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

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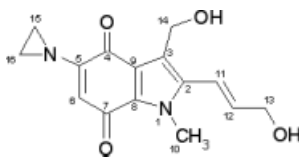
Two new bladder instillations of the investigational anticancer agent EO-9 containing 2-hydroxypropyl-β-cyclodextrin (HPβCD) and the alkalizers sodium bicarbonate ($NaHCO_3$) and tri(hydroxymethyl)-aminomethane (Tris) were developed. During the stability study of these freeze-dried products, formation of new degradation products was seen. We have characterized these products by high performance liquid chromatography in combination with photodiode array detection and mass spectrometry. In total, five new degradation products were identified of which three were detected in both freeze-dried products and two only in the freeze-dried product composed of EO-9/HPβCD/$NaHCO_3$.
Furthermore, the purity profile of two lots of EO-9 drug substance was investigated. Five, probably synthetic intermediates were found. However, the amount of total impurities was very small for both lots of drug substance and below acceptable international limits for pharmaceutical use.

1. Introduction

EO-9, 3-hydroxymethyl-5-aziridinyl-1-methyl-2-(1*H*-indole-4,7-dione)-prop-ß-en-α-ol, is a bioreductive alkylating indoloquinone and a synthetic analogue of the antitumour antibiotic mitomycin C (MMC) (Hendriks et al. 1993). The formulation of EO-9 for intravesical instillation used in phase II clinical trials for the treatment of superficial bladder is a freeze-dried product, which has to be reconstituted with a separate solution prior to administration. This bladder instillation fluid contains 30% v/v propylene glycol after reconstitution and dilution. To avoid the need of a special reconstitution solution and use of the hyper-osmotic organic co-solvent propylene glycol, alternative formulations for intravesical administration were developed. These formulations were freeze-dried products containing EO-9, 2-hydroxypropyl-β-cyclodextrin (HPβCD) and the alkalizers sodium bicarbonate ($NaHCO_3$) and tri(hydroxymethyl)aminomethane (Tris).



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

This article describes the characterization of the degradation products of EO-9 formed in freeze-dried products containing HPβCD and the alkalizers $NaHCO_3$ or Tris using high performance liquid chromatography in combination with photodiode array detection and mass spectrometry. Furthermore, two lots of EO-9 drug substance and EO-9 drug substance after forced degradation in media of pH 6 and pH 11 were analyzed as reference.

2. Investigations, results and discussion

2.1. HPLC-PDA and HPLC-MS analysis

The mobile phase was composed of methanol and 1mM ammonium hydroxide of pH 8.5. This is the pH of the minimum of the log k-pH profile of EO-9 (Jonkman-de Vries et al. 1993) and therefore, no degradation of EO-9 was expected during analysis. Methanol was selected as the eluent organic modifier, because of its limited interference with cyclodextrin inclusion activity (Fujimura et al. 1986). Furthermore, a wide range in percentage of methanol in the gradient was chosen to be able to detect both polar and apolar synthetic intermediates and degradation products of EO-9 in drug substances and freeze-dried products. With PDA detection UV-spectra of detected compounds were recorded. With these UV-spectra an indication of the chromophore was obtained. However, this technique alone was insufficient to fully elucidate the molecular structure of intermediates and/or degradation products. Therefore, LC-MS analysis was also performed.

2.2. Identification of intermediates and degradation products

Two lots of EO-9 drug substance (Lot A and B), EO-9 drug substance Lot A in alkaline solution (pH 11), EO-9 drug substance Lot A in mild acidic/neutral solution (pH 6) and both freeze-dried products fresh and after storage at 40 ± 2 °C/75 ± 5% RH were analyzed using HPLC-PDA and HPLC-MS analysis. The TIC spectra are depicted in Fig. 1. The TIC of blank HPβCD showed broad signals at 1–4 and 24–37 min (data not shown). These broad signals were also present in the TIC of the freeze-dried products and therefore, some small signals of degradation products in these areas may not have been detected with HPLC-MS analysis. The m/z values of the Q1 spectra and the λ_{max} values of the UV spectra of the degradation products and putative intermediates found in these products are summarized in the Table.

The effect of HPβCD on the stability of alkylating indoloquinones was studied earlier. Bekers et al. (1991) showed a positive effect of HPβCD on the stability of mitomycin A, mitomycin B, and mitomycin C in alkaline solution. However, after freeze drying of EO-9 and HPβCD in alkaline environment we found two new compounds, G and M. The origin of compound G, the large

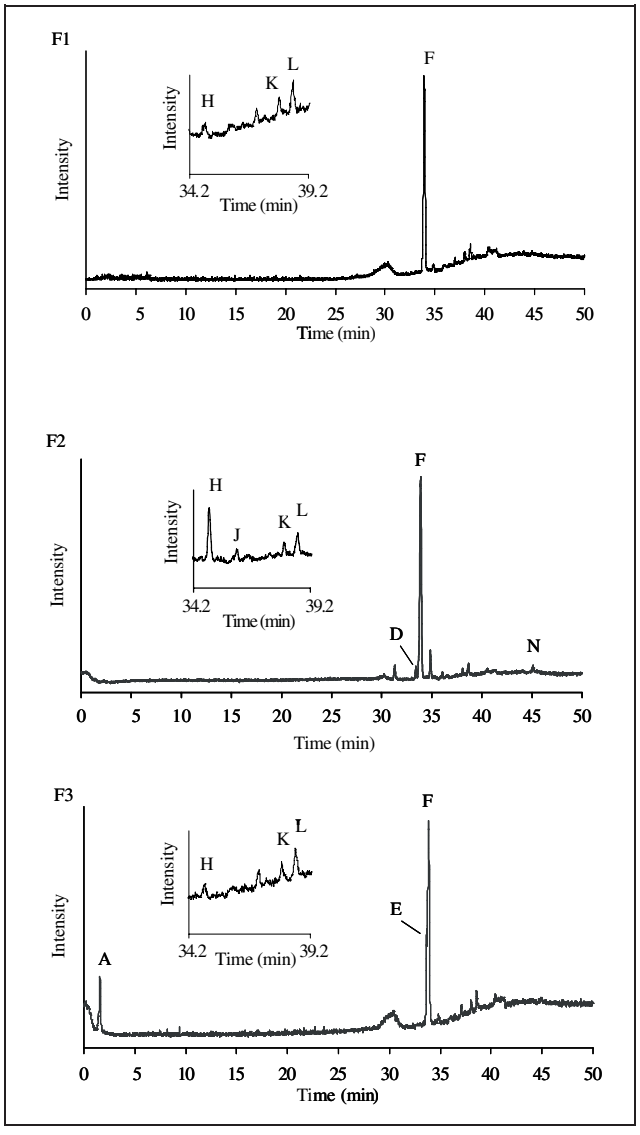


Fig. 1: F1–F3

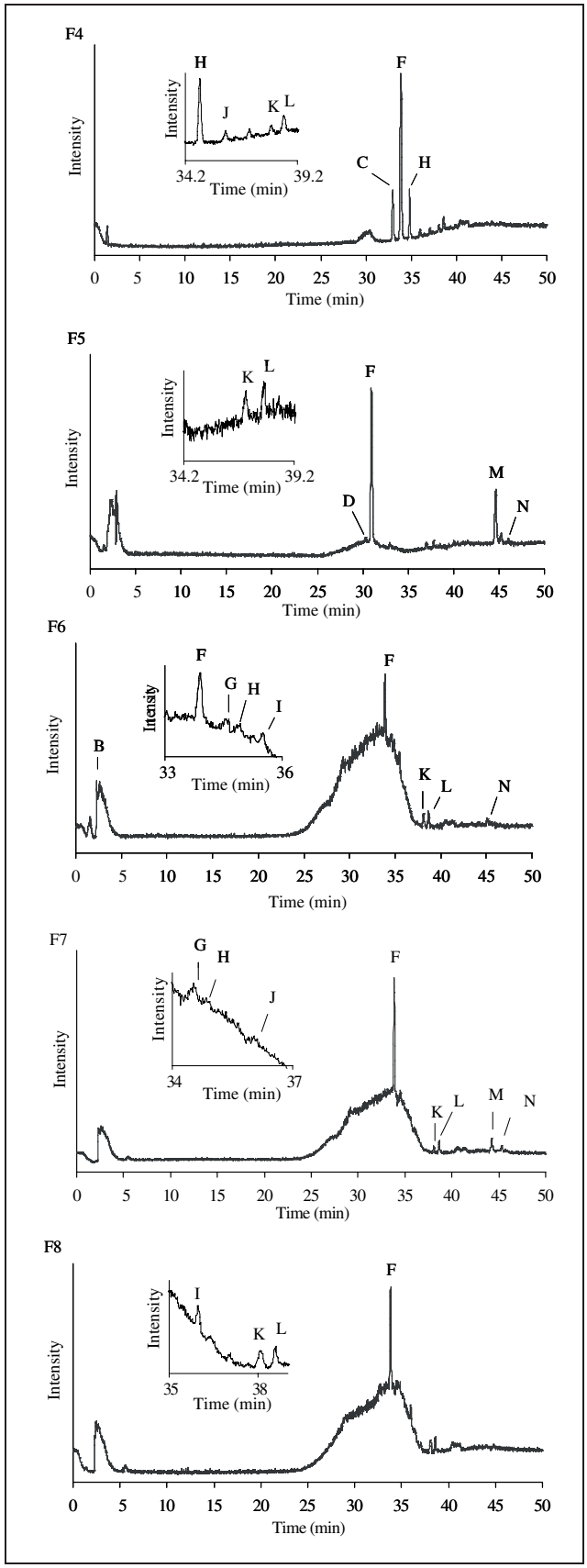


Fig. 1: TIC of EO-9 drug substance Lot A (Figure 1, F1), EO-9 drug substance Lot B (F2), degradation of EO-9 at pH 11 (F3), degradation of EO-9 at pH 6 (F4), fresh freeze-dried product composed of EO-9/HPβCD/$NaHCO_3$ (F5), freeze-dried product composed of EO-9HPβCD/$NaHCO_3$ stored at 40 °C/75% RH for 3 months (F6), fresh freeze-dried product composed of EO-9/HPβCD/Tris (F7), freeze-dried product composed of EO-9/HPβCD/Tris stored at 40 °C/75% RH for 2 months (F8)

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

Signal	Retention time (min)	EO-9 Lot A[1] F1		EO-9 Lot B[2] F2		EO-9 pH 11[3] F3		EO-9 pH 6[4] F4		EO-9/CD/Bic[5] F5		EO-9/CD/Bic[6] 40 °C/75% RH F6		EO-9/CD/Tris[7] F7		EO-9/CD/Tris[8] 40 °C/75% RH F8	
		m/z	λ	m/z	λ	m/z	λ	m/z	λ	m/z	λ	m/z	λ	m/z	λ	m/z	λ
A	1.6	– [a]	– [b]	–	–	**246** 286 492 550	210 281 311 365 541	–	–	–	209 280 311 359 541	–	209 280 311 360 544	–	–	–	213 281 311 363 552
B	2.3	–	–	–	–	–	–	–	–	–	–	257 **287** 305	–	–	–	–	–
C	33.0 EO-5a	–	212 277 323 363 541	–	–	–	–	**307** 329 636	212 277 323 364 545	–	213 278 324 365 546	–	212 277 321 365 547	–	212 277 323 363 547	–	212 277 323 365 546
D	33.4	–	–	229 **273** 313	–	–	–	–	–	229 **273** 291 313	204 252 307	–	–	–	–	–	–
E	33.7	–	–	–	–	231 **260** **272** 300 559 578	272 353 487	–	–	–	–	–	207 251 276 322 357 541	–	–	–	–
F	33.9 EO-9	**241** **271** 289 312 542 **560** 578 595	203 270 313 365 505	241 **272** **289** 311 542 **560** 578	269 313 363 507	241 260 **272** 289 312 542 **559** 578 600	270 312 363 506	241 **272** **289** 312 542 **559** 600	201 270 313 366 506	241 **271** **289** 312 542 **559** 578 595	270 313 364 506	241 **271** **289** 311 542 **559** 578 595	208 271 313 364 512	241 **271** **289** 542 559 578 **595**	270 313 363 506	272 **289** **542** **559** 578 595	201 270 313 366 506
G	34.5	–	–	–	–	–	–	–	–	–	–	**164** 441 500 720 940 1160	–	**163** 280 **441** 720 940 1160	–	–	–
H	34.9	119 179 321	208 278 319 365 544	119 **179** **321** 343 664	253 273 321 369 534	119 **179** 321 343	203 252 277 324	179 304 **321** 343 664	206 270 321 363 544	–	252 314	179 270 287 309	227 279 376	119 **179**	201 252	–	201 252 275 321 369 527
I	35.5	–	–	–	–	–	–	–	–	–	–	291	212 278 322 364 549	–	–	326 347	–
J	36.1	–	270 317 367 544	117 **145** **163** **335** 357	226 268 318 364	–	–	145 163 **326** 347 559 599	201 276 320 361 544	–	–	–	–	145 **163** 325 335	230 264 318	–	201 276 321 366 540
K	38.1	129 148 171 229	–	**129** 148 171 230	–	**129** 148 **172** 191 229	–	**129** 148 **171** 189 **229**	–	**129** 148 **171** 239	–	**129** 148 171 239 **364** 385	210 278 322 368	**129** 147 171 229	–	**364** 385	210 277 325 369 547

Table: (Continued)

Signal	Retention time (min)	EO-9 Lot A[1] F1		EO-9 Lot B[2] F2		EO-9 pH 11[3] F3		EO-9 pH 6[4] F4		EO-9/CD/Bic[5] F5		EO-9/CD/Bic[6] 40 °C/75% RH F6		EO-9/CD/Tris[7] F7		EO-9/CD/Tris[8] 40 °C/75% RH F8	
		m/z	λ	m/z	λ	m/z	λ	m/z	λ	m/z	λ	m/z	λ	m/z	λ	m/z	λ
L	38.6	141 183 241	–	129 141 **183** 241	–	131 **183** 241	–	155 **183** 241	–	129 **183** 241	–	129 **183** 241 650 672	–	129 141 **183** 241	–	141 **183** 241	–
M	44.3	–	–	–	–	–	–	–	–	106 **288** 311	–	–	–	106 **289** 311 598	–	–	–
N	45.1	–	–	**149** 205 280	–	–	–	–	–	149 205 279	–	149 205 279	–	130 149 205 280	–	–	–

[a] m/z values seen in the Q1 spectrum
[b] λ_{max} values seen in the UV/VIS spectrum
[1] EO-9 drug substance manufactured by Kyowa Hakko (Japan) in July 1993
[2] EO-9 drug substance manufactured by Irix Pharmaceuticals, Inc. (USA) in November 2003
[3] EO-9 drug substance Lot A incubated for approximately 1h at room temperature at pH 11
[4] EO-9 drug substance Lot A incubated for approximately 1h at room temperature at pH 6
[5] Freeze-dried product prepared from EO-9 drug substance Lot B, HPβCD and $NaHCO_3$ (4/600/20 mg/vial respectively)
[6] Product 5 stored at 40 °C/75% RH for 3 months
[7] Freeze-dried product prepared from EO-9 drug substance Lot B, HPβCD and Tris (4/600/1 mg/vial respectively)
[8] Product 7 stored at 40 °C/75% RH for 2 months

compound with 3 hydroxypropyl glucose units is uncertain. However, the existence of this molecule cannot only be explained by formation in the ion spray. Compound M is an analogue of EO-9 which was not found in other freeze dried products of EO-9 composed of EO-9/lactose/sodium hydroxide (Jonkman-de Vries et al. 1994) or EO-9/mannitol/$NaHCO_3$ (prepared in-house, data not shown). This indicates that the presence of HPβCD in alkaline environment might influence the degradation pathway of EO-9.

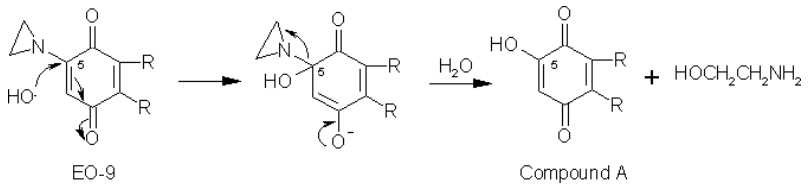
Beside the presence of HPβCD, an effect due to the alkalizers was seen as well. Results showed that the presence of $NaHCO_3$ rather than Tris triggered the formation of alkaline compounds (A and E). Furthermore, formation of compound G was more favoured by Tris than by $NaHCO_3$. In total fourteen different compounds were detected and given letter A-N. These compounds are discussed in paragraph 2.2.1 to 2.2.14.

2.2.1. Compound A

A signal at 1.6 min was only seen in the TIC after degradation of EO-9 in alkaline solution. With HPLC-PDA analysis, this compound was detected in EO-9 in alkaline solution, but also in fresh and stored EO-9/HPβCD/$NaHCO_3$ freeze-dried product, and only in stored EO-9/HPβCD/Tris freeze-dried product. This indicates that the formation of this compound is favored by $NaHCO_3$ rather than by Tris. The UV spectra obtained with HPLC-PDA analysis of this compound in these freeze-dried products resemble the UV spectrum of the product that was seen in EO-9 samples in alkaline medium, indicating that it is the same compound. Jonkman-De Vries et al. (1993) showed that in alkaline environment ring opening of the aziridine ring of EO-9 and replacement of the 5-substituent by a hydroxyl group occurs (Scheme 1). Furthermore, it was seen that initial degradation of mitomycin A, mitomycin B, and mitomycin C is the replacement of the 7-substituent (corresponding to the 5-substituent in EO-9) by a hydroxyl group (Beijnen et al. 1985a, b). The proposed compound (Fig. 2A) has a molecular mass (mw) of 263 Da. The m/z values found at 1.6 min correspond to this molecule: 246 $(MH-H_2O)^+$, 286 $(M+Na)^+$, 492 (dimer of fragment with mw = 245 $(MH)^+$), and 550 (dimer of fragments with mw = 263 and mw = 285 $(MH)^+$). Formation of dimers was reported earlier for mitomycin C (Paz M.M. et al. 2004).

The pK_a of the enolic hydroxyl group situated at the 5-position is approximately 4. Due to the high pH of the mobile phase (pH 8.5) the hydroxyl moiety is deprotonated,

Scheme 1: Alkaline degradation pathway of EO-9



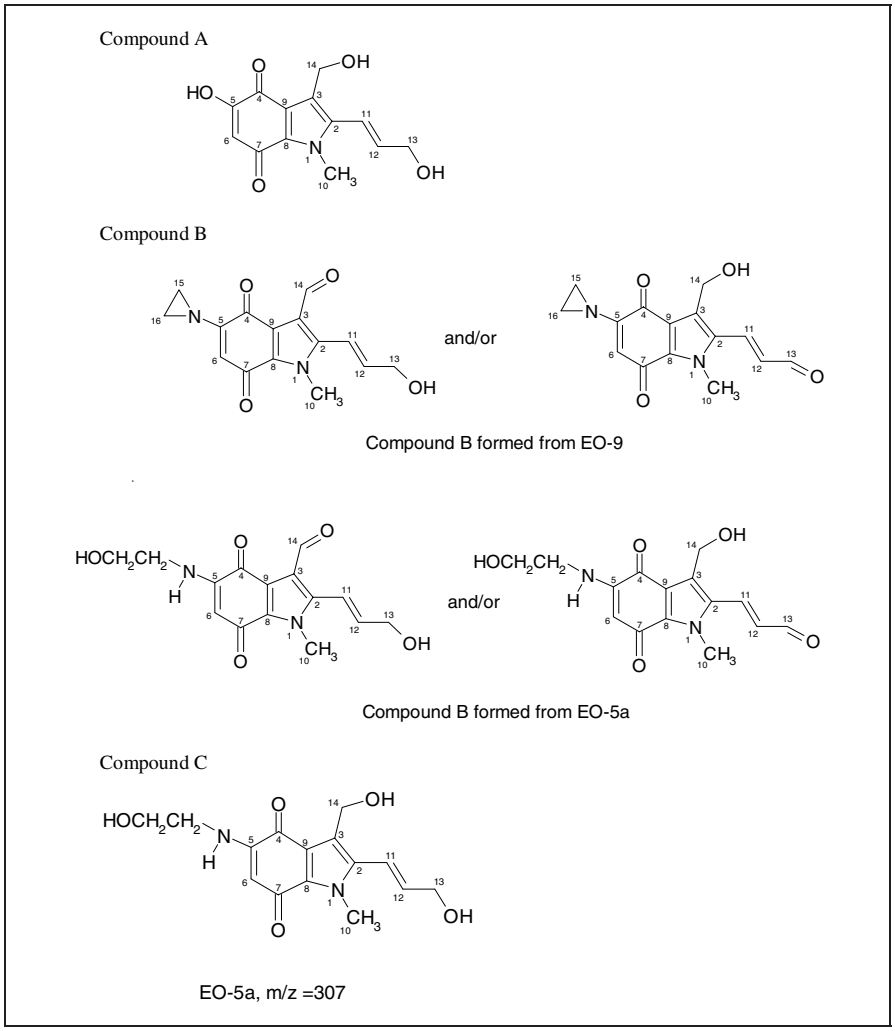


Fig. 2a: Proposed molecular structures A–C

resulting in a negatively charged analyte. This explains why this compound elutes almost with the solvent front.

2.2.2. *Compound B*

The signal of this compound is superimposed on the cyclodextrin signal and was only seen in the TIC of EO-9/HPβCD/$NaHCO_3$ freeze-dried product stored at 40 °C/75% RH. No signal was found with HPLC-PDA analysis. The m/z values correspond to EO-9 and EO-5a minus two hydrogen atoms ($MH^+ = 287$ Da and 305 Da, respectively). This could be due to oxidation of one of the hydroxyl groups in EO-9 or EO-5a to a carbonyl function (Fig. 2B) by $NaHCO_3$. This oxidation process is probably accelerated by the relatively high pH of the formulation. Remarkable is the short retention time of this compound.

2.2.3. *Compound C*

This compound is EO-5a (Fig. 2C), the main degradation product of EO-9 in (mild) acidic medium formed by opening of the aziridine ring. The mechanism of ring opening proposed by Jonkman-De Vries et al. (1993) is depicted in Scheme 2. This ring opening in mild acidic environment was also seen for the bioreductive alkylating indoloquinone Mitomycin C (Beijnen et al. 1986). The Q1 spectrum of EO-5a showed characteristic m/z values of 307 $(MH)^+$, 329 $(M + Na)^+$, and 636 (dimer of EO-5a + $Na)^+$ (Fig. 3). The UV spectrum of EO-5a showed signals at approximately $\lambda = 212$, 278, 323, 365, and 547 nm (Fig. 4). EO-5a was found in EO-9 drug substance Lot A (manufactured in 1993) and in all freeze-dried products, but not in EO-9 drug substance Lot B. Because the freeze-dried products were manufactured with EO-9 drug substance Lot B, the amount of EO-5a found in the freeze-dried products is probably due to degradation of EO-9 during manufacture. The relative area of EO-5a found with HPLC-PDA analysis of the fresh freeze-dried products composed of EO-9/HPβCD/$NaHCO_3$ and EO-9/HPβCD/Tris was 0.6 and 1%, respectively.

2.2.4. *Compound D*

Compound D is present in Lot B of EO-9 drug substance and in the fresh freeze-dried product composed of EO-9/HPβCD/$NaHCO_3$. This compound is probably an intermediate formed during synthesis of EO-9 drug substance and because it was not seen anymore after storage of EO-9/HPβCD/$NaHCO_3$ freeze-dried product, it is assumed that it degrades upon storage at 40 °C/75% RH. An m/z value of 273 Da corresponds to EO-9 minus oxygen. The proposed structures are given in Fig. 2D.

2.2.5. *Compound E*

A signal at a retention time of 33.7 min was seen with LC-MS and HPLC-PDA analysis of EO-9 degraded in alkaline solution and with HPLC-PDA analysis of EO-9/HPβCD/$NaHCO_3$ freeze-dried product after storage at 40 °C/75%RH. However, the UV spectra of these two pro-

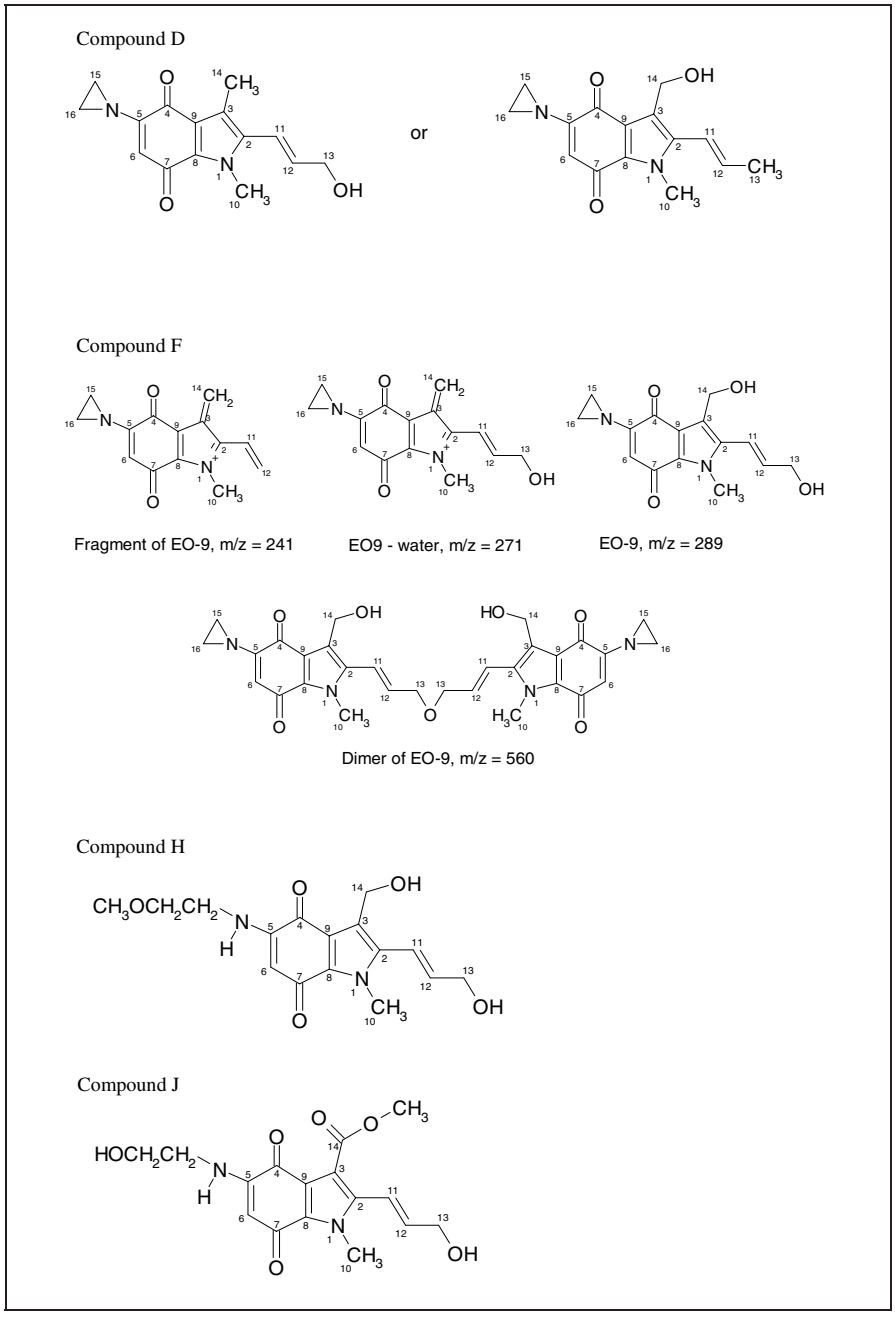
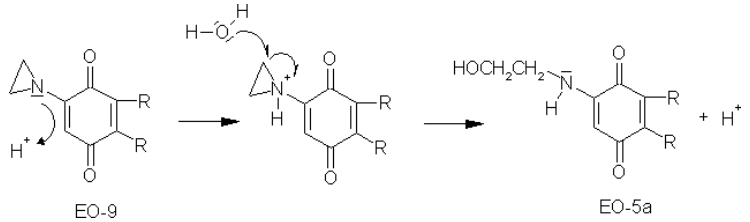


Fig. 2b: Proposed molecular structures D–J

ducts are different. The UV spectrum of the compound in the freeze-dried product corresponds to EO-5a, with an extra (and most abundant) signal at $\lambda = 251$ nm. Furthermore, a hypsochromic shift of the first signal from 212 to 207 nm was seen. This could be due to analytical variation. However the extra signal at $\lambda = 251$ nm in combination with the hypsochromic shift could indicate that more electrons are incorporated in the chromophore. Because the retention time slightly increased compared to EO-5a, it is assumed that the ring-system remained intact and that substituents changed the chromophore and made it less polar. No structural assignments could be made.

Scheme 2: Acidic degradation pathway of EO-9



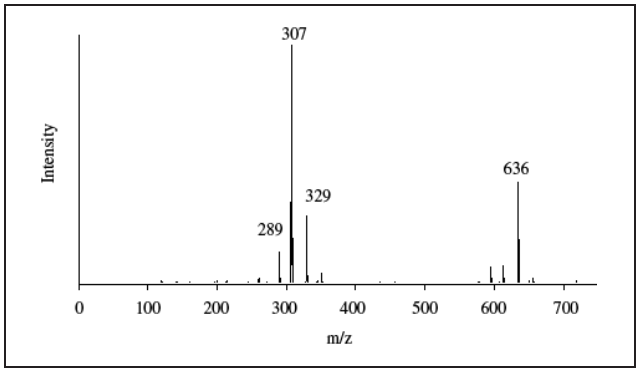


Fig. 3: Q1 spectrum of EO-5a formed from EO-9 in a solution with pH 6

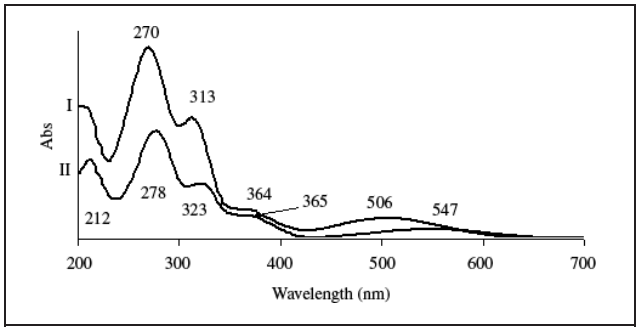


Fig. 4: UV spectra of EO-9 (I) and EO-5a (II) obtained with PDA detection

2.2.6. Compound F

Compound F corresponds to EO-9 drug substance with characteristic m/z values of 241 (−OH on the 14-position and $-CH_2OH$ on the 13-position are spliced off), 271 $(MH-H_2O)^+$, 289 (MH^+), and 312 $(M + Na)^+$ (Fig. 2F). However, m/z values of 542, 560, 578, 595 Da were also found (Fig. 5). The mass of 542 Da corresponds to a dimer of the fragment of EO-9 with an m/z value of 271. The mass of 560 Da corresponds to a dimer of EO-9 minus H_2O. H_2O is probably split off during the formation of the dimer (Fig. 2F). In this figure a dimer formed between the C13 hydroxyl groups is depicted. Furthermore, dimerization between C14 hydroxyl groups might also be possible. An indication for this is a compound detected at m/z 595 Da right next to the peak of the dimer of EO-9 (minus H_2O) at m/z 578. If those dimers were already present in the products they would have different retention times than EO-9. Because these dimers are detected in the same signal as EO-9, it is obvious that these dimers are formed in the ion spray during analysis. Furthermore, with HPLC-PDA analysis a characteristic spectrum of EO-9 was found with maximum signals at approximately $\lambda = 270$, 313, 364, and 506 nm (Fig. 4).

2.2.7. Compound G

Compound G was only found in the freeze-dried product composed of EO-9/HPβCD/$NaHCO_3$ after storage at 40 °C/75% RH and in fresh freeze-dried product composed of EO-9/HPβCD/Tris. The Q1 spectrum showed m/z values of 164, 441, 500, 720, 940 and 1160. Between the last four signals a difference in m/z of 220 is seen. This mass corresponds to a hydroxypropyl moiety conjugated to a glucose-unit. βCD is a cyclic sugar composed of seven glucose units. HPβCD is obtained by conjugation of βCD with hydroxypropyl groups. The mean degree of substitution of the cyclodextrin we used is 5. However, this conjugation process follows a Gaussian distribution also resulting in molecules with a different degree of substitution and thus different masses. The mass of one hydroxypropyl group is 59 Da. Therefore, groups of signals with the same number of glucose units, but a difference in mass of 59 are seen with MS analysis of HPβCD. These groups of signals are indicated as an "envelope". However, the signals we found are not part of such an envelope. Perhaps it is a complex of compound A with one EO-9 molecule and 1–3 glucose units. The proposed, speculative, structure has a molecular weight of 1162. Because it is not very likely that HPβCD falls apart in the freeze-dried product and because formation of dimers of EO-9 and EO-5a in the ion spray was seen earlier, it was first assumed that this compound is formed in the ion spray. It is a very remarkable compound with a retention time equal to that of HPβCD and therefore, a lot of cyclodextrin was in the spray when this compound was formed and may explain its formation. However, this compound was only seen in two of the four drug products. Therefore, it is likely that the composition of the drug products play also an important role in the formation of compound G. Unfortunately, no articles regarding the stability of HPβCD in alkaline environment during freeze drying could be found.

2.2.8. Compound H

The UV spectrum of this compound resembles the UV spectrum of EO-5a. However, it has a longer retention time than EO-5a, indicating that it is less polar. The small hypsochromic shift of the first signal from $\lambda = 212$ to 208 nm could be due to analytical variation. Furthermore, m/z values of 321, 343, and 664 Da are found. The compound at $m/z = 321$ Da is probably EO-9 after reaction with H_3COH $(MH)^+$ in the same way as it reacts with H_2O to form EO-5a (Fig. 2H). The m/z values also exhibit the formation of a sodium adduct $(M + Na)^+$ at $m/z = 343$ Da and dimerization (dimer + $Na)^+$ at $m/z = 664$ Da. The mobile phase is composed of 1 mM ammonium hydroxide (pH 8.5) mixed with methanol and it is likely that this is the source of H_3COH to react with EO-9. The small signals of this compound found in the TIC spectra, indicate that probably only a very small amount of EO-9 reacts with H_3COH.

2.2.9. Compound I

This compound is found in both freeze-dried products after storage at 40 °C/75% RH and is neither present in EO-9 drug substance nor after acidic or alkaline degradation. Due to the very minor amount found, it was not possible to characterize the compound found in the freeze-dried products composed of EO-9/HPβCD/Tris, but it is assumed that it is the same as the compound formed EO-9/HPβCD/$NaHCO_3$. The UV spectrum of this degradation product in the EO-9/HPβCD/$NaHCO_3$ product is similar to the UV spectrum of EO-5a. Furthermore, an m/z value of 291 Da was found. Because a longer retention time and the same UV spectrum as EO-5a are found, it is proposed that this compound is an analogue of EO-5a and that one oxygen atom has been lost.

2.2.10. Compound J

This compound is found in both lots EO-9 drug substance, and in EO-9/HPβCD/Tris freshly prepared and to a lesser extent after storage at 40 °C/75% RH. Because this compound was found in both EO-9 drug substances, it is assumed that it is an intermediate or by-product formed during synthesis. Several m/z values are found. The m/z value of 335 Da is in correspondence with EO-5a having a $-COOCH_3$ substituent instead of $-CH_2OH$ (Fig. 2J). This also explains the retention time being longer than EO-5a. Formation of intermediates with this substituent on the 3-position during synthesis of EO-9 drug substance was reported earlier by Jonkman-de Vries et al. (1996). Other indications for this compound being an analogue of EO-5a are the UV spectra showing resemblance with that of EO-5a.
This compound was not seen in EO-9 in alkaline solution nor in the freeze-dried product with $NaHCO_3$ probably due to hydrolysis of the ester bond.

2.2.11. Compound K

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

2.2.12. Compound L

Of this product no UV spectra could be recorded due to the minor amount present. This product is traced in both Lots of EO-9 drug substance and all freeze-dried products. The m/z value of 241 is also seen in the Q1 spectrum of EO-9 (Fig. 5), which may be indicative for a structural resemblance with EO9. The proposed structure of the fragment with m/z 241 is depicted in Fig. 2F.

2.2.13. Compound M

Compound M is only found in both freeze-dried products, but was not seen after storage at 40 °C/75% RH, indicating that this compound is quite unstable. In the Q1 spectra m/z values of 106, 289, 311, and 598 Da were seen.

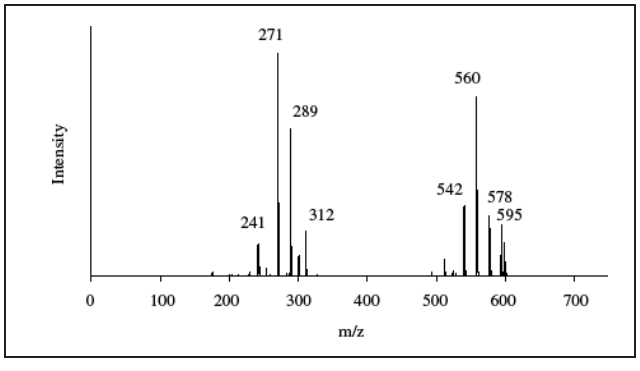


Fig. 5: Q1 spectrum of EO-9 from EO-9 drug substance Lot A

The fragments with m/z 289 and 311 correspond to EO-9 $(MH)^+$ and the sodium adduct $(M + Na)^+$. However, the fragments with m/z 106 and 598 were not seen in the Q1 spectrum of EO-9. Furthermore, this compound was not seen with UV analysis at 270 nm. This could indicate that this compound possesses some structural features of an indoloquinone, but lacks some double bonds in the characteristic chromophore of indoloquinones.

2.2.14. Compound N

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

2.3. Composition and degradation of EO-9 drug substance and drug products

In this part the by-products, intermediates and degradation products found are discussed per drug substance and drug product.

2.3.1. EO-9 drug substance Lot A

EO-9 drug substance Lot A has been manufactured in July 1993. Analysis shows the presence of EO-5a (degradation product, compound C), EO-5a reacted with methanol (probably during analysis, Compound H) and two intermediates with a chromophore resembling that of EO-5a (compound J and K). Furthermore, one intermediate with an EO-9 like chromophore was seen (compound L). This indicates that this lot did not contain other degradation products besides EO-5a.

2.3.2. EO-9 drug substance Lot B

This lot has been manufactured in November 2003. Analysis revealed the same compounds (compounds H, J, K, L) as found in Lot A and two intermediates Compound D and N. Drug substance Lot B was manufactured according to another manufacturing process than Lot A, what might explain the difference in intermediates found. Furthermore, no EO-5a was found in Lot B. This could indicate that the EO-5a present in Lot A may be due to the age of that Lot.

2.3.3. EO-9 drug substance Lot A incubated at pH 11

After incubation of EO-9 Lot A in alkaline environment, compound A was found. This corresponds to the findings of Jonkman-De Vries et al. (1993). The intermediates H, K, and L found in the fresh drug substance were still present. One new compound (compound E) was found. This compound is more polar than EO-9 and less polar than EO-5a. It probably has the same ring system as EO-5a and EO-9.

2.3.4. EO-9 drug substance Lot A incubated at pH6

Incubation of Lot A in slightly acidic environment increased the amount of EO-5a (compound C). This was seen earlier by Jonkman-De Vries et al. (1993). The intermediates H, J, K, and L remained intact. Furthermore, an increase in the amount of compound H was seen. This is probably due to a higher amount of EO-5a available to react with methanol.

2.3.5. Drug product EO-9/HPβCD/$NaHCO_3$ freshly prepared

Compounds D, H, K, L, and N were found in Lot B and are still present in this drug product. Furthermore, compound A was found in this drug product. This compound is formed due to alkaline degradation. The formulation solution (prior to freeze drying) of this product had a pH of 8.5–9 which is very close to the optimal pH of EO-9 regarding degradation. Therefore, formation of compound A in this solution is not likely and therefore it is assumed that compound A is formed due to the combination of freeze drying of EO-9 in presence of $NaHCO_3$. Furthermore, a minor amount of EO-5a (compound C) was found. Compound M was not seen earlier and is probably a less polar analogue of EO-9 lacking a chromophore.

2.3.6. Drug product EO-9/HPβCD/$NaHCO_3$ after storage at 40 °C/75% RH

The compounds A, C, H, K, L, and N found in this product were also present in the freshly prepared freeze dried product composed of EO-9/HPβCD/$NaHCO_3$. Besides these compounds the compounds B, E, G, and I were found, indicating degradation of the freeze dried product within 3 months of storage at accelerated condition. Formation of compound E was also found for EO-9 Lot A in alkaline environment. Compound B, G and I were not present in the drug substance or after incubation in acidic or alkaline environment. Analysis revealed that compound B is formed by oxidation of EO-9 and/or EO-5a. HPLC-MS analysis for compound G revealed a repetitive unit with an m/z value of 220 corresponding to a glucose unit with one hydroxypropyl moiety. Furthermore, analysis showed that both EO-9 and compound A were present in the drug product and thus available for formation of compound G. The structure of compound I could not be elucidated, but is probably an analogue of EO-5a.

2.3.7. Drug product EO-9/HPβCD/Tris freshly prepared

The compounds C, G, H, J, K, L, M, and N were found in this drug product. Of these compounds, the compounds H, J, K, L, and N were already present in the drug substance. Compound C corresponds with EO-5a. Compound G and M were found earlier in the EO-9/HPβCD/$NaHCO_3$ product after storage and the fresh EO-9/HPβCD/$NaHCO_3$ product, respectively. This indicates that the formation of these compounds is not due to the presence of $NaHCO_3$.

2.3.8. Drug product EO-9/HPβCD/Tris after storage at 40 °C/75% RH:

Analysis of this drug product showed the presence of the compounds A, C, H, I, J, K, and L.
The compounds C, H, J, K, and L were also present in the drug product prior to storage. Compound A, an alkaline degradation product, and compound I (EO-5a analogue) were not detected in the fresh drug product and therefore, it is concluded that they are formed during storage.

2.4. Conclusion

In conclusion it can be stated that in total, five new degradation products were found of which three were found in both freeze-dried products and two only in the freeze-dried product composed of EO-9/HPβCD/$NaHCO_3$. The formation of these extra degradation products in this freeze-dried product could be an explanation for the lower stability of this product compared to the Tris-containing product seen during earlier stability studies. The degradation products only formed in EO-9/HPβCD/$NaHCO_3$ freeze-dried product were shown to be more polar products than EO-9, but the three compounds formed in both freeze-dried products were all less polar than EO-9. Probably, parts of HPβCD interact with EO-9 (what could be an explanation for the increase in solubility of EO-9 in presence of HPβCD), resulting in the formation of less polar and probably larger compounds. Unfortunately, the exact structures could not be elucidated possibly due to splicing of those larger molecules in the ion spray.
Furthermore, the purity profile of two lots of EO-9 drug substance was investigated. Three intermediates were found in both products of which two intermediates are probably analogues of EO-5a and one of EO-9. Furthermore, EO-5a was found in the oldest lot (Lot A, manufactured in 1993), probably due to degradation in time. Two more intermediates were found (in very small amounts) in Lot B of EO-9 drug substance (manufactured in 2003).
In general the levels of al degradation products/impurities in the pharmaceutical products were very low and when quantifiable with PDA less than 1%.

3. Experimental

3.1. Materials

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

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

HPLC was performed using a system composed of a HP1100 Series binary HPLC pump and degasser (Agilent Technolgies, Palo Alto, CA, USA), a Model SpectraSERIES AS3000 automatic sample injection device equipped with a 100 µl sample loop (Thermo Separation Products, Breda, The Netherlands). Gradient chromatography was performed using a Synergi 4U Fusion-RP 80A column (150 × 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% 1mM ammonium hydroxide. This condition was maintained for 15 min. After 15 min, the amount of methanol was linearly increased to 70% in 15 min and subsequently increased to 80% in 5 min. This condition was maintained for 10 min. After a run time of 45 min the gradient was returned to 5% methanol in 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, Sunnyvale, CA, USA).

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

Analysis was performed using a HPLC system consisting of a HP1100 Series binary HPLC pump, degasser and HP1100 autosampler (Agilent Technolgies, Palo Alto, CA, USA). The same analytical column, mobile phase, gradient and flow were used as described for the HPLC-PDA analysis. The HPLC eluate was fed directly into an API 365 triple quadrupole MS equipped with an electrospray ion source and controlled with Analyst™ 1.2 software (Sciex, Thornhill, ON, Canada). The quadrupoles were operated in the positive ion mode. Nebulizer gas (compressed air), turbo gas (compressed air) and curtain gas (N2) were operated at 8 psi, 7 ml/min and 15 psi, respectively. The ionspray voltage was kept at 5500 V, with a source temperature of 250 °C. Sample injections of 5 µl were carried. Total Ion Current (TIC) and Q1 spectra were used for identification of intermediates and degradation products utilizing Analyst™ software version 1.2 (Sciex). For the Q1 spectra, background subtraction was performed.

3.4. Manufacture of freeze-dried products

Jonkman-de Vries et al. (1994) showed that a stable final product of EO-9 can be obtained with freeze drying. Therefore, freeze drying was selected as manufacturing process.

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

3.5. Identification of intermediates and degradation products

Both freeze-dried products were stored at the accelerated storage condition of 40 ± 2 °C and $75 \pm 5\%$ relative humidity (RH). Samples of EO9/HPβCD/Tris and EO9/HPβCD/$NaHCO_3$ freeze-dried product were taken after 2 and 3 months of storage, respectively. Furthermore, two lots of EO-9 drug substance were analyzed, Lot A and B (stored at 2–8 °C, in the dark). Lot A and B were manufactured by different suppliers and via different pathways. Lot A was synthesized in July 1993 and was used as reference standard. Lot B was synthesized in November 2003 and used for the manufacture of both freeze-dried products. Of both lots of EO-9 drug substance a stock solution of 100 µg/ml was prepared in methanol and diluted one hundred times with a solution composed of 1mM NH_4OH : methanol 7 : 3 v/v prior to analysis. Furthermore, the pH of the diluted stock solution of drug substance Lot A was adapted to pH 6 and pH 11 with 1M HCl and 1M NaOH, respectively, to study degradation of EO-9 in absence of excipients (the optimal pH of EO-9 is 8.75 (Jonkman-de Vries et al. 1993)). EO-9 was incubated in these solutions for approximately one hour at room temperature. Subsequently, intermediates (i.e. compounds assumed to be formed during synthesis of EO-9 drug substance) and degradation products of EO-9 were characterized with use of HPLC-PDA and LC-MS analysis.

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