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Stability experiments in human urine with EO9 (apaziquone): A novel anticancer agent for the intravesical treatment of bladder cancer

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Abstract

EO9 (apaziquone) is a novel, promising anticancer agent, which is currently being investigated for the intravesical treatment of bladder cancer. EO9 contains a highly reactive aziridine ring in its structure that limits its chemical stability in acidic aqueous solutions. The stability of the pharmaceutically formulated EO9 in human urine, including the effects of several parameters such as temperature, buffer strength and pH have been investigated. Urine extracts were analyzed by high-performance liquid chromatography coupled to electrospray tandem mass spectrometry (HPLC–MS/MS) using a TurboIonspray interface and positive-ion multiple reaction monitoring. EO9 was unstable in urine at 43 °C during the instillation for longer than 1 h. However, the drug was stable in human urine for 3 h at 37 °C. EO9 is stable in urine stabilized with TRIS buffer (pH 9.0; 5 mM) for up to three freeze/thaw cycles at −20 and −70 °C and 3 months of storage at −70 °C. The results also illustrated that with the lower pH in urine, EO9 became more unstable. Furthermore, a new degradation product of EO9 was discovered and successfully identified as EO9-Cl.

The outcomes of these stability experiments will be implemented to insure proper sample handling at the clinical sites, transport, storage, and sample handling during analysis in the forthcoming preclinical studies of EO9 in superficial bladder cancer, supported by bioanalysis and pharmacokinetic monitoring.

Keywords: Bladder cancer; EO9; Apaziquone; EO5a; EO9-Cl; Urine; Metabolites; Stability experiments; Mass spectrometry

1. Introduction

The indoloquinone compound apaziquone (3-hydroxy-5-azir-idinyl-1-methyl-2[indole-4,7-dione]-prop-β-en-α-ol; EO9) is a bioreductive drug that was selected for clinical evaluation on the basis of a novel mechanism of action and good preclinical anti-tumor activity [1,2]. Currently ongoing clinical trials investigating EO9 in superficial bladder tumors with local drug delivery show promising response rates [3].

EO9 is an inactive pro-drug that undergoes redox cycling leading to the formation of alkylating intermediates [4–6]. These alkylating intermediate species are capable of forming adducts with DNA (single-strand breaks and DNA cross-linking) leading to cell kill [7]. Particularly, the acid-catalyzed degradation plays an important role in the activation of EO9 [8].

EO9 has a very short half-life (<10 min in humans) after intravenous administration. It is extensively metabolized. One of the principal known metabolites is EO5a, which has an open aziridine ring and shows less cytotoxicity than EO9 [9]. Another degradation product, which was discovered by us and introduced for the first time in this article, is EO9 with covalently attached chlorine (EO9-Cl). It is formed in the acid-catalyzed reaction of EO9 in the presence of chloride anions in urine.

Studies have been conducted dealing with the bioactivation and mechanism of action [8,10–25], pharmacokinetics [25–32], distribution and metabolism of EO9 [25,33–36] and the bio-

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analysis [37–39] of EO9. An extensive study on the chemical stability of EO9 in aqueous solution using reversed-phase high-performance liquid chromatography with ultraviolet detection and ultraviolet spectrometry has been performed by de Vries et al. [6]. They studied the degradation of EO9 as a function of pH, buffer composition, ionic strength and temperature, and found it followed pseudo-first-order kinetics. Moreover, the degradation rate was strongly affected by phosphate buffer components, but not by acetate and carbonate buffers. Both the degradation rate and activation mechanism of EO9 were found to be strongly pH-dependent: the further the pH shifted in either direction from 8.5, the more unstable EO9 became.

No study, however, was performed to describe the stability of EO9 in human urine under various conditions. Since EO9 will be administered intravesically, knowledge on the stability of EO9 in urine is of considerable importance to understand conditions in the bladder during instillation and to ensure correct sample handling during transport, storage, and bioanalysis.

This study was initiated with the objective of obtaining detailed knowledge on the stability of EO9 in human urine, including the effect of the several parameters, such as pH, buffer, and temperature in this matrix, in order to support clinical studies of EO9 in bladder instillations.

2. Experimental

2.1. Materials

EO9 ($C_{15}H_{16}N_2O_4$; Fig. 1A), EO9-d_3 internal standard ($C_{15}H_{13}D_3N_2O_4$; Fig. 1B), and EO5a-d_4 internal standard ($C_{15}H_{14}D_4N_2O_5$; Fig. 1D) were supplied by Spectrum Pharmaceuticals Inc. (Irvine, CA, USA). The metabolite EO5a ($C_{15}H_{18}N_2O_5$; Fig. 1C) was synthesized from the EO9. Methanol (LC gradient grade) was obtained from Bissolve Ltd. (Amsterdam, The Netherlands). All other solvents or chemicals were analytical grade or better. Distilled water was used throughout the analyses. Drug-free human urine was obtained from volunteers from the laboratory of the Department of Pharmacy&Pharmacology at the Slotervaart Hospital (Amsterdam, The Netherlands).

2.2. Preparation of stock and working solutions

A stock solution of EO9 was prepared in ethanol at a concentration of 1 mg/mL. The solution had to be placed in an ultrasonic bath for 2 h in order to dissolve the compound. The same procedure was repeated each time this solution was thawed.

This solution was further diluted with ammonium acetate buffer (pH 8.5; 0.1 M)–methanol (7:3, v/v) to obtain working solutions.

EO5a was prepared by adding 500 μL $HClO_4$ (pH 2.0; 10 mM) to 10 mg/mL (500 μL) EO9 in DMSO. After incubating for 1 min at ambient temperature, 4 mL of ammonium acetate buffer (pH 8.5; 0.1 M)–methanol (7:3, v/v) was added to yield 1 mg/mL EO5a (the purity was verified by HPLC).

This solution was further diluted with ammonium acetate buffer (pH 8.5; 0.1 M)–methanol (7:3, v/v) to obtain working solutions. The working solutions of EO9 and EO5a were further diluted in ammonium acetate buffer (pH 8.5; 0.1 M)–MeOH (7:3, v/v) to yield concentrations ranging from 100 to 15,000 ng/mL. These working solutions were used to prepare the calibration standards.

Separate stock solutions of EO9-d_3 and EO5a-d_4 were prepared in ethanol at a concentration of 1 mg/mL.

A working solution containing the internal standards was prepared by transferring 500 μL of EO9-d_3 stock solution and 500 μL of EO5a-d_4 stock solution to a 50.0 mL volumetric flask

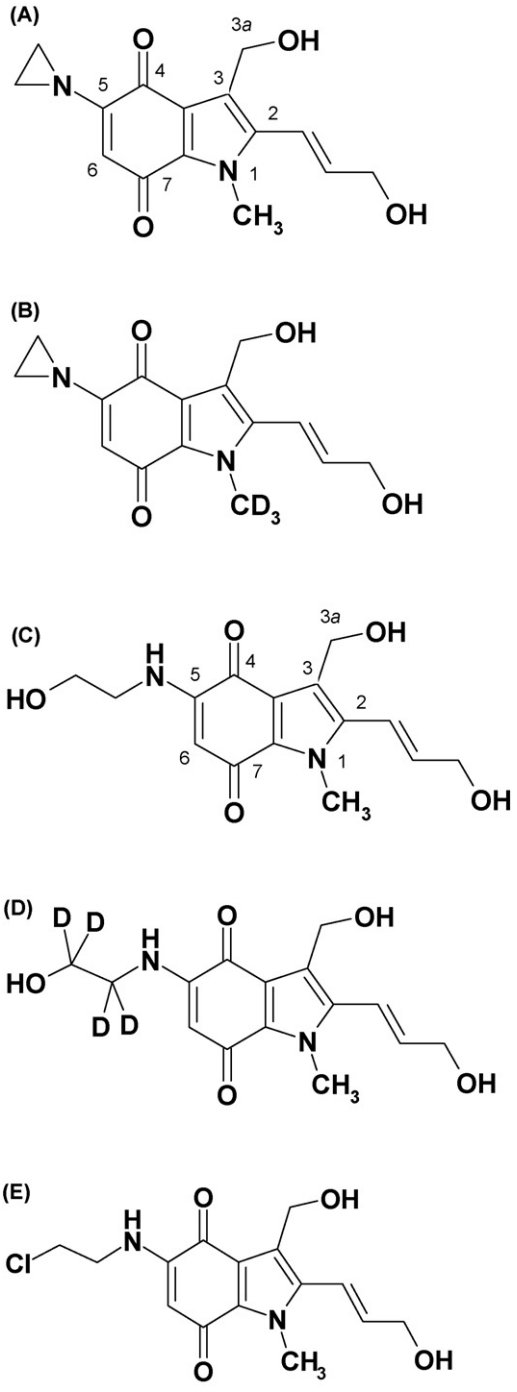

Fig. 1. Chemical structure of EO9, EO9-d_3, EO5a, EO5a-d_4 and EO9-Cl.

and adding ammonium acetate buffer (pH 8.5; 0.1 M)–methanol (7:3, v/v) to give a final concentration of 10,000 ng/mL for EO9-d_3 and EO5a-d_4.

All solutions were stored at −20 °C.

2.3. Preparation of calibration standards

Calibration standards containing both EO9 and EO5a were prepared freshly in ammonium acetate buffer (pH 8.5; 0.1 M)–methanol (7:3, v/v), ranging from 10 to 1500 ng/mL, from the working solutions of EO9 and EO5a, and vortex-mixed for approximately 30 s before analysis. Standards were analyzed in duplicate.

2.4. Reconstitution of formulated product of EO9 (EOquin™)

One vial of lyophilized product, containing 4 mg of EO9, 25 mg mannitol and 10 mg sodium bicarbonate was reconstituted with 2× 10 mL of the diluent. The diluent formulation contained 10 mg/mL sodium bicarbonate, 0.2 mg/mL EDTA, and 0.6 mL propylene glycol in 1.0 mL sterile water for injection (SWFI). This 20 mL solution was further diluted with 20 mL of SWFI to yield 40 mL of EO9 drug solution.

2.5. Preparation of non-stabilized urine

A volume of 5 mL of the EO9 solution was transferred to a 30.0 mL polypropylene tube and placed on ice. A volume of 10 mL of urine was added to the tube and vortex-mixed for 10 s. The batch was immediately divided into 20 portions of 500 μL each. Another two batches of 5 mL EO9 solution and 10 mL urine were prepared in the same way. Three batches of non-stabilized urine (60 samples) were acquired. Three samples from each batch were analyzed at time zero and the other samples were used for the experiments with non-stabilized urine (Section 2.10).

The average volume collected after 60 min of instillation is approximately 120 ± 47 mL. The total volume of instillation fluid is 40 mL (Section 2.4). Therefore, in order to mimic the clinical situation, a volume of control human urine was added to the EOquin™ formulation in the ratio of 2:1 (v/v) as described above.

2.6. Preparation of urine stabilized with TRIS buffer (pH 9.0; 5 mM)

A volume of 2 mL of EO9 drug solution was transferred to a 30.0 mL polypropylene tube and placed on ice. A volume of 4 mL of urine and 6 mL of TRIS buffer (pH 9.0; 5 mM) were added to the tube and vortex-mixed for 10 s. The batch was immediately divided into 24 portions of 500 μL each. Another two batches of 2 mL EO9 solution, urine and TRIS buffer (pH 9.0; 5 mM) were prepared in the same way. Three batches of non-stabilized urine (72 samples) were obtained. Three samples from each batch were analyzed at time zero and the other samples were used for the experiments with the stabilized urine (Section 2.10).

Table 1
The evaluated conditions

Matrix	Conditions	Initial EO9 concentration (μM)	Time points
Non-stabilized urine[a]	43 °C	115.6	0, 1, 2 and 3 h
Non-stabilized urine	37 °C	115.6	0, 1, 2 and 3 h
Non-stabilized urine	Room temperature	115.6	0, 1, 2 and 3 h
Non-stabilized urine	On ice	115.6	0, 1, 2, 3, 6 and 24 h
Stabilized urine[b]	On ice	57.8	0, 1, 2, 3, 6 and 24 h
Stabilized urine	−20 °C	57.8	0, 1, 2 and 3 months
Stabilized urine	−70 °C	57.8	0, 1, 2 and 3 months
Stabilized urine	Freeze/thaw at −20 °C	57.8	0, 1, 2 and 3 cycles
Stabilized urine	Freeze/thaw at −70 °C	57.8	0, 1, 2 and 3 cycles

[a] Urine collected from the bladder.
[b] Urine collected from the bladder and stabilized with TRIS buffer (pH 9.0; 5 mM).

2.7. Sample pretreatment

At each time point (Table 1), 150 μL of a working solution containing the internal standards was added to 50 μL of human urine aliquots and diluted 30 times (1200 μL) in ammonium acetate (pH 8.5; 0.1 M)–methanol (7:3, v/v) to yield initial EO9 analyte concentrations between 1.93 and 3.65 μM.

2.8. HPLC

The HPLC system comprise an HP1100 (Agilent Technologies, Palo Alto, CA) binary pump, degasser and HP1100 auto sampler (Agilent Technologies). 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; 1 mM) 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. To stabilize the column, 40% B was used for 2 min. Sample injections of 25 μL were carried out and the autosampler temperature was gauged at 10 °C.

2.9. Mass spectrometry

The LC eluate was directed 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, 274 → 244, 307 → 231, 311 → 231, 325 → 241 were optimized for EO9, EO9-d_3, EO5a, EO5a-d_4 and EO9-Cl ($C_{15}H_{17}N_2O_4Cl$; Fig. 1E), respectively, with dwell times of 150 ms. The response of EO9-Cl was quantified using the EO9 calibration standards due to the absence of EO9-Cl reference 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 ion spray voltage was kept at 5500 V, with a source temperature of 250 °C.

2.10. The evaluated conditions

Sample stability was evaluated under the conditions listed in Table 1. Three different urine batches with a pH in the range from 5 to 7 were tested (three measurements/batch/time point).

EO9 is considered stable in the biological matrix when 85–115% of the initial concentration is recovered [40,41].

3. Results and discussion

3.1. Sample pretreatment

Liquid–liquid extraction (LLE) was investigated for the extraction of EO9 an EO5a from urine, but the recoveries of EO9 were low and not reproducible (data not shown). Therefore, the urine samples were diluted with ammonium acetate (pH 8.5; 0.1 M)–methanol (7:3, v/v) to examine whether the recovery of EO9 could be increased more reproducibly with a direct injection. This approach was indeed successful and interestingly, we also detected another compound in the urine, which was later identified as EO9-Cl (Section 3.4).

Therefore, dilution of the urine samples was chosen as the sample pretreatment. The samples were diluted 30 times prior to analysis to ascertain that the final concentration would fit into the linear calibration range and to prevent contamination of the mass spectrometer with salts present in urine. The chromatographic system provided excellent peak shapes and the LC run time was only 6 min.

3.2. Detection of EO9 and EO5a by the HPLC–MS/MS

Apart from the protonated species at *m/z* 289 for EO9 and *m/z* 307 for EO5a, sodium adducts were also visible (*m/z* 311 and *m/z* 329, respectively) in the Q1 mass spectra of EO9 and respectively EO5a (spectra not shown). An ion corresponding to the elimination of water (*m/z* 271) from the molecular ion of EO9 was the most prominent, indicating that the elimination of water is a favorable reaction for the molecule, which occurs immediately in Q1. An ion corresponding to the loss of water from the molecular ion is also observed for EO5a (*m/z* 289). The protonated ion after the elimination of water (iminium ion) from EO9 (*m/z* 271) and the protonated molecular ion of EO5a were induced to fragment in the collision cell. The resulting product ion spectra and the proposed fragmentation patterns are

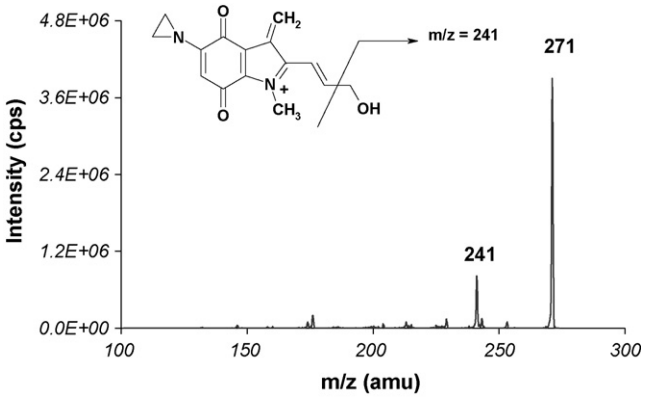

Fig. 2. MS/MS product ion scan of EO9 (precursor ion *m/z* 271).

presented in Fig. 2 for EO9 and Fig. 3 for EO5a. The main fragment ion in the spectrum of EO9 corresponds to the loss of the CH_2O from the allylic alcohol (*m/z* 241). The main fragment ion in the spectrum of EO5a corresponds to the cleavage of the amine moiety and the elimination of water (*m/z* 231). After optimization of the MS parameters, the fragment ion at *m/z* 241 for EO9 and the fragment ion at *m/z* 231 for EO5a were the most abundant and used for quantitative multiple reaction monitoring (MRM) of EO9 and EO5a, respectively.

For EO9-d_3 and EO5a-d_4 similar transitions as for EO9 and EO5a, respectively, were selected and optimized.

3.3. The identification of EO9-Cl, a new degradation product of EO9

The EO9 molecule owes its significant anti-tumor activity to the reactive aziridine moiety. However, because of its reactivity, it can react with other nucleophiles, e.g. solvents or salts prior to its biological target.

After diluting an acidic urine sample (pH ∼ 4) containing EO9 with ammonium acetate buffer (pH 8.5; 0.1 M)–MeOH (7:3, v/v) and recording the total ion current (TIC) scan of the injected sample, the presence of EO9, the degradation product EO5a, and an other unknown compound was accounted for. A closer look at the newly formed compound, the natural isotopic peak abundances (see further below), and the recognition that

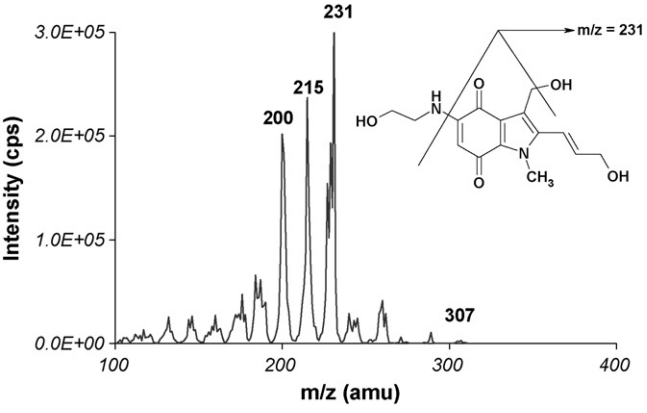

Fig. 3. MS/MS product ion scan of EO5a (precursor ion *m/z* 307).

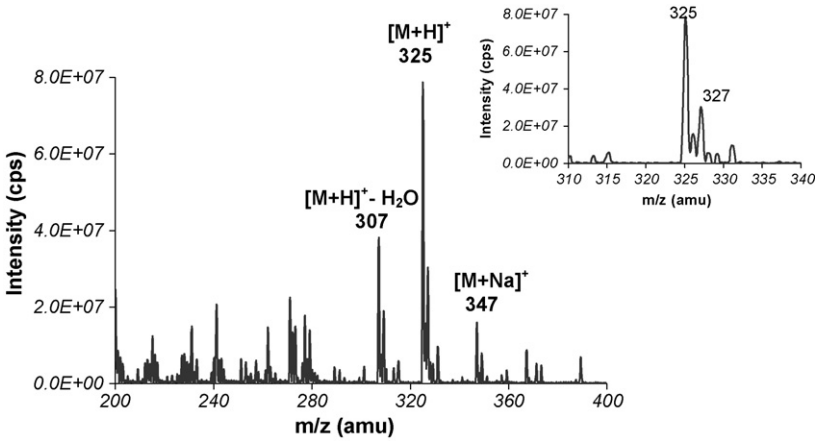

Fig. 4. Q1 (*m/z* 200–400) mass spectrum of EO9-Cl.

the urine contains high levels of nucleophilic chloride anion, pointed out that the new degradation product of EO9 could be the chloride adduct of EO9.

In Fig. 4, a Q1 mass spectrum of EO9-Cl is presented. Apart from the protonated species at *m/z* 325, sodium adducts are also visible (*m/z* 347). Special attention should be given to the characteristic Cl^- isotope abundances in the MS. Since ions are separated in mass spectrometry according to their *m/z* values, the mass spectrum exhibits a peak for each of these ions. Mass spectrometry offers one of the best ways to identify and quantify the presence of different isotopes in a sample [42]. The elemental composition of an ion can be determined from the ratio of the intensity of the isotope peaks to the intensity of the nominal mass peak. The chlorine atom has two naturally occurring stable isotopes that differ by 2 amu. In addition, ^{35}Cl accounts for about 75% of natural Cl; while ^{37}Cl accounts for the rest. If the notation $P(X)$ is used to denote the probability that a given isotope or set of isotopes will occur, the presence of one Cl atom in an ion can be written as the probability of finding each of the individual isotopes, for example $P(^{35}Cl) = 0.75$ and $P(^{37}Cl) = 0.25$, where the numerical probabilities are the approximate natural isotopic abundances of ^{35}Cl and ^{37}Cl, adjusted so that their sum is 1.0. The ratio of the intensities of the two peaks caused by the presence of the Cl atom is given by the equation $[X]/[X+2] = [X^+]/[(X+2)^+] = P(^{35}Cl)/P(^{37}Cl) = 0.75/0.25 = 3/1 = 100/33.3$, where [X] is the intensity of the peak corresponding to the ion having the lower *m/z* value (X^+), and $[X+2]$ is the intensity of the peak 2 amu higher. These intensities are directly proportional to the relative abundances of the corresponding ions, which are denoted by the terms $[X^+]$ and $[(X+2)^+]$ [42]. This is shown in the Q1 mass scan in the Fig. 4. The ratio of the intensities of the peak corresponding to *m/z* 325.1 and the peak corresponding to *m/z* 327.1 is 8.1E7 cps/2.8E7 cps = 100/34.6 and consequentially indicates that the chlorine ion is indeed present in the molecule of EO9.

An ion corresponding to the elimination of water from the molecular ion is also observed (*m/z* 307). It is clear from the isotopic pattern of the ions around *m/z* 307 that chlorine is still present in the remainder of the molecule.

The protonated molecular ion of EO9-Cl was induced to fragment in the collision cell and the resulting product ion spectrum is presented in Fig. 5. The main fragment ion corresponds to the loss of the CH_2O from the allylic alcohol and the elimination of the Cl atom (*m/z* 241). As expected, no typical Cl pattern is seen at the *m/z* of 241. After optimization of the MS parameters, the fragment ion at *m/z* 241 was the most abundant and used for quantitative MRM of EO9-Cl.

3.4. Non-stabilized urine

The stability of EO9 in non-stabilized urine was assessed at an initial concentration of 115.6 μM of EO9 by comparing the mean calculated concentrations against the concentration measured at time zero. The results are summarized in Table 2. EO9 was not stable in non-stabilized acidic urine at pH 5.1 at 43 °C. After 3 h, only 82.0% of the initial EO9 concentration was recovered. The main degradation products were EO5a (13.3%) and EO9-

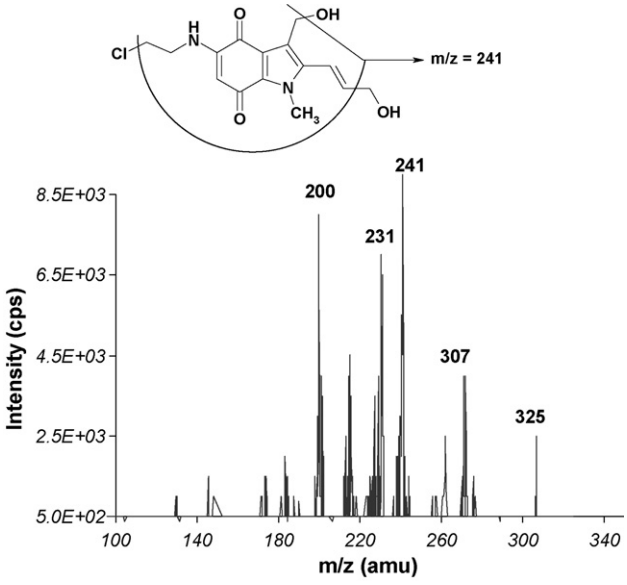

Fig. 5. MS/MS product ion scan of EO9-Cl (precursor ion *m/z* 325).

Table 2
Stability data of EO9 in non-stabilized urine

Condition	Time point (h)	Final pH	EO9 recovered (%)	EO5a recovered (%)	EO9-Cl recovered (%)	Mass balance (%)
43 °C	3	5.1	82.0	13.3	4.35	99.7
43 °C	3	5.9	86.2	8.60	4.54	99.3
43 °C	3	7.3	101	2.43	0.950	104
37 °C	3	5.1	92.5	9.58	3.56	106
37 °C	3	5.9	93.6	6.27	3.41	103
37 °C	3	7.3	92.1	1.61	0.637	94.3
Room temperature	3	5.10	98.8	4.41	2.35	106
Room temperature	3	5.90	95.1	2.60	1.33	99.0
Room temperature	3	7.30	103	0.780	0.210	104
Ice	24	5.1	95.6	3.18	1.26	100
Ice	24	5.9	97.2	0.732	0.393	98.3
Ice	24	7.3	102	0.347	0.158	102

Cl (4.35%). Since the total recovery was around 100% (99.7%), it can be concluded that under these conditions EO9 degrades exclusively into EO5a and EO9-Cl. However, during the 1-h instillation (43 °C, pH 5.1) it can be expected that approximately 6.6% of EO9 will be converted into EO5a and 2.1% into EO9-Cl (data not shown).

EO9 was stable under other tested conditions in non-stabilized urine at pH 5.1–7.3. The coefficient of variation (CV) values for EO9 were less than 10.4%.

3.5. Stabilized urine

Stability of EO9 in urine stabilized with TRIS buffer (5 mM, pH 9.0) (1:1, v/v) was assessed at an initial concentration of 57.8 μM of EO9 by comparing the mean calculated concentrations against the concentration measured at time zero. The results are summarized in Table 3.

EO9 was not stable in a stabilized urine with the final pH 7.3 and 7.4 after 3 months of storage at −20 °C. Only 72.1% of the initial EO9 concentration at pH 7.3 was recovered. The main degradation products were EO5a (3.49%) and EO9-Cl (16.2%). When the recoveries of EO9, EO5a, and EO9-Cl were added together, the total recovery ended up being 91.8%. This may suggest that other unknown degradation products were formed along with EO5a and EO9-Cl. Further research is needed to clarify the lack of mass balance.

At a higher pH level (urine batch 3; pH 8.3), the percent difference from time zero was less than 15%, indicating that EO9 can be considered stable for 3 months during storage at

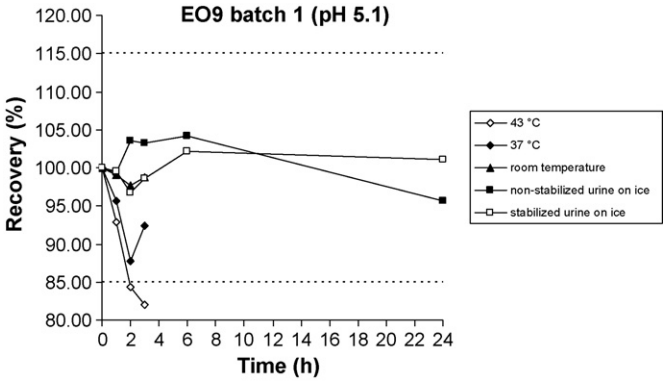

Fig. 6. Stability plot for EO9 in urine batch 1 (pH 5.1).

Table 3
Stability data of EO9 in stabilized urine

Condition	Time point	Final pH	EO9 recovered (%)	EO5a recovered (%)	EO9-Cl recovered (%)	Mass balance (%)
Ice	24 h	7.3	101	1.61	0.347	103
Ice	24 h	7.4	96.1	0.902	0.695	97.7
Ice	24 h	8.3	104	0.693	0.0279	105
−20 °C	3 months	7.3	72.1	3.49	16.2	91.8
−20 °C	3 months	7.4	83.3	2.32	13.8	99.4
−20 °C	3 months	8.3	88.3	0.447	0.961	89.7
−70 °C	3 months	7.3	107	1.05	1.15	109
−70 °C	3 months	7.4	96.1	0.920	0.997	98.0
−70 °C	3 months	8.3	110	0.444	−0.219	110
Freeze/thaw at −20 °C	3 cycles	7.30	98.7	0.702	0.669	100
Freeze/thaw at −20 °C	3 cycles	7.40	99.3	1.09	0.772	101
Freeze/thaw at −20 °C	3 cycles	8.30	112	0.0927	0.0853	112
Freeze/thaw at −70 °C	3 cycles	7.30	98.5	1.03	0.515	100
Freeze/thaw at −70 °C	3 cycles	7.40	106	0.706	0.561	108
Freeze/thaw at −70 °C	3 cycles	8.30	110	0.0995	0.0585	110

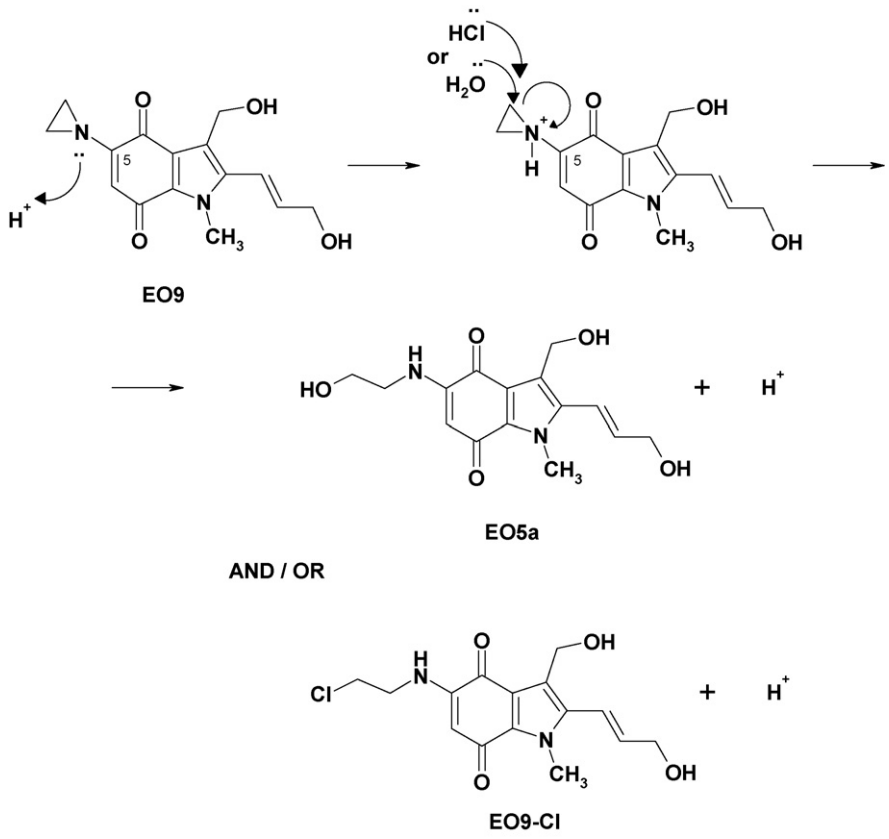

Fig. 7. Proposed degradation scheme of EO9 in (acidic) urine.

−20 °C. Less than 1% of EO9-Cl was formed in comparison to the more acidic urine, however, the total recovery was also around 90%.

EO9 was stable for the rest of the tested conditions in stabilized urine at pH 7.3–8.3. The CV values for EO9 were less than 6.74%.

The graph in Fig. 6 displays the stability plot of EO9 in urine at pH 5.1 for analyzed conditions.

3.6. Buffer strength

When the urine at an initial pH of 5.1 was stabilized with an equal volume of TRIS buffer (pH 9.0; 5 mM), the resulting urine pH was 7.3. With more acidic urine, it can be expected that higher buffer strength is needed.

3.7. Proposed degradation scheme of EO9

In acidic medium, EO9 decomposes principally into EO5a [6]. The degradation scheme for EO9 in acidic medium is proposed in Fig. 7. The initial step of the reaction is thought to be protonation of the trivalent nitrogen in the aziridine ring, yielding an iminium ion. This electrophilic center is then attacked by nucleophiles, such as water molecules, resulting in a degradation product with an ethanolamine group at C5 [6].

In urine that contains high content of chloride anions, an EO9-Cl product is formed (especially at the lower pH) (Fig. 7). The initial step of the reaction is again the protonation of the trivalent nitrogen in the aziridine ring, followed by a nucleophilic chloride attack resulting in the formation of EO9-Cl.

In time, there is substantially more EO9-Cl found in urine than EO5a, indicating that Cl^- is far more reactive than water molecules in this regard. To proof that EO9-Cl is not formed from EO5a, we incubated urine with EO5a. The experiments showed that EO5a is fairly stable in urine and was not converted into EO9-Cl.

4. Conclusion

Stability experiments with EO9 in human urine revealed that during instillation (1 h, 43 °C, pH 5.1) it can be expected that approximately 6.6% of the drug would be converted into EO5a and 2.1% into EO9-Cl. After the collection of urine (pH 5.1–7.3) and stabilization with TRIS buffer (pH 9.0; 5 mM), urine samples can be frozen (at −20 and −70 °C) and thawed at ambient temperature for up to three times and stored for up to 3 months at −70 °C.

A newly found degradation product of EO9, EO9-Cl, has been identified. The proposed degradation mechanism of EO9 in (acidic) urine is supported by the degradation mechanism described earlier by de Vries et al. in aqueous buffer solutions [6]. Results illustrate that EO9 becomes more stable in urine with increased pH, which is also in accordance with previous presented results [6].

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