Development of a Bladder Instillation of the Indoloquinone Anticancer Agent EO-9 Using Tert-Butyl Alcohol as Lyophilization Vehicle

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ABSTRACT

The purpose of this research was to develop a stable bladder instillation of EO-9 for the treatment of superficial bladder cancer. First, stability and dissolution studies were performed. Subsequently, the freeze-drying process was optimized by determination of the freeze-drying characteristics of the selected cosolvent/water system 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. This study revealed that tert-butyl alcohol (TBA) can be used to both dramatically improve the solubility and stability of EO-9 and to shorten the freeze-drying cycle by increasing the sublimation rate. During freeze drying, 3 TBA crystals were found: TBA hydrate-ice crystals, crystals of TBA hydrate, and a third crystal, probably composed of TBA hydrate crystals containing ~90% to 95% TBA. Furthermore, it was shown that crystallization of TBA hydrate was inhibited in the presence of both sodium bicarbonate ($NaHCO_3$) and mannitol. Addition of an annealing step resulted in a minor increase in the crystallinity of the freeze-dried product and formation of the δ-polymorph of mannitol. A stable bladder instillation was obtained after reconstitution of the freeze-dried product (containing 8 mg of EO-9, 20 mg of $NaHCO_3$, and 50 mg of mannitol per vial) to 20 mL with a reconstitution solution composed of propylene glycol/water for injection (WfI)/$NaHCO_3$/sodium edetate 60%/40%/2%/0.02% vol/vol/wt/wt, followed by dilution with WfI to a final volume of 40 mL.

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INTRODUCTION

EO-9, 3-hydroxymethyl-5-aziridinyl-1-methyl-2-(1*H*-indole-4, 7-dione)-prop-β-en-α-ol (Figure 1), is a bioreductive alkylating indoloquinone and a synthetic analog of the antitumor antibiotic mitomycin C (MMC). The use of MMC is limited because of its dose-limiting toxicities. To improve the applicability of MMC, analogs such as EO-9 have been developed. EO-9 is an inactive prodrug, like MMC, that is activated by reduction of the quinone moiety to semiquinone or hydroquinone, generating an intermediate with an electrophilic aziridine ring system. This electrophilic ring system serves as a target for nucleophilic DNA. Reduction occurs in the presence of high levels of specific reducing enzymes (eg, nicotinamide adenine dinucleotide phosphate-oxidase (NADPH):cytochrome P450 reductase[1] and NADPH:quinone oxidoreductase[2]) or under conditions of low oxygen tension (hypoxic areas). This may explain why lack of bone marrow toxicity and preferential cytotoxicity of EO-9 toward hypoxic tumor cells were seen.[3] Since solid tumors are hypoxic because capillary growth cannot meet the oxygen needs of the growing tumors,[4] EO-9 is considered a promising agent for the treatment of these tumors.

However, formulation development of EO-9 was hampered by low solubility and low stability of EO-9 in neutral and acidic environments. The low stability is due to protonation of the aziridine moiety of EO-9 followed by nucleophilic attack of water and formation of EO-5a (Figure 2). Jonkman-de Vries et al developed a freeze-dried product of EO-9 for intravenous administration.[5] The drug substance was ground prior to dissolution to increase the dissolution rate. Subsequently, EO-9 and lactose were dissolved in water for injection (WfI). To increase the stability, the researchers added sodium hydroxide (NaOH) to bring the formulation solution to pH 9 to 9.5 and freeze-dried it. Aliquots of 40 mL were put into 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 was likely due to instability of EO-9 in a physiological

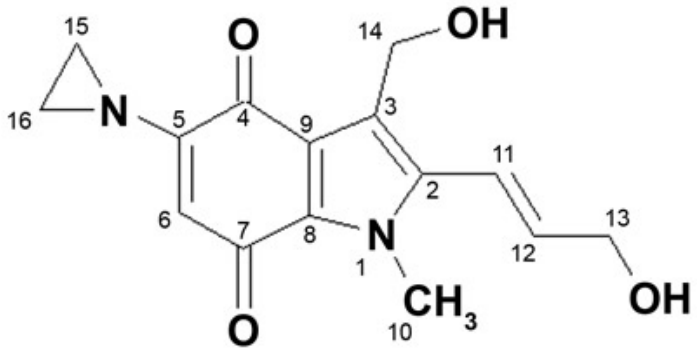

Figure 1. Molecular structure of EO-9 (molecular weight = 288 Da).

environment of pH 7.4 (ie, 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. The new formulation would need to avoid the disadvantages associated with intravenous administration of the formulation: instability of EO-9 after administration, grinding of a hazardous drug substance, and large filling volume per vial.

This article describes the development of a freeze-dried intravesical formulation of EO-9 using the organic solvent tert-butyl alcohol (TBA) in the manufacturing process. Furthermore, optimization of the freeze-drying program by determination of the freeze-drying characteristics of TBA/water systems and use of DSC analysis is described. Subsequently, the influence of the freeze-drying process on the crystallinity of the freeze-dried product was determined with x-ray diffraction (XRD) analysis, and a reconstitution solution was designed to obtain a stable bladder instillation.

Materials and Methods

Chemicals

EO-9 was supplied by Spectrum Pharmaceuticals, Inc (Irvine, CA). Propylene glycol (PG), mannitol, and sodium bicarbonate ($NaHCO_3$) (PhEur grade) were purchased from BUFA (Uitgeest, The Netherlands). TBA, NaOH, and dimethylsulfoxide (DMSO) were obtained from Merck (Darmstadt, Germany). Sterile WfI was purchased from B Braun (Melsungen, Germany). Phosphate buffer (5 mM) was prepared in-house at the Department of Pharmacy and Pharmacology of the Slotervaart Hospital (Amsterdam, The Netherlands). Methanol was purchased from Biosolve BV (Amsterdam, The Netherlands). All chemicals obtained were of analytical grade and used without further purification. For freeze drying, hydrolytic class 1 glass vials (8 mL, Fiolax-clear, Aluglas, Uithoorn, The Netherlands) and gray butyl rubber lyophilization stoppers (Type FM157/1, Helvoet Pharma NV, Alken, Belgium) were used.

High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) analysis was performed using an isocratic P1000 pump, an AS 3000 autosampler, and a UV 1000 UV/Vis detector, all from Thermo Separation Products (Breda, The Netherlands). The mobile phase consisted of 5 mM phosphate buffer pH 7/methanol 70%/30% wt/wt. A Zorbax SB-C18 analytical column (750 × 4.6 mm internal diameter (ID), particle size 3.5 µm, Agilent Technologies, Palo Alto, CA) preceded by a guard column (reversed phase 10 × 3 mm, Varian, Palo Alto) was used. Detection was performed at 270 nm. An injection volume of 10 µL, a flow rate of 0.7 mL/min, and a run time of 10 minutes were used.

Gas Chromatography

Traces of residual TBA in freeze-dried products were identified by gas chromatography (GC) analysis. The GC system was composed of a Model 5890 gas chromatograph equipped with a flame ionization detector, a split-splitless injector, and a Model 6890 series autosampler (Hewlett-Packard, Amstelveen, The Netherlands). Separation was achieved with a Crossbond 6% cyanopropylphenyl–94% dimethylpolysiloxane (30 m × 0.53 mm ID × 3.0 µm film thickness) column. Calibration solutions of 30 to 500 µg/mL TBA in DMSO were used.

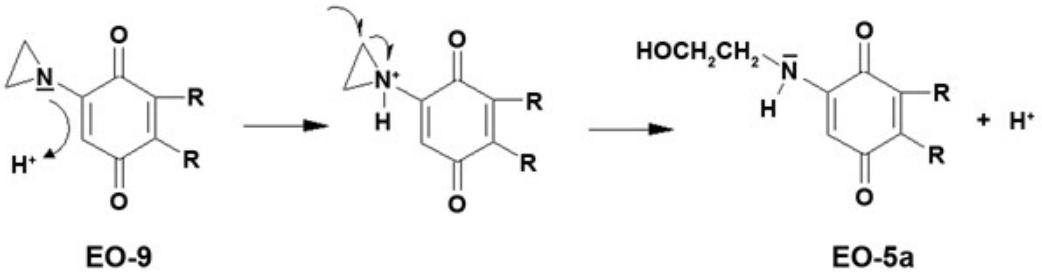

Figure 2. Acid degradation pathway of EO-9.

Pharmaceutical Formulation Development

Solubility and Stability of EO-9

Solutions of 0%, 10%, 20%, 30%, 40%, and 50% vol/vol TBA in WfI containing 1% wt/vol $NaHCO_3$ were saturated with EO-9. The solutions were shaken for 24 hours at room temperature and ambient light. Samples were taken after 6 and 24 hours and filtered using Millex HV filters (0.45 μm × 4 mm, Millipore, Etten-Leur, The Netherlands). The content and purity of the filtrates were determined with HPLC-UV.

Furthermore, the stability of EO-9 in freeze-dried products was determined. These freeze-dried products contained EO-9, a bulking agent, and an alkalizing agent. The bulking agents mannitol (crystalline bulking agent) and polyvinylpyrrolidone (PVP, an amorphous bulking agent) and the alkalizing agents $NaHCO_3$, NaOH, and meglumine were tested. For each solution, the bulking agent, the alkalizing agent, and EO-9 were dissolved in 40% vol/vol TBA to concentrations of 25 mg/mL, 10 mg/mL, and 4 mg/mL, respectively. NaOH was added to pH 8.5. Hydrolytic class I glass vials (8 mL) 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°C ± 3°C/ambient relative humidity (RH), 25°C ± 2°C/60% ± 5% RH, and 40°C ± 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 most stable formulation was selected for further development.

Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) was used to characterize the formulation solution composed of EO-9 4 mg/mL, mannitol 25 mg/mL, and $NaHCO_3$ 10 mg/mL in 40% vol/vol 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 for low temperature in the T4P mode (TA Instruments, New Castle, DE). Temperature scale and heat flow were calibrated with indium. Samples of ~10 mg were transferred into aluminum 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 at 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 $NaHCO_3$ 10 mg/mL in 40% vol/vol TBA, was filtered through a 0.2-μm filter (Midisart 2000, Sartorius, Goettingen, Germany). Aliquots of 2.0 mL were put into 8-mL colorless hydrolytic class 1 glass vials, partly closed with rubber lyophilization stoppers, and loaded into the freeze dryer (Model Lyovac GT4, GEA Lyophil GmbH, Hürth, Germany). Three test batches were prepared. Vials of the first batch were frozen to –43ºC in 1 hour at ambient pressure. The temperature and pressure were maintained for 2 hours. Subsequently, primary drying was performed at a chamber pressure of 0.20 mbar and a shelf temperature of –43ºC for 45 hours, followed by an increase in temperature to 25ºC in 15 hours for secondary drying. The chamber pressure was maintained at 0.20 mbar. These conditions were held constant for another 3 hours. The settings of secondary drying were changed for the next 2 batches into a shelf temperature of 30ºC and a chamber pressure of 0.05 mbar. All vials were closed pneumatically under vacuum and stored at 5ºC ± 3ºC and 25ºC ± 2ºC/60% ± 5% RH, in the dark. Analysis was performed after 3, 6, 12, and 18 months of storage with HPLC-UV analysis.

Reconstitution and Dilution

Solutions containing various amounts of PG in WfI were tested as reconstitution solutions for the freeze-dried product. $NaHCO_3$ (2% wt/vol) was added as alkalizing agent and sodium edetate (0.02% wt/vol) as chelating agent. Subsequently, the solubility and stability of EO-9 after reconstitution and dilution of the freeze-dried product at ambient temperature and light were tested. To check whether all EO-9 was dissolved after reconstitution and dilution, HPLC analysis of the reconstituted product before and after filtration using Millex HV filters (0.45 μm × 4 mm) was performed.

Optimization of the Freeze-Drying Program

After DSC analysis, the influence of shelf temperature on product temperature was determined. Vials filled with 2 mL of the EO-9 formulation solution composed of EO-9 4 mg/mL, mannitol 25 mg/mL, and $NaHCO_3$ 10 mg/mL in 40% vol/vol TBA were freeze-dried using different shelf temperatures. The product temperature was continuously recorded during freeze drying using platinum electrodes.

Furthermore, the freeze-drying characteristics of TBA/WfI solutions were studied to optimize the primary drying step for the formulation solution. Mixtures of 0%, 5%, 7.5%, 10%, 15%, 20%, 30%, and 40% vol/vol of TBA in WfI were

prepared. Hydrolytic class 1 glass vials were filled with aliquots of 2 mL and partly closed with rubber lyophilization stoppers. Per time point, 10 vials of each solution were loaded at random on 1 shelf of the freeze dryer at ambient temperature. The vials were frozen to –35ºC in 1 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 after 1, 1.5, 2, 4, 5, and 6 hours of primary drying. 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 (Abbe refractometer 302, Atago, Tokyo, Japan) using a calibration curve of 1%, 5%, 10%, 20%, 30%, and 40% vol/vol TBA in WfI. The density and amount (g) of solution in the vials were used to calculate the total volume in the vials.

The secondary drying phase was optimized after optimization of the primary drying phase. Batches composed of 25 vials containing 2 mL of EO-9 formulation solution were frozen 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. During secondary drying different shelf temperatures and chamber pressures were selected. Vials were closed at different time points during secondary drying with a maximum of 24 hours. At each time point, residual moisture content and residual TBA content were determined in duplicate using Karl Fischer titration and GC 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

Scanning electron microscopy (SEM) was performed on an 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 of a platinum/palladium mixture. Freeze-dried products before and after optimization of the freeze-drying program were analyzed. Furthermore, a sample of untreated EO-9 was analyzed as a reference.

XRD

XRD of the freeze-dried product, the EO-9, and the excipients was performed using a model PW 3710 PC-APD diffractometer (Philips, Eindhoven, The Netherlands) at atmospheric humidity in the angular range of 5 to 40° (2 theta). The CuKalpha radiation from the anode operating at 40 kV and 50 mA was monochromized using a 15-µm Ni foil. Scan step size was 0.02° (2 theta), and step time was 0.05 to 5.0 s.

Results and Discussion

HPLC and GC Analysis

The calibration curve of the HPLC analysis showed a linear relationship with a correlation coefficient > 0.999. The retention time of EO-9 was ~8.2 minutes. The method was shown 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.

GC analysis showed a sharp signal of TBA at 2 minutes and a large signal of DMSO 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 of EO-9

The maximum solubility, solubility rate, and stability of EO-9 in aqueous solutions were the main items to be improved, based on what the problems with the formulation for intravenous administration had been.[5] Organic solvents have positive effects on these 3 items.[9] TBA is one of the organic solvents that has been used often in freeze drying.[9-15] The benefits of TBA are shorter freeze-drying times and significantly higher specific surface areas of freeze-dried products.[14] TBA increased the maximum solubility of EO-9 significantly. The maximum solubility of EO-9 was 4.4, 6.0, and 8.1 mg/mL for solutions containing 30%, 40%, and 50% vol/vol TBA/WfI, respectively, vs 0.5 mg/mL in WfI alone. As EO-9 is most stable at a pH of around 9, $NaHCO_3$ was added to all solutions.[16] Further stabilization of EO-9 by TBA was shown in concentrations of 20% vol/vol TBA or more.

The dosing range for phase I clinical study was expected to be 0.5 to 32 mg EO-9 per 40 mL of intravesical instillation. A target content of 8 mg per vial was selected for the pharmaceutical product designated for the phase I clinical trial. As a 4 mg/mL EO-9 solution was readily obtained using a 40% vol/vol TBA vehicle without grinding, this concentration was selected and resulted in a 20-fold reduction of filling volume compared with the original formulation.[5]

Three alkalizing agents (NaOH, meglumine, $NaHCO_3$) were tested. Mannitol and PVP were selected as bulking agents. Rapid degradation of EO-9 was seen in both formulations containing meglumine after storage for 1 week at 40ºC/75% RH (Table 1). The formulations containing $NaHCO_3$ were the most stable. Furthermore, it was shown that addition of $NaHCO_3$ to MMC bladder instillations increased the time to recurrence of patients with superficial bladder cancer.[17] Additional stability experiments showed that the freeze-dried product composed of 8 mg EO-9, 50 mg mannitol, and 20 mg $NaHCO_3$ per vial was the most stable

Table 1. Stability of EO-9 in 6 Freeze-Dried Products*

		1 Week		2 Weeks		4 Weeks		8 Weeks	
Product†	Storage Condition‡	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	103.20	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	101.70	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			100.30	100.10	100.10	98.98	96.50	99.99
	5°C					99.12	100.20	102.40	100.20
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					101.40	99.85	101.80	99.73
5	40°C/75% RH	80.94	98.30						
	25°C/60% RH								
	5°C					not			
6	40°C/75% RH	24.93	90.28			analyzed			
	25°C/60% RH								
	5°C								

*The content and purity are calculated as a percentage of the initial values. RH indicates relative humidity; NaOH, sodium hydroxide; qs, quantum sufficit; PVP, polyvinylpyrrolidone; $NaHCO_3$, sodium bicarbonate.

†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.

(Table 2). A concentration of 4 mg EO-9/mL was readily obtained in 40% vol/vol TBA. Therefore, this formulation was chosen for further development.

DSC

DSC analysis showed a recrystallization endotherm at –20°C during reheating of 40% vol/vol TBA. This incomplete crystallization of TBA has been reported earlier.[12,18] Complete crystallization of TBA during the freeze-drying process, however, is considered more beneficial as it reveals a porous cake structure resulting in decreased cake resistance, increased sublimation rate, and decreased freeze-drying time. Furthermore, a porous cake structure facilitates reconstitution of the pharmaceutical product. We obtained complete crystallization of TBA with incorporation of an isothermal step at –20°C into the freeze-drying program. All thermograms of the solutions containing 40% vol/vol TBA (corresponding to 34% wt/wt TBA) showed 3 endothermic signals with onsets at approximately –14°C, –8°C, and –3ºC (Figure 3). The endotherms at –14°C and –8°C are due to melting of the eutectic of TBA hydrate-ice at 20% wt/wt TBA and the melting of TBA hydrate, respectively.[11] A less sharp onset of the endotherm at –14ºC, probably indicating inhibition of TBA crystallization,[13] was seen only in the presence of both mannitol and $NaHCO_3$ (Figure 3). Inhibition of TBA crystallization is probably due to the presence of amorphous

Table 2. Stability of EO-9/Mannitol/$NaHCO_3$ Freeze-Dried Product With and Without PVP at 40ºC/75% RH*

	1 Week		4 Weeks		8 Weeks		12 Weeks	
Product†	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 a percentage of the initial values. $NaHCO_3$ indicates sodium bicarbonate; PVP, polyvinylpyrrolidone; RH, relative humidity.

†Composition of the freeze-dried products: (I) EO-9 8 mg, mannitol 50 mg, $NaHCO_3$ 20 mg; (II) EO-9 8 mg, mannitol 100 mg, $NaHCO_3$ 20 mg; (III) EO-9 8 mg, mannitol 50 mg, PVP 50 mg, $NaHCO_3$ 20 mg.

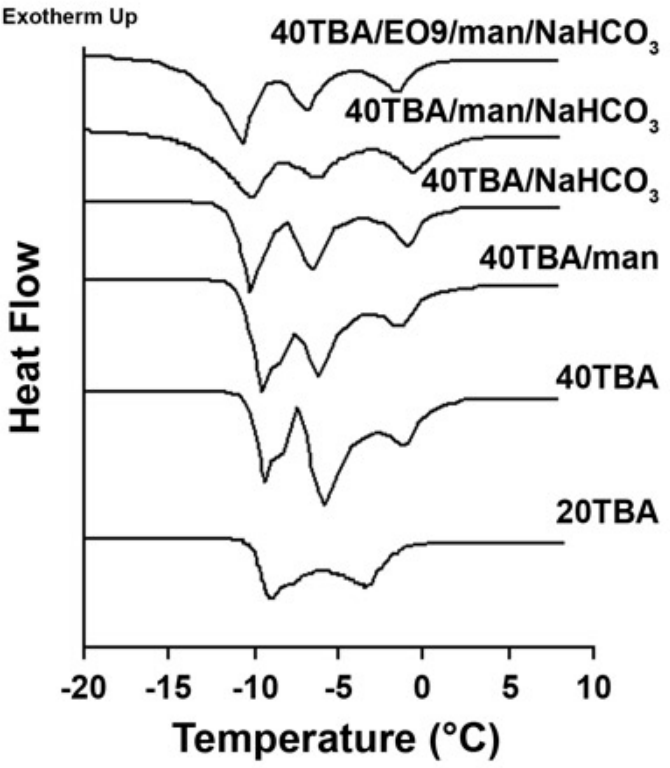

Figure 3. Differential scanning calorimetry thermal analysis. 40TBA = 40% vol/vol tert-butyl alcohol; man = mannitol; $NaHCO_3$, sodium bicarbonate; 20TBA, 20% vol/vol tert-butyl alcohol.

mannitol.[12,18] Normally, mannitol crystallizes easily, but our results showed that crystallization of mannitol is inhibited by $NaHCO_3$ (Figure 4). This finding, also seen by Telang et al,[19] explains the less complete crystallization of TBA. Inhibition of mannitol crystallization during freeze drying may be favorable to the pharmaceutical product, because during storage crystalline mannitol may release water, which can reduce product stability.[20] According to the phase diagram of Kasraian et al,[11] the –3°C endotherm is due to the presence of TBA/TBA hydrate crystals containing 90% to 95% TBA. With cryoscopy, pure TBA crystals were found in liquid 40% vol/vol TBA/WfI solution, probably resulting in TBA/TBA hydrate crystal formation during freeze drying.

Based on these results, an isothermal step at –20ºC was added to the freeze-drying program to obtain complete crystallization of TBA. Furthermore, we decided to keep the product temperature below –20ºC during primary drying to obtain a homogeneous freeze-dried product.

Manufacture and Stability

To prevent scarring and inflammation of bladder tissue by TBA[21] and to enhance the shelf life of the product, freeze drying was chosen for the removal of TBA and WfI. Lowering the chamber pressure and increasing the shelf temperature during secondary drying decreased the moisture levels in the freeze-dried product (Table 3). All batches were stable for at least 18 months at 5ºC (data not shown), but at 25ºC/60% RH, the purity of the batch with high moisture content decreased more rapidly, indicating that the EO-9 freeze-dried product is susceptible to moisture.

Reconstitution and Dilution

The EO-9 final product exhibits poor solubility and stability in aqueous solutions (eg, 0.9% wt/vol sodium chloride, 5% wt/vol glucose). Therefore, a reconstitution solution with improved solubility and stability in these solutions had to be developed. PG was found to be a suitable cosolvent, with a maximal solubility of EO-9 of 3.8 mg/mL in a 60% vol/vol aqueous solution. More $NaHCO_3$ was added to increase stability and ensure equal dosing of $NaHCO_3$ during the phase I dose-escalating study. Sodium edetate was added as a chelating agent for complexation of metal ions probably extracted from the walls of the glass vials because of high pH. Clear and stable instillations at a pH of around 9 were obtained using a reconstitution solution composed of PG/WfI/$NaHCO_3$/sodium edetate of 60%/40%/2%/0.02% vol/vol/wt/wt. The required dose of EO-9 was dissolved in 20 mL of this solution and then diluted to 40 mL with WfI. EO-9 tended to be less stable in the solution of 0.5 mg/40 mL (the lowest dose for phase I) than it was in the 32 mg/40 mL solution (the highest dose for phase I), but in both cases the purity was greater than or equal to 98.5% 6 hours after reconstitution and dilution, which was considered acceptable for clinical practice.

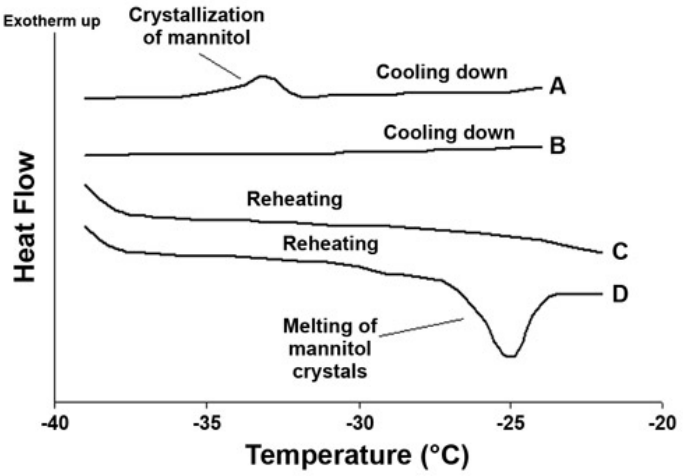

Figure 4. Differential scanning calorimetry thermograms of solutions containing 40% vol/vol tert-butyl alcohol and 2.5% wt/vol mannitol with (B and C) and without (A and D) 1% wt/vol sodium bicarbonate.

Table 3. Results of the Quality Control of the First 3 Batches of EO-9 Freeze-Dried Product (8 mg EO-9 per Vial)*

Test Item	Batch 1†	Batch 2‡	Batch 3‡
Reconstitution	No visible residues after reconstitution, with reconstitution solution resulting in clear, purple solutions	No visible residues after reconstitution, with reconstitution solution resulting in clear, purple solutions	No visible residues after reconstitution, with reconstitution solution resulting in 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

*EU indicates endotoxin units.

†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.

Optimization of the Freeze-Drying Program

Optimization was performed to minimize the residual moisture and TBA content and to prevent long and expensive freeze-drying cycles. During primary drying the product temperature (T_p) needed to be kept below –20ºC to prevent thermal events during sublimation (see DSC results). This was obtained with a shelf temperature of –10ºC and a chamber pressure of 0.20 mbar.

A linear correlation (R^2 = 0.998) was found between the TBA content (expressed as % vol/vol) and the refractive index of TBA/WfI solutions. This correlation was used to determine 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 (% vol/vol) of TBA/WfI solutions (R^2 = 0.981).

Figure 5 shows a positive effect of both primary drying time and TBA content on the sublimation process. A positive effect of TBA on the sublimation rate of TBA/WfI solutions was reported earlier by Kasraian et al.[14] 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 in the vials, we calculated the sublimation rates as fractions sublimed of the initial contents of WfI and TBA. Results of the subtraction of the fraction of sublimed WfI from the fraction of sublimed TBA of TBA/WfI solutions during primary drying are depicted in Figure 6. 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% vol/vol TBA no correlation was seen between the differences in fractions sublimed and TBA content after 1 hour of primary drying. Increasing the primary drying time resulted in a more uniform sublimation.

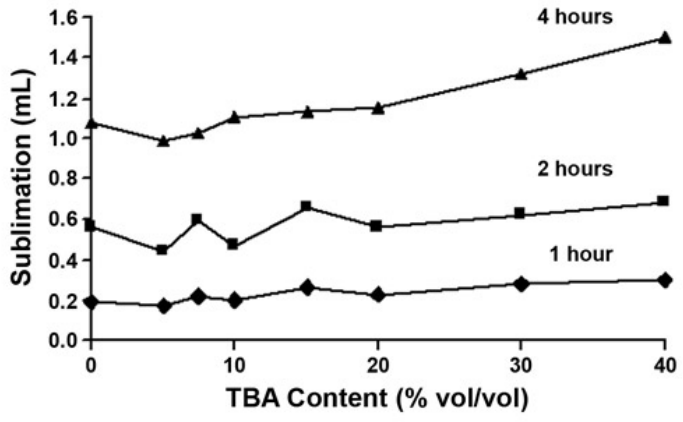

Figure 5. Sublimation of solutions containing 0% to 40% vol/vol tert-butyl alcohol after 1 to 4 hours of primary drying.

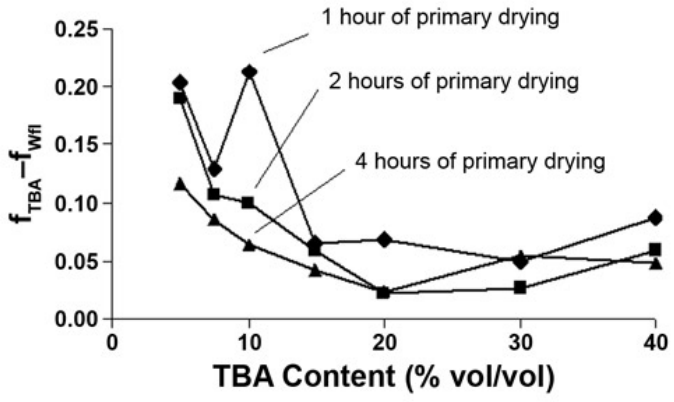

Figure 6. Fraction of TBA sublimed (f_{TBA}) minus fraction of WfI sublimed (f_{WfI}) of solutions containing 0% to 40% vol/vol TBA. TBA indicates tert-butyl alcohol; WfI, water for injection.

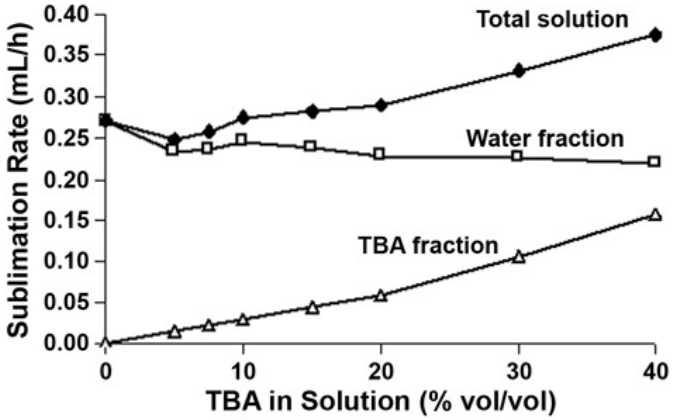

Figure 7. Sublimation rate of solutions containing 0% to 40% vol/vol TBA. TBA indicates tert-butyl alcohol.

Furthermore, the sublimation rates of the total solution, the TBA fraction, and the 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 7). 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 the TBA content was seen for solutions containing 0% to 10% vol/vol TBA. However, a slightly positive effect of TBA on the sublimation rate was seen for solutions with a TBA content of 10% to 20% vol/vol, and a clearly positive effect was seen for solutions containing more than 20% vol/vol TBA. This indicates that a positive effect of TBA on sublimation during primary drying can be obtained only with TBA concentrations higher than 10% vol/vol.

Because 40% vol/vol TBA was selected as the vehicle for the formulation solution, the sublimation of this solution was studied more thoroughly. The sublimation of the TBA fraction, the WfI fraction, and the total solution during primary drying was determined. The results show that WfI sublimes somewhat faster than TBA (Figure 8). Almost all WfI and TBA sublimed in 6 hours of primary drying. To compensate for a probable minor decrease in sublimation rate because of 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% vol/vol TBA.

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% wt/wt was found. Further increases of the shelf temperature to 35ºC and 45ºC resulted in moisture contents of 4.59% ± 0.76% wt/wt and 3.64% ± 0.38% wt/wt, respectively. This effect of chamber pressure and temperature on the residual moisture content was described earlier.[22] The TBA content was not affected by chamber pressure or shelf temperature and remained ~0.6% wt/wt, corresponding to 0.47 mg TBA per vial containing 8 mg EO-9. TBA is a class 3 organic solvent, according to the International Conference on Harmonization guideline Q3C.[23] 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 the phase I clinical trial was 0.5 to 32 mg EO-9 per instillation, corresponding to 0.03 to 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 a 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, so 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 was equal to the moisture content of batches 2 and 3, manufactured before the optimization of the freeze-drying time, indicating that lowering the moisture content to below 3.5% wt/wt is difficult. Stability results of the freeze-dried product showed that batches 2 and 3, containing 3.9% and 3.5% wt/wt moisture, respectively, were stable for at least 18 months at 5ºC, so 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 the length of the freeze-drying cycle dropped from 66 to 35 hours.

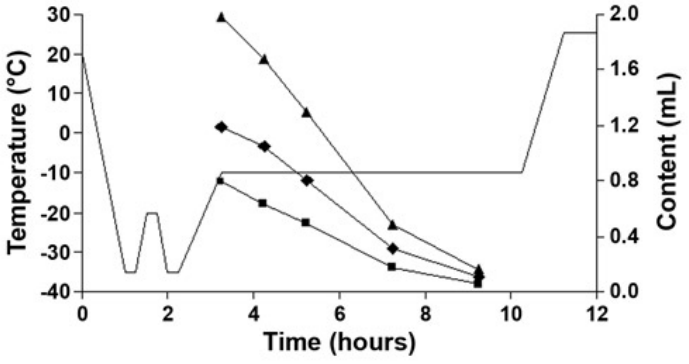

Figure 8. TBA (■), water for injection (◆), and total (▲) content (right Y-axis) of a 40% vol/vol TBA solution remaining in the vials as a function of the freeze-drying program (left Y-axis). TBA indicates tert-butyl alcohol.

SEM

The results of the SEM analysis are depicted in Figure 9. In Figure 9A, before freeze-drying, EO-9 appears as small, irregularly formed crystals. 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. The freeze-dried product before optimization (Figure 9B) clearly shows the presence of crystals. It is assumed that these are $NaHCO_3$ crystals formed because of supersaturation resulting in precipitation and crystallization of $NaHCO_3$ during freeze drying. A more homogeneous product was obtained with the optimized freeze-drying program (Figure 9C). More complete crystallization of TBA probably inhibits $NaHCO_3$ crystal formation.

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

XRD

The freeze-drying process that we developed 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, 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.

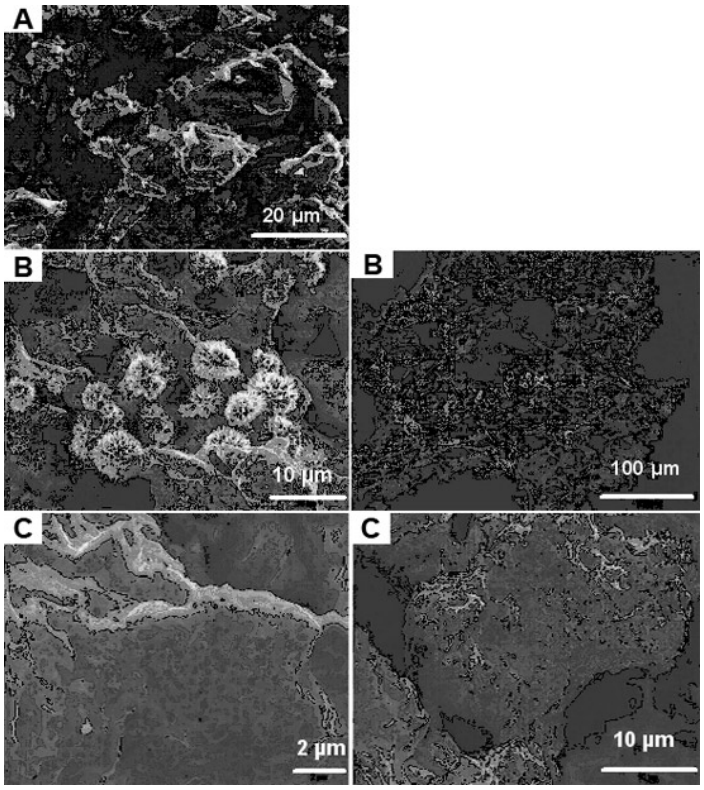

Figure 9. Scanning electron micrographs of EO-9 (A) and of EO-9 freeze-dried product before (B) and after (C) optimization of the freeze-drying program.

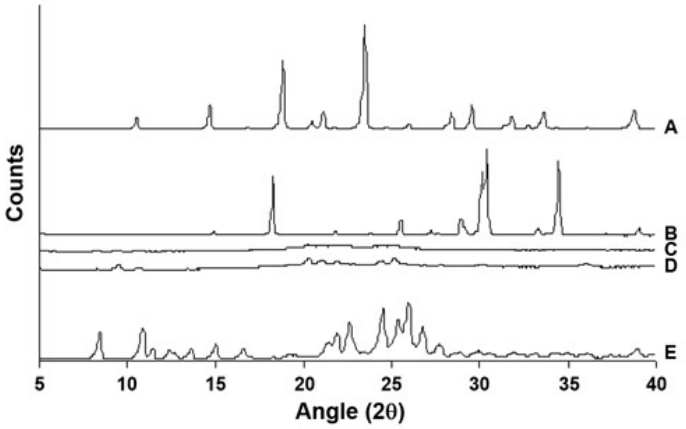

Figure 10. X-ray diffractometry spectra of mannitol (A), sodium bicarbonate (B), EO-9 freeze-dried without annealing (C), EO-9 freeze-dried with annealing at –20°C (D), and EO-9 (E).

Analysis was performed on EO-9, 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 initial freeze-drying program). The XRD diffractograms (Figure 10) clearly show that mannitol, $NaHCO_3$, 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 ~9.5, is not present in any other sample. This was also found by Telang et al.[24] They assigned this signal to the δ-polymorph structure of mannitol, formed in the presence of sodium chloride. Probably, this polymorph is also formed in the presence of $NaHCO_3$ when crystallization is more complete because of annealing. Since just a few small signals are present in the XRD spectrum of the final product after annealing, it was not expected that annealing would prolong 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).

CONCLUSION

This study revealed that a stable bladder instillation of EO-9 can be obtained by freeze drying from an alkaline TBA/WfI solution followed by reconstitution with a solution composed of PG/WfI/$NaHCO_3$/sodium edetate 60%/40%/2%/

0.02% vol/vol/wt/wt. Phase II studies using EO-9 lyophilized products and reconstitution solution are currently ongoing.

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