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EO-9 bladder instillations: Formulation selection based on stability characteristics and *in vitro* simulation studies

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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 h. 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.

Keywords: EO-9; Cyclodextrin; Stability; Urine

1. Introduction

EO-9 is a bioreductive alkylating indoloquinone (Fig. 1) 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 (Hoey et al., 1988; Cera et al., 1989; Naylor et al., 1997). For the treatment of superficial bladder cancer an investigational pharmaceutical product of EO-9 (EOquin^{TM}) 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 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. To be able to select the best formulation for further development, stability data are required. Therefore, the next step in the pharma-

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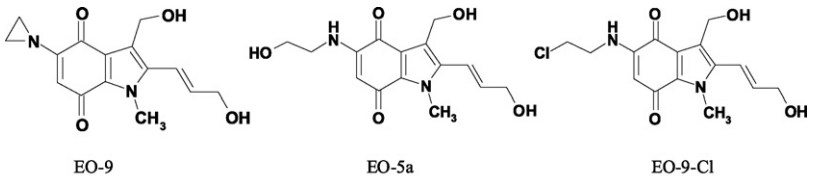

Fig. 1. Molecular structures of EO-9, EO-5a and EO-9-Cl.

ceutical development was a stability study. For this product three kinds of stability are of importance: the stability of the freeze dried product in the primary packaging material 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 how these stability studies and the selection of the best formulation are performed. Furthermore, it also provides practical instructions how to handle this new investigational anticancer agent for the treatment of superficial bladder cancer.

2. Materials and methods

2.1. 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).

2.2. 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) *tert*-butyl alcohol (TBA) were sonicated for 2 h. 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 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 raised to +25 °C in 15 h. The chamber pressure of 0.20 mbar was maintained. Vials were closed at a chamber pressure of 0.20 mbar after 3 h of secondary drying. Subsequently, the freeze dried products were stored at −20 ± 3 °C, 5 ± 3 °C and at the accelerated storage conditions 25 ± 2 °C/60 ± 5% relative humidity (RH) and 40 ± 2 °C/75 ± 5%RH, all in the dark. Samples were taken in time and analyzed using HPLC–UV.

2.3. 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 (0.45 μm × 4 mm, 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 h and analyzed with HPLC–UV.

2.4. In vivo simulation experiment

The exact compositions of the bladder instillations tested are given in Table 1.

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/h (Moffett et al., 2006). Because the bladder instillations (with a volume of 40 ml) are administered into empty bladders and must be hold there for 1 h, the mean amount of urine present during this hour will be approximately 30–60 ml. 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. The pH was analyzed using a pH meter Model 654 (Metrohm, Herisau, Switzerland) equipped with a 3 M KCl electrode (Bonaduz, Switzerland). 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 min and stored immediately at −70 °C in the dark to prevent further degradation. Furthermore, the stability of all

Table 1
Composition of five freeze-dried products and reconstitution solutions used in the stability study

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

[a] EOquin™ is a freeze-dried product composed of EO9/mannitol/$NaHCO_3$ 4/25/10 mg/vial.
[b] PG = propylene glycol; SE = sodium edetate.

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.

2.5. Analysis

2.5.1. High performance liquid chromatography with UV detection (HPLC–UV)

2.5.1.1. 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 five-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.

2.5.1.2. 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 5 mM phosphate buffer pH 7/methanol 70/30% (w/w). A Zorbax SB-C18 analytical column (750 mm × 4.6 mm i.d., particle size 3.5 μm, Agilent Technologies, Palo Alto, California, USA) preceded by a guard column (reversed phase 10 mm × 3 mm, 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 min 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).

2.5.2. High performance liquid chromatography with tandem mass spectrometry (HPLC–MS/MS)

2.5.2.1. 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; 0.1 M)/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; 0.1 M)/methanol (70/30%, v/v) was added to obtain a final concentration of 1000 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 2500 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; 0.1 M)/methanol mixture (70/30%, v/v), followed by vortex-mixing for approximately 30 s. The calibration standards were analyzed in duplicate.

2.5.2.2. 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 mm × 2.1 mm i.d., 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 1320 μl ammonium acetate (pH 8.5; 0.1 M)/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 (N_2), 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 °C.

3. Results and discussion

3.1. Stability of freeze dried products

Due to the chemical instability of EO-9 (Jonkman-de Vries et al., 1993) 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™, 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 2. A product is defined as "stable" if the degradation is less than or equal to 5%. This limit is derived from The Council of European Communities in which it is stated that the API content of finished products at time of release should not exceed ±5% (European Council, 1975). According to this definition, the freeze-dried product with 6 mg Tris/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/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 Products 3 and 4. Product 5 was stable for 1 month at 25 °C/60%RH and less than 1 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 2 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 5 °C were transferred to −20 °C for long term stability testing. After 1 year of storage at −20 °C no significant decrease in purity was seen, indicating that this product is stable for at least 1 year at −20 °C, in the dark. In all products, EO-5a was one of the main degradation products formed (Fig. 1).

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 1 year at −20 °C 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 1 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 1 year at −20 °C, considered as an acceptable storage time.

3.2. 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 recon-

Table 2
Stability of the freeze-dried products

Product[a]	Storage time (months)	Purity (%)[b]			
		−20 °C	5 °C	25 °C/60%RH	40 °C/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

[a] 1/2 = EOquin™ (see Table 1) 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).
[b] Area of the peak of EO-9 expressed as % of the total area of all peaks detected with HPLC–UV analysis.

stituted products were 8.6, 8.3, and 9.9 for the freeze-dried products containing 6 mg Tris (Table 1, Product 3), 1 mg Tris (Table 1, Product 4), and 20 mg $NaHCO_3$ (Table 1, 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 Products 3–5, respectively. The results of the stability study of the reconstituted and diluted products are depicted in Fig. 2. We defined a maximum allowed degradation limit of 5%, indicated in the figure. The results clearly show that both reconstituted products containing Tris (Products 3 and 4) are stable for at least 8 h at room temperature and ambient light. Reconstituted Product 5 (the HPβCD/$NaHCO_3$-containing product) however, is only stable for 4 h. On the other hand, this product is stable for at least 8 h after further dilution. These differences in stability are all due to a pH-effect. The pH of Products 3 and 4 is very near the optimal pH (i.e. maximum stability) of EO-9 (pH 8.75) (Jonkman-de Vries et al., 1993). 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

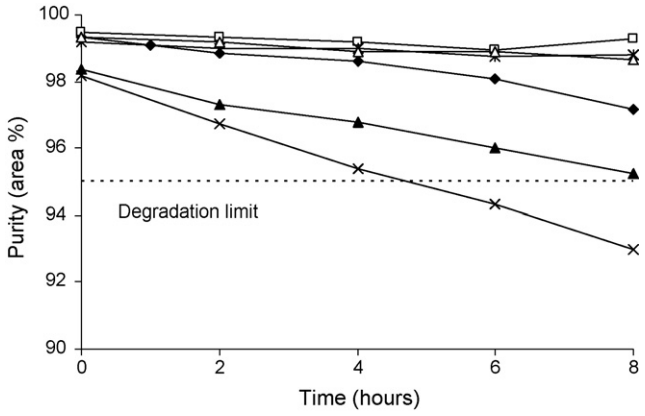

Fig. 2. Stability of freeze dried products composed of EO-9/HPβCD/Tris (4/600/6 mg/vial, Product 3) after reconstitution with WfI (⋆) and after dilution in normal saline (△), EO-9/HPβCD/Tris (4/600/1 mg/vial, Product 4) after reconstitution with WfI (□) and after dilution in normal saline (⧫), and EO-9/HPβCD/$NaHCO_3$ (4/600/20 mg/vial, Product 5) after reconstitution in WfI (×) and dilution in normal saline (▲). Degradation limit is the maximum allowed degradation.

Table 3
Degradation of EO-9 in the bladder instillation and after mixing with urine pH 4, 6 and 8 in the ratios bladder instillation:urine of 40:30 and 40:60 after storage for 1 h at 37 °C in the dark

Product[b]	Mixture instillation/urine	pH urine	pH mixture	EO-9 (%)[a]	EO-5a (%)[a]	EO-9-Cl (%)[a]	Mass balance (%)[a]
1	100/0	–	9.2	103 ± 5.3	0.2	0.0	103
2	100/0	–	9.3	102 ± 7.4	0.2	0.0	103
3	100/0	–	8.8	102 ± 4.9	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 ± 4.7	0.4	0.4	97.7
1	40/30	4	8.2	95.9 ± 2.6	0.3	0.0	96.2
2	40/30	4	7.6	94.8 ± 3.6	0.5	0.0	95.3
3	40/30	4	4.5	1.5 ± 0.1	42.1	49.3	92.9
4	40/30	4	4.2	1.2 ± 0.1	40.9	54.8	97.0
5	40/30	4	5.7	18.6 ± 1.4	39.1	34.5	92.2
1	40/30	6	8.7	97.4 ± 4.0	0.3	0.0	97.6
2	40/30	6	8.4	98.1 ± 2.8	0.3	0.0	98.4
3	40/30	6	6.5	78.0 ± 5.1	8.6	24.6	111
4	40/30	6	6.2	47.0 ± 2.0	14.6	46.7	108
5	40/30	6	6.9	95.7 ± 1.9	3.8	6.8	106
1	40/30	8	8.9	96.7 ± 2.1	0.1	0.0	96.8
2	40/30	8	8.9	97.9 ± 3.9	0.1	0.0	98.0
3	40/30	8	8.2	105 ± 2.9	0.6	0.7	107
4	40/30	8	7.9	102 ± 3.0	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 ± 3.8	0.5	0.0	97.8
2	40/60	4	7.2	93.1 ± 1.2	1.5	0.3	95.0
3	40/60	4	4.3	1.3 ± 0.1	45.3	46.5	93.1
4	40/60	4	4.1	1.2 ± 0.1	43.1	50.6	94.9
5	40/60	4	5.0	1.2 ± 0.1	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 ± 1.6	0.6	0.0	97.3
3	40/60	6	6.1	56.6 ± 0.8	15.7	29.3	102
4	40/60	6	6.2	61.2 ± 2.0	16.9	28.3	106
5	40/60	6	6.6	83.1 ± 5.5	6.0	7.2	96.4
1	40/60	8	8.7	97.4 ± 2.3	0.1	0.0	97.5
2	40/60	8	8.7	94.8 ± 1.5	0.3	0.0	95.1
3	40/60	8	8.2	99.2 ± 6.2	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 ± 5.8	0.6	1.7	100

[a] The amounts of EO-9, EO-9-Cl and EO-5a are given as percentages of the initial molar amounts of EO-9.
[b] Composition of products as indicated in Table 1.

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 h of storage respectively. With 6 mg Tris/vial, no change in pH was seen after dilution and therefore, EO-9 was most stable in this formulation.

Because in the clinic the instillation duration is 1 h, a bladder instillation must be stable for at least 1 h plus the additional time necessary for preparation at the hospital pharmacy and transfer to the bedside. Therefore, a stability of 8 h (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 h after reconstitution and dilution and are therefore suited for the clinic.

3.3. 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 1) 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 1) 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 1 h at 37 °C in the dark (Table 3). No significant differences were seen between the five formulations. However, the bladder instillation composed of EO-9/HPβCD/$NaHCO_3$ (Product 5) showed a tendency 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 Fig. 3A–C for urine of pH 8, 6 and 4, respectively. All curves start at $t = 5$ min because 5 min were required to measure the pH before the mixtures were placed in the water bath. Fig. 3A 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 (Jonkman-de Vries et al., 1993). However, after mixing with urine of pH 6, the bladder instillations containing EO-9/HPβCD/Tris (Products 3 and 4) were less stable than Product 5. Furthermore, all HPβCD-containing instillations (Products 3–5) were less stable than both EOquin™ (Products 1 and 2) bladder instillations (Fig. 3B). 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 3). 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 (Fig. 3C). However, it is likely that this was not due to the presence of

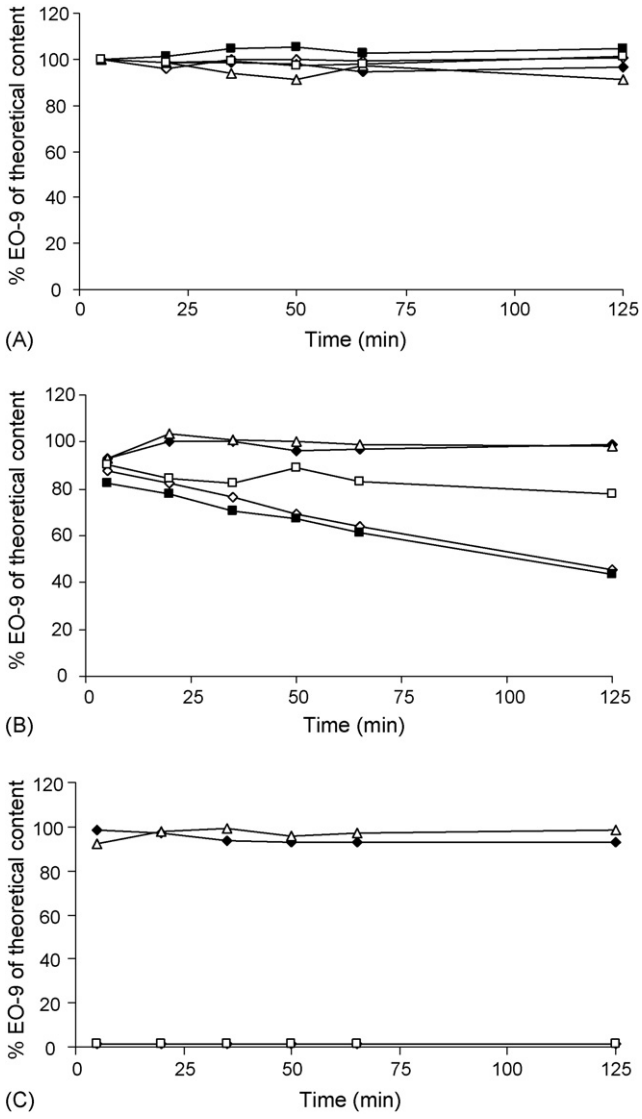

Fig. 3. Stability of five bladder instillations (EO-9/HPβCD/Tris 4/600/6 mg/vial (◊), EO-9/HPβCD/Tris 4/600/1 mg/vial (■), EO-9/HPβCD/$NaHCO_3$ 4/600/20 mg/vial (□), EOquin™ reconstituted with PG/WfI/$NaHCO_3$/sodium edetate 60/40/2/0.02% (v/v/w/w) (△), and EOquin™ reconstituted with PG/WfI/$NaHCO_3$/sodium edetate 60/40/1/0.02% (v/v/w/w) (⧫)) mixed with urine pH 4, 6 and 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.

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 (Fig. 3C).

Degradation of EO-9 in the bladder instillation of Product 4 mixed with urine pH 6 in the ratio 40:60 is depicted in Fig. 4. This figure shows that degradation of EO-9 in urine results in the formation of EO-9-Cl and EO-5a (Fig. 1). 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

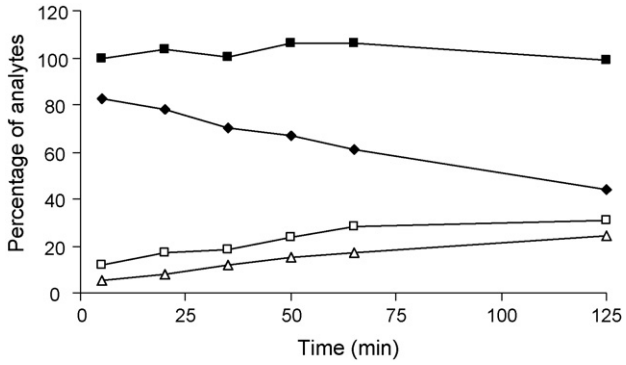

Fig. 4. Degradation of EO-9 bladder instillation prepared from the freeze-dried product composed of EO-9/HPβCD/Tris 4/600/1 mg/vial mixed with urine pH 6 in the volume ratio bladder instillation:urine = 40:60. The amounts of EO-9 (♦), EO-5a (△), EO-9-Cl (□) and the total mass balance (■) are depicted.

other HPβCD-containing bladder instillations (Table 3). 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 3). 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 3).

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 1 h. Alkalizing of patients was also performed in a randomized clinical trial to study the efficacy of intravesical Mitomycin C (Au et al., 2001).

4. Conclusion

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^{TM}. 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 1 year at −20 °C, in the dark. Furthermore, this product is stable for 8 h 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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