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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.
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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

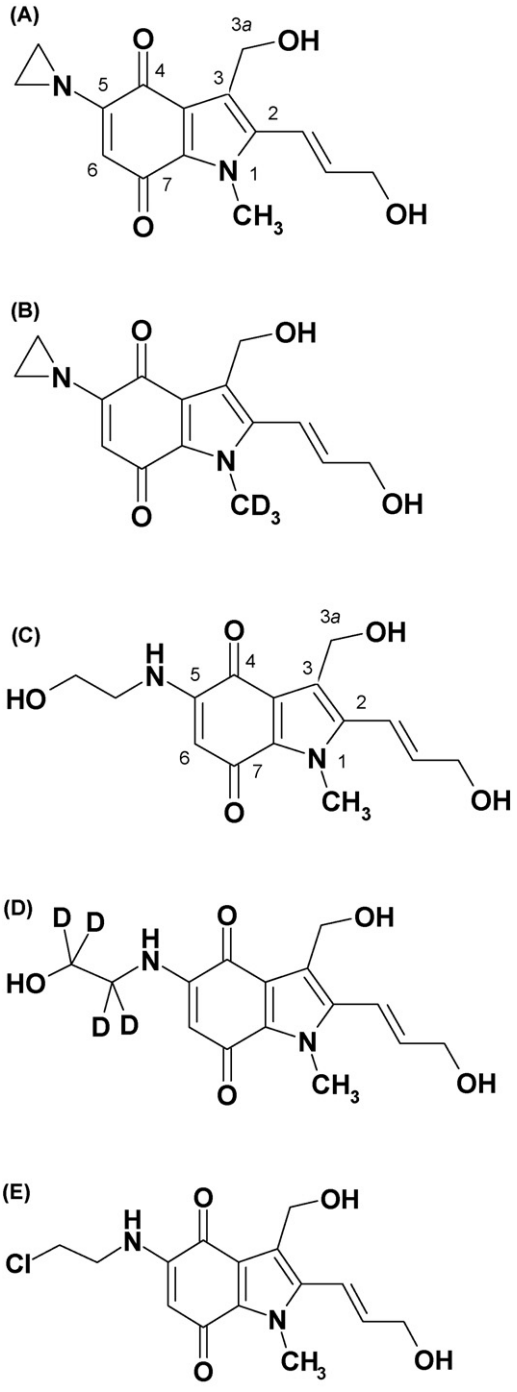

Fig. 1. Chemical structure of EO9, EO9-d_3, EO5a, EO5a-d_4 and EO9-Cl.

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