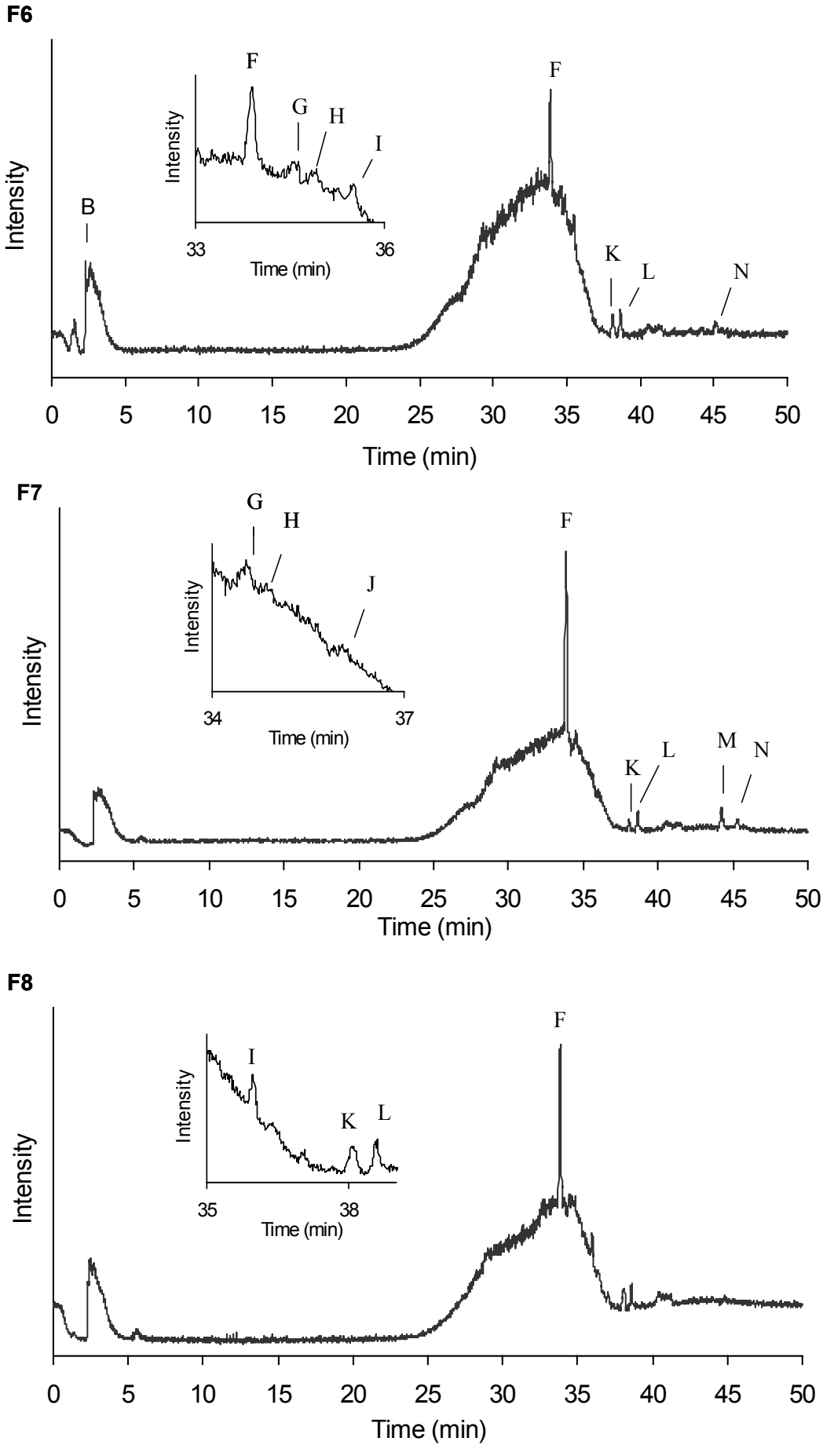





F3
Intensity
A
H
K
L
E
F
34.2
39.2
Time (min)
0
5
10
15
20
25
30
35
40
45
50
Time (min)


F4
Intensity
H
J
K
L
C
F
H
34.2
39.2
Time (min)
0
5
10
15
20
25
30
35
40
45
50
Time (min)


F5
Intensity
K
L
F
D
M
N
34.2
39.2
Time (min)
0
5
10
15
20
25
30
35
40
45
50
Time (min)


F6
Intensity
B
F
G
H
I
33
36
Time (min)
F
K
L
N
0
5
10
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20
25
30
35
40
45
50
Time (min)
F7
Intensity
G
H
J
34
37
Time (min)
F
K
L
M
N
0
5
10
15
20
25
30
35
40
45
50
Time (min)
F8
Intensity
I
K
L
35
38
Time (min)
F
0
5
10
15
20
25
30
35
40
45
50
Time (min)

The TIC of blank HPβCD showed broad signals at 1- 4 and 24 - 37 minutes (data not shown). These broad signals were also present in the TIC of the freeze-dried products and therefore, some small signals of degradation products in these areas may not have been detected with HPLC-MS analysis. In total fourteen different compounds were detected and given letter A-N. These compounds are discussed below.

Compound A:

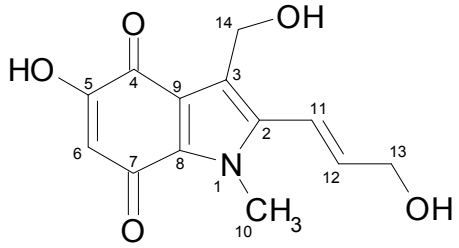
A signal at 1.6 min was only seen in the TIC after degradation of EO-9 in alkaline solution. With HPLC-PDA analysis, this compound was detected in EO-9 in alkaline solution, but also in fresh and stored EO-9/HPβCD/$NaHCO_3$ freeze-dried product, and in stored EO-9/HPβCD/Tris freeze-dried product. The UV spectra obtained with PDA analysis of this compound in these freeze-dried products resemble the UV spectrum of the product that was seen in EO-9 samples in alkaline medium. This indicates that it concerns the same compound. The compound was not seen in fresh EO-9/HPβCD/Tris freeze-dried product, thus it was formed in time. Furthermore, it was found in fresh EO-9/HPβCD/$NaHCO_3$ indicating that the formation of this compound is favored by $NaHCO_3$ rather than by Tris. Jonkman-De Vries et al [1] showed that the degradation product of EO-9 formed in alkaline solution has a molecular mass (mw) of 263 Da. In this product, the aziridine ring is replaced by a hydroxyl group (Figure II, compound A). The m/z values found at 1.6 min correspond to this molecule: 246 $(MH\text{-}H_2O)^+$, 286 $(M+Na)^+$, 492 (dimer of fragment with mw = 245 $(MH)^+$), and 550 (dimer of fragments with mw = 263 and mw = 285 $(MH)^+$). The pK_a of such an enolic hydroxyl group is approximately 4. Due to the high pH of the mobile phase (pH 8.5) the hydroxyl moiety is deprotonated, resulting in a negatively charged analyte that elutes almost with the solvent front. This was also seen for 7-hydroxymitosane, a degradation product of mitomycin C [2]. In strong acidic environment EO-9 is probably first degraded into EO-5a (Fig. II, compound C) and subsequently into compound A.

Compound B:

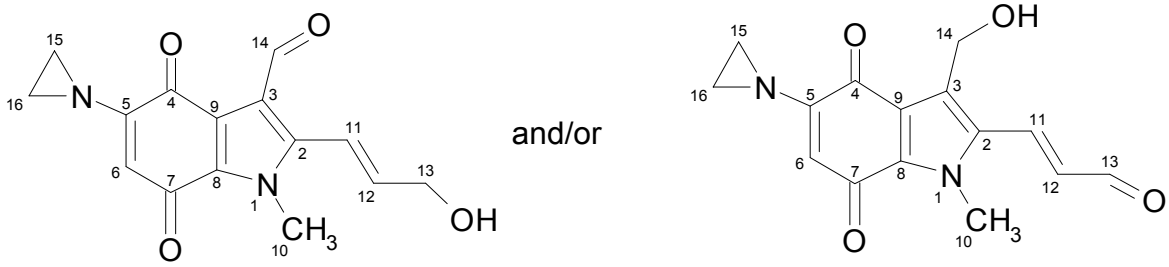
The signal of this compound is superimposed on the cyclodextrin signal and was only seen in the TIC of EO-9/HPβCD/$NaHCO_3$ freeze-dried product stored at 40ºC/75%RH. No signal was found with HPLC-PDA analysis. The m/z values correspond to EO-9 and EO-5a minus two hydrogen atoms (MH^+ = 287 Da and 305 Da, respectively). This could be due to oxidation of one of the hydroxyl groups in EO-9 or EO-5a to a carbonyl function (Fig. II, compound B) by $NaHCO_3$. This oxidation process is probably accelerated by the relatively high pH of the formulation. The short retention time of this compound is Remarkable.

Figure II. Proposed molecular structures

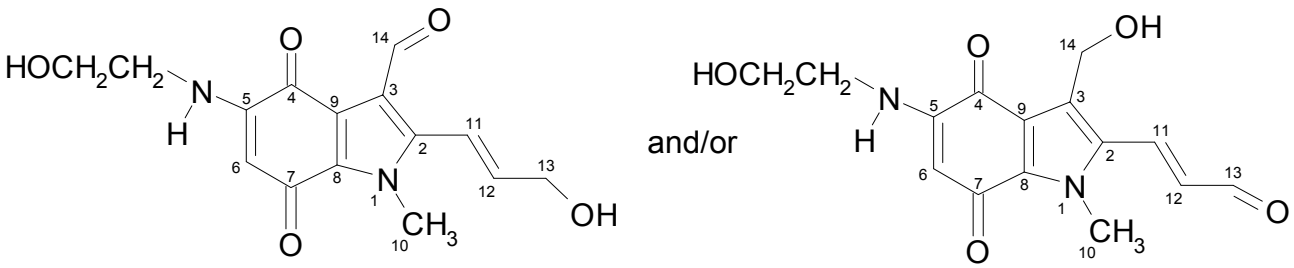
Compound A



Compound B

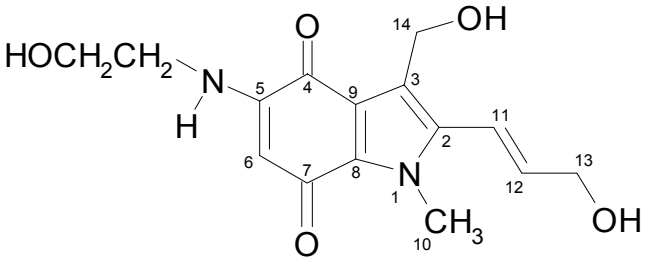


Compound B formed from EO-9



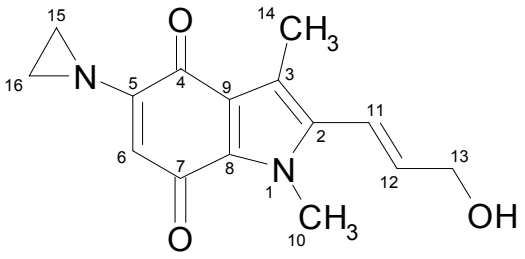
Compound B formed from EO-5a

Compound C

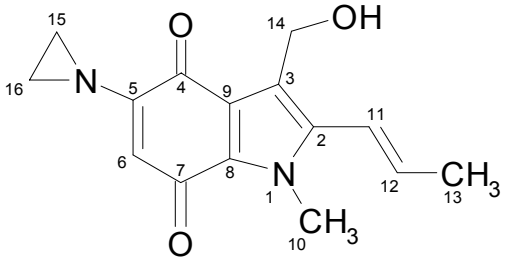


EO-5a, m/z =307

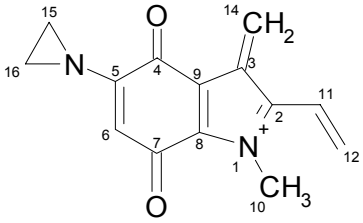
Compound D



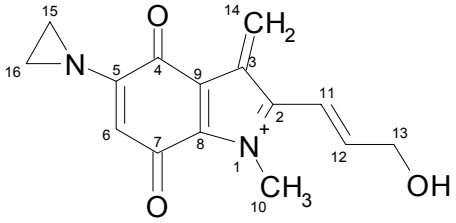
or



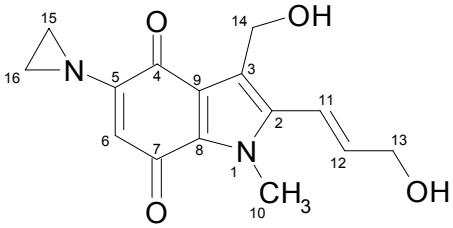
Compound F



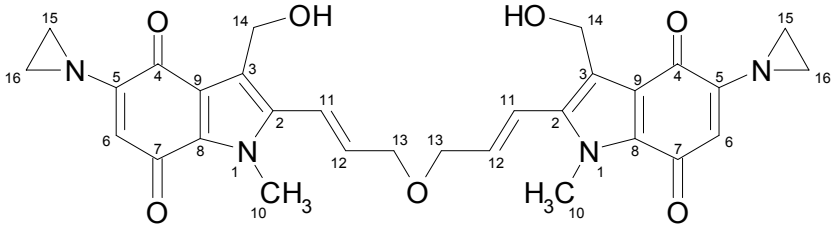
Fragment of EO-9, m/z = 241



EO9 - water, m/z = 271

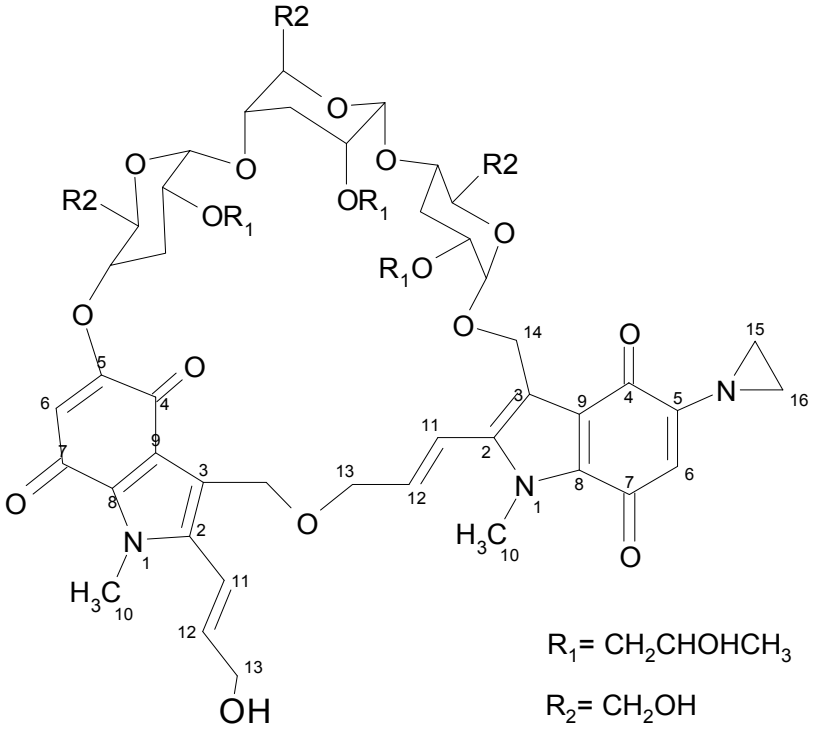


EO-9, m/z = 289



Dimer of EO-9, m/z = 560

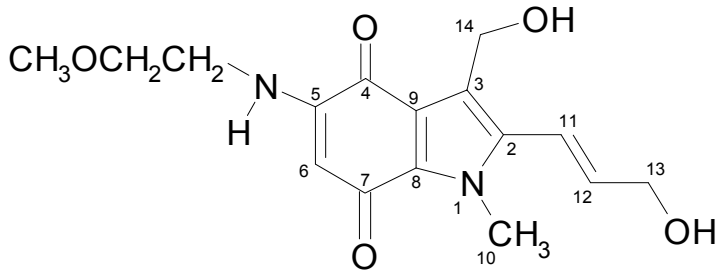
Compound G



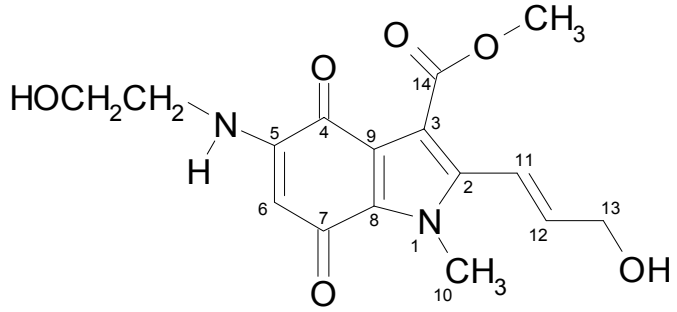
R_1= $CH_2CHOHCH_3$

R_2= CH_2OH

Compound H



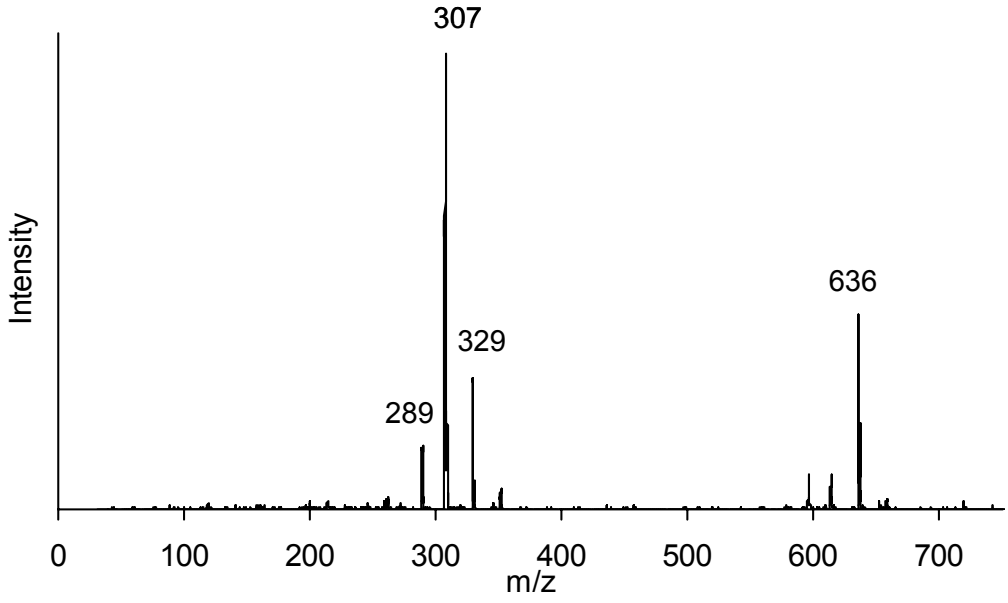
Compound J



Compound C:

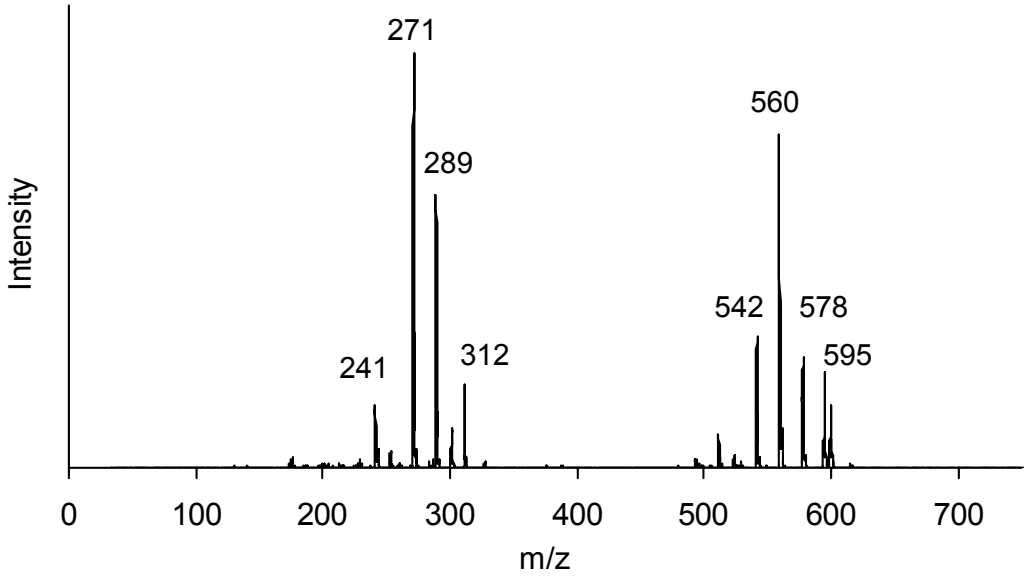
This compound is EO-5a (Fig. II, compound C), the main degradation product of EO-9 in (mild) acidic medium, with characteristic m/z values of 307 $(MH)^+$, 329 $(M+Na)^+$, and 636 (dimer of EO-5a + $Na)^+$ (Fig. IIIa). The UV spectrum of EO-5a showed signals at approximately λ = 212, 278, 323, 365, and 547 nm (Fig. IV).

Figure IIIa. Q1 spectrum of EO-5a formed from EO-9 in a solution with pH 6.



EO-5a was found in EO-9 drug substance Lot A (manufactured in 1993) and in all freeze-dried products, but not in EO-9 drug substance Lot B. Because the freeze-dried products were manufactured with EO-9 drug substance Lot B, the amount of EO-5a found in the freeze-dried products is probably due to degradation of EO-9 during manufacture. The relative area of EO-5a found with HPLC-PDA analysis of the fresh freeze-dried products composed of EO-9/HPβCD/$NaHCO_3$ and EO-9/HPβCD/Tris was 0.6 and 1%, respectively.

Figure IIIb. Q1 spectrum of EO-9 from EO-9 drug substance Lot A.



Compound D:

Compound D is present in Lot B of EO-9 drug substance and in the fresh freeze-dried product composed of EO-9/HPβCD/$NaHCO_3$. This compound is probably an intermediate formed during synthesis of EO-9 drug substance and because it was not seen anymore after storage of EO-9/HPβCD/$NaHCO_3$ freeze-dried product, it is assumed that it degrades upon storage at 40ºC/75%RH. An m/z value of 273 Da corresponds to EO-9 minus oxygen. The proposed structures are given in Fig. II, compound D.

Compound E:

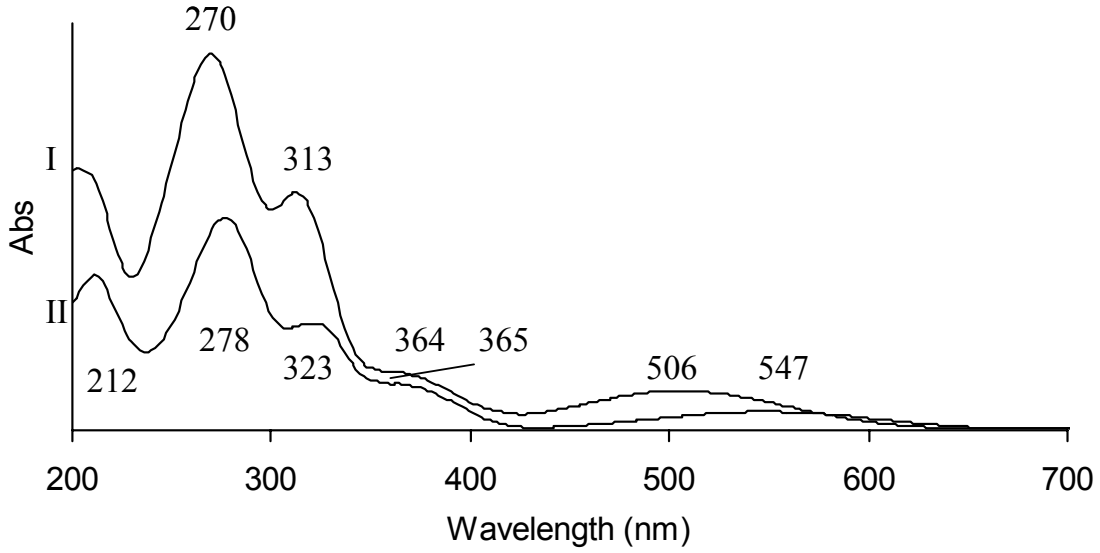
A signal at a retention time of 33.7 min was seen with LC-MS and HPLC-PDA analysis of EO-9 degraded in alkaline solution and with HPLC-PDA analysis of EO-9/HPβCD/$NaHCO_3$ freeze-dried product after storage at 40°C/75%RH. However, the UV spectra of these two products are different. The UV spectrum of the compound in the freeze-dried product corresponds to EO-5a, with an extra (and most abundant) signal at λ = 251 nm. Furthermore, a hypsochromic shift of the first signal from 212 to

207 nm was seen. This could be due to analytical variation. However the extra signal at λ = 251 nm in combination with the hypsochromic shift could indicate that more electrons are incorporated in the chromophore. Because the retention time slightly increased compared to EO-5a, it is assumed that the ring-system remained intact and that substituents changed the chromophore and made it less polar. No structural assignments could be made.

Compound F:

Compound F corresponds to EO-9 drug substance with characteristic m/z values of 241 (-OH on the 14-position and $–CH_2OH$ on the 13-position are spliced off), 271 $(MH–H_2O)^+$, 289 (MH^+), and 312 $(M+Na)^+$ (Fig. II, compound F). However, m/z values of 542, 560, 578, 595 Da were also found (Fig. IIIb). The mass of 542 Da corresponds to a dimer of the fragment of EO-9 with a m/z value of 271. The mass of 560 Da corresponds to a dimer of EO-9 minus H_2O. H_2O is probably split off during the formation of the dimer (Fig. II, compound F, dimer). A dimer of EO-9 (minus H_2O) was detected with m/z 578 Da and this dimer with an extra H_2O was detected at m/z 595 Da. If those dimers were already present in the products they would have had other retention times compared to EO-9. Because these dimers are detected in the same signal as EO-9, it is obvious that these dimers are formed in the ion spray during analysis. Furthermore, with HPLC-PDA analysis a characteristic spectrum of EO-9 was found with maximum signals at approximately λ = 270, 313, 364, and 506 nm (Fig. IV).

Figure IV. UV spectra of EO-9 (I) and EO-5a (II) obtained with PDA detection.



Compound G:

Compound G was only found in the freeze-dried product composed of EO-9/HPβCD/$NaHCO_3$ after storage at 40°C/75%RH and in fresh freeze-dried product composed of EO-9/HPβCD/Tris. The Q1 spectrum showed m/z values of 164, 441, 500, 720, 940 and 1160. Between the last four signals a difference in m/z of 220 is seen. This mass corresponds to a hydroxypropyl moiety conjugated to a glucose-unit. βCD is a cyclic sugar composed of seven glucose units. HPβCD is obtained by conjugation of βCD with hydroxypropyl groups. The mean degree of substitution of the cyclodextrin we used is 5. However, this conjugation process follows a Gaussian distribution also resulting in molecules with a different degree of substitution and thus different masses. The mass of one hydroxypropyl group is 59 Da. Therefore, groups of signals with the same number of glucose units, but a difference in mass of 59 are seen with MS analysis of HPβCD. These groups of signals are indicated as an "envelope". However, the signals we found are not part of such an envelope. Perhaps it is a complex of compound A with one EO-9 molecule and 1-3 glucose units (Fig. II, compound G). The proposed, speculative, structure has a molecular weight of 1162. Because it is not very likely that HPβCD falls apart in the freeze-dried product and because formation of dimers of EO-9 and EO-5a in the ion spray was seen earlier, it is assumed that this compound is also formed in the ion spray. It is a very remarkable compound, with a retention time equal to HPβCD. Therefore, a lot of cyclodextrin was in the spray when this compound was formed, what may explain its formation.

Compound H:

The UV spectrum of this compound resembles the UV spectrum of EO-5a. However, it has a longer retention time than EO-5a, indicating that it is less polar. The small hypsochromic shift of the first signal from λ = 212 to 208 nm could be due to analytical variation. Furthermore, m/z values of 321, 343, and 664 Da are found. The compound at m/z = 321 Da is probably EO-9 after reaction with H_3COH $(MH)^+$ in the same way as it reacts with H_2O to form EO-5a (Fig. II, compound H). The m/z values also exhibit the formation of a sodium adduct $(M+Na)^+$ at m/z = 343 Da and dimerization $(dimer + Na)^+$ at m/z = 664 Da. The mobile phase is composed of 1mM ammonium hydroxide (pH 8.5) mixed with methanol and it is likely that this is the source of H_3COH to react with EO-9. The small signals of this compound found in the TIC spectra, indicate that probably only a very small amount of EO-9 reacts with H_3COH.

Compound I:

This compound is found in both freeze-dried products after storage at 40ºC/75%RH and is neither present in EO-9 drug substance nor after acidic or alkaline degradation.

Due to the very minor amount found, it was not possible to characterize the compound found in the freeze-dried products composed of EO-9/HPβCD/Tris, but it is assumed that it is the same as the compound formed EO-9/HPβCD/$NaHCO_3$. The UV spectrum of this degradation product in the EO-9/HPβCD/$NaHCO_3$ product is similar to the UV spectrum of EO-5a. Furthermore, an m/z value of 291 Da was found. Because a longer retention time and the same UV spectrum as EO-5a are found, it is proposed that this compound is an analogue of EO-5a and that one oxygen atom has been lost.

Compound J:

This compound is found in both Lots EO-9 drug substance, and in EO-9/HPβCD/Tris freshly prepared and to a lesser extent after storage at 40ºC/75%RH. Because this compound was found in both EO-9 drug substances, it is assumed that it is an intermediate or by-product formed during synthesis. Several m/z values are found. The m/z value of 335 Da is corresponding with EO-5a having a $–COOCH_3$ substituent instead of $–CH_2OH$ (Fig. II, compound J). This also explains the retention time being longer than EO-5a. Formation of intermediates with this substituent on the 3-position during synthesis of EO-9 drug substance was reported earlier by Jonkman-de Vries et al[3]. Other indications for this compound being an analogue of EO-5a are the UV spectra showing resemblance with that of EO-5a.

This compound was not seen in EO-9 in alkaline solution nor in the freeze-dried product with $NaHCO_3$ probably due to hydrolysis of the ester bond.

Compound K:

This compound is present in both Lots of EO-9 drug substance and therefore it is assumed that it is an intermediate or by-product formed during synthesis of the drug substance. This intermediate is stable in strong alkaline and mild acidic environment and is also found in all freeze-dried products. The UV spectrum resembles the spectrum of EO-5a, but the retention time is much longer than EO-5a, indicating the introduction of apolar substituents. Remarkable are the small m/z values seen with LC-MS analysis compared to EO-5a. Perhaps the substituents are unstable and separate easily from the molecule in the ion spray, making structural assignment impossible.

Compound L:

Of this product no UV spectra could be recorded due to the minor amount present. This product is traced in both Lots of EO-9 drug substance and all freeze-dried products. The m/z value of 241 is also seen in the Q1 spectrum of EO-9 (Fig. IIIb), which may be indicative for a structural resemblance with EO9. The proposed structure of the fragment with m/z 241 is depicted in Fig. II, Compound F.

Compound M:

Compound M is only found in both freeze-dried products, but was not seen after storage at 40°C/75%RH, indicating that this compound is quite unstable. In the Q1 spectra m/z values of 106, 289, 311, and 598 Da were seen. The fragments with m/z 289 and 311 correspond to EO-9 $(MH)^+$ and the sodium adduct $(M+Na)^+$. However, the fragments with m/z 106 and 598 were not seen in the Q1 spectrum of EO-9. Furthermore, this compound was not seen with UV analysis at 270 nm. This could indicate that this compound possesses structural features of an indoloquinone, but lacks some double bonds in the characteristic chromophore of indoloquinones.

Compound N:

This compound is found in EO-9 drug substance Lot B and in all freeze-dried products except the product composed of EO-9/HPβCD/Tris stored at 40ºC/75%RH. The freeze-dried products were manufactured with EO-9 drug substance Lot B, explaining the presence of this compound in the freeze-dried products. Obviously, this compound is not stable in presence of Tris at 40ºC/75%RH. Possibly, it is a synthetic intermediate or by-product formed during the synthesis of EO-9 drug substance. Only low m/z values were found in the Q1 spectrum and therefore, structural assignments could not be made.

Conclusion

This study was performed to investigate the influence of the complexing agent HPβCD in the formulation on the degradation of EO-9. Therefore, degradation products formed in the freeze-dried products were compared to degradation products formed from EO-9 in alkaline and mild acidic/neutral solution. In total, five new degradation products were found of which three were found in both freeze-dried products and two only in the freeze-dried product composed of EO-9/HPβCD/$NaHCO_3$. The formation of these extra degradation products in this freeze-dried product could be an explanation for the lower stability of this product compared to the Tris-containing product seen during earlier stability studies. The degradation products only formed in EO-9/HPβCD/$NaHCO_3$ freeze-dried product were shown to be more polar products than EO-9, but the three compounds formed in both freeze-dried products were all less polar than EO-9. Probably, parts of HPβCD are interacting with EO-9 (which could be an explanation for the increase in solubility of EO-9 in presence of HPβCD), resulting in the formation of less polar and probably larger compounds. Unfortunately, the exact structures could not be elucidated possibly due to splicing of those larger molecules in the ion spray.

Furthermore, the purity profile of two lots of EO-9 drug substance was investigated. Three intermediates were found in both products of which two intermediates are probably analogues of EO-5a and one of EO-9. Furthermore, EO-5a was found in the oldest lot (Lot A, manufactured in 1993), probably due to degradation in time. Two more intermediates were found (in very small amounts) in Lot B of EO-9 drug substance (manufactured in 2003).
In general the levels of al degradation products/impurities in the pharmaceutical products were very low and when quantifiable with PDA less than 1%.

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Chapter 1.4

EO-9 Bladder instillations: stability characteristics and in vitro simulation studies

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D. Mirejovsky, L. Lenaz, B. Nuijen

Submitted for publication

Abstract

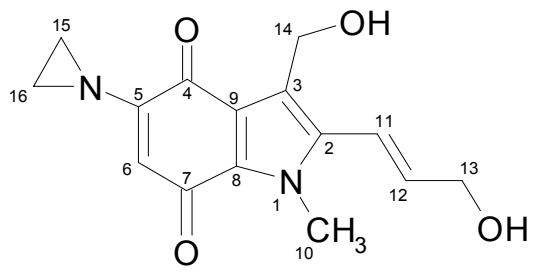
A bladder instillation of EO-9 (EOquin^{TM}) is currently used in phase II clinical trials for the treatment of superficial bladder cancer. Three alternative formulations were developed to improve its pharmaceutical properties and clinical acceptability. Freeze-dried products composed of EO-9, 2-hydroxypropyl-β-cyclodextrin (HPβCD), tri(hydroxymethyl)aminomethane (Tris), and sodium bicarbonate ($NaHCO_3$) were tested. Selection of one formulation for further development was based on stability studies. These studies comprised stability of the freeze dried products, stability after reconstitution and dilution and stability during bladder instillation in an experimental set-up. The stability study of the freeze dried products showed that the formulation composed of EO-9/HPβCD/Tris (4/600/1 mg/vial) was most stable. After reconstitution and dilution all products were stable for at least 8 hours. The product composed of EO9/HPβCD/$NaHCO_3$ (4/600/20 mg/vial) was the least stable product both as freeze-dried formulation and after reconstitution and dilution. The bladder instillation simulation experiment showed that all products were stable when mixed with urine of pH 8 and unstable in urine of pH 4 and 6. The degradation products formed in urine were EO-5a and EO-9-Cl.
Based on these results, the product composed of EO-9/HPβCD/Tris (4/600/1 mg/vial) was selected for further pharmaceutical development.

Introduction

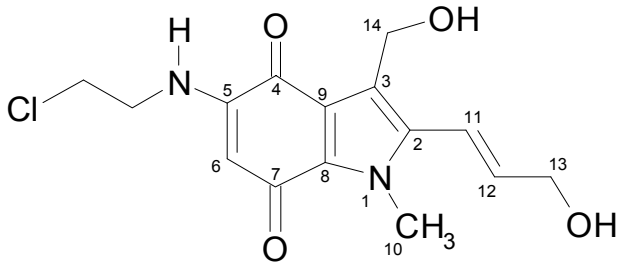
EO-9 (EOquin^{TM}) is a bioreductive alkylating indoloquinone (Figure I) and an analogue of the antitumour antibiotic mitomycin C. EO-9 is an inactive prodrug, which is activated by reduction of the quinone moiety to semiquinone or hydroquinone, generating an intermediate with an electrophilic aziridine ring system, which serves as a target for nucleophilic DNA. This reaction mechanism is common for bioreductive alkylating indoloquinones[1-3]. For the treatment of superficial bladder cancer an investigational pharmaceutical product of EO-9 is currently used successfully in phase II clinical trials. This formulation is a freeze dried product which has to be reconstituted with a separate solution composed of propylene glycol/water for injection (WfI)/sodium bicarbonate ($NaHCO_3$)/sodium edetate 60/40/2/0.02% v/v/w/w. The need of this special reconstitution solution results in higher costs, requires more planning in the logistic field and is less user friendly than reconstitution

with WfI and/or normal saline. Furthermore, the bladder instillation contains 30% v/v propylene glycol after reconstitution and dilution, which is hyper-osmotic and could cause local irritation of the bladder tissue. Therefore, efforts were made to design a new pharmaceutical product for intravesical administration of EO-9. This resulted in three prototype freeze-dried products containing per vial 4 mg EO-9, 600 mg 2-hydroxypropyl-β-cyclodextrin (HPβCD) and one of the alkalizers $NaHCO_3$ (20 mg) or tri(hydroxymethyl)aminomethane (Tris, 1 or 6 mg). HPβCD was selected as complexing agent, because it dramatically increases the solubility of EO-9 in aqueous solutions. The next step in the pharmaceutical development was a stability study. For this product three kinds of stability are of importance: the stability of the freeze dried product to determine storage conditions between manufacture and administration, the stability of the product after reconstitution and dilution to determine storage and handling conditions between preparation of the bladder instillation and administration, and the "in vivo" stability of the product during bladder installation.
This article describes these stability studies and provides practical instructions how to handle this new investigational anticancer agent for the treatment of superficial bladder cancer.

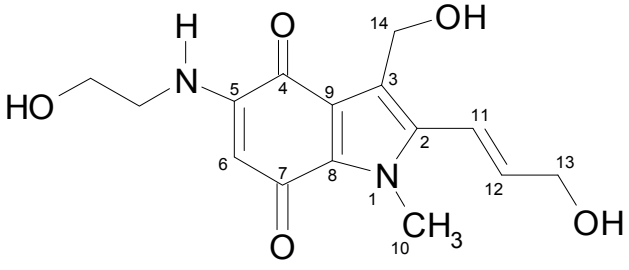
Figure I. Molecular structures of EO-9, EO-5a and EO-9-Cl.



EO-9



EO-9-Cl



EO-5a

Materials and methods

Materials

EO-9 drug substance ($C_{15}H_{16}N_2O_4$, mw = 288 Da) originated from IRIX, Inc. (Irvine, CA, USA). EO-9-d3 internal standard ($C_{15}H_{13}D_3N_2O_4$), EO-9-Cl ($C_{15}H_{17}N_2O_4Cl$, degradation product of EO-9), EO-5a ($C_{15}H_{18}N_2O_5$, degradation product of EO-9), and EO-5a-d4 internal standard ($C_{15}H_{14}D_4N_2O_5$) were all kindly supplied by Spectrum Pharmaceuticals, Inc. (Irvine, USA). WfI and normal saline were originated from B. Braun (Melsungen, Germany). HPβCD (Mw = 1399 Da) was purchased from Roquette Freres (Lestrum, France). Methanol (LC gradient grade) was obtained from Biosolve Ltd. (Amsterdam, The Netherlands). All other solvents or chemicals used were of analytical grade. Distilled water was used throughout the analyses. Drug free human urine was obtained from a healthy volunteer from the laboratory of the Pharmacy at the Slotervaart Hospital (Amsterdam, The Netherlands). All freeze dried products were prepared in-house (Slotervaart Hospital, Amsterdam, The Netherlands).

Manufacturing and stability of the freeze dried product

Formulation solutions composed of EO-9/HPβCD/$NaHCO_3$ (2/300/10 mg/ml), EO-9/HPβCD/Tris (2/300/0.5 mg/ml), and EO-9/HPβCD/Tris (2/300/3 mg/ml) in 20% v/v TBA were sonicated for 2 hours. Aliquots of 2 ml were filled in 8 ml glass vials (hydrolytic class I type Fiolax-clear, Aluglas, Uithoorn, The Netherlands), partially closed with grey butyl rubber lyophilization stoppers (Type FM157/1, Helvoet Pharma N.V., Alken, Belgium) and subsequently freeze dried (Model Lyovac GT4, STERIS, Hürth, Germany). The solutions were frozen to –35°C in one hour. The primary drying phase started after 2 hours and was performed at a shelf temperature of –35°C and a chamber pressure of 0.20 mbar for 45 hours. The product temperature during primary drying was -30ºC. For secondary drying the temperature was raised to +25°C in 15 hours. The chamber pressure of 0.20 mbar was maintained. Vials were closed at a chamber pressure of 0.20 mbar after 3 hours of secondary drying. Subsequently, the freeze dried products were stored at -20±3°C, 5±3ºC and at the accelerated storage conditions 25±2ºC/60±5% relative humidity (RH) and 40±2ºC/75±5%RH, all in the dark. Samples were taken in time and analyzed using HPLC-UV.

Stability after reconstitution and dilution

The stability after reconstitution and dilution was determined in triplicate. Three vials of each product were reconstituted with 1.45 ml WfI and shaken manually. Part of the

reconstituted solutions was filtered using Millex® HV filters (0.45µm x 4mm, Millipore, Etten Leur, The Netherlands) and diluted 20 times with normal saline to a final concentration of 100 µg/ml EO-9, corresponding to the target dose of 4 mg EO-9 per bladder instillation of 40 ml. All solutions were stored in glass containers at room temperature and ambient light. Samples were taken after 2, 4, 6, and 8 hours and analyzed with HPLC-UV.

In vivo simulation experiment

The exact compositions of the bladder instillations tested are given in Table I. To mimic the situation in the bladder of the patient, the instillations were mixed with urine. Typically, a wide variation in urine production is seen, with a mean of approximately 60-120 ml/hour [4]. Because the bladder instillations (with a volume of 40 ml) are administered into empty bladders and must be hold there for one hour, the mean amount of urine present during this hour will be approximately 30-60 ml.

Table I. Composition of five freeze dried products and reconstitution solutions used in the stability study.

Product		Reconstitution solution		Dilution solvent		Final EO-9	Final
		Composition	Volume	Solvent	Volume	concentration	volume
1	EOquin™ *	PG/Wfl/$NaHCO_3$/SE** 60/40/2/0.02% v/v/w/w	20 ml	Wfl	20 ml	100 µg/ml	40 ml
2	EOquin™ *	PG/Wfl/$NaHCO_3$/SE** 60/40/1/0.02% v/v/w/w	20 ml	Wfl	20 ml	100 µg/ml	40 ml
3	EO9/HPβCD/Tris 4/600/6 mg/vial	Wfl	1.45 ml	Normal saline	38 ml	100 µg/ml	40 ml
4	EO9/HPβCD/Tris 4/600/1 mg/vial	Wfl	1.45 ml	Normal saline	38 ml	100 µg/ml	40 ml
5	EO9/HPβCD/$NaHCO_3$ 4/600/20 mg/vial	Wfl	1.45 ml	Normal saline	38 ml	100 µg/ml	40 ml

* EOquin™ is a freeze dried product composed of EO9/mannitol/$NaHCO_3$ 4/25/10 mg per vial
** PG = propylene glycol, SE = sodium edetate

Therefore, bladder instillation:urine ratios of 40:30 ml and 40:60 ml were chosen for this experiment. Because a wide pH range is common in urine, the stability was tested in urine with pH 4, 6, and 8. The pH of the urine was adjusted to pH 4, 6, and 8 using

HCl and NaOH. Immediately after preparation of the bladder instillation, urine was added, the pH was determined and the mixtures were stored at 37°C in the dark in a water bath. Samples were taken after 0, 15, 30, 45, 60 and 120 minutes and stored immediately at -70°C in the dark to prevent further degradation.
Furthermore, the stability of all bladder instillations (i.e. after reconstitution and dilution of the freeze dried products) was determined at the same conditions and samples were taken at the same time points. Samples were analyzed using HPLC-MS/MS. This experiment was performed in triplicate.

Analysis

High performance liquid chromatography with UV detection (HPLC-UV)

Preparation of calibration standards
Two calibration standards were prepared by dissolving EO-9 drug substance in methanol to a concentration of 500 µg/ml followed by a 5-fold dilution with mobile phase, resulting in a final EO-9 concentration of 100 µg/ml. Subsequently, a system suitability test was performed. The requirements of this test were a deviation ≤ 1% between six repetitive injections from one calibration standard and a deviation ≤ 1.5% between the response factors of both calibration standards.

Analysis
HPLC-UV analysis was performed using an isocratic P1000 pump, AS 3000 autosampler and an UV 1000 UV/VIS detector, all from Thermo Separation Products (Breda, The Netherlands). The mobile phase consisted of 5mM phosphate buffer pH 7/methanol 70/30% w/w. A Zorbax SB-C18 analytical column (750 x 4.6mm ID, particle size 3.5 µm, Agilent Technologies, Palo Alto, California, USA) preceded by a guard column (reversed phase 10 x 3mm, Varian, Palo Alto, California, USA) was used. Detection was performed at 270 nm. An injection volume of 10 µL, flow rate of 0.7 ml/min and run time of 10 minutes were applied. Samples were diluted prior to analysis with mobile phase to a concentration of 100 µg/ml. Chromatograms were processed using Chromeleon software (Dionex Corporation, Sunnyvale, CA, USA).

High performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS)

Preparation of calibration standards
Stock solutions of 1 mg/ml of EO-9, EO-5a and EO-9-Cl in ethanol were prepared. Working solutions of EO-9, EO-5a and EO-9-Cl were obtained by dilution of the stock solutions with ammonium acetate buffer (pH 8.5; 0.1 M)/methanol (70/30% v/v).

Subsequently, the working solutions of EO-9, EO-5a and EO-9-Cl were further diluted in ammonium acetate buffer (pH 8.5; 0.1M)/methanol (70/30% v/v) to concentrations ranging from 100 to 25,000 ng/ml. These diluted working solutions were used to prepare the calibration standards.
Furthermore, stock solutions of 1 mg/ml of the internal standards EO-9-d3 and EO-5a-d4 in ethanol were prepared. Subsequently, one working solution of the internal standards was prepared by transferring 500 µl of EO-9-d3 stock solution and 500 µl of EO-5a-d4 stock solution to a 50.0 ml volumetric flask. Subsequently, a mixture of ammonium acetate buffer (pH 8.5; 0.1M)/methanol (70/30% v/v) was added to obtain a final concentration of 1,000 ng/ml for both EO-9-d3 and EO-5a-d4. All solutions were stored at -20±3°C.
Prior to analysis, calibration standards containing EO-9, EO-5a and EO-9-Cl were freshly prepared in a range from 10 to 2,500 ng/ml by dilution of the working solutions of EO-9, EO-5a and EO-9-Cl ten times with ammonium acetate buffer (pH 8.5; 0.1M)/methanol mixture (70/30% v/v), followed by vortex-mixing for approximately 30 seconds. The calibration standards were analyzed in duplicate.

Analysis
The HPLC system comprised an HP1100 (Agilent Technologies, Palo Alto, CA) binary pump, degasser and HP1100 auto sampler (Agilent Technologies, CA). Gradient chromatography was performed using a Gemini C18 column (150 x 2.1 mm ID, particle size 5 µm). The mobile phase consisted of ammonium hydroxide (pH 8.5; 1mM) in water (A) and methanol (B), pumped at a flow-rate of 0.2 ml/min. In the first 0.3 min, the eluent consisted of 60% A and 40% B, followed by 90% B for 2.7 min. The column was stabilized with 40% B for 2 min. The autosampler temperature was 10 °C and 25 µl of pre-treated samples were injected into the HPLC system. Sample pre-treatment was performed by mixing aliquots of 30 µl of the bladder instillation/urine samples with 150 µl working solution of the internal standards and 1,320 µl ammonium acetate (pH 8.5; 0.1M)/methanol (70/30% v/v) solution.
The HPLC eluate was fed directly into an API 2000 triple quadrupole MS equipped with an electrospray (ESI) ion source (Sciex, Thornhill, ON, Canada). Positive ions were created at atmospheric pressure and the mass analyzer was operated in the multiple reaction monitoring (MRM) mode using unit resolution for the quadrupoles. The resulting MRM chromatograms were used for quantification utilizing Analyst™ software version 1.2 (Sciex). Mass transitions of *m/z* 271 → 241 and 274 → 244 were optimized for EO-9 and EO-9-d3, respectively, with dwell times of 150 ms. Mass transitions of *m/z* 307 → 231 and 311 → 231 were optimized for EO-5a and EO-5a-d4, respectively, with dwell times of 150 ms. Mass transition of *m/z* 325 → 241 was optimized for EO-9-Cl with a dwell time of 150 ms. EO-9 and EO-9-Cl were

quantified using EO-9-d3 as internal standard and EO-5a using EO-5a-d4 as internal standard. Nebulizer gas (compressed air), turbo gas (compressed air), curtain gas (N2), and collision activated dissociation gas (N_2) were operated at 40, 65, 20, and 4 psi, respectively. Finally, the ionspray voltage was kept at 5500 V, with a source temperature of 250 ^{o}C.

Results and discussion

Stability of freeze dried products

Due to the chemical instability of EO-9 [5] freeze drying was selected to keep EO-9 pharmaceutical product stable for a longer period of time. The stabilities of the freeze dried products EOquin^{TM}, EO-9/HPβCD/Tris (4/600/6 mg/vial), EO-9/HPβCD/Tris (4/600/1 mg/vial), and EO-9/HPβCD/$NaHCO_3$ (4/600/20 mg/vial) are given in Table II. A product is defined as "stable" if the purity level is more or equal to 95%.

Table II. Stability of the freeze dried products.

Product*	Storage time (months)	Purity (%)**			
		-20ºC	5ºC	25ºC/60%RH	40ºC/75%RH
1/2	0	99.01 ± 0.19	-	-	-
3	0	99.30 ± 0.02	-	-	-
4	0	99.48 ± 0.03	-	-	-
5	0	98.84 ± 0.07	-	-	-
1/2	1	-	-	-	97.89 ± 0.37
3	1	99.24 ± 0.01	98.83 ± 0.10	91.93 ± 1.45	70.44 ± 2.00
4	1	-	-	99.09 ± 0.04	95.53 ± 0.19
5	1	-	98.99 ± 0.03	95.43 ± 0.44	69.96 ± 6.29
1/2	3	-	99.37 ± 0.05	98.73 ± 0.14	97.20 ± 0.31
3	3	99.37 ± 0.01	98.34 ± 0.46	83.50 ± 1.06	41.59 ± 3.00
4	3	99.42 ± 0.00	99.33 ± 0.03	96.65 ± 0.17	86.80 ± 0.96
5	3	-	-	85.28 ± 0.89	27.82 ± 2.54
4	5	-	99.38 ± 0.09	94.47 ± 0.17	78.83 ± 2.23

* 1/2 = EOquin^{TM} (see Table I)
3 = EO-9/HPβCD/Tris (4/600/6 mg/vial)
4 = EO-9/HPβCD/Tris (4/600/1 mg/vial)
5 = EO-9/HPβCD/$NaHCO_3$ (4/600/20 mg/vial)

** Area of the peak of EO-9 expressed as % of the total area of all peaks detected with HPLC-UV analysis.

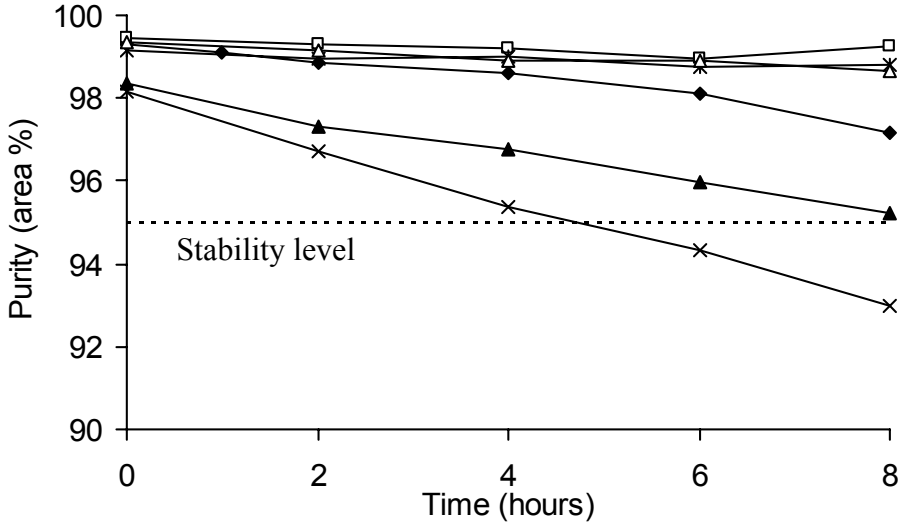
According to this definition, the freeze-dried product with 6 mg Tris per vial (Product 3) was stable for at least 3 months at 5°C, and less than 1 month both at 25°C/60%RH and 40°C/75%RH. The product containing 1 mg Tris per vial (Product 4) is more stable than Product 3, with stabilities of at least 5 months at 5°C, 3-5 months at 25°C/60%RH and 1 month at 40°C/75%RH. The freeze dried product containing HPβCD/$NaHCO_3$ (Product 5) was less stable than Product 3 and 4. Product 5 was stable for 1 month at 25°C/60%RH and less than one month at 40°C/75%RH. At 5°C, this product was stable for at least 2 months at 5°C (data not shown). However, within those two months a slight decrease in purity of 1% was seen, indicating a slow, but measurable degradation in this period of time. Therefore, the vials stored 2 months at 5ºC were transferred to -20ºC for long term stability testing. After one year of storage at -20°C no significant decrease in purity was seen, indicating that this product is stable for at least one year at -20°C, in the dark. In all products, EO-5a was one of the main degradation products formed (Figure I).
These results indicate that of the HPβCD-containing products, Product 4 (EO-9/HPβCD/Tris 4/600/1 mg/vial) is most stable. Long term stability testing is still ingoing, but because Product 5 was stable for at least one year at -20ºC and the accelerated stability study showed that Product 4 is much more stable than Product 5, it is expected that Product 4 will be stable for a longer period of time than one year at -20°C.
As reference, the stability of EOquin™ (Product 1/2) is also given. This product was stable for at least 3 months at 5°C, 25°C/60%RH and 40°C/75%RH. This might indicate that the HPβCD-containing freeze-dried products are all less stable than the currently used freeze-dried product. However, this may not be a problem if the product is stable for at least one year at -20°C, considered as an acceptable storage time.

Stability after reconstitution and dilution

This study was performed to determine the storage condition and storage time in the clinic between preparation of the bladder instillation and administration to the patient. Normally, in the clinic bladder instillations are prepared at room temperature and ambient light and therefore, these conditions were chosen for the stability study.
Reconstitution of the freeze dried products with 1.45 ml WfI resulted in a final volume of 2.0 ml. The pH values of the reconstituted products were 8.6, 8.3, and 9.9 for the freeze dried products containing 6 mg Tris (Table I, Product 3), 1 mg Tris (Table I, Product 4), and 20 mg $NaHCO_3$ (Table I, Product 5), respectively. All solutions remained clear and purple after dilution with normal saline to a final volume of 40 ml. The pH values after dilution were 8.7, 7.2, and 9.5 for the final bladder instillations of Product 3, 4, and 5, respectively. The results of the stability study of the reconstituted

and diluted products are depicted in Figure II. We defined a stability level of ≥95% purity, which is indicated in the figure. The results clearly show that both reconstituted products containing Tris (Product 3 and 4) are stable for at least 8 hours at room temperature and ambient light. Reconstituted Product 5 (the HPβCD/$NaHCO_3$-containing product) however, is only stable for 4 hours. On the other hand, this product is stable for at least 8 hours after further dilution. These differences in stability are all due to a pH-effect. The pH of product 3 and 4 is very near the optimal pH (i.e. maximum stability) of EO-9 (pH 8.75)[5]. For Product 5 however, the pH after dilution (pH 9.5) is closer to the optimal pH of EO-9 than after reconstitution (pH 9.9). Furthermore, it was shown that 1 mg Tris is not sufficient to maintain the pH at 8.3 after dilution. Due to this decrease in pH a decrease in stability was seen in time. The purity of this product after dilution was 99.1, 98.1, 97.1% after 1, 6, and 8 hours of storage respectively. With 6 mg Tris per vial, no change in pH was seen after dilution and therefore, EO-9 was most stable in this formulation.

Figure II. Stability of freeze dried products composed of EO-9/HPβCD/Tris (4/600/6 mg per vial, Product 3) after reconstitution with WfI (∗) and after dilution in normal saline (△), EO-9/HPβCD/Tris (4/600/1 mg per vial, Product 4) after reconstitution with WfI (□) and after dilution in normal saline (◆), and EO-9/HPβCD/NaHCO3 (4/600/20 mg/vial, Product 5) after reconstitution in WfI (×) and dilution in normal saline (▲).



Because in the clinic the instillation duration is one hour, a bladder instillation must be stable for at least one hour plus the additional time necessary for preparation at the hospital pharmacy and transfer to the bedside. Therefore, a stability of 8 hours (a working day) is preferred, which gives a feasible time-span from a logistic point of

view. Both bladder instillations composed of EO-9/HPβCD/Tris are stable for at least 8 hours after reconstitution and dilution and are therefore suited for the clinic.

In vivo simulation experiment

With this experiment a good estimation of drug stability in the bladder instillations after administration into the bladder of the patient can be obtained. The bladder instillation indicated as Product 1 (Table I) is the product which is currently used in phase II clinical trials. The stability of this product was analyzed as reference for the alternative HPβCD-containing formulations. Furthermore, the stability of EOquin™ using a reconstitution solution containing 1% w/v instead of 2% w/v $NaHCO_3$ (Product 2, Table I) was also analyzed to determine the effect of the $NaHCO_3$ concentration on the in vivo stability of EOquin™.

The results show that all formulations are stable (i.e. have an EO-9 content ≥ 95% calculated as percentage of the theoretical content at t=0) after reconstitution and dilution for at least one hour at 37°C in the dark (Table III). The bladder instillation composed of EO-9/HPβCD/$NaHCO_3$ (Product 5) seems to be less stable than the other instillations. This corresponds to the data found in the stability study after reconstitution and dilution. The stability of the five bladder instillations mixed with urine in the ratio bladder instillation:urine of 40:60 stored at 37°C in the dark are depicted in Figure IIIA, B, C for urine of pH 8, 6, 4, respectively. All curves start at t = 5 min. because 5 minutes were required to measure the pH before the mixtures were placed in the water bath. Figure IIIA clearly shows that there was no difference in stability of the bladder instillations when they were mixed with urine pH 8. This was expected because EO-9 is quite stable at this pH [5]. However, after mixing with urine of pH 6, the bladder instillations containing EO-9/HPβCD/Tris (Product 3 and 4) were less stable than Product 5. Furthermore, all HPβCD-containing instillations (Products 3 to 5) were less stable than both EOquin™ (Product 1 and 2) bladder instillations (Figure IIIB). This was due to pH differences.

The pH of the EO-9/HPβCD/$NaHCO_3$ bladder instillation mixed with urine pH 6 in the ratio bladder instillation:urine of 40:60 was 6.6 compared to pH 7.9 and 8.1 for the EOquin™ bladder instillations (Table III). Further decrease of the pH of urine to pH 4 resulted in a dramatic decrease in stability of EO-9 in all bladder instillations containing HPβCD (Figure IIIC). However, it is likely that this was not due to the presence of HPβCD, but due to low concentrations of alkalizer resulting in low pH levels (4.1-5.0) of the mixtures. The EOquin™ bladder instillations are more stable upon dilution with urine due to the relatively high $NaHCO_3$ concentrations of the reconstitution solutions. Both EOquin™ bladder instillations were stable when mixed with urine pH 4 (Figure IIIC).

Table III. Degradation of EO-9 in the bladder instillation and after mixing with urine pH 4, 6, 8 in the ratios bladder instillation:urine of 40:30 and 40:60 after storage for one hour at 37°C in the dark.

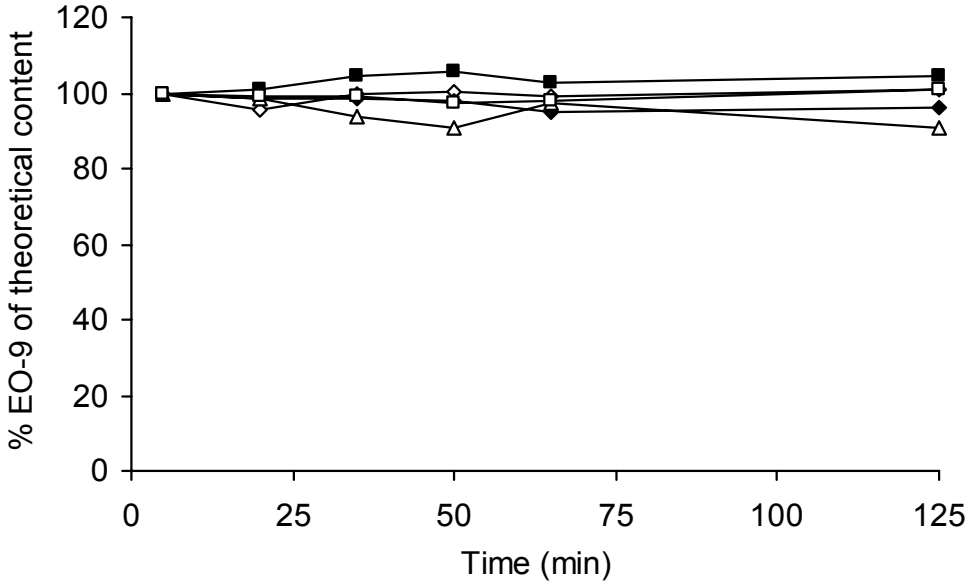
Product **	Mixture Instillation/urine	pH urine	mixture	EO-9 (%)*	RSD (%)*	EO-5a (%)*	EO-9-Cl (%)*	Mass balance (%)*
1	100/0	—	9.2	103	5.1	0.2	0.0	103
2	100/0	—	9.3	102	7.3	0.2	0.0	103
3	100/0	—	8.8	102	4.8	0.3	0.6	103
4	100/0	—	8.1	101	4.2	0.5	1.1	102
5	100/0	—	9.6	97.0	4.8	0.4	0.4	97.7
1	40/30	4	8.2	95.9	2.7	0.3	0.0	96.2
2	40/30	4	7.6	94.8	3.8	0.5	0.0	95.3
3	40/30	4	4.5	1.5	7.4	42.1	49.3	92.9
4	40/30	4	4.2	1.2	12.1	40.9	54.8	97.0
5	40/30	4	5.7	18.6	7.3	39.1	34.5	92.2
1	40/30	6	8.7	97.4	4.1	0.3	0.0	97.6
2	40/30	6	8.4	98.1	2.9	0.3	0.0	98.4
3	40/30	6	6.5	78.0	6.5	8.6	24.6	111
4	40/30	6	6.2	47.0	4.3	14.6	46.7	108
5	40/30	6	6.9	95.7	2.0	3.8	6.8	106
1	40/30	8	8.9	96.7	2.2	0.1	0.0	96.8
2	40/30	8	8.9	97.9	4.0	0.1	0.0	98.0
3	40/30	8	8.2	105	2.8	0.6	0.7	107
4	40/30	8	7.9	102	2.9	0.6	5.7	109
5	40/30	8	8.6	99.8	2.9	0.8	0.7	101
1	40/60	4	7.6	97.4	3.9	0.5	0.0	97.8
2	40/60	4	7.2	93.1	1.3	1.5	0.3	95.0
3	40/60	4	4.3	1.3	5.3	45.3	46.5	93.1
4	40/60	4	4.1	1.2	12.4	43.1	50.6	94.9
5	40/60	4	5.0	1.2	5.8	47.6	34.0	82.8
1	40/60	6	8.1	98.7	2.1	0.1	0.0	98.8
2	40/60	6	7.9	96.7	1.7	0.6	0.0	97.3
3	40/60	6	6.1	56.6	1.5	15.7	29.3	102
4	40/60	6	6.2	61.2	3.2	16.9	28.3	106
5	40/60	6	6.6	83.1	6.6	6.0	7.2	96.4
1	40/60	8	8.7	97.4	2.4	0.1	0.0	97.5
2	40/60	8	8.7	94.8	1.6	0.3	0.0	95.1
3	40/60	8	8.2	99.2	6.3	0.7	0.7	101
4	40/60	8	7.9	102	1.8	0.8	2.3	105
5	40/60	8	8.3	97.9	5.9	0.6	1.7	100

* The amounts of EO-9, EO-9-Cl and EO-5a are given as percentages of the initial molar amounts of EO-9.

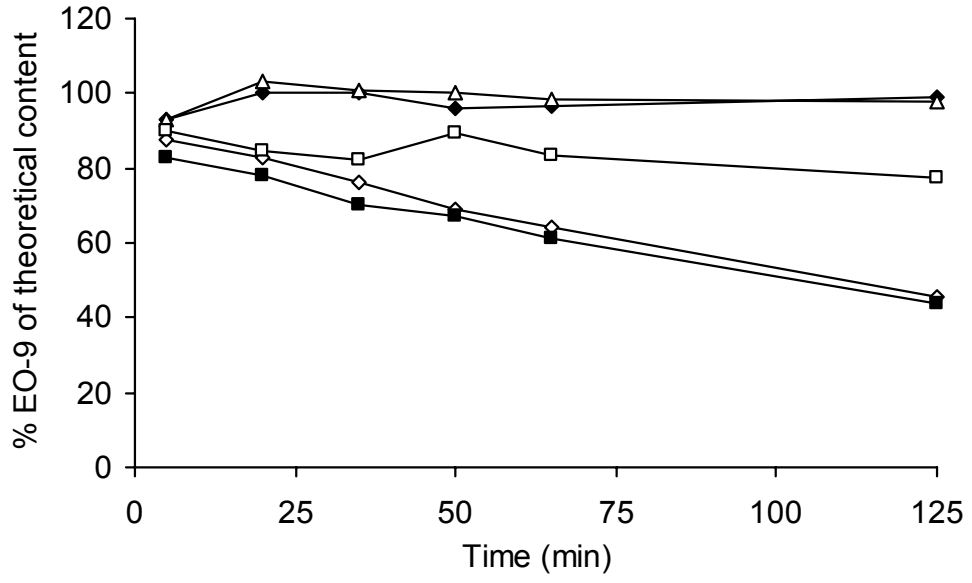
** Composition of products as indicated in Table I.

Figure III. Stability of five bladder instillations (EO-9/HPβCD/Tris 4/600/6 mg/vial (◇), EO-9/HPβCD/Tris 4/600/1 mg/vial (■), EO-9/HPβCD/$NaHCO_3$ 4/600/20 mg/vial (□), EOquin™ reconstituted with PG/WfI/$NaHCO_3$/sodium edetate 60/40/2/0.02% v/v/w/w (△), and EOquin™ reconstituted with PG/WfI/$NaHCO_3$/sodium edetate 60/40/1/0.02% v/v/w/w (◆)) mixed with urine pH 4, 6, 8 in 40:60 ratio.

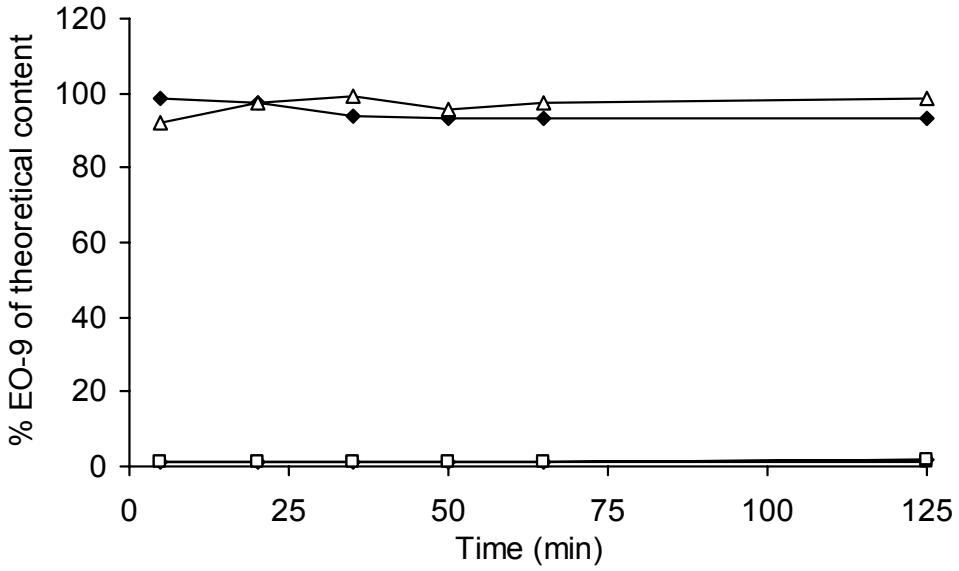
A. Stability of EO-9 bladder instillations mixed with urine pH 8 in 40:60 ratio.



B. Stability of EO-9 bladder instillations mixed with urine pH 6 in 40:60 ratio.



C. Stability of EO-9 bladder instillations mixed with urine pH 4 in 40:60 ratio.

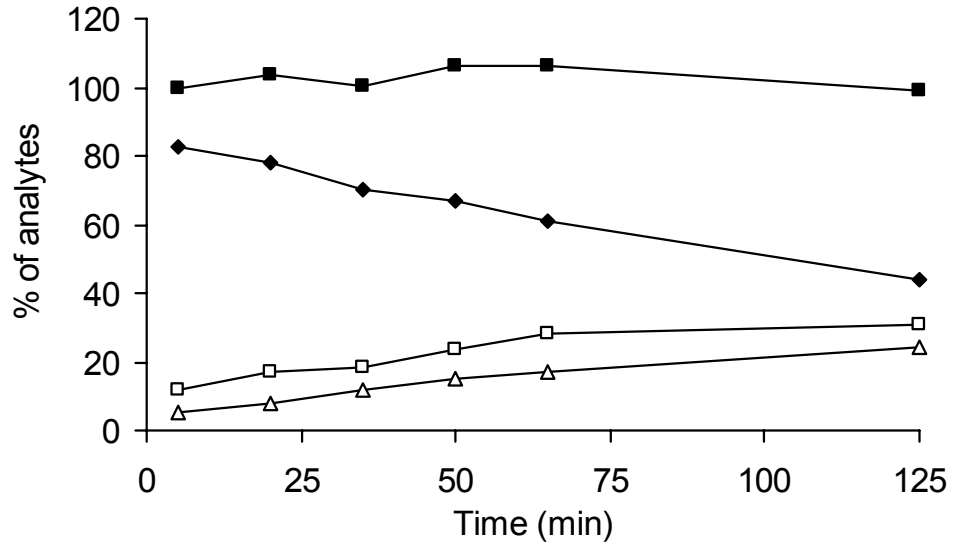


Degradation of EO-9 in the bladder instillation of Product 4 mixed with urine pH 6 in the ratio 40:60 is depicted in Figure IV. This figure shows that degradation of EO-9 in urine results in the formation of EO-9-Cl and EO-5a (Figure I). Furthermore, the mass balance shows that no other degradation products were formed. The formation of EO-9-Cl and EO-5a was also seen for the other HPβCD-containing bladder instillations (Table III). Very minor degradation of the EOquin^{TM} bladder instillations was seen. The formation of EO-5a was seen in both EOquin^{TM} bladder instillations, but the formation of EO-9-Cl was only found in the bladder instillation prepared with the reconstitution solution containing 10 mg/ml $NaHCO_3$ mixed with urine pH 4 in the ratio bladder instillation:urine of 40:60 (Table III). This indicates that EO-5a is probably formed more easily than EO-9-Cl. Furthermore, the formation of EO-9-Cl increases with decreasing pH for all bladder instillations at both bladder instillation:urine ratios (Table III).

Conclusion

These results show that the pH of the urine of patients must be increased (i.e. alkalizing of patients with $NaHCO_3$ tablets) prior to administration of one of the HPβCD-containing bladder instillations to hold EO-9 stable during the instillation of one hour. Alkalizing of patients was also performed in an randomized clinical trial to study the efficacy of intravesical Mitomycin C [6].

Figure IV. Degradation of EO-9 bladder instillation prepared from the freeze dried product composed of EO-9/HPβCD/Tris 4/600/1 mg/vial mixed with urine pH 6 in the volume ratio bladder instillation:urine = 40:60. The amounts of EO-9 (◆), EO-5a (△), EO-9-Cl (□) and the total mass balance (■) are depicted.



Based on the stability studies of the freeze dried products, after reconstitution and dilution and the "in vivo" simulation the pharmaceutical formulation composed of EO-9/HPβCD/Tris 4/600/1 mg/vial was selected as alternative formulation for EOquin™. Long term stability of this freeze dried product is still ongoing. Nevertheless, the product seems to be less stable than the product currently used in phase II clinical trials. However, based on the current stability data it is expected that this product is stable for at least one year at -20°C, in the dark. Furthermore, this product is stable for 8 hours after reconstitution and dilution, indicating that the bladder instillation can be prepared well before administration, which is practical for use in the clinic. A probable disadvantage may be that alkalizing of patients is required as pre-treatment. However, this is a non-invasive procedure which can be performed with administration of $NaHCO_3$ tablets.

The disadvantage of a slightly lower stability of the freeze dried product and the need to alkalize patients is compensated by the advantage of reconstitution and dilution with WfI and normal saline instead of a special reconstitution solution. Furthermore, less irritation of bladder tissue may occur because this alternative instillation is iso-osmotic.

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CHAPTER 2

Pharmaceutical development, analysis & manufacture of AP5346

Chapter 2.1

Pharmaceutical development, quality control, stability and compatibility of a parenteral lyophilized formulation of the investigational polymer-conjugated platinum antineoplastic agent AP5346

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Submitted for publication

Abstract

AP5346 is a low molecular weight polymer-conjugated platinum antineoplastic agent. The lyophilized drug product has completed a phase I clinical trial. In order to guarantee a constant quality of AP5346 pharmaceutical product, quality control and analysis of the drug substance and final product were performed. The identity of AP5346 was confirmed using ^{1}H NMR, ^{195}Pt NMR and IR spectroscopy. Furthermore, the free platinum content, platinum release characteristics, molecular size and size distribution were established. With the selected analytical techniques, AP5346 could be distinguished very well from its polymeric analogues, such as AP5280 and AP5279. The combination of all analytical techniques provides a complete picture of the physical and chemical characteristics of AP5346 drug substance as well as the pharmaceutical end-product. Specifications were set for the analytical results to guarantee a constant quality. Stability experiments revealed that AP5346 final product is stable for 12 months at 5°C, in the dark.

For administration to patients, AP5346 final product is reconstituted with 5% w/v dextrose and diluted in infusion containers. To investigate the influence of container materials, the stability of AP5346 after reconstitution and dilution in infusion containers was determined. The infusion containers investigated were composed of glass, polyvinyl chloride (PVC, Intraflex®) and low density polyethylene (LD-PE, Ecoflac®). AP5346 was shown to be stable after reconstitution and dilution with 5% w/v dextrose in these infusion containers for at least 96 hours at 2-8°C in the dark and at room temperature with ambient light conditions.

Introduction

Since the discovery of cisplatin (cis-diamminedichloroplatinum), platinum-containing drugs have grown to be the most widely prescribed class of anti-cancer agents for the treatment of solid tumors, especially lung, head and neck, ovarian and testicular cancers. Thus far, only cisplatin, carboplatin, and oxaliplatin have been approved for routine clinical treatment [1]. The main problems with the conventional platinum agents are the toxic side effects, including myelosuppression, nephro-, oto-, and neurotoxicity [1].

In the last decade, efforts have been made to overcome these problems and the use of platinum-containing biocompatible macromolecules has been studied. Macromolecules are targeted to tumor tissue in a passive way. Due to the enhanced

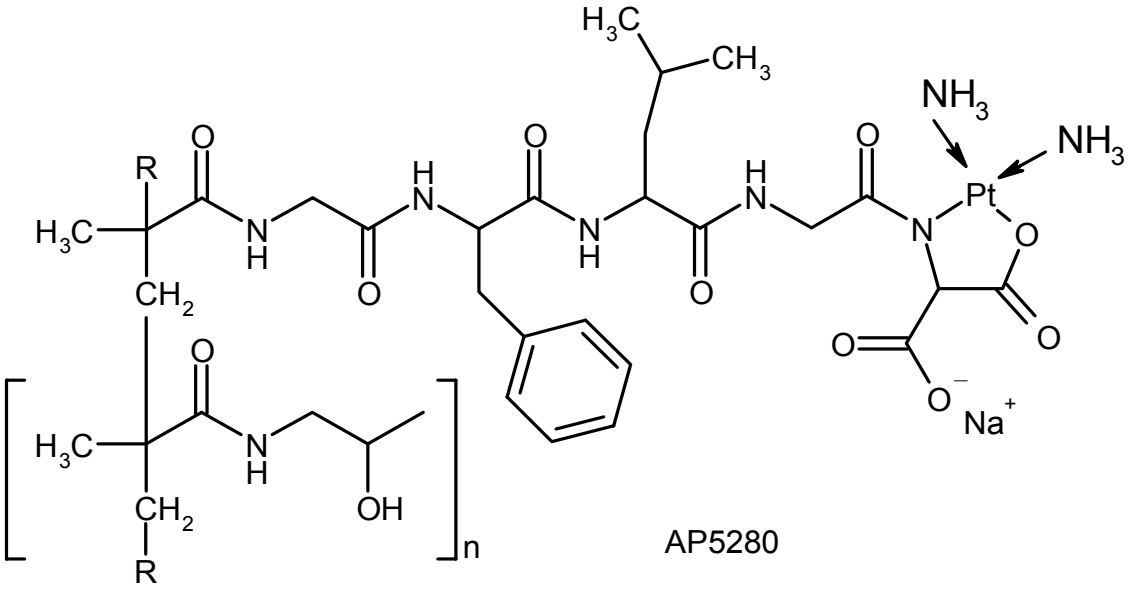
permeation and retention effect (EPR) (i.e. increased permeability of the tumor vasculature and decreased lymphatic drainage) macromolecules extravasate and accumulate mainly at the tumor site [2]. pHPMA (poly-N-(2-hydroxypropyl)-methacrylamide) is a biocompatible, water-soluble macromolecule which has been used for this purpose. In studies with doxorubicin coupled to pHPMA it was shown that this compound was highly toxic for tumor tissue and significantly less toxic for other tissues, overcame multi-drug resistance, and inhibited mechanisms of cellular drug defense, contrary to free doxorubicin [3].

AP5280 is the first of a series of polymer-conjugated platinum agents. It is comprised of platinum bound to pHPMA with a glycyl-phenylalanyl-leucyl-glycine (GFLG) spacer (Figure I). The GFLG spacer was originally chosen because it was thought that a cleavable spacer was needed to exert its effect. This tetrapeptide is degraded by the most important lysosomal cysteine proteinase cathepsin B [3]. The pharmaceutical development of AP5280 has been described elsewhere [4-6].

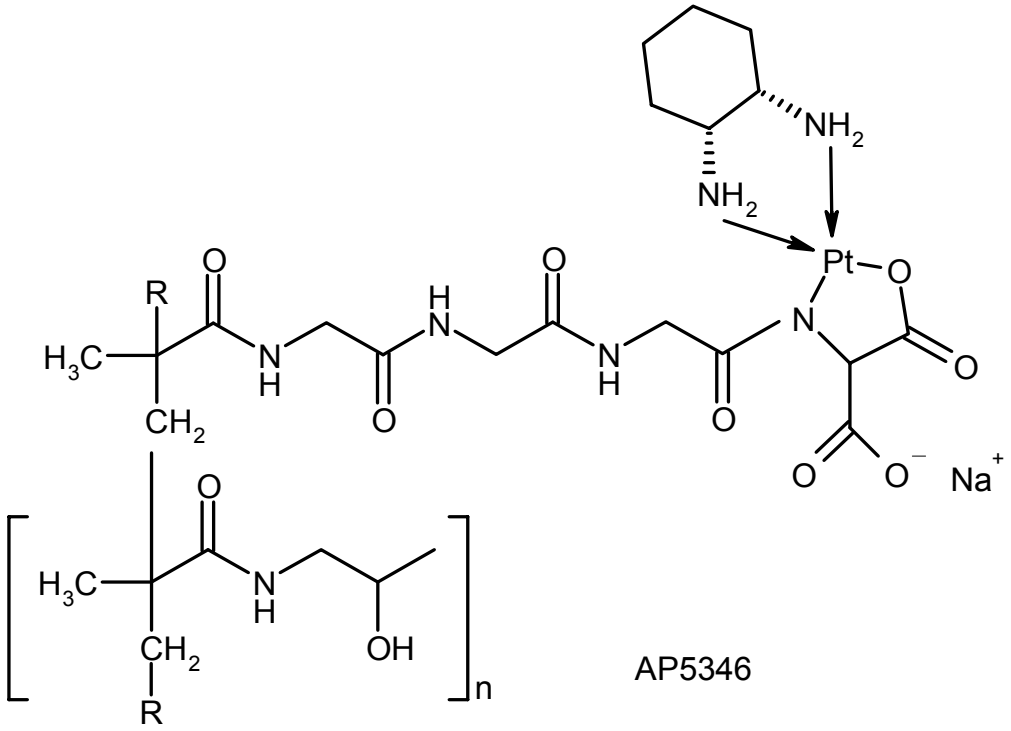
As the DACH (diaminocyclohexane) platinum (II) moiety, exemplified by oxaliplatin, provides a different spectrum of activity compared with the diamino platinum (II) moiety, Access Pharmaceuticals conducted a research program to generate a second-generation platinum-polymer conjugate based upon DACH platinum. From the synthesis of a large number of DACH platinum-polymer conjugates, AP5346 was selected for further development based upon its excellent preclinical efficacy and therapeutic index. Access Pharmaceuticals' structure-activity studies (unpublished data) had shown that a cleavable peptide link was unnecessary, but that a spacer between the polymer backbone and the chelate was required. Hence, the tetrapeptide linker of AP5280 was replaced by a simple triglycine linker. AP5346 (Figure I) belongs to the (1R, 2R) diaminocyclohexane (DACH) platinum(II) compounds, which were shown to be active in resistant murine leukemia cells [7,8]. For AP5346 prolonged tumor growth inhibition was seen in B16F10 melanoma s.c. tumor cells [9]. Compared to equitoxic doses of oxaliplatin, treatment with AP5346 resulted in much greater inhibition of tumor growth [10]. The total average molecular weight of AP5346 was very similar to that of AP5280 and was approximately 25 kDa.

This article describes the development of a parenteral lyophilized product of AP5346, including the quality control, establishment of specifications and performance of stability studies, all of which are needed to ensure that the drug product is stable, robust and well-suited for clinical use.

Figure I. Structures of AP5280 and AP5346 (n = 9 for AP5280 and n = 12 for AP5346)



AP5280



AP5346

Materials and methods

Materials

AP5346 drug substance, containing 9-12 % w/w platinum, was characterized and provided by Access Pharmaceuticals, Inc. (Dallas, Texas, USA). AP5346 lyophilized pharmaceutical product was manufactured in-house (Department of Pharmacy & Pharmacology, Slotervaart Hospital, Amsterdam, The Netherlands). Sterile Water for Injection (Ecotainer®), 0.9 % (w/v) NaCl (Normal Saline), 5 % w/v dextrose, and the glass, Intraflex® and Ecoflac® infusion containers, all filled with 100 ml 5 % w/v dextrose, were obtained from B. Braun (Melsungen, Germany). All chemicals were of analytical grade and used without further purification. 50 ml Colorless CZ-resin vials were obtained from Daikyo-Seiko, Ltd. (Tokyo, Japan), siliconized gray bromobutyl rubber stoppers Type FM 157/1 from Helvoet Pharma (Alken, Belgium), and aluminum caps (Alu-caps 20.3 x 7.5 mm with center tear off) from Aluglas BV (Uithoorn, The Netherlands).

Quality control of AP5346 drug substance

Hydrogen (^{1}H) and platinum (^{195}Pt) nuclear magnetic resonance (NMR) spectroscopy

^{1}H and ^{195}Pt NMR spectra were recorded on a Bruker AVANCE300 300 MHz spectrometer equipped with a z-axis gradient and a 5 mm multinuclear broad band probe.

The system for the ^{195}Pt NMR was calibrated using K_2PtCl_4 as an external reference at -1624 ppm. The temperature was nominally 22 °C. The ^{195}Pt NMR samples were prepared by dissolving 100 mg drug substance in a total volume of 650 µL H_2O/D_2O (93:7). Samples for ^{1}H NMR analysis were prepared by dissolving 20-30 mg in a total volume of 650 µL D_2O containing 0.05 % w/w TMS. The spectra were referenced internally with respect to TMS at 0 ppm. Data acquisition was started within one hour after dissolution. This analysis was developed and performed by Access Pharmaceuticals, Inc.

Graphite Furnace Atomic Absorption Spectrometry (GFAAS)

The free platinum content and the release of free platinum were determined using a GFAAS system consisting of a SOLAAR MQZ Zeeman AAS spectrometer, equipped with a FS95/97 autosampler, Merlyn 33 cooler and a GF95 oven controller (all from Thermo Electron Corporation, Breda, The Netherlands). Absorbances were recorded at 265.9 nm with a bandpass of 0.2 nm. Measure time was 3 sec. Argon was used to purge the graphite tube. A calibration curve of carboplatin corresponding to 0.5 to 20

µM platinum was prepared and analysis was performed using the temperature program given in Table IV. The method for determining free platinum concentration (i.e. all platinum species with a molecular weight < 3 kDa) consisted of the following: AP5346 drug substance was dissolved in water for injections at a concentration of 2.0 ± 0.1 mg/ml and stored at ambient temperature (+ 20-25 ºC) for one hour. Subsequently, 2 ml samples were ultrafiltrated (45 minutes, 41 x g) through a Centricon YM-3 filter (3 kDa cut-off, Millipore, Milford, MA, USA). The platinum concentration in the ultrafiltrate was analyzed in duplicate. For the determination of the platinum release characteristics, AP5346 was dissolved in PBS at a concentration of 2.0 ± 0.1 mg/ml and stored at 37 ºC. Samples were taken after 3 and 24 hours and analyzed as described for the determination of the free platinum concentration. This analysis was developed by Access Pharmaceuticals, Inc and performed at the Slotervaart Hospital.

Table IV. Temperature program of GFAAS analysis

Phase	Temp (°C)	Time (s) a	Ramp (°C s-1) b	Gas Flow (L min-1)
1	50	1.0	0	0.3
2	85	5.0	17	0.3
3	95	30.0	1	0.3
4	120	20.0	2	0.3
5	250	30.0	5	0.3
6	1400	40.0	30	0.3
7	2700	3.0	0	0
8	2800	4.0	0	0.3
9	50	10.0	0	0.3

[a] Time the temperature remains constant
[b] Velocity at which the temperature is reached

Size Exclusion Chromatography

Size exclusion chromatography was performed with an HPLC system consisting of a Spectra System P1000 pump, a Spectra Series AS3000 autosampler and a RI-150 refractive index detector (all from Thermo Separation Products (TSP), Fremont, CA, USA). The mobile phase consisted of 10mM $LiClO_4$ in 35 % methanol (aq.) at a flow rate of 1.0 ml/min. The injection volume was 100 µl and the run time 30 min. Separation was achieved using a PL aquagel-OH guard column (7.5 mm ID x 50 mm, particle size 8 µm, Varian BV, Houten, The Netherlands) and two PL aquagel-OH mixed columns (7.5 mm ID x 30 cm, particle size 8 µm, Varian BV, Houten, The Netherlands) in series kept at a temperature of +35 ºC with a Croco-cil® column oven

(TSP). The SEC system was calibrated using polyethylene glycol and polyethylene oxide standards (Polymer Laboratories) with molecular ranges from 1,080 to 219,300 Da. Samples were prepared by dissolving 2-3 mg AP5346 drug substance in 1.0 ml mobile phase by gentle swirling. This analysis was developed by Access Pharmaceuticals, Inc and performed at the Slotervaart Hospital.

Infrared spectroscopy

IR spectra were recorded with a Model PU 9706 IR spectrophotometer (Philips, Eindhoven, The Netherlands) using the potassium bromide (KBr) pellet technique. The pellet consisted of approximately 2 mg AP5346 drug substance and 300 mg KBr. The ratio recording mode was auto-smooth and the scan time 8 minutes.

Preformulation studies

Sterilization

A solution of AP5346 (Lot AP5346-2-100) 108 mg/ml in water for injection was prepared. Aliquots of 6.0 ml were filled in triplicate in 20 ml type I glass vials. Vials were closed with siliconized grey bromobutyl rubber stoppers and aluminium caps, and sterilized for 15 minutes at 121 ºC in a Model 6.6.15 autoclave (Koninklijke Ad Linden B.V., The Netherlands). Subsequently, the sterilized solutions were inspected visually, and analyzed by Size Exclusion Chromatography.

Differential scanning calorimetry

Differential scanning calorimetry was performed on a Q1000 V9.0 DSC equipped with a refrigerated cooling accessory (RCS) for low temperatures in T4P mode (TA Instruments, New Castle, DE, USA). Samples of approximately 10 mg AP5346 (125 mg/ml in water for injection) were weighed into aluminium pans. Subsequently, the pans were hermetically sealed and measured against an empty pan as reference. Indium was used to calibrate the temperature and heat flux. Analysis was performed under nitrogen purge at 50 ml/min. The solutions were cooled to –40 °C at a rate of 10 °C/min, and subsequently heated to +25 °C with 1 °C/min.

Manufacturing process

The manufacturing process was performed aseptically. A formulation solution of 10 mg Pt (as AP5346)/ml in sterile water for injection was prepared. The formulation solution was sterile filtered using a sterile hydrophilic 0.22 µm filter (Millipak® 40, consisting of modified polyvinylidene fluoride (PVDF) membranes in a polycarbonate housing). Aliquots of 40 ml sterile formulation solution were filled into washed and sterilized 50 ml colorless CZ-resin vials (Daikyo-Seiko, Ltd., Tokyo, Japan). Platinum cured silicone tubing (Watson Marlow, Cheltenham, UK) was used for transportation of the formulation solution during filtration and filling processes. The vials were

partially closed with grey bromobutyl rubber stoppers (Type FM 157/1, Helvoet Pharma NV, Alken, Belgium) and loaded into the freeze dryer (Model Lyovac GT4 freeze drier, STERIS, Hürth, Germany) at ambient temperature. After lyophilization, the vials were closed pneumatically under vacuum and capped with aluminium caps.
The in-process controls consisted of integrity testing of the filter, weight variation of the filling volume, and determination of the bio-burden before filtration. During freeze-drying the product temperature, shelf temperature, chamber pressure and condenser temperature were continuously monitored. Manufacturing was performed according to the Good Manufacturing Practices (GMP) guidelines [11].

Quality control of AP5346 final product

For the quality control of AP5346 final product, ^{1}H NMR and ^{195}Pt NMR spectroscopy, free platinum content, release of free platinum, and Size Exclusion Chromatography (SEC) were executed. Furthermore, the appearance (no visible contamination present), reconstitution characteristics, and residual moisture content were determined. The content and content uniformity were measured using UV/VIS analysis. Sample preparation and analysis of the moisture content, UV/VIS analysis and SEC analysis are described below. All other analyses mentioned above were performed as described for AP5346 drug substance.

Size Exclusion Chromatography
The product was reconstituted with 27.4 ml 5 % dextrose, revealing a final volume of 30 ml and a platinum concentration of 13.3 mg Pt/ml. Samples of the final product were prepared by diluting the solution obtained after reconstitution 40 times with mobile phase. Analysis was performed as described for AP5346 drug substance.

Residual moisture content
The residual moisture content of the final product was determined using the Karl Fisher titration method. Immediately after opening of the vial, 500 mg lyophilized product was transferred into the titration unit of a Model 658 KF Titrino apparatus (Metrohm, Herisau, Switzerland). The moisture content was determined in triplicate.

UV/VIS spectrophotometry
Analysis was performed using a Model UV/VIS 918 spectrophotometer (GBC Scientific Equipment Ld., Victoria, Australia). Calibration curves were prepared with the same batch of drug substance used for the production of the batch to be determined. Samples were prepared by diluting the solution after reconstitution (a solution of approximately 113 mg AP5346/ml 5 % dextrose) with 5 % w/v dextrose to a final concentration of approximately 135 μg AP5346/ml. The absorption was determined at 240 nm and AP5346 content and content uniformity were assessed.

Setting of specifications

To estimate the robustness of AP5346 drug substance and final product "The risk of batch failure" (i.e. frequency of failing to meet the specifications) was calculated. This was performed according to the method described by Stafford [12]. The risk of batch failure was estimated by using the means and standard deviations of the analytical outcomes of 3 Lots of drug substance and 4 batches of final product manufactured out of these three Lots of drug substance: 3 validation batches with a batch size of 40-56 vials containing 150 mg Pt/vial, and 1 batch with a batch size of 79 vials containing 400 mg Pt/vial.

Stability of AP5346 final product

Vials of the first three pilot batches (batch 1, 2, and 3, all containing 150 mg platinum/vial) were stored at 5 ± 3 ºC and 25 ± 2 ºC / 60 ± 5 % relative humidity, both in the dark. Samples were taken after 0, 3, 6, and 12 months of storage. At each time point, the free platinum content, platinum release characteristics, AP5346 content, moisture content, pH, molecular size and size distribution were determined.

Stability and compatibility upon reconstitution and dilution

Vials of AP5346 final product were reconstituted with 5 % w/v dextrose to yield a concentration of 13.3 mg Pt/ml. The reconstituted product was diluted in triplicate to final concentrations of 0.034, 0.34 and 3.4 mg Pt/ml in 5 % w/v dextrose for the compatibility study with infusion containers. The compatibility of AP5346 infusion solution was investigated for three kinds of infusion containers: glass, polyvinyl chloride (PVC, Intraflex®) and low density polyethylene (LD-PE, Ecoflac®). The vials with reconstituted product and the infusion containers were stored at 5 ± 3°C, in the dark and at room temperature, ambient light. The AP5346 concentration and the amount of free platinum were determined 0, 1, 2, 4, 8, 24, 48, 72, and 96 hours after preparation using UV/VIS and GFAAS analysis, respectively. Furthermore, ^{1}H NMR and ^{195}Pt NMR spectra were recorded from the reconstituted products stored at +2-8 ºC and +15-25 ºC after 0 and 96 hours.

Results and discussion

Quality control of AP5346 drug substance

Hydrogen (^{1}H) and platinum (^{195}Pt) nuclear magnetic resonance (NMR) spectroscopy

For the quality control of AP5346 drug substance, it is important to demonstrate the specificity of the analytical techniques. To test the specificity of ^{1}H NMR and ^{195}Pt NMR, the results of the analysis of AP5346 were compared to the analogues AP5280 and AP5279. The differences between AP5346 and AP5280 are the peptidyl spacer and the platinum drug. The spacer of AP5346 is composed of three glycine amino acids. For AP5280 and AP5279, the pHPMA backbone and the platinum chelate are connected with a glycyl-phenylalanyl-leucyl-glycine (GFLG) spacer [5]. AP5279 is an O,O-platinum chelate. AP5346 and AP5280 are both N,O-platinum chelates. Furthermore, in AP5346, a di-aminocyclohexane moiety is coupled to the platinum atom (Figure I). This moiety is absent in AP5280 and AP5279. ^{1}H NMR, and ^{195}Pt NMR appeared powerful techniques to show the differences in spacer and platinum chelate, respectively.

The ^{1}H NMR spectrum provides information about the number of different types of hydrogen, the functional groups, nearby (non)hydrogen neighbours of the hydrogen atoms, relative numbers of each type of hydrogen, and the location of the hydrogens in the macromolecule.

The ^{1}H NMR spectrum of AP5346 drug substance shows 12 signals: at 1.03 ppm assigned to methyl hydrogens along the backbone, at 1.24 ppm assigned to methyl hydrogens of HPMA, at 1.67, 1.82, 1.92, and 2.13 ppm assigned to methylene hydrogens of the backbone and the cyclohexane moiety, at 3.15 and 3.22 ppm assigned to methylene hydrogens of HPMA moiety, at 3.98 ppm assigned to methylene hydrogens of the GGG-spacer and hydrogens of the methine of the HPMA moiety, at 7.61 ppm assigned to hydrogens of the amide of the HPMA moiety, and at 7.72 and 8.09 ppm assigned to hydrogens of the amides of the GGG-spacer. The ^{1}H NMR spectra of AP5346 and AP5280 are depicted in Figure IIa. The main differences between AP5346 and AP5280 are seen at 1.5 – 2.5, 4.4, 7.3 and 7.4 ppm. In the first area, at 1.5 – 2.5 ppm, more signals are seen in the spectrum of AP5346. These signals are due to the methylene hydrogens in the cyclohexane moiety, which are absent in AP5280. At 4.4 ppm a signal is seen in AP5280, which is absent in AP5346. This signal is due to the methine hydrogen on the chiral carbon of leucine. The signals at 7.3 and 7.4 ppm, present in the spectrum of AP5280 and absent in AP5346, are due to the aromatic hydrogens of phenylalanine. Analogue AP5279 can be easily

distinguished from both AP5280 and AP5346 due to the presence of a small signal at 5.6 ppm, assigned to the methine hydrogen at the 2 position of the malonato moiety [5].
Identification of AP5346 was also performed using ^{195}Pt NMR analysis. ^{195}Pt NMR analysis is a very specific method, i.e. small changes in binding of the platinum atom result in large peak shifts. For AP5346, two integrals were seen between - 2200 and -2350 ppm. In AP5279 and AP5280 just one peak was seen at -1725 ppm and -2049 ppm, respectively. This large shift of AP5346 is due to the cyclohexane moiety bound to the platinum. The ^{195}Pt NMR spectra of AP5280 and AP5346 are depicted in Figure IIb.
Besides confirmation of the identity of AP5346, ^{195}Pt NMR analysis can also be used for the determination of platinum impurities. During the manufacturing process [13], of the DACH platinum(II) complex three isomers are formed: the O,O-chelate, N,O-chelate, and the N,N-chelate. The chelate impurities will show only minor differences in the ^{1}H NMR spectra, because the polymer and spacer are the same. In the ^{195}Pt NMR spectra however, large shifts were seen due to different bindings to the platinum atom. The two peaks between - 2200 and -2350 ppm are due to the N,O-chelate [14]. The peaks between approximately -2550 and -2650 ppm are due to the N,N-chelate. Unreacted DACHPt$(OH_2)_2^{2+}$ would appear at approximately -1870 ppm. The O,O-chelate resonance would appear at approximately -1960 ppm, but neither of these O,O-species are observed. Therefore, the purity of AP5346 was determined as the percentage of ^{195}Pt NMR peaks which appear in the N,O-chelate range. The quality control data of AP5346 drug substance showed two signals, at approximately -2282 ppm and - 2260 ppm, corresponding with the N,O-chelate (Table I). The integral of these two signals was for all drug substances more than 90% of the total area.

Figure IIa. ^{1}H NMR spectra of AP5280 and AP5346 drug substance in D_2O containing 0.05 % w/w TMS.

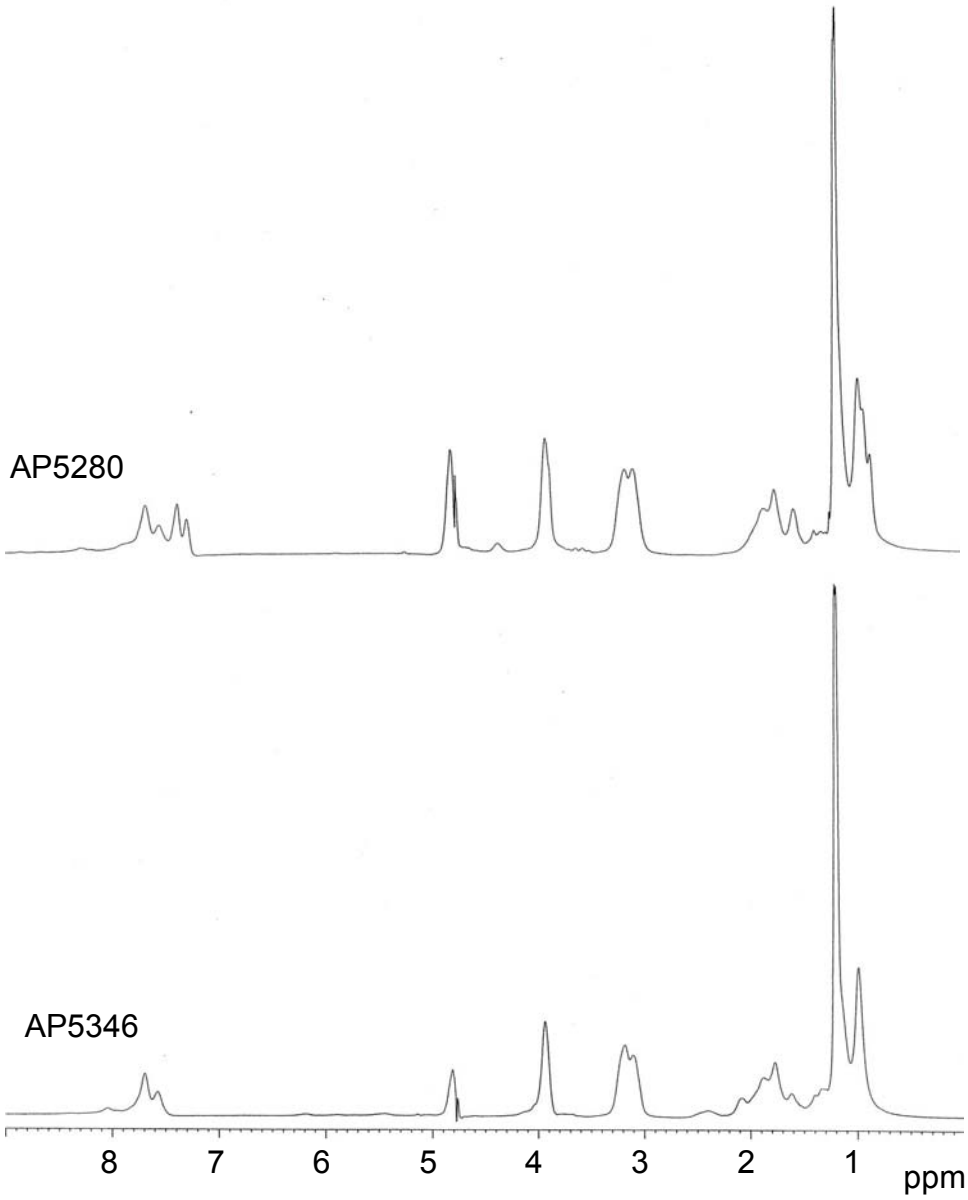
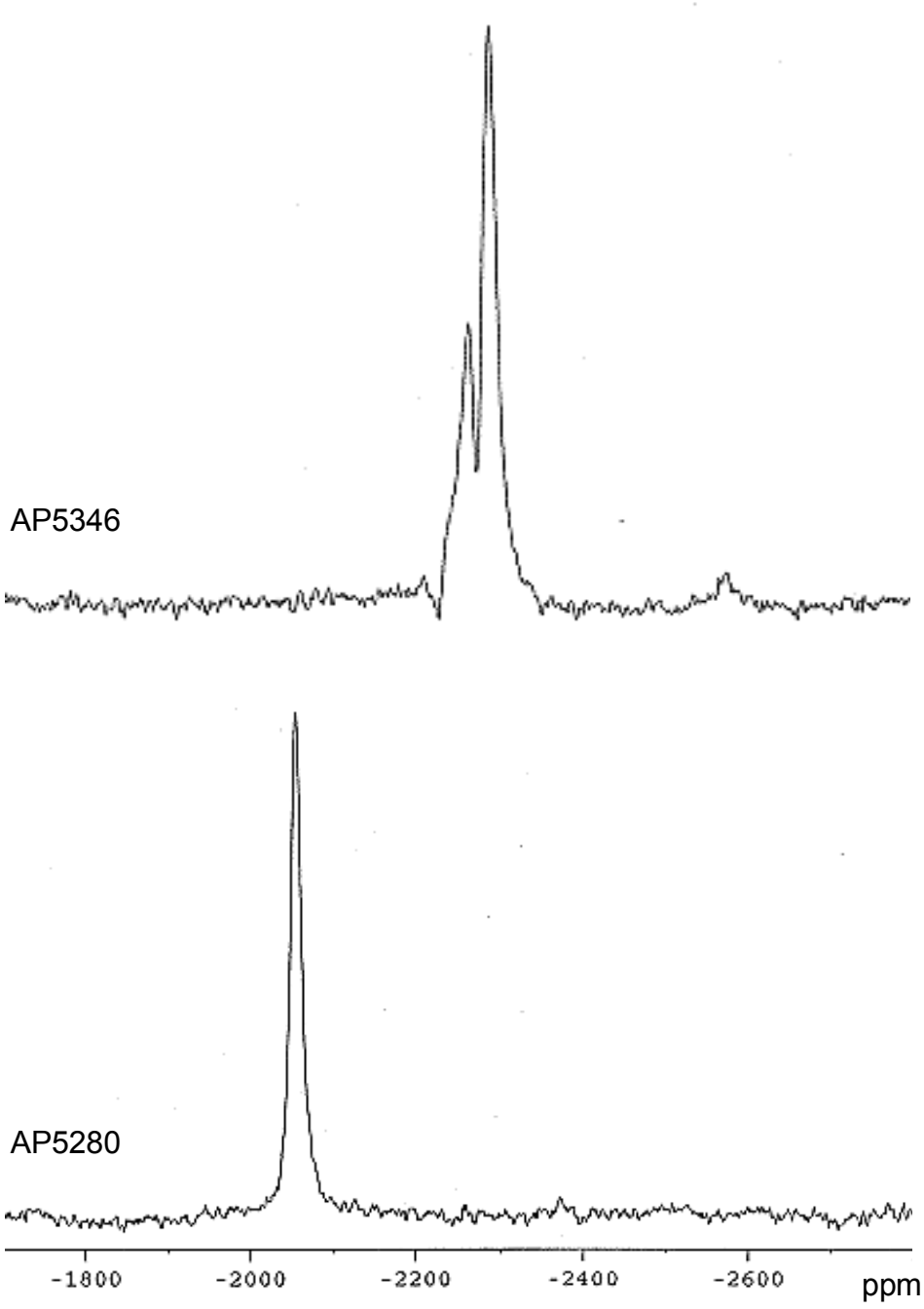


Figure IIb. ^{195}Pt NMR spectrum of AP5280 and AP5346 drug substance in H_2O/D_2O (93:7)



Graphite Furnace Atomic Absorption Spectrometry (GFAAS)

In AP5346, the toxicity of free platinum is reduced by coupling of the platinum chelate to the pHPMA polymer. However, during the production process of AP5346 drug substance, small amounts of platinum may remain weakly associated with AP5346. These platinum species will dissociate from the macromolecule in acidic aqueous solutions and possibly increase the toxicity of the drug substance. Therefore, the amounts of "free" platinum (platinum species smaller than 3kDa) were determined in ultrafiltrate samples from AP5346. Furthermore, the release characteristics of these small platinum species were studied in phosphate buffered saline, pH 7.4 at 37 ºC to mimic the conditions after administration. The use of a furnace instead of a flame for platinum atomization in ultrafiltrate samples resulted in a much more sensitive method. Therefore, furnace AAS was used. The results are depicted in Table I.

No significant differences in free platinum content or release of free platinum were seen for the three Lots of drug substance. All Lots contained 0.2 – 0.3 % w/w free platinum. The release of free platinum in PBS at 37 ºC varied from 1.0 – 1.4 % w/w and from 3.8 – 4.3 % w/w after 3 and 24 hours, respectively.

Size Exclusion Chromatography (SEC)

AP5346 is a DNA crosslinking antitumour compound and exerts its cytotoxic effect by formation of platinum-DNA adducts like other platinum containing agents, e.g. cisplatin, carboplatin and oxaliplatin [15]. In AP5346 the platinum chelate is bound to a polymer backbone to obtain passive targeting (due to the EPR-effect), reducing the toxicity. Therefore, it is important for the efficacy and toxicity of AP5346 that the copolymer does not disintegrate into small fragments. Furthermore, the molecular weight of polymers is also of importance for their elimination after administration. Because pHPMA is a non-biodegradable copolymer, the molecular size must remain below the renal threshold of pHPMA (45 kDa) to prevent accumulation in the body. Therefore, the molecular size distribution was determined using SEC, which is an analytical technique based on differences in molecular size and not molecular weight. However, the molecular weight of polymers is roughly proportional to their molecular size, making SEC a very suited method for the analysis of the molecular size distribution of AP5346.

Due to the production process, synthetic polymers (such as pHPMA) are polydisperse instead of having a single molecular weight. The degree of polydispersity is calculated (Mw/Mn) as the polydispersity index (PDI). The PDI increases with increasing size distribution. The results are depicted in Table I. The quality control results showed that the drug substances had a weight average molecular weight (Mw) of approximately 17 – 22 kDa and a number average molecular weight (Mn) of 7.8 – 10.7 kDa. The resulting PDI was 2.08 – 2.20.

Table I. Quality control data of AP5346 drug substance and final product

	AP5346 drug substance			**AP5346 final product**			
				Batch 1 150 mg Pt/vial	Batch 2 150 mg Pt/vial	Batch 3 150 mg Pt/vial	Batch 4 400 mg Pt/vial
	Lot AP5346-2-100	Lot AP5346-3-325	Lot AP5346-4-325	Lot 300902SS2	Lot 021002SS3	Lot 041002SS4	Lot 111202SS5
^{1}H NMR	Peaks at 1.00, 1.21, 1.63, 1.78, 1.92, 2.10, 3.11, 3.18, 3.94, 7.59, 7.71, 8.05 ppm	Peaks at 0.99, 1.21, 1.63, 1.78, 1.92, 2.09, 3.11, 3.18, 3.94, 7.59, 7.71, 8.05 ppm	Peaks at 0.99, 1.20, 1.62, 1.78, 1.9, 2.08, 3.11, 3.18, 3.94, 7.59, 7.71, and 8.05 ppm	Peaks at 1.13, 1.34, 1.76, 1.92, 2.03, 2.23, 3.25, 3.33, 4.07, 7.54, 7.76, 8.11 ppm	Peaks at 1.00, 1.21, 1.63, 1.78, 1.88, 2.09, 3.11, 3.19, 3.94, 7.59, 7.71, and 8.05 ppm	Peaks at 1.12, 1.33, 1.76, 1.91, 2.02, 2.22, 3.24, 3.32, 4.06, 7.64, 7.75, 8.09 ppm	Peaks at 1.00, 1.22, 1.64, 1.79, 1.90, 2.10, 3.12, 3.19, 3.95, 7.60, 7.72, and 8.10 ppm
^{195}Pt NMR	Peaks at -2285 and –2260 ppm	Peaks at -2282 and δ = -2260 ppm	Peaks at -2277 and -2259 ppm	Peaks at -2278 and -2260 ppm	Peaks at -2283 and –2260 ppm	Peaks at -2277 and -2260 ppm	Peaks at -2284 and -2260 ppm
Free Pt	0.20 ± 0.04 %	0.26 ± 0.06 %	0.33 ± 0.06 %	0.15 ± 0.01 %	0.15 ± 0.05 %	0.15 ± 0.01 %	0.30 ± 0.03 %
Release of free Pt							
3 hours	1.15 ± 0.01 %	1.25 ± 0.03 %	1.00 ± 0.33 %	1.06 ± 0.10 %	0.99 ± 0.03 %	1.02 ± 0.07 %	1.37 ± 0.07 %
24 hours	3.80 ± 0.55 %	4.19 ± 0.36 %	3.80 ± 0.37 %	4.01 ± 0.07 %	4.28 ± 0.49 %	3.85 ± 0.15 %	4.03 ± 0.14 %
AP5346 content	*	*	*	116.9 ± 0.32 %	101.4 ± 0.97 %	100.0 ± 0.25 %	105.9 ± 4.37 %

	AP5346 drug substance			AP5346 final product			
				Batch 1 150 mg Pt/vial	Batch 2 150 mg Pt/vial	Batch 3 150 mg Pt/vial	Batch 4 400 mg Pt/vial
	Lot AP5346-2-100	Lot AP5346-3-325	Lot AP5346-4-325	Lot 300902SS2	Lot 021002SS3	Lot 041002SS4	Lot 111202SS5
SEC							
M_w	21.7 ± 0.86 kDa	19.5 ± 0.2 kDa	17.2 ± 0.08 kDa	21.5 ± 0.5 kDa	19.9 ± 0.09 kDa	19.9 ± 0.06 kDa	17.3 ± 0.06 kDa
M_n	10.7 ± 1.8 kDa	9.4 ± 0.6 kDa	7.8 ± 0.05 kDa	10.6 ± 0.2 kDa	8.9 ± 0.14 kDa	9.0 ± 0.21 kDa	7.9 ± 0.02 kDa
PDI	2.08 ± 0.38	2.08 ± 0.1	2.20 ± 0.58	2.04	2.23	2.21	2.20
PH	*	*	*	6.74	6.75	6.78	6.7
Moisture content	*	*	*	1.33 %	1.43 %	1.28 %	0.74 %
IR spectroscopy	Major absorption bands at app. 3700-3100 cm^{-1}, 2970 cm^{-1}, 2920 cm^{-1}, 1630 cm^{-1}, 1520 cm^{-1}, 1380 cm^{-1}, 1290-1230 cm^{-1}, 1190 cm^{-1}, 1140-1050 cm^{-1}, 960-900 cm^{-1}	Major absorption bands at app. 3700-3100 cm^{-1}, 2970 cm^{-1}, 2920 cm^{-1}, 1630 cm^{-1}, 1520 cm^{-1}, 1380 cm^{-1}, 1290-1230 cm^{-1}, 1190 cm^{-1}, 1140-1050 cm^{-1} and 960-900 cm^{-1}	Major absorption bands at app. 3700-3100 cm^{-1}, 2970 cm^{-1}, 2920 cm^{-1}, 1630 cm^{-1}, 1520 cm^{-1}, 1380 cm^{-1}, 1290-1230 cm^{-1}, 1190 cm^{-1}, 1140-1050 cm^{-1} and 960-900 cm^{-1}	*	*	*	*

* Not analyzed

Infrared spectroscopy

Major absorption bands were seen at 3700–3100 cm^{-1} assigned to O-H and N-H stretching, 2970 cm^{-1} and 2920 cm^{-1} assigned to $–CH_2$ and $–CH_3$ stretching, 1630 cm^{-1} assigned to N-H deformation, 1520 cm^{-1} assigned to N-monosubstituted amide, 1380 cm^{-1} assigned to carboxylate ion deformation, 1290–1230 cm^{-1} assigned to C-O bending, 1190 cm^{-1} assigned to C-O stretching of esters, 1140-1050 cm^{-1} assigned to C-O stretching, and 960-900 cm^{-1} assigned to C-H bending. These assignments are the same as for AP5280 [5] and therefore IR spectroscopy alone is not suited to distinguish between AP5346 and its analogues. However, a combination of IR, ^{1}H NMR and ^{195}Pt NMR provides a complete identification of AP5346 drug substance.

Preformulation studies

Sterilization

After sterilization of a solution of AP5346 in water for injection, no changes in appearance were seen. All vials contained dark brown solutions, free from visible particles before and after sterilization. The SEC data however, showed an increase in polydispersity index (2.15 instead of 2.08) and a decrease in M_w and M_n: 17.5 kDa and 8.1 kDa instead of 21.7 kDa and 10.7 kDa, respectively. These data show that there is a change in the molecular weight and hence distribution of the polymer, but at this time what that change can be attributed to is uncertain. Due to these changes it was decided to manufacture AP5346 aseptically. In order to produce a stable product with sufficient shelf-life, a lyophilized dosage form was developed.

Differential Scanning Calorimetry

Differential scanning calorimetry was performed to study the freeze drying characteristics of AP5346 formulation solution. The DSC thermogram of AP5346 showed two thermal events: a glass transition temperature (T_g) with the onset at –13.5 °C and an ice-melting endotherm. The formulation solution of AP5280 showed a T_g at –13.2 °C. This indicates that the freeze drying characteristics of both compounds are comparable. Therefore, the freezing drying program of AP5280 [4] was used as starting point for the development of the freeze drying program of AP5346.

Manufacturing process

Excipients are often used to improve physical and chemical characteristics of drug substances, such as solubility and stability, and to provide a solid cake with good appearance. However, AP5346 drug substance is highly soluble (due to the pHPMA moiety) in water for injection. Furthermore, freeze drying of a solution of AP5346 in water for injection revealed a voluminous cake with good appearance. Therefore, no

excipients (e.g. bulking agents, cryoprotectants, solubilizing agents) were required and water for injection was chosen as dissolution vehicle.
During manufacturing of the pharmaceutical product, AP5346 drug substance was dissolved in water for injection, and subsequently freeze dried. The freeze drying program was optimized for AP5346 drug product. Vials were frozen to –40 °C in 3 hours, followed by a freeze-hold lasting 5 hours to ensure complete freezing of the solutions in the vials. Subsequently, the primary drying phase was started by lowering the chamber pressure to 0.15mbar, while keeping the temperature at –40 °C. After achieving the vacuum, the shelf temperature was increased to +25 °C in 2 hours. During these 2 hours, the product temperature increased from –40 °C to –30 °C. The chamber pressure was held constant. This secondary drying phase was maintained for 80 hours. During these 80 hours, the product temperature further increased to +25 °C. This manufacturing process revealed a light brown, dry, voluminous freeze dried product, which could be reconstituted easily. Reconstitution was performed with 27.4 ml 5% dextrose solution prior to administration. No meltback of ice and hence cake collapse was seen and no vial breakage occurred. The quality control results showed that this production process did not alter the characteristics of AP5346 drug substance (Table I).

Quality control of AP5346 final product

AP5346 final product was characterized with the same methods as used for AP5346 drug substance. Furthermore, visual inspection, reconstitution characteristics, pH after reconstitution, moisture content, and AP5346 content were determined. The results are given in Table I. All final products were light brown, voluminous cakes. No visible contamination was seen. Reconstitution of the final products with 27.4 ml 5 % w/v dextrose revealed a final volume of 30 ± 0.1 ml for all batches. The pH after reconstitution varied from 6.7-6.8. The moisture content was 0.7-1.4 % w/w. For ^{1}H NMR, ^{195}Pt NMR, free platinum content, release of free platinum, and SEC analysis the results of the quality control of the final product were similar to the results of the drug substances. This indicates that the production process and freeze drying of AP5346 afforded a final product with the same physical and chemical properties as AP5346 drug substance. Therefore, this production process is suited for the manufacture of AP5346 final product.
The platinum content per vial was determined using UV/VIS analysis. These results are discussed below.

UV/VIS spectrophotometry

The effect of AP5346 after administration depends both on the release of platinum at the tumor site and on the platinum content (free and bound platinum) of the final product. Therefore, the dosage is calculated as mg platinum. For AP5280, analysis of the total platinum content was performed with Flame Atomic Absorption Spectrometry [5]. However, with AP5346 a decreased signal was seen. This may be due to interference of the DACH group or the shorter distance between the DACH platinum chelate and the pHPMA backbone compared to AP5280. Therefore, a new method was developed. The total platinum content and platinum content uniformity were indirectly determined by UV/VIS spectrometry at 240 nm. UV/VIS is not a specific method, because all compounds (impurities included) which absorb at 240 nm are measured. However, if no impurities are detected with the specific analytical methods (^{1}H NMR, ^{195}Pt NMR, SEC), UV/VIS is a suitable method for the determination of the AP5346 content.

There was a small variation in platinum content of the drug substance between Lots, resulting in lot-to-lot differences in the amount of total AP5346 per vial (to obtain 400 mg platinum per vial) which in turn gave rise to lot-to-lot differences in concentrations of AP5346 after reconstitution. Therefore, the solutions used for calibration had to be prepared with the drug substance from which the final product was manufactured. The platinum content of the drug substances used for calibration was determined by Inductively Coupled Plasma (ICP) by Access Pharmaceuticals. Subsequently, the platinum content of the final product (as percentage of the theoretical content of 400 mg/vial) was calculated from the AP5346 content with use of the percentage of platinum of the drug substance used for the manufacture. The first batch of final product showed a relatively high platinum content of 116.9 % of the theoretical content. The platinum content of the other three batches varied form 100.0 – 106 %. The results are listed in Table I.

Setting of specifications

The quality control results of the 4 batches of final product resembled the quality control results of the 3 Lots of drug substance, indicating that the production process did not alter the characteristics of AP5346 drug substance. Furthermore, no differences in quality control results were seen between AP5346 final product 150 mg Pt/vial and 400 mg Pt/vial, indicating that the filling volume can be varied without changing the physico-chemical characteristics of the final product. Because of similarity between the 3 Lots of drug substance and the 4 batches of final product, just one set of specifications was established for both the drug substance and final product. First, preliminary specifications were set based on the means and standard deviations of the quality control results. These specifications were transformed into final specifications after completion of the stability study. The preliminary specifications are given in

Table II. Variations in the results between Lots are not only due to variation in characteristics of the Lots, but also due to analytical variation. This analytical variation depends on the analytical method used. Therefore, higher deviations were accepted for less robust analytical methods. Besides the analytical method, the influence of the analyzed parameter on product quality is also of importance. For example, results of SEC are less critical than the free platinum content and release of free platinum because the copolymer acts as a carrier and relative deviations in molecular size are not likely to influence the *in vivo* distribution. Furthermore, a deviation up to 10 % from run to run for SEC chromatography is quite normal. Therefore, a deviation of mean ± 20 % was found acceptable. For the free platinum content, however, the maximum concentration is important due to the toxicity of platinum. For this parameter, a deviation of ± 25 % was set. The same deviation was chosen for the release of free platinum. This deviation may seem high for a critical parameter, but due to the very low free platinum content the absolute deviation remained small. For the moisture content, only an upper limit was set, because increasing water content could lead to changes in the Pt chelate. For ^{195}Pt NMR analysis, small standard deviations were seen and narrow specifications were set. For ^{1}H NMR analysis, specifications were set for 12 signals. For all signals, the accepted deviation was based on the one signal with the highest expected Rate of Failure (ROF). This was the signal at 1.03 ppm, with an expected ROF of almost 12 %. The calculated expected ROF based on these specifications, decreased with increasing mean value of the signal. This indicates that the expected ROF of the first signal is mainly due to variation in the analytical method.

During storage of batches of AP5346 drug substance and final product at –20 °C, analysis of these batches in time showed that the SEC parameters exceeded the limits of the preliminary specifications. An increase in M_n and the subsequent decrease in PDI, per its definition, were seen in both the drug substances and the final products, indicating that these changes are inherent to the AP5346 molecule and not the manufacturing process. The M_w remained constant, which cannot be easily explained based on the definition of M_w and M_n. The SEC data (not shown) simply indicate that the copolymer changes during storage at –20 °C. To avoid batch rejection during storage, specifications must be recalculated Obviously, in these cases, changes of the preliminary specifications are only allowed when it can be guaranteed that the new specifications do not influence product quality. The copolymer however, is not the active part of the drug. It serves as a carrier of the DACH- platinum compound.

Table II. Preliminary setting of specifications

	SEC			Free Pt	Pt release		pH	Moisture	^{195}Pt NMR	
	M_w	M_n	PDI		3h	24h			Peak 1	Peak 2
Specification limits	± 20 %	± 20 %	± 20 %	± 25 %	± 25 %	± 25 %	± 7.5 %	± 10 %	± 0.5 %	± 0.5 %
Mean	19.6	9.1	2.15	0.22	1.14	4.06	6.74	0.65	-2264	-2289
Sd	1.85	1.16	0.08	0.08	0.13	0.23	0.03	0.07	1.02	0.80
Upper Specification Limit (USL)	23.6 kDa	11.0 kDa	2.58	1.00 %	1.42 %	5.07 %	7.25	3.00 %	-2270	-2300
Lower Specification Limit (LSL)	15.7 kDa	7.3 kDa	1.72	0.00 %	0.85 %	3.04 %	6.24	0.00 %	-2250	-2280
Expected ROF (%)	3.40	11.44	$6.09*10^{-6}$	0.16	3.14	0.00	$4.29*10^{-50}$	$1.71*10^{-21}$	$8.52*10^{-8}$	$6.21*10^{-30}$
Reliability index	2.30	1.45	$2.69*10^{4}$	8.02	2.38	522.19	$1.44*10^{48}$	$6.97*10^{18}$	$5.89*10^{6}$	$1.83*10^{28}$

	^{1}H NMR											
	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Peak 7	Peak 8	Peak 9	Peak 10	Peak 11	Peak 12
Specification limits	± 10 %	± 10 %	± 10 %	± 10 %	± 10 %	± 10 %	± 10 %	± 10 %	± 10 %	± 10 %	± 10 %	± 10 %
Mean	1.03	1.24	1.67	1.82	1.92	2.13	3.15	3.22	3.98	7.61	7.72	8.09
Sd	0.07	0.06	0.06	0.06	0.07	0.06	0.07	0.07	0.06	0.02	0.02	0.02
Upper Specification Limit (USL)	1.14	1.37	1.83	2.00	2.11	2.34	3.47	3.55	4.38	8.37	8.50	8.89
Lower Specification Limit (LSL)	0.93	1.12	1.50	1.64	1.73	1.92	2.84	2.90	3.58	6.85	6.95	7.28
Expected ROF (%)	11.98	4.74	0.91	0.47	0.85	0.10	$1.35*10^{-4}$	$1.78*10^{-4}$	$3.97*10^{-8}$	$4.45*10^{-294}$	$1.13*10^{-277}$	$7.49*10^{-239}$
Reliability index	1.43	2.00	4.41	6.37	4.60	17.07	$2.14*10^{3}$	$1.72*10^{3}$	$1.95*10^{6}$	$6.06*10^{287}$	$2.84*10^{271}$	$6.70*10^{232}$

Therefore, it is not to be expected that the toxicity or efficacy of this drug are influenced by small deviations in the size distribution of the copolymer, provided that the free platinum content and platinum release are not affected. Analyses showed that the free platinum content and platinum release were not affected during storage at –20 °C (data not shown). Therefore, the changes and recalculation of the specifications for M_n, and PDI are justified and the preliminary specifications of both SEC parameters were recalculated The recalculation was performed with the analytical data of 4 additional batches and revealed a M_n of 7.1-16.5 kDa and a PDI of 1.11-2.31. The specifications of the M_w were not changed, because this parameter remained essentially constant during storage. The change in these specifications only concerned the lyophilized product and not the drug substance.
The specifications set for AP5346 are based on a limited set of data, resulting in relative wide ranges for the specifications. While this is common for new products, it is expected in the future that the specifications will be recalculated with data from additional batches of AP5346 final product, resulting in smaller specification ranges.

Stability of AP5346 final product

The results of the stability study of the final product are given in Table III.
During 12 months of storage at 5 ± 3 °C (ambient RH) a tendency was observed of increasing M_n and decreasing PDI. These deviations were not significant and smaller than the deviations seen at a storage condition of –20 °C. Both parameters are within the final specification (see "Setting of specifications"). Furthermore, a small increase in pH was seen. After twelve months of storage the pH of the samples stored at 5 ± 3 °C (ambient RH) was on the limit of the specification (pH 6.2 – 7.2). These limits seem very small, but the calculated ROF with these specifications was $4.29 * 10^{-50}$. However, the stability data showed a change in pH of unknown origin. Calculations based on the stability data showed that a change of specifications from pH 6.2-7.2 to pH 6.4-7.4 is required to prevent batch rejection. This minor change in specification will not influence the quality of the product and is therefore allowed. No changes were seen in the appearance, ^{1}H NMR or ^{195}Pt NMR (data not shown).
At 25 ± 2 °C / 60 ± 5 % RH a significant increase in pH and moisture content was seen. The free platinum content and release of free platinum remained constant, indicating that the binding of the platinum was not influenced. The increase in pH is likely due to the higher moisture content. Furthermore, an increase in M_n and a decrease in PDI were seen at 12 months. The deviations of both parameters were larger at 25 ± 2 °C / 60 ± 5 % RH than at 5 ± 3 °C / ambient RH. Based on these results and the results of storage at –20 °C, a storage condition of 5 ± 3 °C / ambient

RH, in the dark, is preferred. AP5346 final product was given a storage time for 12 months at a storage condition of 5 ± 3°C, in the dark.

Stability and compatibility upon reconstitution and dilution

To investigate the stability of AP5346 final product after reconstitution and dilution, vials with final product were reconstituted with 27.4 ml 5 % w/v dextrose and diluted in three kinds of infusion containers with 5 % dextrose solution. Because the presence of free platinum (i.e. platinum bound to molecules smaller than 3 kDa) increases the toxicity of AP5346, the free platinum content and total platinum content were determined 96 hours after reconstitution or dilution. No changes in free platinum content and total platinum content were seen after reconstitution of the product in the vials (data not shown), indicating that AP5346 is stable for at least 96 hours after reconstitution and that no adsorption of platinum to the walls of the vials occurred.
The results of the analysis of the amount of free platinum after dilution in the infusion containers are depicted in Figure IIIa to IIIc. No difference was seen in compatibility of AP5346 between the different infusion containers. In all containers, the percentage of free platinum was highest for the solution of 0.034 mg Pt/ml stored at + 15-25 ºC. This content however, was for all containers still less than 1 % after 20 hours of storage. Furthermore, no differences were seen in the total platinum contents for the different containers (data not shown).
Besides the analysis of the free platinum content and release, any formation of degradation products was analyzed using ^{1}H NMR and ^{195}Pt NMR. No changes were seen in ^{1}H NMR and ^{195}Pt NMR spectra during the stability study, indicating that no structural changes occurred.

Table III. Stability data of AP5346 final product. The given values are the means and standard deviations of 3 Pilot batches.

		5 ± 3 °C			25 ± 2 °C / 60 ± 5 % RH		
	0 months	3 months	6 months	12 months	3 months	6 months	12 months
Free Pt (%)	0.44 ± 0.24	0.22 ± 0.11	0.17 ± 0.08	0.15 ± 0.01	0.24 ± 0.07	0.21 ± 0.05	0.30 ± 0.02
Pt release (%)							
3 hours	1.01 ± 0.17	1.22 ± 0.08	1.14 ± 0.05	1.28 ± 0.07	1.29 ± 0.08	1.34 ± 0.10	1.43 ± 0.12
24 hours	*	3.81 ± 0.30	4.18 ± 0.10	4.77 ± 0.22	3.96 ± 0.34	4.18 ± 0.10	4.80 ± 0.29
AP5346 content (%)**	100.0	93.4	99.8	104.4	95.3	99.1	104.1
SEC							
M_w	20.9 ± 1.40	21.8 ± 1.10	19.6 ± 0.83	21.6 ± 1.78	21.6 ± 1.10	19.4 ± 0.81	20.7 ± 1.15
M_n	10.5 ± 0.12	10.6 ± 0.84	9.8 ± 0.59	11.5 ± 0.55	10.4 ± 0.92	9.5 ± 0.64	13.2 ± 0.75
PDI	2.01 ± 0.14	2.05 ± 0.06	2.00 ± 0.03	1.73 ± 0.27	2.08 ± 0.07	2.68 ± 1.09	1.57 ± 0.04
Moisture content % w/w	1.35 ± 0.08	*	2.50 ± 0.21	1.55 ± 0.27	*	4.20 ± 0.33	5.05 ± 0.67
pH	6.8 ± 0.06	6.9 ± 0.06	6.9 ± 0.06	7.2 ± 0.06	7.1 ± 0.06	7.3 ± 0.06	7.7 ± 0.06

* Not analyzed

** Calculated as percentage of the initial AP5346 content

Figure IIIa. Free platinum content (as % of the total Pt content) in the Ecoflac® infusion containers of 5 % dextrose solutions containing 0.034 mg Pt/ml stored at + 2-8°C (◆), 0.034 mg Pt/ml stored at +15-25°C (■), 0.34 mg Pt/ml stored at + 2-8°C (●), 0.34 mg Pt/ml stored at +15-25°C (x), 3.4 mg Pt/ml stored at + 2-8°C (+) and 3.4 mg Pt/ml stored at +15-25°C (△).

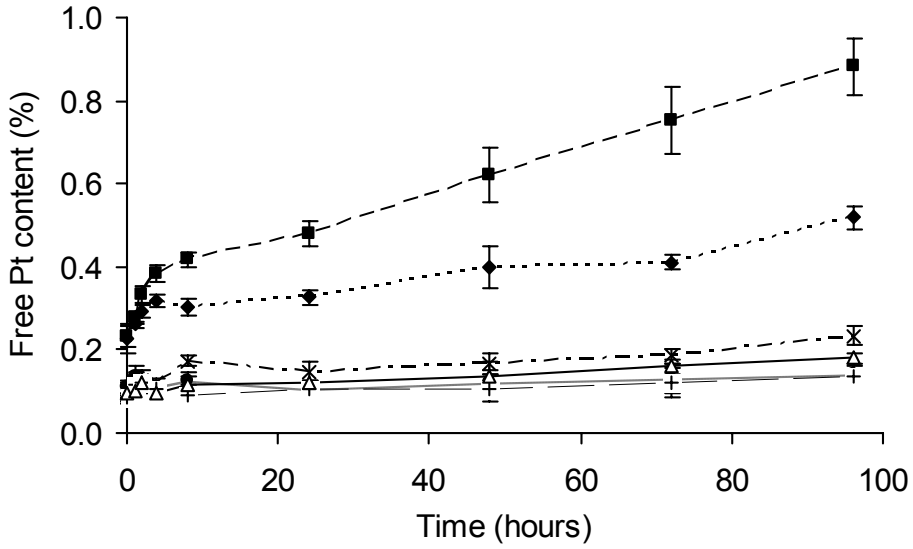


Figure IIIb. Free platinum content (as % of the total Pt content) in the Intraflex® infusion containers of 5 % dextrose solutions containing 0.034 mg Pt/ml stored at + 2-8°C (◆), 0.034 mg Pt/ml stored at +15-25°C (■), 0.34 mg Pt/ml stored at + 2-8°C (●), 0.34 mg Pt/ml stored at +15-25°C (x), 3.4 mg Pt/ml stored at + 2-8°C (+) and 3.4 mg Pt/ml stored at +15-25°C (△).

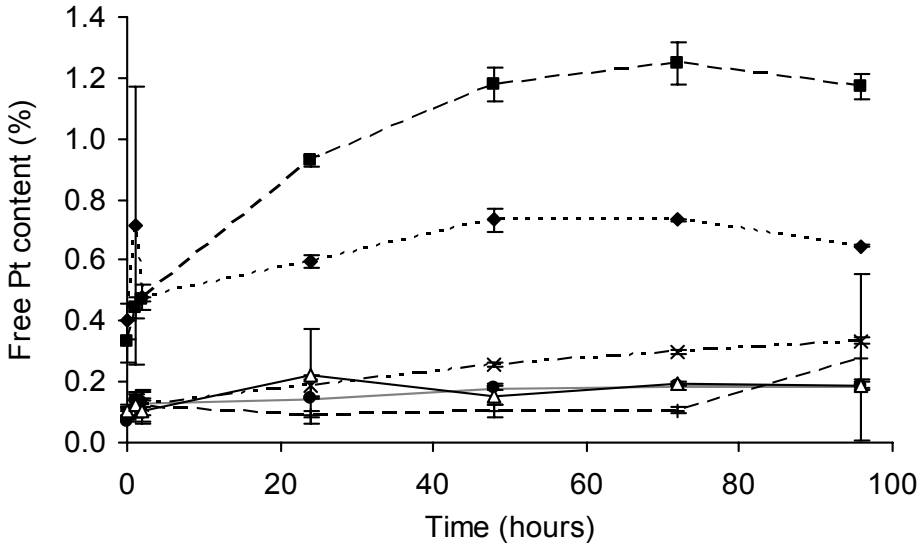
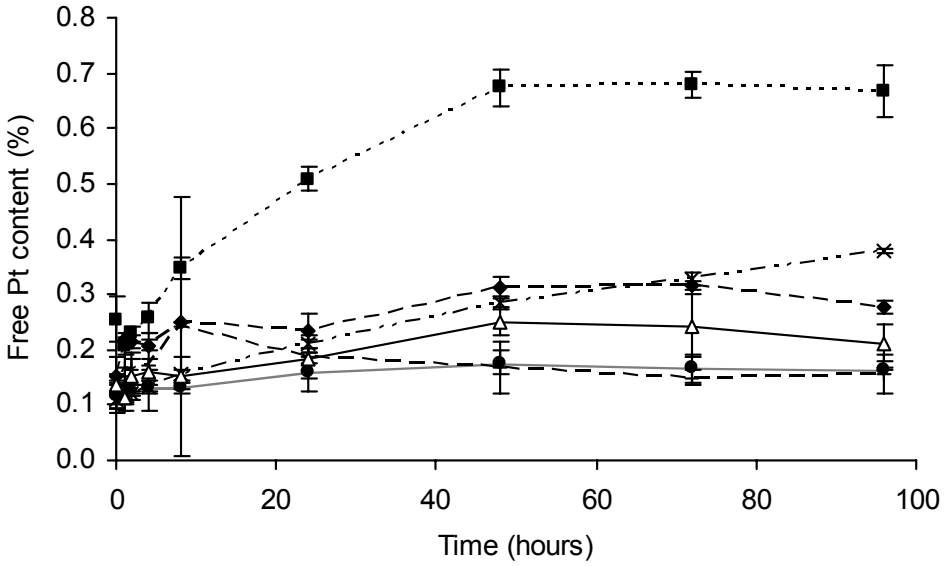


Figure IIIc. Free platinum content (as % of the total Pt content) in the glass infusion containers of 5 % dextrose solutions containing 0.034 mg Pt/ml stored at + 2-8°C (◆), 0.034 mg Pt/ml stored at +15-25°C (■), 0.34 mg Pt/ml stored at + 2-8°C (●), 0.34 mg Pt/ml stored at +15-25°C (x), 3.4 mg Pt/ml stored at + 2-8°C (+) and 3.4 mg Pt/ml stored at +15-25°C (△).



Conclusion

Several different analytical methods were used to characterize AP5346 final product and drug substance. It was shown that the final lyophilization process did not alter these characteristics. Based on the analytical methods, a list of specifications was set. These specification limits were chosen carefully, taking into account the risk of batch failure and the characteristics of the analytical method: what is the analytical variation of the analytical method and do large specification limits influence the quality of the product? These specifications were also used for the stability study. The results of the stability study of the final product showed that AP5346 final product is stable for at least 12 months at a storage condition of 5 ± 3 °C, in the dark. Furthermore, AP5346 final product was shown to be stable after reconstitution and compatible with infusion containers composed of glass, polyvinyl chloride (PVC, Intraflex®) and low density polyethylene (LD-PE, Ecoflac®).

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CHAPTER 3

Manufacturing of experimental anticancer agents

Chapter 3.1

2-Hydroxypropyl-β-cyclodextrin extracts 2-phenylphenol from silicone tubing

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Abstract

Cyclodextrins are capable to solubilize lipophilic drugs via (partial) inclusion in their lipophilic cavity. This, however, also provides the potential for the extraction of small molecules from production materials. In the present study, the potency of the commercially available and used cyclodextrin, 2-hydroxypropyl-β-cyclodextrin (HPβCD) to extract the preservative 2-phenylphenol (2-PP) from platinum cured silicone tubing was tested. The presence of 2-PP was structurally confirmed with HPLC-UV and LC/MS/MS in HPβCD solutions after incubation with platinum cured silicone tubing. HPβCD concentration and prior tubing sterilisation were found not to influence the levels of 2-PP extracted. Interestingly, extraction to ethanol was 15-fold higher than observed for HPβCD solutions.

2-PP was extracted from silicone tubing during routine manufacture of a blank dosage form formulated with only HPβCD, resulting in detectable levels of 2-PP in the final product. In a freeze-dried dosage form containing HPβCD and an active pharmaceutical ingredient (exhibiting a stability constant for HPβCD /drug of 1045 L/mole), on the other hand, 2-PP was undetectable.

Introduction

Cyclodextrins have been studied extensively for their ability to improve various physico-chemical properties such as solubility and stability of drugs by forming inclusion complexes [1-5]. Cyclodextrins are cone-shaped, cyclic oligosaccharides consisting of covalently (α-1,4)-linked α-D-glucopyranose rings with a relatively hydrophilic outer surface and lipophilic cavity. Currently, the cyclodextrins 2-hydroxypropyl-β-cyclodextrin (HPβCD) and sulfobutylether-β-cyclodextrin (SBEβCD) are used in the commercially available intravenous formulations of itraconazol and voriconazol, respectively [6,7]. As cyclodextrins have the capacity to solubilise lipophilic drugs via (partial) inclusion in their lipophilic cavity, they also have the potential for the extraction of small molecules from production materials and administration devices.

Extraction of extraneous substances from these materials into a drug product is undesirable in view of toxicity and/or compatibility issues. An example is the leaching of the plasticizer di-2-etylhexyl-phthalate (DEHP) from polyvinyl chloride (PVC) by co-solvents and surfactants [8,9]. Recently, the extraction potency of SBEβCD was

confirmed by Zimmerman, et al., who recovered 2-phenylphenol (2-PP) from a SBEβCD formulation after exposure to platinum cured silicone tubing [10].
Platinum cured silicone tubing is routinely used in the manufacture of several pharmaceutical dosage forms in our facility. In the present study, the potency of HPβCD to extract 2-PP from silicone tubing was tested. Furthermore, platinum cured tubing was evaluated for its applicability in the manufacture of a pharmaceutical dosage form for intravenous use of an active pharmaceutical ingredient (API) with an apparent stability constant ($K_{1:1}$) for HPβCD /drug of 1045 L/mole, formulated with HPβCD .

Materials and methods

Chemicals

HPβCD (USP grade, average Mw of 1399 and a mean degree of substitution of 0.65) was purchased from Roquette Freres (Lestrem, France) and phenylphenol (PP) isomers (2-PP, 3-PP, and 4-PP) were purchased from Sigma Aldrich Chemie (Zwijndrecht, The Netherlands). Silicone tubing was platinum cured and complied with the requirements of the United States Pharmacopoeia (USP) <87> [11] and European Pharmacopoeia (Ph.Eur.) <3.1.9> [12], (article no. 913.A048.016, Watson Marlow B.V., Rotterdam, The Netherlands). Ethanol absolute and Water for Injection (WfI) were of Ph.Eur. grade and purchased from Biosolve B.V. (Valkenswaard, The Netherlands) and B.Braun (Melsungen, Germany), respectively. All reagents used were of analytical grade and used without further purification.

Phase solubility diagram

A phase solubility diagram of 2-PP in HPβCD solutions was generated according to the method of Higuchi and Connors [13]. An excess amount of 2-PP was suspended in 5.0ml of solutions containing 0, 5, 10, 15, 20, and 40 % w/v HPβCD in 20ml glass vials. The vials were closed with siliconised gray bromobutyl rubber stoppers and subsequently shaken at room temperature and ambient light for 120 hours. Experiments were conducted in duplicate. The resulting suspensions were filtered (Millex®-LCR PTFE Syringe filter, 0.45 µm, Millipore, The Netherlands) and analysed for 2-PP concentration.

Extraction

Silicone tubing with an inner diameter of 4.8mm and a wall thickness of 1.6mm was used. Prior to extraction studies the tubing was rinsed for 1 minute with WfI. Part of

the silicone tubing was sterilised by autoclaving for 3 minutes at 134ºC. Extraction studies were performed in triplicate by filling silicone tubes over a length of 50 cm, corresponding to 9.05 ml of test solution per tube. Both ends of the test tube were closed with clips. The solutions tested were ethanol absolute and 0, 10, 20, and 40% (w/v) HPβCD in WfI. The silicone tubes were stored at room temperature (15-25°C) and ambient light. Samples were taken after 0.5, 1, 2, 4, 8, and 24 hours. Contents of the silicone tubes were emptied into 30 ml polypropylene tubes, mixed to assure homogeneity, sampled, and replaced in the same tubing. Two lots (lot 16549274 and 14936984) of silicone tubing were tested. Moreover, silicone tubes were filled with solutions containing 20% (w/v) HPβCD and 0.3, 3.1 and 31 mM of API ($K_{1:1}$ of 1045 mol/L). The sampling procedure was the same as described for the other solutions.

High performance liquid chromatography (HPLC)

The HPLC system consisted of an 1100 Series binary HPLC pump, Model G1312A (Agilent Technologies, Palo Alto, CA, USA), a Model SpectraSERIES AS3000 automatic sample injection device, equipped with a 100 µl sample loop (Thermo Separation Products, Breda, The Netherlands), and a photodiode array detector (PDA) Model Water™ 966 (Waters Chromatography B.V., Etten-Leur, The Netherlands). Chromatograms were processed using Chromeleon software (Dionex Corporation, Sunnyvale, CA, USA). Separation was achieved using an Inertsil ODS-2 analytical column (100mm x 3mm i.d., particle size 5 µm, Chrompack, Middelburg, The Netherlands), which was protected by a guard column packed with reversed-phase material (3 mm x 10 mm, Chrompack). The mobile phase consisted of 40/60% (v/v) acetonitrile/water (identification studies) or 60/40% (v/v) acetonitrile/water (quantification studies). A flow rate of 0.4 ml/min, an injection volume of 20 µl and run times of 20 min (identification studies) or 5 min (quantification studies) were employed. For the identification studies, stock solutions of 2-PP, 3-PP and 4-PP of 100 µg/ml in mobile phase were prepared. UV spectra of the PP isomers were recorded from 190 to 400 nm.

For the quantification of 2-PP, a stock solution of 2-PP of 100 µg/ml was prepared in 20% (w/v) HPβCD in water. The stock solution was diluted with 20% (w/v) HPβCD to give standard solutions of 0.02, 0.1, 1.0, 10, 25, and 50 µg/ml 2-PP. Calibration and quality control samples were prepared from two separately weighed stock solutions. UV-detection was performed at 245 nm. 2-PP eluted as a single, sharp peak with a retention time of approximately 3.6 min in the chromatogram. Linear calibration curves were obtained (correlation coefficients > 0.9999) with accuracies between 93.2 and 103%, within- and between-run precisions ≤ 2.2% and a limit of detection for 2-PP of 0.02 µg/ml, based on a signal-to-noise ratio of 3:1. Test solutions were injected

into the HPLC without further dilution, with the exception of the samples generated for the phase solubility diagram, which were diluted in WfI.

Mass spectrometry (MS)

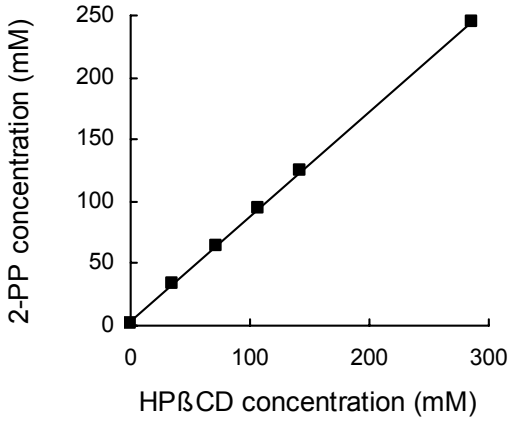
MS experiments were performed on a Sciex API 365 triple quadrupole LC/MS/MS spectrometer (Sciex, Thornhill, ON, Canada) equipped with an electrospray interface (ESI) ionisation source operating in the negative ion mode. Other parameters were: spray voltage 4.5 kV, heated capillary 250ºC. LC conditions were as described above. For determination of 2-PP/HPβCD complexation, samples were directly infused into the MS system. Product ion scans were obtained by mass-selecting the precursor ion from the Q1 scan. For the optimisation, stock solutions of the PP isomers of 10 µg/ml in mobile phase were prepared. Spectra were processed using Analist^{TM} software (version 1.2, Sciex).

Results and discussion

Phase solubility diagram

Figure I shows that HPβCD is indeed capable of forming inclusion complexes with 2-PP. The aqueous solubility of 2-PP increased up to a factor 90 in HPβCD 40% (w/v), corresponding to 82.4 ± 0.6 mg/ml. The linear curve of the phase solubility diagram can be classified as type A_L, indicating that the complex is first order with respect to HPβCD and first or higher order with respect to 2-PP. The slope of the curve was found to be greater than unity (1.67), which could indicate that the complexes are of second or higher order with respect to 2-PP. Although, slopes greater than unity do not always reflect higher order complexation with respect to the drug. It has been shown that drug/cyclodextrin complexes form aggregates or micelles in aqueous solution, which can further solubilize the drug molecule [14]. For 2-PP and SBEβCD, an A_L type phase solubility diagram with a slope of 1.26 was acquired [10]. A 20% (w/v) HPβCD solution was able to solubilise 42 mg/ml 2-PP in comparison with approximately 20 mg/ml 2-PP by 20% (w/v) SBEβCD [10]. These results indicate that 2-PP complexation is more favourable with HPβCD than SBEβCD, which could result in more extensive 2-PP extraction from silicone tubing.

Figure I. Phase solubility diagram for the 2-PP / HPβCD system in water.



Identification of the extractable

To examine the possible extraction of 2-PP, silicone tubing was subjected to a 40% (w/v) HPβCD solution for 5 days. The resulting solution was injected into the HPLC system. Indeed, an extraneous peak was obtained in the chromatogram with a retention time of 13.2 min corresponding to 2-PP (Figure II). The two other isomers of phenylphenol 3-PP and 4-PP were shown to elute ahead of 2-PP with base-line resolution. The PDA UV spectra of 2-PP, 3-PP, 4-PP, and the peak obtained for the extractable are depicted in Figure III. Identical UV-spectra were found for 2-PP and the extractable.

For further identification LC/MS/MS analysis was used. A strong signal (*m/z* 169.0, $[M-H]^-$) was seen in the LC/MS spectra of the PP isomers. The product ion scan of 2-PP shows strong signals at *m/z* 140.9, 115.0, 93.0 and 65.0 (Figure IV). The ion at *m/z* 140.9 was probably formed by the loss of a C-OH group, resulting in a phenylcyclopentadiene fragment. Cleavage of the bonds between atoms C7-C8 and C7-C12 (Figure V) rendered heptanol with a molecular weight of 116 ($[M-H]^{-1}$ = 115). The presence of an electrophilic hydroxyl group at the *ortho* position may favour the formation of this fragment. Indeed, both 3-PP and 4-PP showed only a weak signal at *m/*z 115, corresponding to the positioning of the hydroxyl group on the *meta* and *para* positions, respectively. The fragments at *m/z* 93 and *m/z* 65 indicate the formation of a phenol-like structure and pentadiene fragment, respectively.

The LC/MS spectrum of test solution containing the extractable showed a strong signal at *m/z* 169, indicating the presence of PP. The fragmentation pattern was indicative for the presence of 2-PP, confirming the results found with HPLC-PDA analysis.

No evidence for the formation of a complex between HPβCD and 2-PP was obtained when a test solution with both compounds was directly infused into the MS interface.

Though signals originating from HPβCD were observed in the positive ion mode and from 2-PP in the negative ion mode, no signals originating from the complex between both molecules were observed.

Figure II:. Chromatogram of a 40% (w/v) HPβCD solution prior to incubation with silicone tubing (top graph) and after 5 days of incubation (bottom graph).

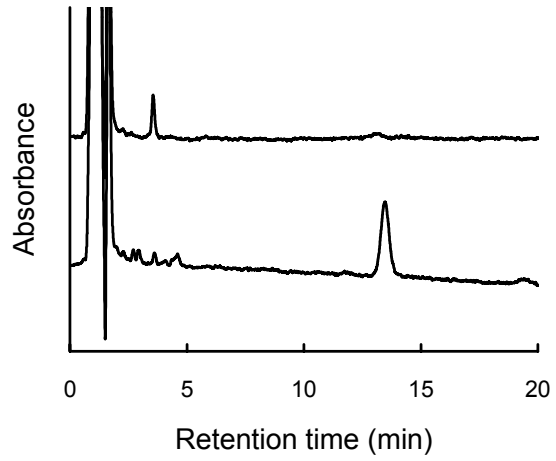


Figure III. UV absorbance spectra of 4-PP (1), the extractable (2), 2-PP (3), and 3-PP (4).

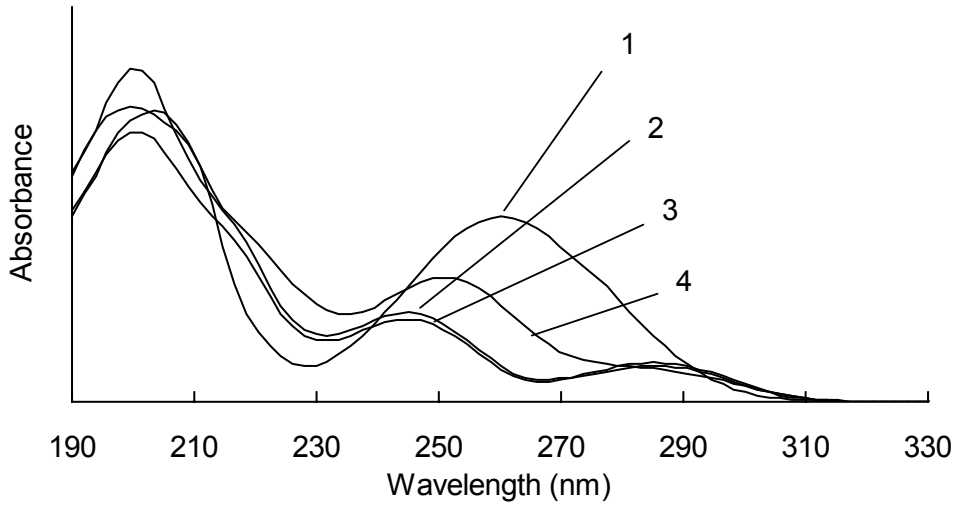


Figure IV. MS/MS spectrum of 2-PP.

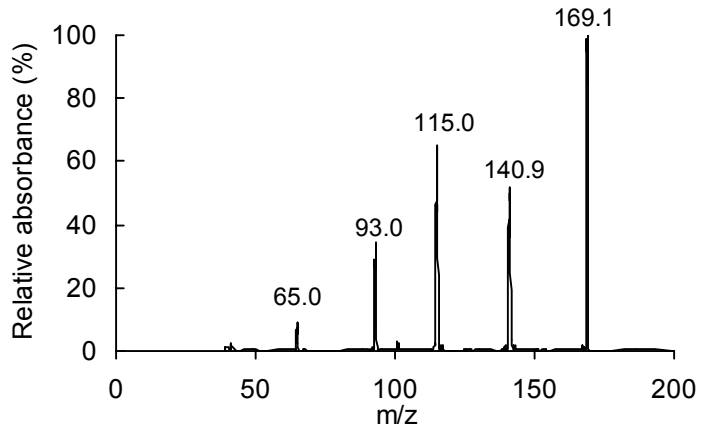
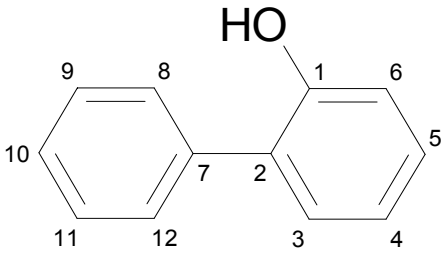


Figure V. Chemical structure of 2-PP ($C_{12}H_{23}O$, Mw = 170).



Extraction simulations

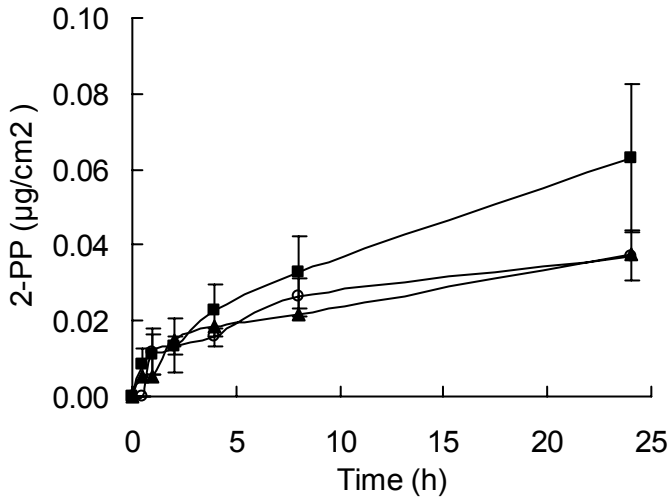
To better understand the implications of the extracting potency of HPβCD during routine manufacture, 2-PP extraction as a function of contact time and HPβCD concentration was investigated. Figure 6 shows that the extraction process is relatively slow and the amount extracted continuously increases for at least 24 hours, with decreasing extraction rate. The maximal 2-PP concentration in the cyclodextrin solutions observed was 0.80 µg/ml, corresponding to 0.096 µg/cm^2 tubing surface area. No 2-PP was recovered from the tubes containing pure water as extraction medium. 2-PP concentrations obtained were in the same order of magnitude as found earlier for SBEβCD (maximal 2-PP concentrations of 0.09-3.89 µg/cm^2 extracted over 24 hours into 20% (w/v) SBEβCD for different lots of tubing) [10]. Although it would be likely that more 2-PP is extracted from tubing exposed to higher HPβCD concentrations, differences were not significant due to the large standard deviations observed. The amount of 2-PP extracted was found to vary with the piece of tubing of the same lot used, suggesting inhomogeneous distribution of the extractable. The same phenomenon was observed between two different lots of tubing extracted with 20%

(w/v) HPβCD (Table I). The effect of exposure of the silicone tubing to one sterilisation cycle was shown not to reduce the 2-PP extraction in 20% (w/v) HPβCD solution (Table I). Similar results were obtained for 10, and 40% (w/v) HPβCD solutions. Interestingly, extraction with ethanol absolute resulted in an approximately 15-fold increase in the amount of 2-PP extracted (Table I). This illustrates that the decrease in extraction rate in time observed for the HPβCD solutions was not due to depletion of 2-PP from the tubing, but rather the effect of approaching equilibrium. The partition coefficient of 2-PP between silicone tubing and solution would be significantly higher for ethanol with a solubility of > 1000 mg/ml for 2-PP, than for HPβCD solutions with a maximal solubility of 82 mg/ml for 2-PP.
HPβCD is used as an excipient in drug formulations to solubilize or stabilise the drug by complexation. The presence of a drug competing with 2-PP for complexation could reduce the amount of 2-PP extracted from silicone tubing during manufacture. To test this, an API with a $K_{1:1}$ for HPβCD/drug of 1045 L/mole and an aqueous solubility of 0.9 mM was added to the HPβCD 20% (w/v) solution in concentrations of 0.3, 3.1, and 31.0 mM. Although only single pieces of one lot of silicone tubing were tested for each drug concentration, the amounts of 2-PP extracted in the presence of 3.1 mM and 31.0 mM of drug molecule were well below those found for all other pieces of silicone tubing tested in the presence of any of the HPβCD solutions (Table I). The drug was shown not to influence the 2-PP amount extracted when solubilized in a concentration of 0.3 mM in 20% (w/v) HPβCD. This was expected because the drug concentration was below its aqueous solubility.

Table I. Extraction of 2-PP (μg/cm^2) from silicone tubing after eight hours of incubation. Standard deviations are given between parenthesis.

Silicone tubing	Solution	Extracted 2-PP
Lot 16549274	20% (w/v) HPβCD	0.022 (0.010)
	20% (w/v) HPβCD + 0.3 mM API	0.021
	20% (w/v) HPβCD + 3.1 mM API	0.007
	20% (w/v) HPβCD + 31 mM API	0.006
	Ethanol absolute	0.37 (0.14)
Lot 16549274 autoclaved	20% HPβCD	0.017 (0.002)
Lot 14936984	20% HPβCD	0.009 (0.001)

Figure VI. Extraction of 2-PP from silicone tubing as a function of contact time and HPβCD concentration, with 10% w/v HPβCD (■), 20% w/v HPβCD (▲), and 40% w/v HPβCD (○).



Two test batches were manufactured to evaluate the amounts of 2-PP extracted from silicone tubing during routine manufacture. For the first test batch, blank dosage forms were freeze-dried from a formulation solution containing 20% (w/v) HPβCD. A second test batch was manufactured from a formulation solution containing API in a concentration of 31 mM in 20% (w/v) HPβCD. During both manufacturing processes, formulation solution was exposed to approximately 150 cm^2 of tubing surface area during filtration and filling for a maximum of four hours. When analysing the blank dosage form, 0.06 µg of 2-PP per vial was recovered. The presence of 2-PP was identified using LC/MS/MS. The maximum level of 2-PP retrieved from the silicone tubing after four hours of exposure in the extraction simulation study was found to be 0.036 $µg/cm^2$ tubing surface area, which would correspond to a maximum extraction of 5.4 µg of 2-PP during manufacturing. For the manufacturing batch size of 100 vials, this would result in 0.05 µg 2-PP per vial. These calculations do not take into account the dynamic flow and constant renewal of formulation solution in the silicone tubing during manufacture, which is expected to result in higher extraction values than found during the static extraction simulation studies.

No 2-PP, however, was recovered from the dosage form containing the API using either HPLC or LC/MS/MS analysis. This result confirms the observation in the static extraction simulation studies that there may be indeed a competition between API and 2-PP for complexation with HPβCD. Therefore, depending on the type of drug, the amount of 2-PP extracted during a manufacturing process could be far less than the amounts extracted without the presence of the drug.

Toxicity

2-PP is used as a preservative in numerous industrial applications, as a fungicide for citrus fruits, in the rubber industry, as well as a component of household and commercial disinfectant formulations. No information on the parenteral toxicity of 2-PP is available in literature. The only route of administration to man described in literature is dermal exposure [15,16]. Following dermal exposure to a single 8 h dose of 0.4 mg ^{14}C-2-PP, 43% of the applied compound was absorbed. Elimination was found quite rapid ($t_{1/2}$ of 0.8 hours), with 99% of the radioactivity excreted in the urine in the first 48 hours [16]. In animal toxicity studies, the no-observed-adverse-effect level (NOAEL) for 2-PP was found to be 36 mg/kg and 750 mg/kg body weight per day for rat and mice, respectively. Acute toxicity of 2-PP in rats, mice, and cats treated orally was low, with LD_{50} values ranging from 500 to 3000 mg/kg body weight [17]. The metabolism of 2-PP was found similar in man, mouse, and rat [15]. Based on the animal toxicology studies, the joint FAO/WHO meeting on pesticide residues (JMPR) established an oral acceptable daily intake (ADI) of 0.4 mg/kg body weight per day using a hundred-fold safety factor [18]. Assuming almost complete oral absorption, as also observed in mouse and rat with 80-98% of the administered radioactivity excreted in the urine within 48 hours [15], acceptable daily parenteral exposure to 2-PP could be in the same order of magnitude.

The levels of 2-PP extracted from silicone tubing by SBEβCD in the study of Zimmerman, et al. [10] and in the present study with HPβCD, both executed with platinum cured silicone tubing but originating from two different manufacturers, are well below the oral ADI. Though, 2-PP levels were found to be very inhomogeneous distributed within and between different lots of tubing. No specific test for the determination of the extractable amount of 2-PP is described in the Ph.Eur. monograph "silicone tubing elastomer for closures and tubing" <3.1.9> [12]. However, the amount of 2-PP present in tubing would influence the results of two tests specified. The test on "phenylated compounds" is based on the ultraviolet absorption of phenylated compounds present in a hexane extract of silicone tubing. Assuming that 2-PP is the only phenylated compound present in silicone tubing, the limit of specification would correspond to 400 µg 2-PP per gram of silicone tubing extracted (calculated with ε_{244} = 8450 in hexane), corresponding to 110 µg/cm^2 for the tubing used in the present extraction studies. For the test on "substances soluble in hexane" the maximum value for 2-PP would be 30 mg/g of silicone tubing, corresponding to 51 mg/cm^2 2-PP for the silicone tubing used. These amounts of extractable 2-PP in silicone tubing could result in levels of 2-PP in the final dosage form close to or exceeding the acceptable daily intake. Therefore, monitoring of 2-PP concentration in the starting material(s)

(possibly the source of 2-PP) used for the manufacture of silicone tubing or monitoring during in-process control or final control of the tubing is advisable.

Conclusions

2-PP was recovered from 10, 20, and 40% (w/v) HPβCD solutions after incubation with platinum cured silicone tubing, with no significant differences found in the extracted amount of 2-PP with increasing HPβCD concentrations. Addition of a selected API able to complex with HPβCD was shown to decrease the amount of 2-PP extracted. Extraction of 2-PP from silicone tubing to ethanol was shown to be 15-fold higher than observed for HPβCD solutions.
2-PP was extracted from silicone tubing during routine manufacture of a blank dosage form freeze-dried from a formulation solution containing 20% (w/v) HPβCD, resulting in a level of 0.06 µg of 2-PP in the final product.
Considering the oral ADI of 0.4 mg/kg body weight per day, values of 2-PP extracted from silicone tubing seem only marginal. Though, no in-vivo studies conducted to the effect of intravenously administered 2-PP have been conducted so far. Therefore, extraction of 2-PP from silicone tubing to formulations containing HPβCD and especially formulations containing ethanol should be carefully evaluated.
A freeze-dried dosage form containing HPβCD and an API (exhibiting a stability constant for HPβCD /drug of 1045 L/mole) was found compatible with platinum cured silicone tubing with respect to 2-PP as the API was shown to decrease the amount of 2-PP extracted during manufacture to undetectable, probably due to competition between API and 2-PP for HPβCD complexation.

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Chapter 3.2

Assessment of performance of manufacturing procedures in a unit for production of investigational anticancer agents, using a mixed effects analysis

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Submitted for publication

Abstract

The first steps in the clinical development of investigational anticancer agents are the phase I and II clinical trials. It is evident that product quality is very significant at this stage of development as it may, besides patient safety, affect the outcomes of these studies and thus the development program of the drug. Product quality should be built in by design, which is only possible if all aspects of the manufacturing process that present a significant risk to product quality are identified and controlled, based on validation studies. At this early stage of development, however, elaborate validation studies are generally precluded due to e.g. limited availability of the active pharmaceutical ingredient and number of (small) batches required. The aim of this article was to identify the magnitude and sources of variability of a generic, aseptic manufacturing process for experimental anticancer agents we employ at our facility, and to estimate the effects on product quality. Therefore, we retrospectively analyzed in-process and quality control data of all products manufactured according to this generic process (composed of weighing, dissolution, filtration, filling, semi-stoppering and lyophilization) over a 3-year period using mixed-effects analysis. This analysis showed that variability in the filling process was marginal and of minor importance for product quality in terms of content and content uniformity. An overall content of 101% was found with batch-to-batch and vial-to-vial variability up to 4.21% and 2.57%, respectively. Estimation of the overall batch failure revealed that structural bias in content and a high batch-to-batch variability in content were the most prominent factors determining batch failure. Furthermore, content and not content uniformity was shown to be most important parameter influencing batch failure. Calculated Process Capability Indices (CpKs) were calculated for each product, which showed that our manufacturing process is capable of manufacturing products which will routinely comply with the specification of 90-110% for content. However, the CpK values decreased dramatically using the specification of 95-105% as required for approved drug products. These results indicate that at the early stage of product development less tight specification limits must be applied to prevent unnecessary batch rejection of investigational agents.

Introduction

The first steps in clinical development of experimental anticancer agents are phase I and II trials. Product quality, evidently, is very significant at this stage of development as it may, apart from patient safety, affect the outcomes of clinical trials which will determine whether or not the product will be selected for further development [1]. Therefore, guidelines have been established with respect to Good Manufacturing Practice (GMP) of investigational agents [1-4]. Product quality starts off with drug synthesis, followed by scientific sound formulation-, compatibility- and stability studies. This also involves the development and validation of a set of analytical techniques and methods to enable characterization and quality control of both the active pharmaceutical ingredient (API) and the final pharmaceutical product. Consistent manufacturing of the pharmaceutical product of the required quality should be possible then and proven by validation studies [5].

Anticancer drug formulations for experimental use are generally intended for intravenous administration to obtain absolute bio-availability, to circumvent possible disturbance of or degradation in the gastrointestinal tract and to be able to adjust or to stop administration of the drug immediately in case of acute toxicity. Consequently, the development of a pharmaceutical formulation of a novel anticancer agent is focussed on issues associated with the design of sterile and stable injectable products.

General characteristics in this early phase of development are: often limited availability of API precluding elaborate validation studies; small scale (both in number of units per batch as well as in number of batches); flexibility (due to relatively many changes in strength and/or composition of batches); aseptical manufacturing process as the instability of the APIs often does not allow heat sterilization.

For the manufacture of sterile anticancer agents in the early phase of development, we established a small manufacturing unit at our institute in which we apply a generic production process capable of handling intravenous investigational anticancer drugs with the characteristics inherent to this phase. In this unit, we have a long experience in the pharmaceutical development and manufacture of experimental anticancer agents for Phase I and II clinical trials [6-13]. The generic production process is composed of six steps: weighing, dissolution, filtration, filling, semi-stoppering, and lyophilization.

The aim of this study was to identify the magnitude and sources of variability of the production process and to estimate the effects on product quality. Therefore, we performed a retrospective validation of this generic manufacturing process using

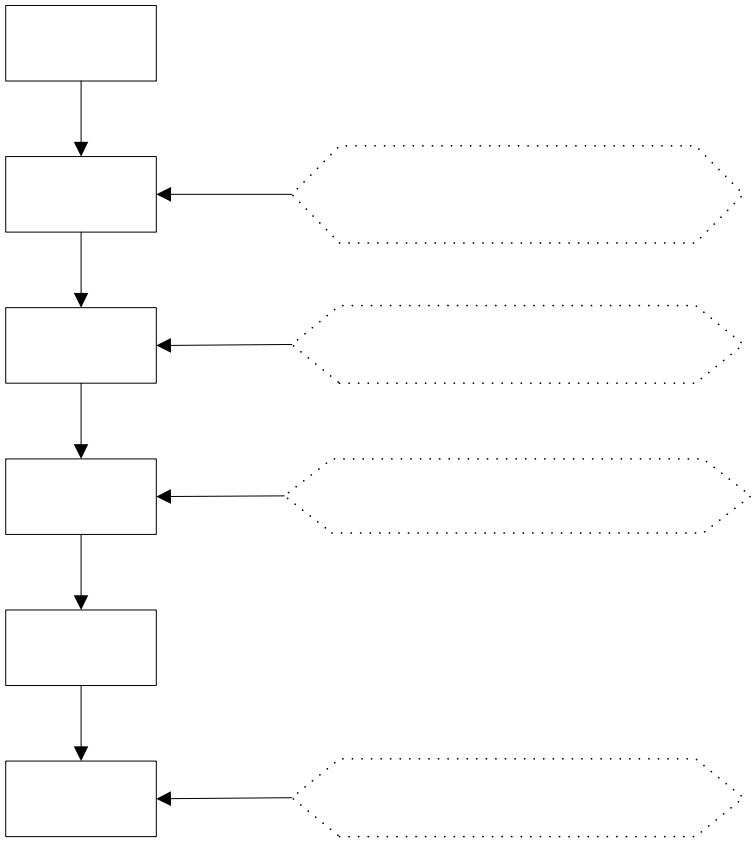
mixed effects analysis. Based on this analysis, we evaluated the specifications for investigational products.

Materials and method

Manufacturing process

A flow chart of the generic production process, including the in-process controls which are performed at the different processing phases, is depicted in Figure I.

Figure I. Schematic representation of the generic production process



All manufacturing steps are performed in a class 100 (B) clean room facility. Manipulations with open product (filtration, filling, semi-stoppering, lyophilization) are performed in a class 100 (A) environment. The clean room facility is regularly validated with respect to viable (settle plates, air sampling, contact plates) and non-viable particles, both in the "at rest state" and "at operating state" situation. Also, the

aseptic manufacturing process and personnel is regularly validated by media fills by simulating the complete manufacturing process. This is all performed according to GMP-guidelines [14]. The facility holds a manufacturer's licence for the manufacture of experimental anticancer agents since 1999.

Data Collection

Data (i.e. batch characteristics and quality control data) were collected of all batches manufactured between January 2003 and November 2005. The following batch characteristics were used as input variables: product, batch size, dissolution vehicle, and theoretical filling volume. Table I gives an overview of the batch characteristics and their ranges. Output variables were: filling weight, content, and content uniformity.

Table I. Batch characteristics of all batches manufactured from 2003 to 2005 used for validation.

Batch characteristic	Range
No. of products	7
No. of batches	97
Batch Size	75 – 800 vials
Dissolution vehicle	DMSO, WfI, TBA/WfI *
API concentration **	0.5 – 150 mg/ml
Filling volume	1 – 40 ml

* DMSO = dimethyl sulfoxide, WfI = water for injection, TBA/WfI = 40% v/v TBA in WfI

** Concentration of API in the formulation solution

Data analysis

For all batches, two types of observations were available:

- In-process controls during the filling process (= filling weight)
- Overall content (n=13) of the vials calculated from both the content uniformity (n=10) and content (n=3) data.

Both parameters were expressed as percentage of the theoretical values in order to facilitate comparison of the different batches and products.

Retrospective data analysis: basic model

Three distinct types of variability were assumed to exist:

- Structural bias: The occurrence of a structural deviation from the theoretical content may be due to e.g. the production process, characteristics of the API (e.g. absorbance to materials used during manufacture) or filling of very low volumes.
- Batch-to-batch variation (i.e. variation between different batches of one product)
- Vial-to-vial variation (i.e. variation within one batch)

The basic model applied for data obtained from the filling process was:

$$F_{ij} = \theta_{fill} + \eta_{fill,j} + \varepsilon_{fill,ij}$$

in which F_{ij} is the measured filling weight in vial i of batch j, θ_{fill} is the typical value of the filling weight (if no structural bias is present θ_{fill}=100%), $\eta_{fill,j}$ is a random effect describing batch-to-batch variability with mean 0 and a standard deviation of ω_{fill}, and $\varepsilon_{fill,ij}$ is the random effect describing vial-to-vial variability with mean 0 and standard deviation σ_{fill}.

The predicted filling weight for an unknown vial in batch j equals $\hat{F}_j = \theta_{fill} + \eta_{fill,j}$

Similarly, the content data were modelled as:

$$C_{ij} = WT \times \hat{F}_j \times \theta_{cont} + \eta_{cont,j} + \varepsilon_{cont,ij}$$

in which C_{ij} is the measured content of vial i of batch j, WT is the weighted amount of API (expressed as percentage of theoretical), θ_{cont} is the typical value of content, $\eta_{cont,j}$ is a random effect describing batch-to-batch variability with mean 0 and standard deviation ω_{cont} and $\varepsilon_{cont,ij}$ is the random effect describing vial-to-vial variability with mean 0 and standard deviation σ_{cont}. Because weighing is performed on a calibrated balance, it was assumed that bias and precision of the weighing could be neglected compared to the other sources of variability.

Both models were simultaneously applied to the data containing both types of observations (in-process controls during the filling process, and Overall content of the vials calculated from the content uniformity and content). Non-linear mixed effects modelling (NONMEM, version V, double precision, level 1.1, Globomax, Ellicott City, MD, USA) was used for the data analysis. NONMEM applies a maximum likelihood criterion to simultaneously estimate fixed effects (i.e. the typical values of content and the filling process) and random effects (i.e. the different variability terms). The first-order conditional estimation method with interaction between different types of variability (INTERACTION option of NONMEM) was used throughout. The following fixed effects were estimated for the basic model: θ_{fill} and θ_{cont}. The following random effects were estimated: ω_{fill}, σ_{fill}, ω_{cont} and σ_{cont}. Precision of parameter estimates was obtained with the COVARIANCE option of NONMEM.

Retrospective data analysis: influence of production parameters

For all batches the following co-variates were recorded: product (PROD), batch size (SIZE), filling volume (FILL), and vehicle (VEH). The influence of these co-variates was tested on the different terms in the model. For instance, a product may have a systematic bias, an increased batch-to-batch variability or an increased vial-to-vial variability. The influence of these co-variates on the random effects was tested by introduction of different random effects terms for data with and without the co-variate (i.e. one product compared to the other products). The influence on the fixed effects was tested by introduction of a separate fixed effect describing the systematic bias for that co-variate. Significance was tested using the likelihood ratio test. The difference in objective function (minus twice the log likelihood of the data) between two nested models (i.e. models with and without a co-variate influence) has a chi-square distribution with one degree of freedom. Therefore, a difference of 3.84 points corresponds with a p-value of 0.05.

Possible co-variates were introduced separately on the different terms of the basic model. Subsequently, all possible significant co-variates were introduced in an intermediate model. Stepwise backward elimination was used to retain only the significant co-variates in the final model.

Furthermore, the Process Capability Index (CpK) was calculated. This parameter is often used to measure the reproducibility as a function of the specification limits [15]. CpK values were calculated for each product assuming a content equal to the average content for this product (optimal situation resulting in an "ideal" batch) and for each product assuming a content equal to the average content ± 1 R.S.E. batch-to-batch variability, using equation 1 and 2, whichever gives the lowest number.

$$\text{CpK} = \frac{\text{upper limit of specification - mean}}{\text{3 x standard deviation}} \qquad (1)$$

or

$$\text{CpK} = \frac{\text{mean - lower limit of specification}}{\text{3 x standard deviation}} \qquad (2)$$

For the calculation of the CpK values the specification limits for content of 90-110% and 95-105% were used.

Simulation studies

Based on the results of the retrospective data analysis, several simulations were performed in order to characterize the influence of different production parameters on batch failure (both content and content uniformity). For content uniformity the specifications according to the European Pharmacopoeia [16] were used, for content an

average of 90-110% was used as specification. Several scenarios were investigated in which different sets of production parameters were defined and the influence of these parameters on batch failure was investigated. For each scenario, 1000 batches were simulated and subsequently the batch failure based on content, content uniformity and overall batch failure was recorded. Simulations were performed using the SIMULATION option of NONMEM and the model as developed in the retrospective data analysis.

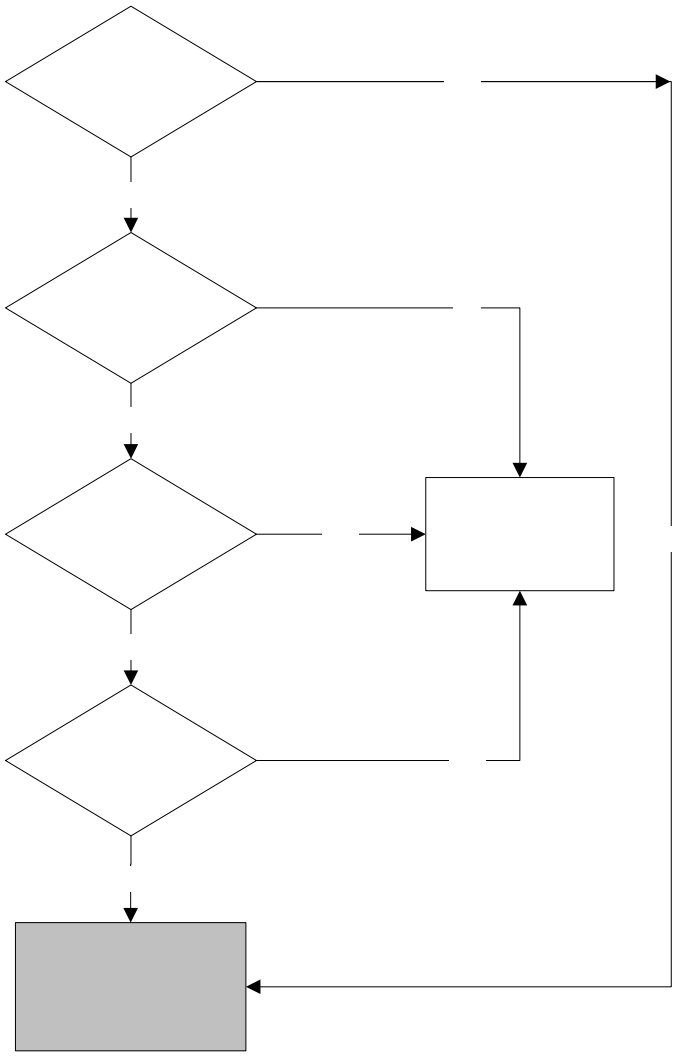
Results and discussion

Manufacturing process

Table II gives a list of standard quality control test items for sterile, lyophilized products for parenteral use and the variables which may affect these. From these, the items and in-process controls were selected which are critical for product quality and are indicative for the general performance of the production process in relation to batch characteristics (product, batch size, dissolution vehicle, theoretical filling volume) were selected. The decision tree used to designate a variable as critical is given in Figure II. The test items Appearance, Reconstitution characteristics, and Residual moisture or solvent content are all output variables which are inherent to the selected freeze-drying process and are product-specific. Also, they influence more critical product quality items like content and purity only indirectly and are for this reason considered non-critical. Therefore, these items were not included in the assessment. For the same reason, pH after reconstitution was not used. Purity was not used because for all products analyzed the stability was studied well during formulation development and the compounds of interest were shown to be stable during processing time. Of all batches analyzed, no deviations in the manufacturing process occurred which may have affected purity or content. The test items content and content uniformity, however, were selected for the assessment because they are critical items for product quality. As shown from Table II, both content and content uniformity are directly influenced by filling weight and filling weight uniformity. Therefore, the in-process control filling weight was used in the analysis as well. Controls for producing an investigational new drug are primarily aimed at patient safety [4]. Because it concerns the manufacture of parenteral drugs, sterility and pyrogenicity immediately affect product safety and are therefore critical items. The sterility and bacterial endotoxins content of all batches used for the assessment were according to the specifications. However, the tests performed to determine sterility and pyrogenicity are only performed on a selected number of vials and therefore, a

negative result does not completely guarantee that all vials are sterile and free of endotoxins. This emphasizes that minimizing bioburden during manufacture and validation of production personnel, clean room facilities and production process is of utmost importance. No critical deviations with respect to these items occurred during the period of analysis.

Figure II. Decision tree for determination of product quality



* Generally unknown at early stage of drug development

Affects safety?

Table II. Variables which may influence product quality.

Product quality item	Process variable
Appearance	Freeze drying process
Reconstitution characteristics	Freeze drying process
content	Filling weight (IPC)
content uniformity	Filling weight uniformity (IPC)
Purity	Purity API
	Processing time
pH after reconstitution	Concentration/characteristics of API and/or excipients
Residual moisture/solvent content	Freeze drying process
Sterility	API/excipients/bioburden of formulation solution (IPC)
	Filter integrity (IPC)
	clean room/personnel performance
Bacterial endotoxins	Contamination of API and/or excipients
	clean room/personnel performance

IPC = in-process control

Data analysis

Retrospective data analysis: basic model

In total, data of 97 batches of 7 products were used for the retrospective risk assessment. Variation in content or content uniformity found by quality control analysis is the result of an addition of errors in weighing, filling, and analytical variation. Due to the performance of extensive formulation and stability studies, it can be assumed that no degradation or loss during dissolution, filtration and freeze-drying occurs during the production process. The overall accuracy of the filling process was 99.6%, with a batch-to-batch and vial-to-vial variability of 0.796% and 0.988%, respectively (Table III). During manufacture of each batch, the pump was calibrated using the freshly prepared formulation solution which might explain the relatively small batch-to-batch variability compared to the vial-to-vial variability, although this difference is small.

For content, an overall accuracy of 101% was found, while batch-to-batch variability and vial-to-vial variability were approximately 4% and 2%, respectively. This indicates that variability in the filling process is only marginal compared to the variability in content and therefore of minor relevance for the overall product quality.

Table III. Results of the retrospective data analysis: basic model

	Typical value (%) (%R.S.E)	Batch-to-batch variability (%) (%R.S.E)	Vial-to-vial variability (%) (%R.S.E)
Filling process	99.6 (0.089)	0.796 (20)	0.988 (23)
Content	101 (0.40)	3.89 (19)	1.96 (16)

%R.S.E. = % relative standard error of estimate

Retrospective data analysis: influence of production parameters

With this analysis, it was tested whether different production parameters had impact on the performance of the production process. This analysis, however, was hampered by the fact that several co-variates showed some degree of co-linearity. For instance, the dissolution vehicle dimethyl sulfoxide (DMSO) was only used as vehicle in a single product. Therefore, it was impossible to differentiate between the influence of DMSO as vehicle or other specific properties of this product (e.g. quality control methods). In these cases, it was chosen to use the product first as co-variate, except for co-variates related to the filling process where filling volume and vehicle were used preferentially. Table IV shows the results of this analysis. As can be seen, two products (3 and 5) had a systematic lower filling weight and one product (product 7) showed a lower batch-to-batch variability in the filling process than the other products. In product 3 the excipient 2-hydroxypropyl-β-cyclodextrin was used, resulting in a slight increase in viscosity of the formulation solution, probably resulting in a decrease in filling volume. For the vial-to-vial variability almost a 3-fold increase (from 0.735% to 1.99%) was seen when a fill volume of 1 ml was used compared to higher filling volumes of 2-40 ml. Furthermore, the use of DMSO as vehicle increased the vial-to-vial variability with 53%, while the use of a mixture of water and tert-butyl alcohol (TBA) reduced this variability with 33.9%.

The typical value of the overall content was exact 100%, indicating that no general structural bias was present. However, product 2 and 6 had a systematic and significant bias of –5.04% and +7.14%, respectively. This bias is not due to variation in filling weight, because no deviation in the typical value of the overall filling of these products was seen (Table IV). No specific product characteristics could be related to the structural bias of these products. Further investigation of the cause of the structural bias is required.

The batch-to-batch variability of the overall content was 4.21% while product 3 and 7 had a significant lower variability of less than 2%. Vial-to-vial variability was estimated for all products separately in order to account for differences in quality control methods. Vial-to-vial variability in content ranged from 1.51% to 2.57%. The highest vial-to-vial variability was found for product 4. Remarkable is the low vial-to-

vial variability of Product 3 compared to the other products, because this product is analyzed using a derivatization step, which may inherently increase variability. Apparently, this derivatization process is robust.

Furthermore, the Process Capability Index (CpK) was calculated for each product. There are generally accepted rules to relate the CpK value to the robustness of the process. A CpK value of less than or equal to 0.8 indicates that the process is not capable of meeting the specification limits routinely and therefore, further efforts have to be made in developing a more robust process. CpK values of 0.9-1.0 indicate a marginal process, of 1.0–1.25 are satisfactory, of 1.25-1.5 are good, and values higher than 1.5 are excellent. The calculated CpK values of our products are given in Table V. Within the limits of 90-110% and a deviation of one batch-to-batch variability, Product 2 and Product 6 had a CpK value of less than 0.8, indicating that the process was not able to meet the specifications routinely for these products. The CpK values of the "ideal" batches of these products, however, were not much better. This is due to the structural bias found for these products. The presence of a structural bias can be a pitfall when using CpK values, because it can result in a low CpK value while the manufacturing process is acceptable and vice versa [15]. The CpK values were satisfactory for Product 1 and 4, good for product 7 and excellent for Product 3 and 5.

As expected, using the specification limits of 95-105% according to the European guidelines for approved drugs [17], the CpK values were lower. The CpK values were less than 0.8 for all products with a deviation of one batch-to-batch variability. For the "ideal" batches, the CpK values of four of the seven products were still less than 0.8. It should be noted that the estimates for batch-to-batch variability were based on a limited number of batches. These values may therefore be highly dependent on single outlying batches. Further process optimization may be required before the products can fulfil the specifications for approved drug products routinely, especially when no outliers were found.

Table IV. Results of the retrospective data analysis: influence of production parameters

	Typical value (%) (%R.S.E)	Batch-to-batch variability (%) (%R.S.E)	Vial-to-vial variability (%) (%R.S.E)
Overall filling process	99.8 (0.063)	0.857 (19)	0.735 (20)
Product 3	-0.552[a] (37)		
Product 5	-0.892[a] (40)		
Product 7		0.251[b] (59)	
FILL = 1 ml			1.99[b] (49)
VEH = DMSO			+53%[a] (87)
VEH = TBA/WfI			-33.9%[a] (75)
Overall content	100 (0.28)	4.21 (22)	
Product 1			1.65[b] (11)
Product 2	-5.04[a] (32)		2.04[b] (64)
Product 3		1.95[b] (28)	1.51[b] (21)
Product 4			2.57[b] (33)
Product 5			1.52[b] (16)
Product 6	+7.14[a] (28)		2.06[b] (21)
Product 7		1.53[b] (30)	2.12[b] (30)

a: relative difference compared to typical value, b: absolute value, FILL = filling volume, VEH = dissolution vehicle, DMSO = dimethyl sulfoxide, TBA/WfI = 40% v/v tert-butyl alcohol in water for injection, %R.S.E. = % relative standard error of estimate

Table V. Calculated CpK values for content

Product	Limit 90-110%		Limit 95-105%	
	Mean *	Mean ± 1 bbv **	Mean *	Mean ± 1 bbv **
1	2.02	1.17	1.01	0.16
2	0.81	0.12	-0.01	-0.69
3	2.21	1.78	1.10	0.67
4	1.30	1.04	0.65	0.40
5	2.19	1.77	1.10	0.67
6	0.46	0.15	-0.35	-0.66
7	1.57	1.33	0.79	0.55

* CpK value calculated for the most ideal batch with content equal to the mean content of this product.

** CpK value calculated for a content equal to the mean ± 1 batch-to-batch variability.

Simulation studies

From the retrospective data analysis, it was established between which limits the different terms of the model varied according to the different production parameters. The ranges of the different terms were used in simulation studies to assess the influence of the different parameters on batch failure. The ranges used in the simulation study are shown in Table VI. The lower limits were defined as the best case scenario and the upper limits were defined as the worst case scenario.

In a first series of simulations, it was investigated to what extent parameters derived from the filling process influenced the results. In the best case scenario as well as the worst case scenario no batches (out of the 1000 simulated batches) were out of specifications for content and/or content uniformity. Therefore, it was concluded that the filling process was not a critical step in the manufacturing process within our current assessment.

The results of the same series of simulation for content are shown in Figure III. As can be seen, an increased batch-to-batch variability in content (1.5% to 5%, Table VI) was the most prominent factor determining batch failure in the absence of a structural bias. Furthermore, it was observed that the batch-to-batch variability had more impact on content than on content uniformity. It should be noted that overall batch failure was only determined by failure on content and not on content uniformity.

In a next step, the influence of a structural bias in content and content uniformity on the chance of batch failure was tested. This was performed using a best and a worst case scenario (indicated in Table VI). The results of these simulations are shown in

Figure IV and V. Again, overall batch failure was only determined by batch failure due to content being out of specification. In the worst case scenario defined in our retrospective analysis (for all parameters involved) a structural bias of 7% in content could be found (Table VI), resulting in an overall content of 93 or 107%. In this situation approximately 30% and 15% of the batches were out of specification for content and content uniformity, respectively (Figure V). Caution should be taken in interpretation of these results as it is a simulation of the worst case scenario for all parameters simultaneously, which has not been encountered in practice thus far. Nonetheless, it indicates that structural bias has an important influence on batch failure.

Overall it was found that within the limits as found in our retrospective data analysis, a structural bias in content and a high batch-to-batch variability in content are the most prominent factors determining batch failure. A variation in structural bias from -5 to +7% was seen. The presence of such a bias would result in a dramatic increase in batch failure if the limits 95-105% for content, according to the European guidelines for approved drugs, were used [17].

These results indicate that at the early stages of product development, less tight specification limits must be applied to prevent unnecessary batch rejection of investigational agents. It is generally accepted that product specifications will evolve during development, starting off with relative wide limits followed by tightening of these specifications in the course of product development as more information is gained and manufacture and analysis becomes more qualified [18-20]. It is shown that the 90-110% specification limits we apply for content is feasible in this respect.

Table VI. Parameter ranges for simulation study

	Filling process	Content
Absolute structural bias	0% - 1%	0% - 7%
Batch-to-batch variability	0.25% - 1%	1.5% - 5%
Vial-to-vial variability	0.75% - 3%	1.5% - 2.5%

Figure III. Calculated effect of vial-to-vial and batch-to-batch variability on the risk of batch failure based on the content and content uniformity in absence of structural bias.

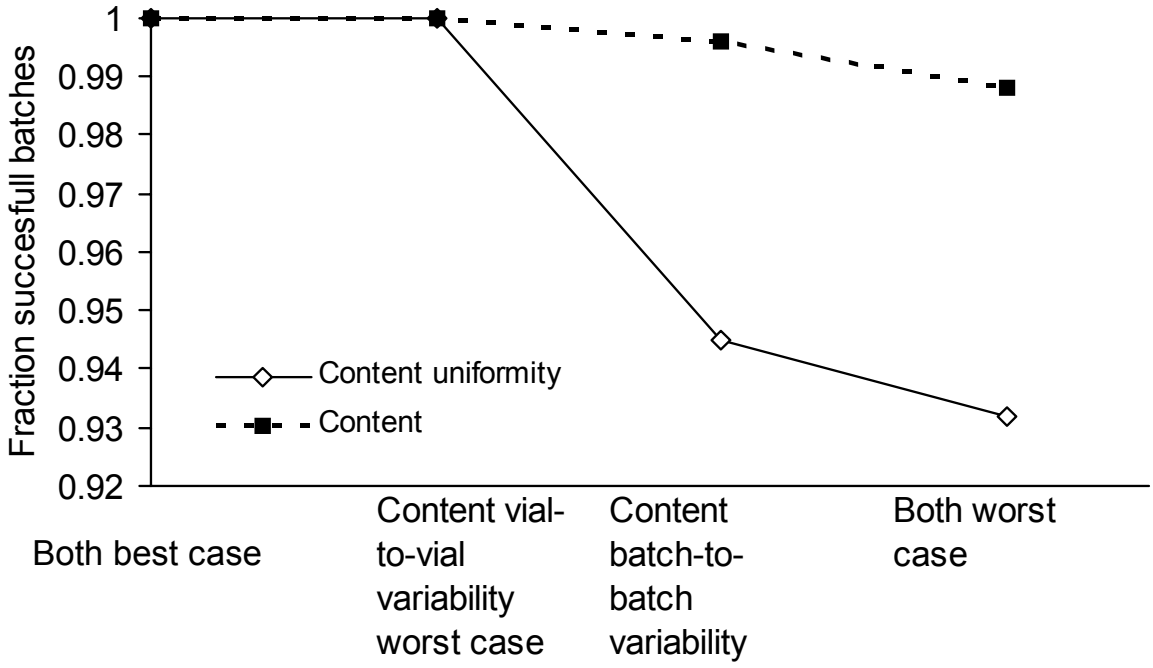


Figure IV. Estimated fraction of successful batches as function of the overall bias in the best case scenario.

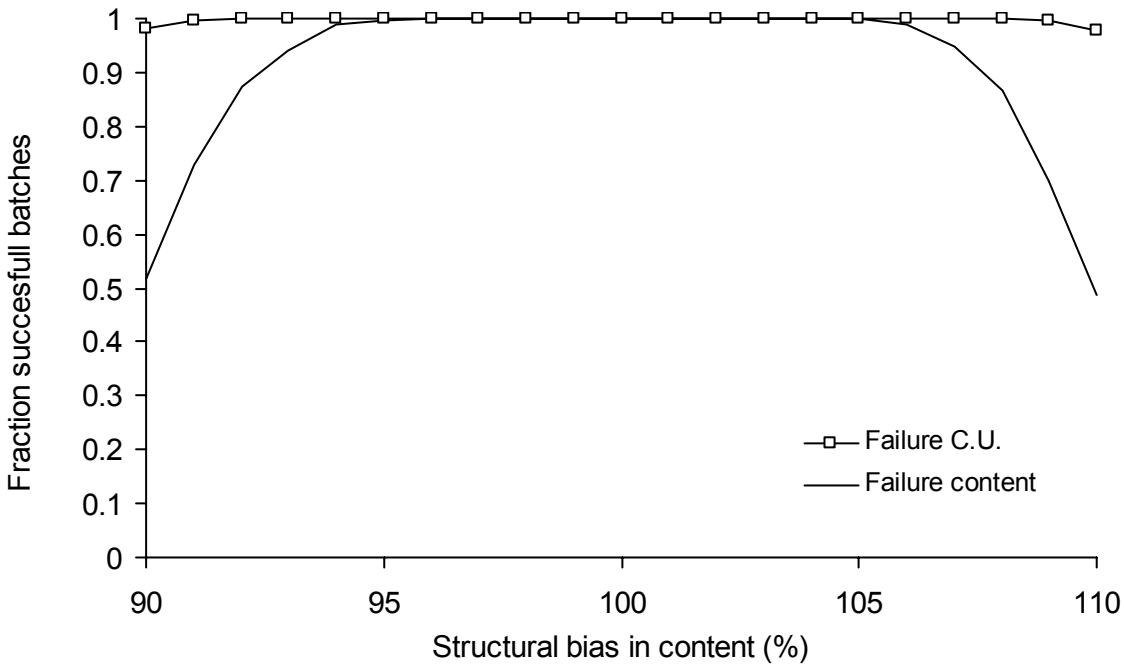
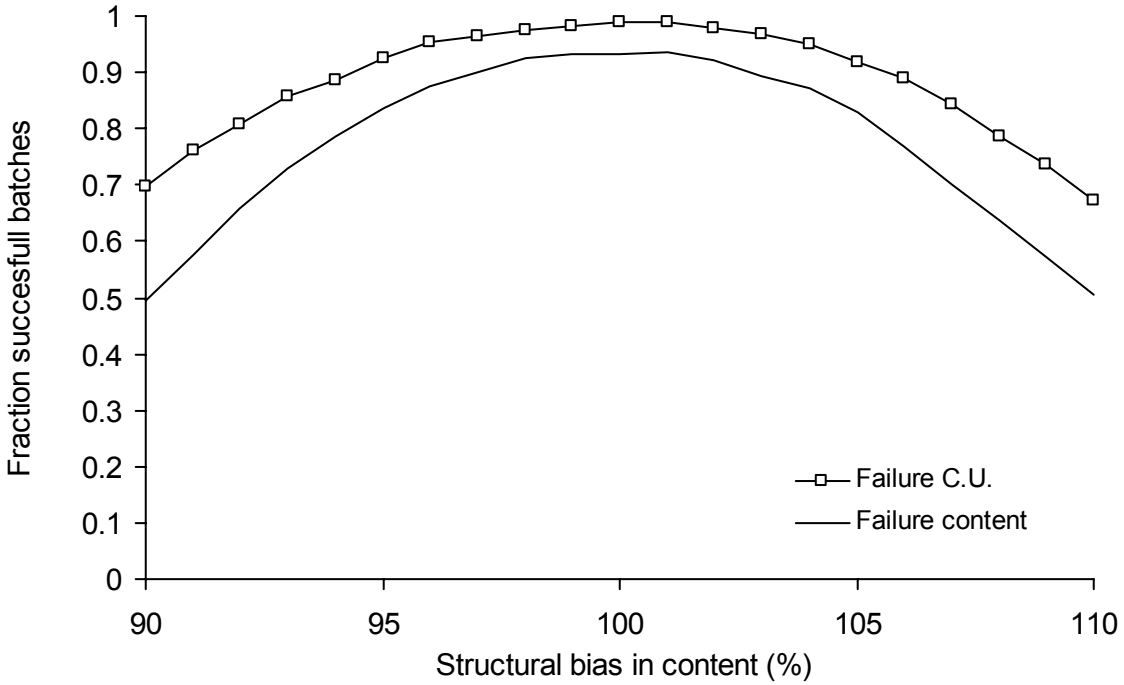


Figure V. Estimated fraction of successful batches as function of the overall bias in the worst case scenario.



Conclusion

This analysis showed that for our generic manufacturing process, variability in the filling process was marginal and of minor importance for product quality in terms of content and content uniformity although it can be influenced by production parameters like dissolution vehicle and filling volume. An overall content of 101% was found with batch-to-batch and vial-to-vial variability up to 4.21% and 2.57%, respectively. Estimation of the overall batch failure revealed that structural bias in content and a high batch-to-batch variability in content were the most prominent factors determining batch failure. Furthermore, content and not content uniformity was shown to be most important parameter influencing batch failure. Calculated Process Capability Indices (CpKs) were calculated for each product, which showed that our manufacturing process is capable of manufacturing products which will routinely comply with the specification of 90-110% for content. However, the CpK values decreased dramatically using the specification of 95-105% as required for approved drug products. These results indicate that at the early stages of product development, less

tight specification limits must be applied to prevent unnecessary batch rejection of investigational agents. Mixed effect analysis was shown to be a valuable tool in the assessment of the performance of our manufacturing procedures in this regard.

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Summary

Summary

Despite the progress already made in cancer research, cancer is still a major public health problem in western countries. For patients with advanced disease, chemotherapy is the best option. Therefore, more cancer research and the development of innovative anticancer agents are required. The development of anticancer agents for chemotherapy is a complex process and starts with the acquisition of anticancer drugs. Nature is still the primary source of anticancer drugs with compounds isolated from plants and micro-organisms. However, due to the increasing knowledge of tumor pathology and drug resistance mechanisms, rational development of anticancer agents (i.e. chemical synthesis of derivatives of natural compounds) increases dramatically.
In most cases, administration of the pure compound to patients is often hampered by poor aqueous solubility and stability characteristics of anticancer agents. Therefore, complex pharmaceutical formulations have to be developed before the anticancer agent can be administered to patients. Anticancer drug formulations for experimental use are generally intended for intravenous use to obtain absolute bio-availability, circumvent possible disturbance of or degradation in the GI-tract (nausea and vomiting) and to be able to adjust or stop administration of the drug in case of acute toxicity. Consequently, the development of a pharmaceutical formulation of a novel anticancer agent is focussed on issues associated with the design of sterile and stable injectable products.

This thesis describes the pharmaceutical development of two innovative anticancer agents EO-9 (chapter 1) and AP5346 (chapter 2). For EO-9, the emphasis is on the pharmaceutical development and stability of the compound. For AP5346, besides the pharmaceutical development, the structural and analytical characterization of this compound is discussed. Furthermore, issues regarding the manufacture of investigational anticancer agents for clinical trials are discussed in chapter 3.

EO-9

EO-9 is a bioreductive alkylating indoloquinone and an analogue of the antitumor antibiotic mitomycin C. EO-9 is an inactive prodrug, which is activated by reduction of the quinone moiety to semiquinone or hydroquinone, generating an intermediate with an electrophilic aziridine ring system, which serves as a target for nucleophilic DNA. EO-9 is used for the treatment of superficial bladder cancer.
Chapter 1.1 describes the pharmaceutical development of a bladder instillation of EO-9. To improve solubility and stability of EO-9, tert-butyl alcohol was chosen as co-

solvent for the solution vehicle in the freeze-drying process. Because EO-9 is most stable at alkaline pH sodium bicarbonate was used as alkalizer. Stability and dissolution studies revealed an optimal formulation solution for freeze-drying composed of 4 mg/ml EO-9, 10 mg/ml sodium bicarbonate ($NaHCO_3$), and 25 mg/ml mannitol in 40% v/v tert-butyl alcohol in water for injection. Optimization of the freeze drying process was performed by determination of the freeze drying characteristics of tert-butyl alcohol/water systems and differential scanning calorimetry analysis of the formulation solution. Furthermore, the influence of the freeze drying process on crystallinity and morphology of the freeze dried product was determined with X-ray diffraction analysis and scanning electron microscopy, respectively. Subsequently, a reconstitution solution was developed. A stable bladder instillation was obtained after reconstitution of freeze dried product containing 8 mg of EO-9 per vial to 20 ml with a reconstitution solution composed of propylene glycol/water for injection/$NaHCO_3$/sodium edetate 60/40/2/0.02% v/v/w/w, followed by dilution with water for injection to a final volume of 40ml. This pharmaceutical product of EO-9, named EOquin^{TM}, is currently used in Phase II clinical trials.

Chapter 1.2 describes the complexation and pharmaceutical formulation of EO-9 with the complexing agent 2-hydroxypropyl-β-cyclodextrin (HPβCD). Complexation was studied in aqueous solution and in solid freeze dried products. A phase solubility study, UV/VIS analysis and analysis of the effect of HPβCD on the stability of EO-9 were performed. With the phase solubility study a K1:1 of 32.9, a CE of 0.0457 and an U_{CD} of 38.3 were calculated. These K1:1 and CE values indicate a weak complex, but the U_{CD} shows that HPβCD can be very useful as solubilizer in the desired formulation. Furthermore, a positive effect of HPβCD on the chemical stability of EO-9 in solution was seen. Subsequently, complexation in the freeze dried products was studied more thoroughly using FTIR, DSC, XRD, and SEM analysis. HPβCD was found to be an excellent pharmaceutical complexing agent for application in formulations for EO-9 bladder instillations. Reconstitution prior to use of the developed freeze dried products can be simply accomplished with water for injection, making a separate reconstitution solution redundant.

Chapter 1.3 describes the characterization of the degradation products found in bladder instillations of EO-9 containing HPβCD in combination with the alkalizers $NaHCO_3$ or tri(hydroxymethyl)aminomethane (Tris). During the stability study of these freeze-dried products, formation of new degradation products was seen. We have characterized these products by using high performance liquid chromatography in combination with photodiode array detection and mass spectrometry. In total, five new degradation products were identified of which three were detected in both freeze-dried

products and two only in the freeze-dried product composed of EO-9/HPβCD /$NaHCO_3$.
Furthermore, the purity profile of two lots of EO-9 drug substance was investigated. Five, probably synthetic intermediates were found. However, the amount of total impurities was very small for both lots of drug substance and below acceptable international limits for pharmaceutical use.
Chapter 1.4 describes the stability study of freeze-dried products composed of EO-9, HPβCD, Tris, and $NaHCO_3$. These studies comprised stability of the freeze dried products, stability after reconstitution and dilution and stability during bladder instillation in an experimental set-up. The stability study of the freeze dried products showed that the formulation composed of EO-9/HPβCD/Tris (4/600/1 mg/vial) was most stable. After reconstitution and dilution all products were stable for at least 8 hours. The product composed of EO9/HPβCD/$NaHCO_3$ (4/600/20 mg/vial) was the least stable product both as freeze-dried formulation and after reconstitution and dilution.
The bladder instillation simulation experiment showed that all products were stable when mixed with urine of pH 8 and unstable in urine of pH 4 and 6. The degradation products formed in urine were EO-5a and EO-9-Cl. Based on these results, the product composed of EO-9/HPβCD/Tris (4/600/1 mg/vial) was selected for further pharmaceutical development.

AP5346

AP5346 is a diaminocyclohexane (DACH) platinum(II) compound bound to the low molecular weight polymer poly-N-(2-hydroxypropyl)-methacrylamide, a biocompatible, water-soluble macromolecule. AP5346 is used for the treatment of solid tumors, especially lung, head and neck, ovarian and testicular cancers.
Chapter 2.1 describes the characterization of the drug substance AP5346, and the development and quality control analysis of the pharmaceutical product. The identity of AP5346 was confirmed using ^{1}H NMR, ^{195}Pt NMR and IR spectroscopy. Furthermore, the free platinum content, platinum release characteristics, molecular size and size distribution were established. With the selected analytical techniques, AP5346 could be distinguished very well from its polymeric analogues, such as AP5280 and AP5279. The combination of all analytical techniques provides a complete picture of the physical and chemical characteristics of AP5346 drug substance as well as the pharmaceutical end-product. Specifications were set for the analytical results to guarantee a constant quality. Stability experiments revealed that AP5346 final product is stable for 12 months at 5°C, in the dark.

To investigate the influence of container materials, the stability of AP5346 after reconstitution and dilution in infusion containers was determined. AP5346 was shown to be stable after reconstitution and dilution with 5% w/v dextrose in infusion containers for at least 96 hours at 2-8°C in the dark and at room temperature with ambient light conditions. This lyophilized pharmaceutical product has completed a phase I clinical trial.

Manufacture

The first steps in the clinical development of investigational anticancer agents are the phase I and II clinical trials. It is evident that product quality is very significant at this stage of development as it may, besides patient safety, affect the outcomes of these studies and thus fate of the drug. Therefore, it is important to investigate the compatibility of the pharmaceutical formulation with the manufacturing materials and the performance of the manufacturing process.

Chapter 3.1 describes the compatibility of the excipient HPβCD with the platinum cured silicone tubing used during aseptic manufacturing. Cyclodextrins are capable to solubilize lipophilic drugs via (partial) inclusion in their lipophilic cavity. This, however, also provides the potential for the extraction of small molecules from production materials (e.g. 2-PP from silicone tubing). The presence of 2-PP in HPβCD solutions was structurally confirmed with HPLC-UV and LC/MS/MS after incubation with platinum cured silicone tubing. HPβCD concentration and prior tubing sterilization were found not to influence the levels of 2-PP extracted. Interestingly, extraction to ethanol was 15-fold higher than observed for HPβCD solutions.

2-PP was extracted from silicone tubing during routine manufacture of a blank dosage form formulated with only HPβCD, resulting in detectable levels of 2-PP in the final product. In a freeze-dried dosage form containing HPβCD and a drug substance (exhibiting a stability constant for HPβCD /drug of 1045 L/mole), on the other hand, 2-PP was undetectable. This indicates competition of the drug substance and 2-PP for complexation with HPβCD.

Chapter 3.2 describes the identification of the magnitude and sources of variability of a generic aseptic manufacturing process for investigational anticancer agents. Furthermore, the effects on product quality were estimated. This was obtained by retrospectively analysis of in-process and quality control data of all products manufactured according to this generic process over a 3-year period using mixed-effects analysis. This analysis showed that variability in the filling process was marginal and of minor importance for product quality in terms of content and content uniformity. An overall content of 101% was found with batch-to-batch and vial-to-vial variability up to 4.21% and 2.57%, respectively. Estimation of the overall batch failure

revealed that structural bias in Content and a high batch-to-batch variability in Content were the most prominent factors determining batch failure. Furthermore, Content and not Content Uniformity was shown to be most important parameter influencing batch failure. To measure the reproducibility of the manufacturing process, the Process Capability Index (CpK) was calculated for each product. The calculated CpK values showed that our manufacturing process is capable of manufacturing products which will routinely comply with the specification of 90-110% for content. However, the CpK values decreased dramatically using the specification of 95-105% for content as required for approved drug products, indicating that first less strict specification limits must be applied to prevent unnecessary batch rejection of investigational agents.

Samenvatting

Samenvatting

Ondanks de vooruitgang die bereikt is in de behandeling van kanker, zorgt deze ziekte in de westerse landen nog steeds voor een groot maatschappelijk gezondheidsprobleem. Voor patiënten met een vergevorderd stadium van kanker is chemotherapie tot op heden de beste behandelingsmethode. Meer onderzoek naar de behandeling van kanker en de ontwikkeling van nieuwe innovatieve geneesmiddelen tegen kanker is daarom nodig. De ontwikkeling van nieuwe geneesmiddelen tegen kanker is een ingewikkeld proces en begint met het verkrijgen/ontdekken van moleculen (verbindingen) die deze ziekte bestrijden. Deze moleculaire verbindingen vinden hun oorsprong in de natuur en worden geïsoleerd uit o.a. planten en micro-organismen. Door de toegenomen kennis over de pathologie van tumoren en mechanismen die ten grondslag liggen aan geneesmiddelenresistentie, worden steeds meer geneesmiddelen met behulp van "rational drug design" ontwikkeld (d.w.z. chemische synthese van derivaten van de moleculen afkomstig uit de natuur).
De verbindingen die momenteel gebruikt worden voor de behandeling van kanker zijn vaak instabiel en lossen meestal slecht op. Hierdoor is het vrijwel nooit mogelijk om deze verbindingen direct aan patiënten te verstrekken en moet men eerst complexe farmaceutische formuleringen ontwikkelen voordat de verbindingen als geneesmiddel kunnen worden toegediend. Nieuwe geneesmiddelen voor de behandeling van kanker worden meestal toegediend via een infuus. Op deze manier worden problemen met de biologische beschikbaarheid (d.w.z. verminderde absorptie vanuit de darmen naar de bloedbaan) vermeden. Bovendien wordt eventuele schade aan het maagdarmkanaal, veroorzaakt door het geneesmiddel, voorkomen. Een ander voordeel van deze manier van toedienen is dat deze direct gewijzigd of gestopt kan worden indien ernstige bijwerkingen optreden.

In dit proefschrift wordt de farmaceutische ontwikkeling van twee innovatieve geneesmiddelen tegen kanker, EO-9 (hoofdstuk 1) en AP5364 (hoofdstuk 2), beschreven. Bij de bespreking van EO-9 is de nadruk gelegd op de farmaceutische ontwikkeling en de stabiliteit van deze verbinding. Bij AP5346 wordt, naast de farmaceutische ontwikkeling, de karakterisering van de verbinding uitgebreid behandeld Van deze twee geneesmiddelen zijn tevens producten bereid voor fase 1 en 2 klinische trials. In hoofdstuk 3 worden de problemen rond het bereiden van nieuwe geneesmiddelen belicht.

EO-9

EO-9 behoort tot de bioreductieve alkylerende indolochinonen en is een analoog van het antitumor antibioticum mitomycine C. EO-9 is een inactieve verbinding die geactiveerd wordt door reductie van de quinone groep tot een semiquinone of hydroquinone. Hierbij ontstaat een tussenproduct met een electrofiel aziridine ringsysteem, waarop nucleofiel DNA aangrijpt. EO-9 wordt gebruikt voor de behandeling van kanker aan het oppervlak van de blaaswand.

Hoofdstuk 1.1 beschrijft de farmaceutische ontwikkeling van een blaasspoeling van EO-9. Om de oplosbaarheid en stabiliteit van EO-9 te verbeteren, is voorafgaand aan het vriesdrogen tert-butyl alcohol aan de formuleringsoplossing toegevoegd. Omdat EO-9 vooral stabiel is in een basisch milieu, is natrium bicarbonaat toegevoegd aan de formuleringsoplossing. Stabiliteits- en oplosbaarheidsstudies resulteerden in een optimale formuleringsoplossing bestaande uit 4 mg/ml EO-9, 10 mg/ml natrium bicarbonaat, en 25 mg/ml mannitol in 40% v/v tert-butyl alcohol in water voor injecties. Voor de optimalisatie van het vriesdroogproces zijn de vriesdroogkarakteristieken van tert-butyl alcohol/water systemen en "differential scanning calorimetry" analyse van de formuleringsoplossing bestudeerd. Daarnaast is ook nog de invloed van het vriesdroogproces op de kristallijnvorm en morfologie van het gevriesdroogde product onderzocht met respectievelijk röntgendiffractie analyse en "scanning electron microscopy". Vervolgens is een reconstitutieoplossing ontwikkeld om het gevriesdroogde product voor toediening geschikt te maken. Een stabiele blaasinstallatie is verkregen na reconstitutie van het gevriesdroogde product (8mg EO-9 per flacon) tot een volume van 20ml met een oplossing bestaande uit propyleen glycol/water voor injecties/natrium bicarbonaat/natrium edetaat 60/40/2/0.02% v/v/w/w, gevolgd door verdunning met water voor injecties tot een eindvolume van 40ml. Dit farmaceutische product van EO-9, EOquinTM, wordt momenteel in fase 2 klinische onderzoeken gebruikt.

Hoofdstuk 1.2 beschrijft de complexering en farmaceutische ontwikkeling van een formulering met EO-9 en 2-hydroxypropyl-β-cyclodextrin (HPβCD). Het complexeringsmechanisme tussen EO-9 en HPβCD is zowel in een waterig milieu als in gevriesdroogde producten onderzocht. Een oplosbaarheidstudie, UV/VIS analyse en analyse van de effecten van HPβCD op de stabiliteit van EO-9, zijn uitgevoerd. Uit de data van de oplosbaarheidstudie zijn een K1:1 van 32.9, een CE van 0.0457 en een UCD van 38.3 berekend. Deze K1:1 en CE waarden duiden op een zwak complex, maar de waarde van de UCD geeft aan dat HPβCD toch zeer nuttig kan zijn als een oplosbaarheidverhogende hulpstof in de gewenste formulering. Daarnaast is een positief effect van HPβCD op de stabiliteit van EO-9 geconstateerd. Vervolgens is de complexering nader bestudeerd met behulp van FTIR, DSC, XRD en SEM analyses.

Bovenvermelde studies toonden aan dat HPβCD een excellente oplosbaarheidverhogende hulpstof is voor toepassing in formuleringen voor blaasspoelingen met EO-9. Reconstitutie van het gevriesdroogde product kan eenvoudig worden uitgevoerd met water voor injecties.

Hoofdstuk 1.3 beschrijft de karakterisering van de ontledingsproducten in twee blaasspoelingen bestaande uit EO-9, HPβCD en één van de basen natrium bicarbonaat of tri(hydroxymethyl)aminomethane (Tris). Tijdens de stabiliteitsstudie van de gevriesdroogde producten is de vorming van nieuwe ontledingsproducten geconstateerd. Deze ontledingsproducten zijn gekarakteriseerd met behulp van de hoge druk vloeistof chromatografie (HPLC) in combinatie met "photodiode array (PDA)" detectie en massa spectrometrie (MS). In totaal zijn vijf nieuwe ontledingsproducten gekarakteriseerd in beide gevriesdroogde producten. Van deze vijf gekarakteriseerde ontledingsproducten zijn er drie terug te vinden in beide gevriesdroogde producten en twee alleen in het gevriesdroogde product bestaande uit EO-9/HPβCD/natrium bicarbonaat. Daarnaast is het zuiverheidprofiel van twee lots van EO-9 grondstof onderzocht. Vijf vermoedelijk synthetische tussenproducten zijn gevonden. Echter, de totale hoeveelheid aanwezige onzuiverheden is in beide lots erg klein en voldoet aan de internationale eisen voor farmaceutische toepassing.

Hoofdstuk 1.4 beschrijft de stabiliteitstudie van gevriesdroogde producten bestaande uit EO-9, HPβCD, Tris en natrium bicarbonaat. Tijdens deze studie zijn de stabiliteit van het gevriesdroogde product, van het gevriesdroogde product na reconstitutie en doorverdunnen (= blaasspoeling) en van de blaasspoeling in de blaas "in vitro" onderzocht. De resultaten van deze studie tonen aan dat het gevriesdroogde product bestaande uit EO-9/HPβCD/Tris (4/600/1 mg/vial) het meest stabiel is.. Alle producten blijken na reconstitutie en doorverdunnen voor minstens 8 uur stabiel. Het product bestaande uit EO-9/HPβCD/natrium bicarbonaat (4/600/20 mg/vial) is het minst stabiel, zowel als gevriesdroogd product als na reconstitutie en doorverdunnen. Uit de "in vitro"-studie blijkt dat alle blaasspoelingen stabiel zijn na mengen met urine pH 8 en instabiel na mengen met urine pH 4 en pH 6. EO-5a en EO-9-Cl zijn de enige gevormde ontledingsproducten. Op basis van deze resultaten, is het product bestaande uit EO-9/HPβCD/Tris (4/600/1 mg/vial) geselecteerd voor verdere farmaceutische ontwikkeling.

AP5346

AP5346 is een diaminocyclohexane (DACH) platinum(II) verbinding gebonden aan het laag moleculaire polymeer poly-N-(2-hydroxypropyl)-methacrylamide (pHPMA). Dit polymeer is biocompatibel en wateroplosbaar. AP5346 wordt gebruikt voor de

behandeling van vaste tumoren, vooral long-, hoofd- en hals-, eierstok en testikelkanker.
Hoofdstuk 2.1 beschrijft de karakterisering van de grondstof AP5346 en de ontwikkeling alsook de kwaliteitscontrole van het farmaceutische product. De identiteit van AP5346 is bevestigd met behulp van 1H NMR, 195Pt NMR en IR spectroscopie. Daarnaast zijn ook het gehalte aan vrij platinum, de platinum afgifte, de molecuul grootte en de spreiding in molecuul grootte bepaald. Met al deze technieken kan een goed onderscheid worden gemaakt tussen AP5346 en analogen, zoals AP5280 en AP5279. De combinatie van al deze analytische technieken zorgt voor een complete karakterisering van de fysische en chemische eigenschappen van de grondstof AP5346 en van het farmaceutische product. Om een constante kwaliteit van het farmaceutische product te kunnen garanderen, zijn specificaties vastgesteld. Stabiliteitsexperimenten tonen aan dat het farmaceutische product gedurende 12 maanden bij 5°C (in het donker) stabiel is. Vervolgens is de stabiliteit van het product na reconstitutie en verdunnen in verschillende infusiematerialen getest. AP5346 is stabiel na reconstitutie en verdunnen met 5% w/v dextrose in infusiecontainers voor minstens 96 uur bij 2-8°C in het donker en bij kamertemperatuur met normale licht/donker cycli. Met dit gevriesdroogde farmaceutische product zijn de fase 1 studies inmiddels afgerond.

Productie

De eerste stappen in de ontwikkeling van nieuwe geneesmiddelen voor de kliniek zijn de fase 1 en 2 klinische studies. De kwaliteit van deze nieuwe geneesmiddelen is in dit stadium van het onderzoek van belang omdat dit naast de veiligheid voor de patiënt, ook de resultaten van de klinische studies bepaalt, op basis waarvan besloten wordt of de ontwikkeling van het geneesmiddel wordt voortgezet. Een deel van de kwaliteit van het geneesmiddel is al bepaald in voorgaande formuleringsstudies. Echter, de kwaliteit van een geneesmiddel wordt niet alleen bepaald door de samenstelling, maar ook door het verloop van het productieproces. Daarom is het erg belangrijk de compatibiliteit van het product met de productiematerialen en de kwaliteit van het productieproces te onderzoeken.
Hoofdstuk 3.1 beschrijft de compatibiliteit van de hulpstof HPβCD met platina gecoate siliconenslangen die gebruikt worden tijdens de aseptische bereiding van geneesmiddelen. Cyclodextrines kunnen lipofiele geneesmiddelen in oplossing brengen door (gedeeltelijke) inclusie in hun lipofiele holten. Echter, de mogelijkheid bestaat dat naast geneesmiddelen ook lipofiele verbindingen uit productiematerialen (zoals 2-PP uit siliconenslangen) interactie aangaan met cyclodextrines en hierdoor in het product terecht komen. De aanwezigheid van 2-PP in oplossingen met HPβCD na incubatie met platina gecoate slangen, is bevestigd met behulp van HPLC-UV en

LC/MS/MS analyse. De HPβCD concentratie en sterilisatie van de slangen blijken geen invloed te hebben op de geëxtraheerde hoeveelheid 2-PP. Extractie van 2-PP met ethanol leverde 15x hogere 2-PP concentraties op dan met HPβCD-oplossingen. Verder blijkt dat 2-PP geëxtraheerd wordt uit silicone slangen tijdens een routineproductie met een blanco formuleringsoplossing die alleen HPβCD bevat. Echter, in aanwezigheid van een grondstof is geen 2-PP gedetecteerd. Dit duidt op competitie tussen 2-PP en de grondstof voor de holte van het cyclodextrinemolecuul.

Hoofdstuk 3.2 beschrijft de identificatie van de grootte en bronnen van variabiliteit van een algemeen aseptisch productieproces voor de bereiding van nieuwe geneesmiddelen tegen kanker. Daarnaast zijn ook de effecten van deze variabiliteit op de kwaliteit van het product bekeken. Dit is gedaan met behulp van retrospectieve analyse van de data van in-proces controles en kwaliteitscontroles van producten die geproduceerd waren volgens dit algemene bereidingsproces over een periode van 3 jaar. De retrospectieve analyse is uitgevoerd met "mixed effects analysis". Deze analyse toont aan dat de variabiliteit in het vulproces slechts marginaal en nauwelijks van belang is voor de kwaliteit van het product met betrekking tot het gehalte en de gehaltespreiding. Een gehalte van 101% is gevonden voor het algemene productieproces met een variabiliteit tussen batches tot 4.21% en variabiliteit tussen flacons tot 2.57%. Uit de schatting van de hoeveelheid batches die afgekeurd zullen worden door variabiliteit in het productieproces, blijkt dat een structurele afwijking in gehalte en variatie in gehalte tussen batches van één product, de twee belangrijkste factoren zijn die tot afkeuring leiden. Verder is gebleken dat het aantal af te keuren batches vooral bepaald wordt door afwijkingen in het gehalte en niet in de gehaltespreiding. De reproduceerbaarheid van het productieproces is bepaald door de "Proces Capability Index (CpK)" van alle producten te berekenen. Uit de berekende CpK waarden blijkt dat met dit productieproces producten kunnen worden gemaakt die routinematig voldoen aan de eis voor gehalte van 90-110%. Echter, een grote afname in CpK waarden is geconstateerd als de eis voor gehalte van 95-105% (eis voor geregistreerde producten) is genomen. Dit geeft aan dat aan nieuwe producten eerst ruimere eisen moeten worden gesteld om onnodige afkeuring van batches te voorkomen.

Curriculum vitae

Sabien van der Schoot werd geboren op 26 juli 1977 te ’s-Hertogenbosch. In 1995 behaalde zij haar VWO diploma aan het Rodenborch College te Rosmalen. Aansluitend werd begonnen aan de studie Farmacie aan de Universiteit Utrecht. Tijdens de doctoraalopleiding werd in het Universitair Medisch Centrum Utrecht (UMCU) bij de afdeling Klinische Chemie Research een scriptie geschreven over analysemethodes voor het meten van oxidatieve stress en onderzoek gedaan naar het meten van oxidatieve stress met behulp van parinaarzuur. In augustus 1999 werd het doctoraaldiploma behaald gevolgd door het apothekersdiploma in augustus 2001. Direct na haar studie is ze als tweede apotheker 8 maanden werkzaam geweest bij Apotheek Campagne te Geldermalsen. Aansluitend is ze in april 2002 begonnen als projectapotheker in het Slotervaart Ziekenhuis te Amsterdam. Na 6 maanden als projectapotheker werkzaam te zijn geweest, startte ze in dit ziekenhuis met het in dit proefschrift beschreven promotieonderzoek onder leiding van promotor prof. dr J.H. Beijnen en co-promotor dr B. Nuijen.

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