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Complexation Study of the Anticancer Agent EO-9 with 2-hydroxypropyl-β-Cyclodextrin

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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-visible spectroscopy (UV/VIS), and analysis of the effect of HPβD on the stability of EO-9 were performed. With the phase solubility study, a complexation constant (K1:1) of 32.9, a complexation efficiency (CE) of 0.0457, and a utility number (UCD) of 38.3 were calculated. These K1:1 and CE values indicate a weak complex, but the UCD 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 Fourier transform infrared (FTIR), differential scanning calorimetry (DSC), X-ray diffraction (XRD), and scanning electron microscopy (SEM) analyses. HPβCD was found to be an excellent pharmaceutical complexing agent for application in formulations for EO-9 bladder instillations. Reconstitution before use of the developed freeze-dried products can be simply accomplished with water for injection.

Keywords complexation; cyclodextrin; EO-9

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INTRODUCTION

EO-9, 3-hydroxymethyl-5-aziridinyl-1-methyl-2-(1*H*-indole-4,7-dione)-prop-ß-en-α-ol (Figure 1), is a bioreductive alkylating indoloquinone and a synthetic analog of the antitumor antibiotic mitomycin C (Beijnen, Den Hartigh & Underberg, 1985; Hendriks et al., 1993; McKeown, Cowen, Williams, 2007). For the treatment of superficial bladder cancer, a formulation of EO-9 for intravesical instillation was developed (van der Schoot, Nuijen et al., 2007). However, this formulation is a freeze-dried product that 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 (Rowe, Owen, & Sheskey, 2006). 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 (Arias et al., 2000; Bandi, Wei, Roberts, Kotra, & Kompella, 2004; Echezarreta-Lopez, Torres-Labandeira, Castineiras-Seijo, Santana-Penin, & Vila-Jato, 2000; Gibaud, Zirar, Mutzenhardt, Fries, & Astier, 2005; Loftsson, Hreinsdottir, & Masson, 2005; Pralhad & Rajendrakumar, 2004; Ribeiro, Ferreira, & Veiga, 2003; Ruan, Yu, Fu, & Zhu, 2005; Veiga, Fernandes, & Maincent, 2001; Wen, Tan, Jing, & Liu, 2004; Zheng, Haworth, Zuo, Chow, Chow, 2005; Zingone & Rubessa, 2005). 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 (Uekama, Hirayama, & Irie, 1998). The natural

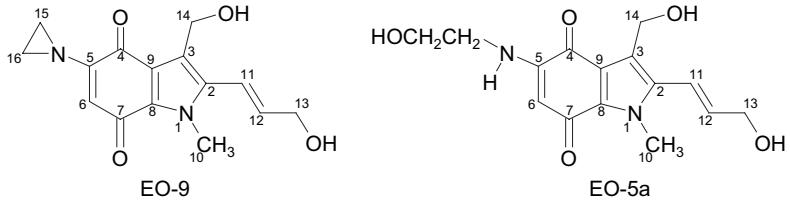

FIGURE 1. Molecular structure of EO-9 and its main degradation product EO-5a.

cyclodextrins (α-, β-, γ-cyclodextrins) have a poor solubility in both water and organic solvents, but their most critical drawback is irreversible renal toxicity owing to crystallization of cholesterol–cyclodextrin complexes in the kidneys (Frijlink et al., 1991; Szente & Szejtli, 1999; Uekama et al., 1998). 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 (Uekama et al., 1998). It was shown that HPβCD has a very low toxicity when administered through parenteral route and that any toxic effects on the kidney are reversible (Gould & Scott, 2005; Irie & Uekama, 1997). This improvement was due to a higher solubility of HPβCD–cholesterol complexes, resulting in a decrease in crystallization of those complexes in the kidneys (Frijlink et al., 1991). 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 (van der Schoot, Vainchtein et al., 2007).

This article describes the complexation study of EO-9 with HPβCD intended as alternative formulation for intravesical administration of EO-9.

MATERIALS AND METHODS

Materials

EO-9 drug substance (MW = 288 Da) was supplied by Spectrum Pharmaceuticals, Inc. (Irvine, CA, United States). HPβCD (MW = 1399 Da, degree of substitution MS_7 = 4.55) 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

High-performance liquid chromatography (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 5 mM phosphate buffer pH 7/methanol 70/30% (wt/wt). A Zorbax SB-C18 analytical column (750 × 4.6 mm ID, particle size 3.5 μm; Agilent Technologies, Palo Alto, CA, USA) preceded by a guard column (reversed phase 10 × 3mm, Varian, Palo Alto, CA, 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 min were used. Chromatograms were processed using Chromeleon software (Dionex Corporation, Sunnyvale, CA, USA).

High-Performance Liquid Chromatography with Photodiode-Array Detection

Samples were analyzed with HPLC using a system composed of a HP1100 Series binary HPLC pump and degasser (Agilent Technologies) and a Model Spectra SERIES 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 × 2.0 mm ID, particle size of 4.0 μm; Phenomenex, Torrance, CA, USA). The mobile phase consisted of ammonium hydroxide (pH 8.5; 1 mM) in water and methanol, pumped at a flow rate of 0.2 mL/min. The gradient started with 5% methanol and 95% 1 mM 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 1 min and the column was stabilized for 4 min, resulting in a total run time of 50 min. 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).

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 Connors (Higuchi & Connors, 1965). 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 h and subsequently shaken for 17 h at room temperature and ambient light. Next, samples were filtered using a hydrophilic Minisart 0.45-μm filter (Millipore, Etten Leur, The Netherlands). Subsequently, the EO-9 content of the filtrates was 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 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 fivefold 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 h 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), and HPβCD/Tris (100/0.5 mg/mL) in 20% (vol/vol) TBA were sonicated for 2 h and mixed shortly to obtain clear homogeneous purple solutions free of visible particles. Aliquots of 2 mL were filled in 8 mL glass vials (hydrolytic class I type Fiolax-clear, Münnerstadter Glaswarenfabrik, Münnerstadt, Germany), partially closed with gray 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 1 h. The primary drying phase started after 2 h and was performed at a shelf temperature of –35°C and a chamber pressure of 0.20 mbar for 45 h. The product temperature during primary drying was –30°C. For secondary drying, the temperature was increased to +25°C in 15 h. The chamber pressure of 0.20 mbar was maintained. Vials were closed after 3 h 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 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 FTIR 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 4,000 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 aluminum pans (TA Instruments) and hermetically closed. Samples were cooled to 0ºC and subsequently heated to 210ºC at 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 × (2θ). The Cu Kα radiation from the anode operating at 40 kV and 50 mA was monochromized using a 15-μm Ni foil. Scan step size was $x \times 2\theta$ and step time 0.05–5.0 s. 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 h to accelerate disintegration of those particles. To prevent degradation of EO-9, 10 mg/mL $NaHCO_3$ was added to all solutions (Jonkman-de Vries, Winkelhorst, Underberg, Henrar, & Beijnen, 1993).

Addition of HPβCD resulted in a linear increase in the solubility of EO-9 (Figure 2) A complexation constant ($K_{1:1}$) of 32.9 M^{-1} was calculated using the formula of Higuchi and Conners (Higuchi and Connors, 1965) (Equation 1):

$$K_{1:1} = \frac{\text{slope}}{S_0 \times (1 - \text{slope})} \tag{1}$$

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 50–2,000 M^{-1} for all cyclodextrins (Connors, 1995). 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 form M to mg/mL revealed maximum solubilities of EO-9 of 2.1, 2.8, and 4.3 mg/mL in presence of 200, 300, and 400 mg/mL HPβCD, respectively.

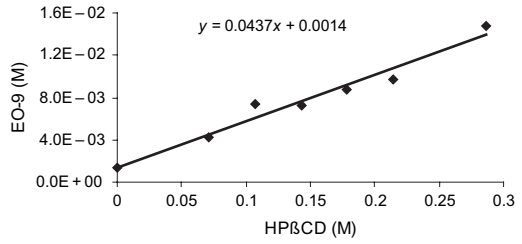

FIGURE 2. Phase solubility diagram of EO-9 with 2-hydroxypropyl-β-cyclodextrin (HPβCD) in water for injection containing 10 mg/mL NaHCO3.

To calculate the number of HPβCD molecules required to obtain one EO-9/cyclodextrin complex, the complexation efficiency (CE) of Lofttson et al. was used (Loftsson et al., 2005). The CE is determined by the ratio of free cyclodextrin and drug–cyclodextrin complex. The CE is calculated according to Equation 2:

$$\text{CE} = \frac{[\text{DCD}]}{[\text{CD}]} = \frac{\text{slope}}{1 - \text{slope}} \tag{2}$$

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. 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 (Rao & Stella, 2003):

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

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% (wt/vol) in the formulation solution is workable because of 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 with 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 (Gibaud et al., 2005; Wen et al., 2004). This indicates interaction of HPβCD with the chromophore of the drug. Because EO-9 has a very high characteristic UV/VIS spectrum and changes in absorption might indicate complexation, UV/VIS analysis was also performed to study complexation. Owing 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 the presence of HPβCD, there is only a very small difference between the duplicates before mixing (Figure 3, curves A and B) and no difference between the duplicates after mixing (Figure 3, curves C and D). 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 toward a bathochromic shift was seen. As expected, for the reference no difference in absorption was seen before and after mixing (Figure 3, curves E and F). 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.

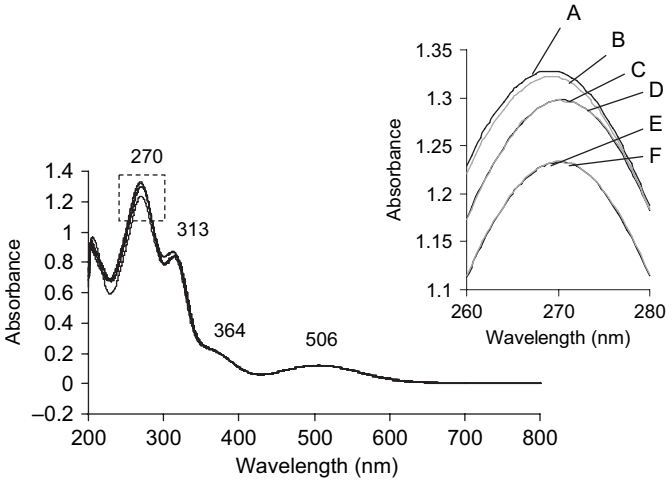

FIGURE 3. UV spectra of EO-9 before and after mixing with 2-hydroxypropyl-β-cyclodextrin (HPβCD) solution.

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 70°C all solutions turned purple, indicating the presence of (or analogs of) EO-5a, the main degradation product of EO-9 (Figure 1) (Jonkman-de Vries et al., 1993). The percentages 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) (Jonkman-de Vries et al., 1993). Furthermore, we showed that the addition of HPβCD increased the stability of EO-9. This increase is likely to be more in the absence of methanol, because methanol may have a negative effect on complexation constants of drug–cyclodextrin complexes (Porras, Sarmini, Fanali, Kenndler, 2003). 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 before using FTIR analysis (Arias et al., 2000; Bandi et al., 2004; Gladys, Claudia, & Marcela, 2003; Ribeiro et al., 2003). The advantage of FTIR 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 because of 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 FTIR 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 4A and B, respectively). Small signals of EO-9 at approximately 1,625 cm^{-1} and 1,580 cm^{-1} were seen in both physical mixtures but were absent in the freeze-dried products and freeze-dried blanks. Disappearance of these signals of EO-9 in both freeze-dried products and freeze-dried blanks could indicate interaction and/or complexation of the quinone (C=O) part of EO-9.

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 5, 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 (Ruan et al., 2005). Owing to this large exotherm, no endotherm due to melting of the 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 (curves 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.

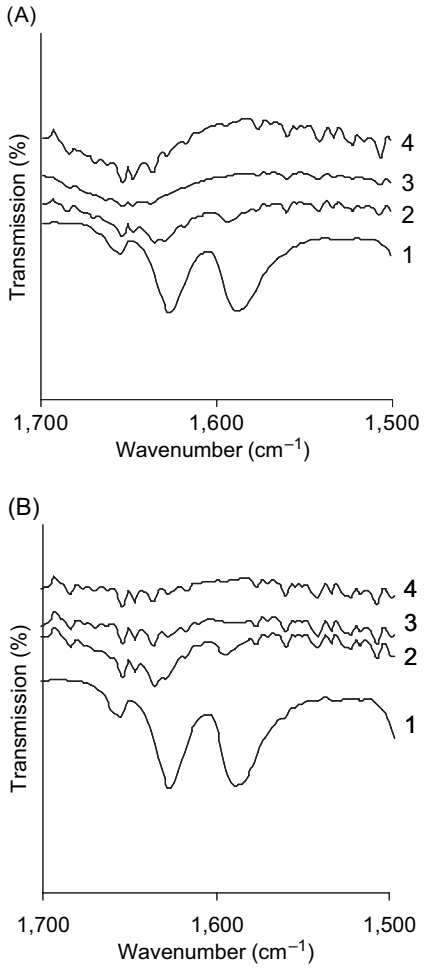

FIGURE 4. (A) Part of the IR spectra of EO-9 (1), of EO-9/HPβCD/NaHCO3 4/200/20 mg physical mixture (2), EO-9/HPβCD/NaHCO3 4/200/20 mg freeze-dried product (3), and freeze dried-blank composed of HPβCD/NaHCO3 200/20 mg (4); (B) Part of the IR spectra of EO-9 (1), of EO-9/HPβCD/Tris 4/200/1 mg physical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

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 the 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 with 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 (Arias et al., 2000; Gladys et al., 2003; Pralhad & Rajendrakumar, 2004; Ribeiro et al., 2003; Veiga et al., 2001; Zingone & Rubessa, 2005).

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 (Echezarreta-Lopez et al., 2000; Zingone & Rubessa, 2005).

The XRD spectra show that EO-9 (Figure 6A and B, curve B), Tris (Figure 6A, curve A), and $NaHCO_3$ (Figure 6B,

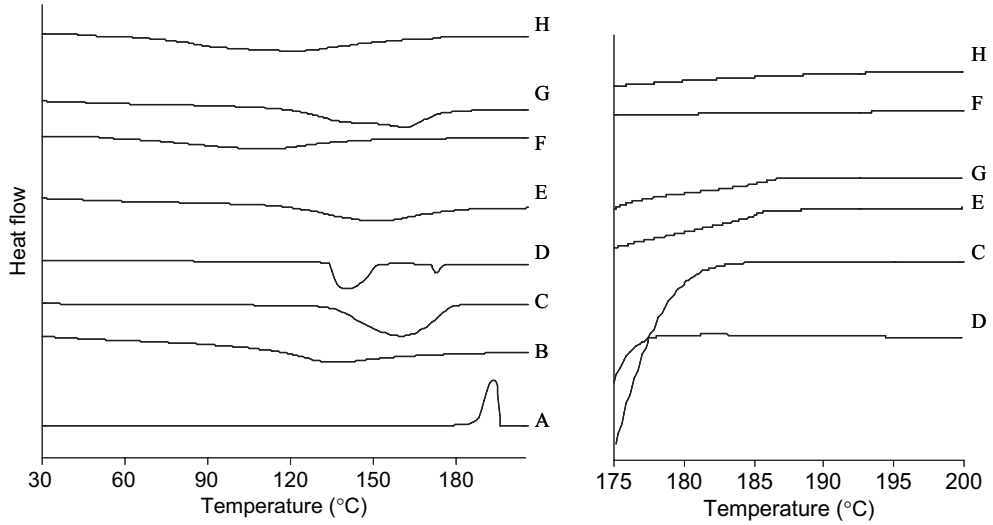

FIGURE 5. Differential scanning calorimetry (DSC) thermograms of EO-9 (A), HPβCD (B), NaHCO3 (C), Tris (D), EO-9/HPβCD/Tris (4/600/1 mg) physical mixture (E), EO-9/HPβCD/Tris (4/600/1 mg/vial) freeze-dried product (F), EO-9/HPβCD/NaHCO3 (4/600/20 mg) physical mixture (G), and EO-9/HPβCD/NaHCO3 (4/600/20 mg/vial) freeze-dried product (H).

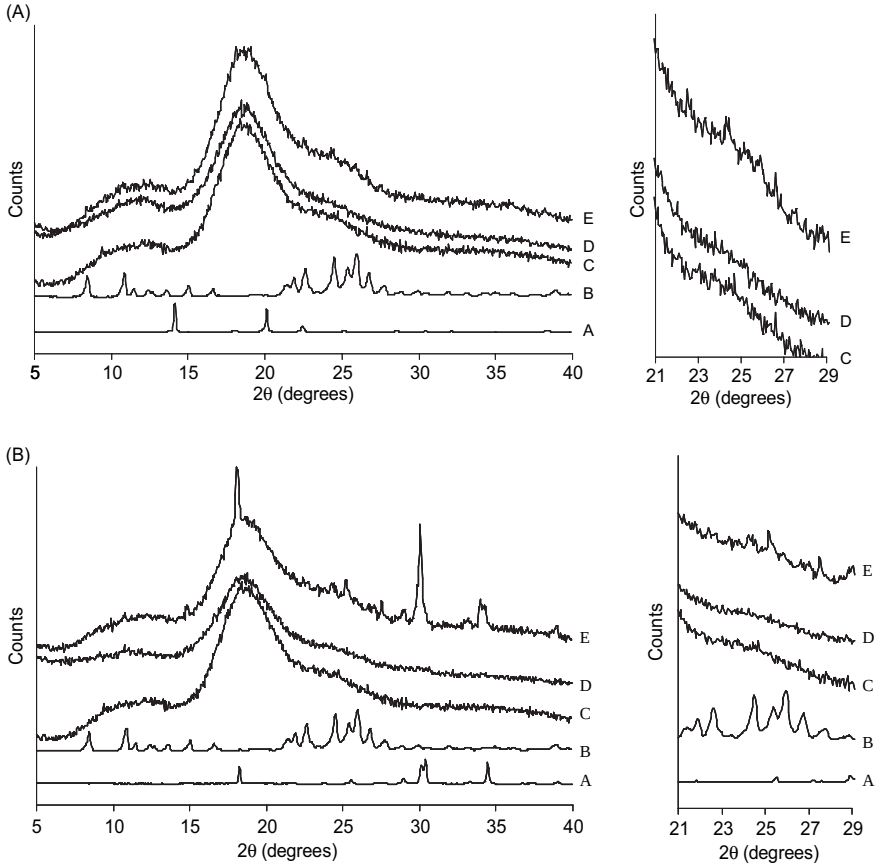

FIGURE 6. (A) X-ray diffraction (XRD) spectrum of Tris (A), EO-9 (B), HPβCD (C), EO-9/HPβCD/Tris freeze-dried product (4/200/1 mg) (D), and EO-9/HPβCD/Tris (4/200/1 mg) physical mixture (E); (B) XRD spectrum of $NaHCO_3$ (A), EO-9 (B), HPβCD (C), EO-9/HPβCD/NaHCO3 (4/200/20 mg) freeze-dried product (D), and EO-9/HPβCD/NaHCO3 (4/200/20 mg) physical mixture (E).

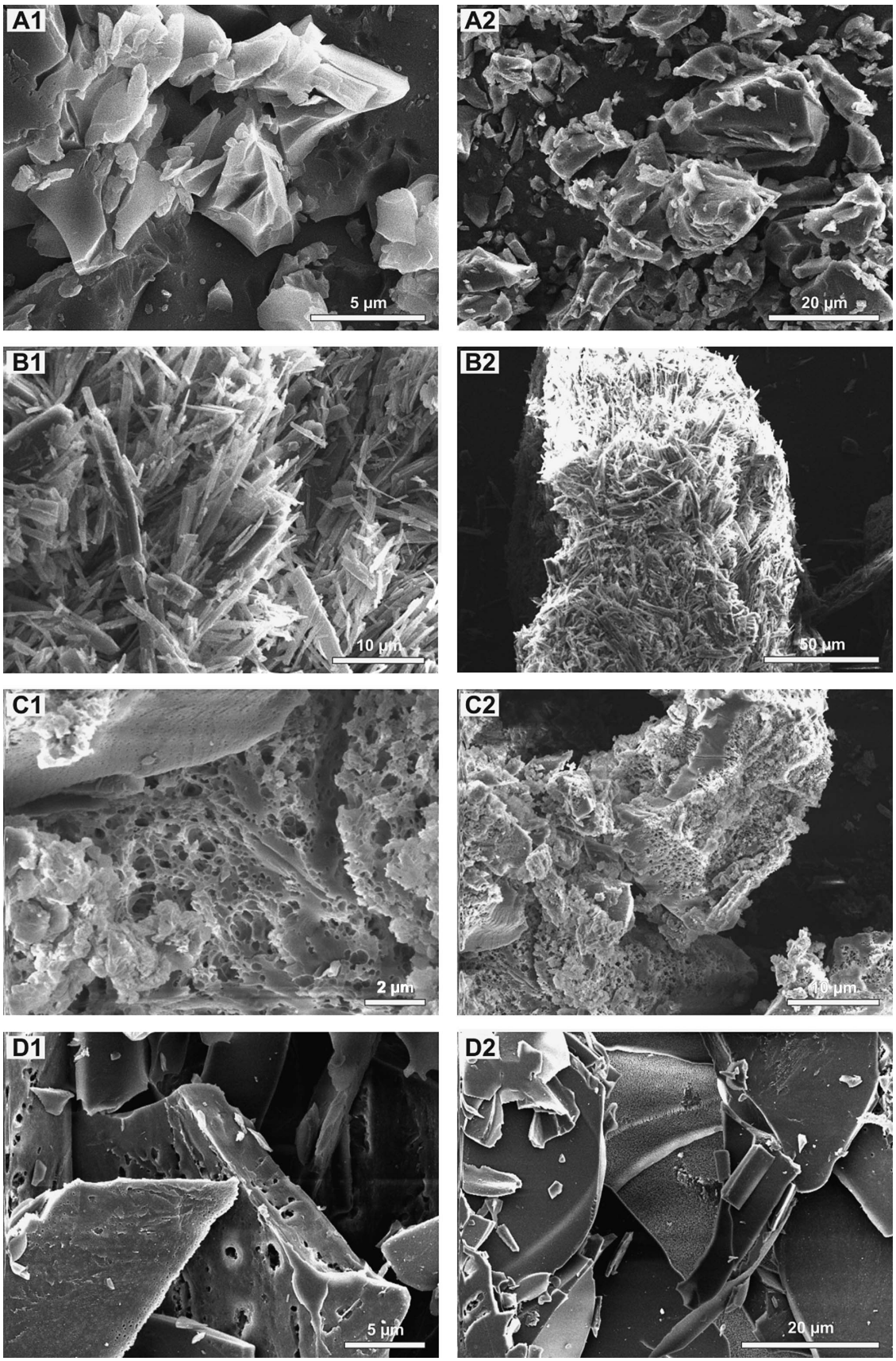

FIGURE 7. Scanning electron microscopy (SEM) data of EO-9 (A1 and A2), HPβCD (B1 and B2), and freeze-dried products composed of EO-9/HPβCD/$NaHCO_3$ (C1 and C2) and EO-9/HPβCD/Tris (D1 and D2).

curve A) are crystalline compounds. The XRD spectrum of the physical mixture composed of EO-9/HPβCD/$NaHCO_3$ (Figure 6B, curve E) showed signals of both EO-9 and $NaHCO_3$. With XRD analysis of the physical mixture composed of EO-9/HPβCD/Tris (Figure 7A, 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 with $NaHCO_3$ (1 mg of Tris compared with 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.

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 7A and B, respectively. EO-9 is composed of irregular formed crystals, whereas HPβCD is composed of large needle-like structures. Neither of both structures is seen in the freeze-dried products containing EO-9/HPβCD/$NaHCO_3$ and EO-9/HPβCD/Tris (Figure 7C 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.

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, owing 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% (vol/vol) 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. Both $NaHCO_3$ and Tris are used as alkalizers, 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. Owing to the chemical instability of EO-9 (Jonkman-de Vries et al., 1993), 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. Because of the low complexation constant, complexation of EO-9 with HPβCD was difficult to prove, but an indication of complexation was obtained with UV/VIS, DSC, XRD, SEM, and IR analyses. 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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