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Simultaneous Quantitative Analysis of EO9 (Apaziquone) and its Conversion Products EO5a and EO9-Cl in Human and Dog Urine by High-Performance Liquid Chromatography Coupled with Electrospray Tandem Mass Spectrometry

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Abstract: A sensitive and specific LC-MS/MS assay for the quantitative determination of anticancer agent EO9 and its conversion products EO5a and EO9-Cl in human and dog urine is presented. A 20-µL-urine aliquot was spiked with a mixture of deuterated internal standards EO9-d3 and EO5a-d4 and diluted with 180 µL 0.1 M ammonium acetate – methanol (7:3, v/v). Next, 25 µL-volumes were injected onto the HPLC system. Separation was achieved on a 150 × 2.1 mm C18 column using an alkaline eluent (1 mM ammonium hydroxide – methanol (gradient system)). Detection was executed by positive ion electrospray followed by tandem mass spectrometry. The assay quantifies a range from 0.1 µg/mL to 50 µg/mL for EO9, from 0.2 µg/mL to 50 µg/mL for EO5a and 0.1 µg/mL to 4 µg/mL for EO9-Cl using 20 µL of stabilized urine samples. Validation results demonstrate that EO9, EO5a and EO9-Cl concentrations can be accurately and precisely quantified in human and dog urine. This assay is used now to support pre-clinical and clinical pharmacologic studies with intravesically administered EO9.

Keywords: Indoloquinones, bioreductive anticancer drugs, intravesical route, bladder cancer, DNA adducts.

1. INTRODUCTION

The indoloquinone compound apaziquone (3-hydroxy-5-aziridinyl-1-methyl-2[indole-4,7-dione]-prop-β-en-α-ol; EO9, see Fig. (**1**) for structure) is a bioreductive anticancer drug that was selected for clinical evaluation on the basis of a novel mechanism of action and good preclinical anti-tumor activity [1-3].

EO9 is an inactive pro-drug that undergoes redox cycling leading to the formation of drug-derived DNA alkylating moieties or DNA-damaging reactive oxygen species. These alkylating intermediate species are capable of forming DNA adducts with single - strand breaks and DNA cross-linking and leading to cell kill [4, 5].

EO9 is extensively metabolized. One of the principal known metabolites/degradation products is EO5a, which has an open aziridine ring and shows less cytotoxicity than EO9. Another degradation product, which was discovered by us and described earlier, 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 [6, 7].

EO9 has a very short half-life in plasma ($t_{½}$ values ranging from 0.8 to 19 min in humans) and a relatively poor ability to penetrate through multicell layers *in vitro*. However, this is an advantage for chemotherapeutic treatment of cancers that arise in a delimited compartment, such as superficial bladder cancer [8, 9]. In this case, drug delivery can be accomplished *via* the intravesical route and drug exposure of tumor tissue can be maintained with therapeutically relevant drug concentrations within the bladder cavity [8, 9]. Consequently, currently ongoing clinical trials investigating EO9 in superficial bladder tumors with local drug delivery show promising response rates. Drug analysis in voided urine provides insight into the pharmacologic behavior of EO9 after intravesical administration [10-14].

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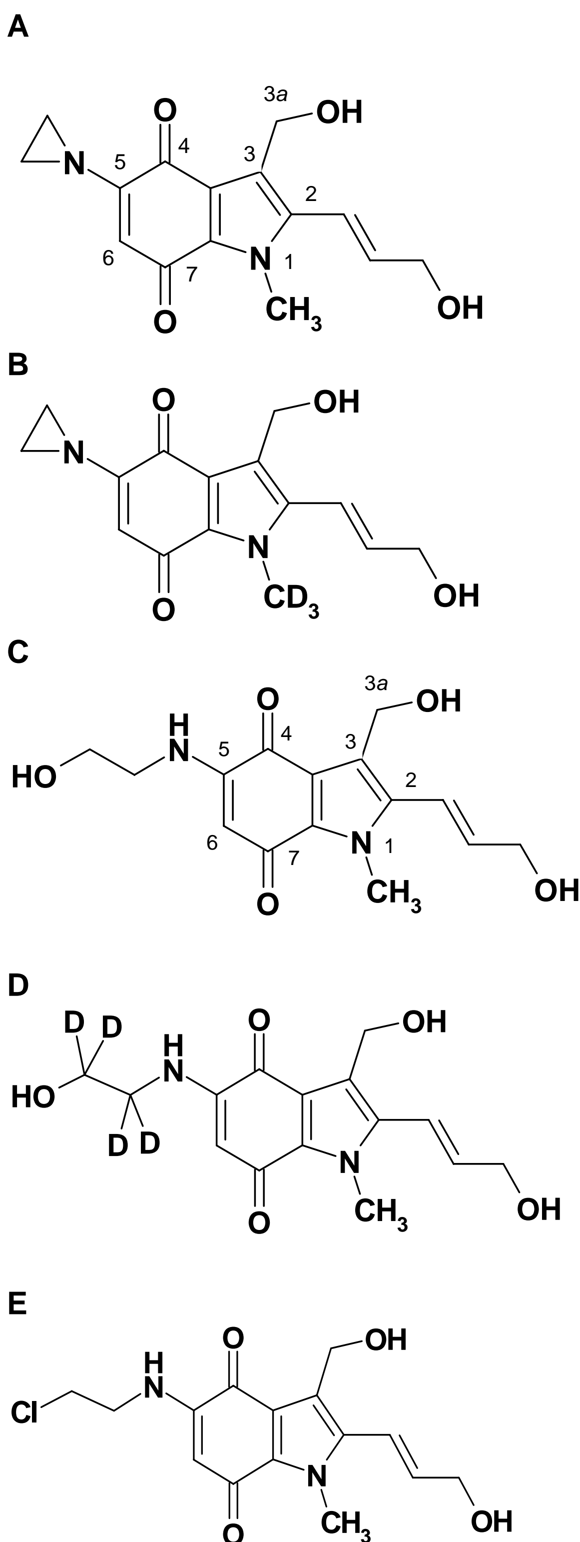


Fig. (1). Chemical structures of EO9, EO9-d3, EO5a, EO5a-d4 and EO9-Cl.

So far, however, only one analytical assay has been published describing the determination of EO9 and EO5a, but not EO9-Cl, in human urine using high-performance liquid chromatography coupled to the ultraviolet detection with low specificity [15]. We have published earlier a high performance liquid chromatography coupled to tandem mass spectrometry method (HPLC-MS/MS) method for the analysis of EO9 and EO5a in human plasma [16]. This assay was, however, not suitable for urine analysis. In order to quantify all three compounds of interest, EO9, EO5a and EO9-Cl in ongoing and upcoming preclinical and clinical studies, we developed a sensitive, specific, accurate, and rapid assay for the quantification of these analytes in human and dog urine using an HPLC-MS/MS. Deuterated internal standards for EO9 and EO5a were used for quantification. The method has been fully validated according to the FDA guidelines on bioanalytical validation [17, 18], and will be applied in the pharmacokinetic studies with intravesically administered EO9.

2. EXPERIMENTAL

2.1. Materials

EO9 ($C_{15}H_{16}N_2O_4$), EO9-d3 internal standard ($C_{15}H_{13}D_3N_2O_4$), EO5a ($C_{15}H_{18}N_2O_5$), EO5a-d4 internal standard ($C_{15}H_{14}D_4N_2O_5$) and the degradation product EO9-Cl ($C_{15}H_{17}N_2O_4Cl$) (see Fig. (**1**) for structures) were supplied by Spectrum Pharmaceuticals, Inc (Irvine, CA, USA). Methanol (LC gradient grade) was 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). Drug free dog urine was obtained from MPI research (Mattawan, MI, USA)

2.2. Preparation of Stock and Working Solutions

Two sets of stock solutions of EO9, EO5a and EO9-Cl were prepared from two independent weighings and dissolution in ethanol at a target concentration of 1 mg/mL. The solutions had to be placed in the ultrasonic bath for 2 hours to dissolve all the compounds in ethanol. The same procedure was repeated each time the solutions were taken out of the freezer. These solutions were further diluted with ethanol to yield separate working solutions for EO9, EO5a and EO9-Cl. One set of each working solutions was used to prepare calibration standards, the other to prepare quality control samples. The obtained concentrations of calibration standards working solutions were 800, 400, 200, 40, 20, 4 and 2 µg/mL for EO9; 800, 400, 200, 40, 20, 10 and 4 µg/mL for EO5a and 400, 100, 80, 60, 40, 30, 20, 10, 4 and 2 µg/mL for EO9-Cl. The quality control working solutions concentrations were 800, 200, 6 and 2 µg/mL for EO9; 800, 200, 12 and 4 µg/mL for EO5a and 60, 40, 20, 6 and 2 µg/mL for EO9-Cl. Separate stock solutions of EO9-d3 and EO5a-d4 were prepared in ethanol at a concentration of 1 mg/mL. An internal standards working solution was prepared by transferring 500 µL of EO9-d3 stock solution and 500 µL of EO5a-d4 stock solution to a 50.0 mL volumetric flask and adding 0.1 M ammonium acetate buffer pH 8.5 - methanol (7:3, v/v) to give a final concentration of 10,000 ng/mL for both internal standards. All solutions were stored at -20°C.

2.3. Preparation of Control Human and Dog Stabilized Urine

The formulated product of EO9 (EOquin™) is used in clinical studies. This formulation consisted of 4 mg of EO9, 25 mg mannitol, 10 mg sodium bicarbonate and 40 mL of the

diluent. The diluent formulation contained 10 mg/mL sodium bicarbonate (20 mg/mL for dogs), 0.2 mg/mL EDTA, and 0.6 mL propylene glycol in sterile water for injection (SWFI). The average volume collected after 60 min of instillation was 120 ± 47 mL, which consisted of EOquin formulation solution – human urine (1:2, v/v) [15]. In dogs however, the total collected urine was approximately 80 mL, which consisted of EOquin formulation solution – dog urine (1:1, v/v). After the collection of samples, an equal volume of TRIS buffer (5mM; pH 9.0) was added to raise the pH of urine and therefore prevent ex vivo degradation (stabilization of urine).

EO9 is most stable at approximately pH 8.5. To mimic the *in vivo* situation, we prepared the stabilized control human urine solutions, which consisted of 100 mL blank formulation (0.525 g sodium bicarbonate, 0.01 g EDTA, 62.5 mg mannitol, 30 mL propylene glycol in 70 mL SWFI), 200 mL control human urine and 300 mL TRIS buffer (5mM; pH 9.0). The stabilized control dog urine solution consisted of 100 mL blank formulation (1.03 g sodium bicarbonate, 0.01 g EDTA, 62.5 mg mannitol, 30 mL propylene glycol in 70 mL SWFI), 100 mL control dog urine and 200 mL TRIS buffer (5mM; pH 9.0).

2.4. Preparation of Calibration Standards and Quality Control Samples in Stabilized Human and Dog Urine

Calibration standards containing EO9, EO5a and EO9-Cl were prepared freshly in a range from 0.1 µg/mL to 50 µg/mL for EO9, from 0.2 µg/mL to 50 µg/mL for EO5a and 0.1 µg/mL to 4 µg/mL for EO9-Cl by adding 25 µL of EO9, EO5a and EO9-Cl working solutions in ethanol (described in section 2.2) to 2 mL-Eppendorf tubes. This solutions were evaporated and the compounds were redissolved in stabilized human urine and stabilized dog urine, respectively, to yield concentrations of 0.1, 0.2, 1.0, 2.0, 10, 20, 40 and 50 µg/mL for EO9; 0.2, 0.5, 1.0, 2.0, 10, 20, 40 and 50 µg/mL for EO5a, and 0.1, 0.2, 0.5, 1.0, 1.5, 2.0, 3.0 and 4.0 µg/mL for EO9-Cl in human and dog urine. The calibration standards were vortex-mixed for approximately 30 sec before processing. Standards were processed in singlicate and analyzed in duplicate.

Quality control samples for EO9, EO5a and EO9-Cl were prepared in stabilized human respectively dog urine at concentrations of 0.1, 0.3, 10 and 40 µg/mL for EO9; 0.2, 0.6, 10 and 40 µg/mL for EO5a, and 0.1, 0.2, 2.0 and 3.0 µg/mL for EO9-Cl using the same procedure as described above for the calibration standards. The calibration standards and quality control samples were prepared fresh for each validation run.

2.5. Sample Preparation

Sample pretreatment was performed at ambient temperatures. 20 µL of a working solution containing the internal standards was added to 20 µL of stabilized human or dog urine aliquots and diluted with 180 µL 0.1 M ammonium acetate buffer pH 8.5 – methanol (7:3, v/v). The samples were vortexed for 10 sec and the clean solution was transferred to a glass autosampler vial with insert.

2.6. HPLC

The HPLC system comprises an HP1100 binary pump, degasser and HP1100 autosampler (Agilent Technologies, Palo Alto, CA). Gradient chromatography was performed using a Gemini C18 column (150 × 2.1 mm ID, particle size 5 µm). The mobile phase consisted of 1 mM ammonium hydroxide in water (A) and 100% methanol (B) and was 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 kept at 10°C.

2.7. 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) [16]. 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-d3, EO5a, EO5a-d4 and EO9-Cl, respectively, with dwell times of 150 ms. EO9-d3 was used as an internal standard for EO9 and EO9-Cl. EO5a-d4 was used to quantify EO5a. 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. Furthermore, declustering potential (DP) for EO9 and EO9-d3 was 66 V and for EO5a, EO9-Cl and EO5a-d4 was 31 V. Focusing potential (FP) for EO9 and EO9-d3 was 310 V and for EO5a, EO9-Cl and EO5a-d4 was 350 V. The optimized collision energy (CE) was 17 V for EO9 and EO9-d3, 45 V for EO5a and EO5a-d4 and 40 V for EO9-Cl. Finally, the ion spray voltage was kept at 5500 V, with a source temperature of 250°C.

2.8. Validation Procedures

A full validation programme according to the FDA guidelines was performed for the assay in human urine [17, 18]. For the assay in dog urine partial validation was executed by means of accuracy and precision, specificity and selectivity and stability in urine under storage conditions according to FDA rules.

2.8.1. Linearity

For the validation of the assay in human urine, calibration standards (8 non-zero standards of the analytes) were prepared in stabilized control human urine and analyzed in duplicate in three analytical runs. For the validation of the assay in dog urine, calibration standards (8 non-zero standards of the analytes) were prepared in stabilized control dog urine and analyzed in duplicate in one analytical run.

The linear regression of the ratio of the areas of the analyte and internal standard peaks versus the concentration were weighed by $1/x^2$ (the reciprocal of the squared concentration). Concentrations were back-calculated from the constructed calibration curve and deviations from the nominal concentrations should be within ± 20% for the lower limit of quantitation (LLOQ) and within ± 15% for other concentrations with coefficient of variation (C.V.) values less than 20% and 15% respectively [19].

2.8.2. Accuracy and Precision

Five replicates of the quality control samples (see section 2.4) were analyzed in each analytical run together with the

calibration standards. Accuracies were determined as the percentage difference of the measured concentration from the nominal concentration and the C.V. was used to report the precision.

The intra and inter-assay accuracies (% bias) should be within ± 20% at the LLOQ level and within ± 15% at the other concentrations. The intra and inter-assay precisions should be less than 20% at the LLOQ level and less than 15% at the other concentrations.

The ability to dilute samples originally above the upper limit of quantitation (ULOQ) of the calibration curve was demonstrated by and analyzing validation samples containing 5 times the ULOQ for EO9, EO5a and EO9-Cl. Five replicates of each sample were analyzed in one analytical run after 20 time dilution in stabilized control human and control dog urine.

2.8.3. Limit of Detection

The limit of detection (LOD) for EO9, EO5a and EO9-Cl, with the responses of the analytes at 2 times the response of the blank was established in three analytical runs in human urine.

2.8.4. Specificity and Selectivity

To investigate whether endogenous matrix constituents interfered with the assay, six individual batches of control drug-free human and dog urine samples containing neither analyte nor internal standard (double blank), samples containing only internal standard (blank), and samples spiked at the LLOQ were prepared. Samples were prepared and analyzed according to the described procedures. Peak areas of compounds co-eluting with the analyte or internal standard should not exceed 20% of the analyte peak area at the LLOQ or 5% of the internal standard area. Deviations from the nominal concentrations should be within ± 20% for the LLOQ samples.

2.8.5. Ion Suppression

Ion suppression was determined by comparing the analytical response of processed quality control samples with the analytical response of blank samples reconstituted with solutions containing the analytes and internal standards in 0.1 M ammonium acetate pH 8.5 - methanol (7:3, v/v). The loss of signal represents the ion-suppression. These experiments were performed in triplicate at three concentration levels. Ion suppression experiments for the internal standards were performed in a similar way [20].

2.8.6. Carry-Over

Carry-over was tested by injecting two blank matrix samples after injecting an ULOQ sample. Carry-over is considered acceptable when response in the first blank matrix at the retention times of the analytes are less than 20% of the response in an LLOQ samples.

2.8.7. Stability

To test the stability of EO9, EO5a and EO9-Cl during processing at the clinical sites, the stability of analytes was evaluated in the non-stabilized control human urine maintained on an ice/water bath. The stability of analytes in stabilized human and dog urine after 3 freeze/thaw cycles was investigated by comparing quality control samples that had been frozen and thawed three times with freshly prepared quality control samples. The stability of EO9, EO5a and EO9-Cl in human and dog urine under processing (ambient temperatures) and storage (-70°C) conditions was evaluated. Furthermore, the stability in the final solution and the re-injection reproducibility in the auto sampler was determined after 24h. Finally, the long-term stability of EO9, EO5a and EO9-Cl was evaluated in the stock solutions, in the working solutions after storage at -70°C and in human and dog urine after storage at -70°C.

Stability experiments in the biomatrix were executed at 2 concentration levels for EO9 (0.3 and 40 μg/mL), for EO5a (0.6 and 40 μg/mL) and for EO9-Cl (0.3 and 3.0 μg/mL) in triplicate. The analytes were spiked separately to the biological matrix. EO9, EO5a and EO9-Cl are considered stable in the stock and working solutions when 90-110% of the fresh sample's ratio is found and they are considered stable in biological matrices or final solutions when 85-115% of the initial concentration is recovered.

3. RESULTS AND DISCUSSION

3.1. Sample Pretreatment

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

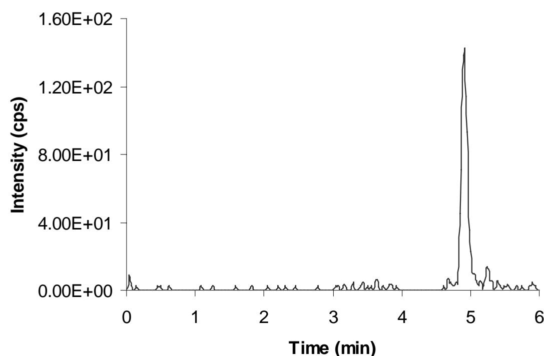
Therefore, dilution of the urine samples was chosen as the sample pretreatment. The samples were diluted 10 times prior to analysis to ascertain that the final concentration would fit into the linear calibration range and to prevent contamination of the mass spectrometer with salts present in urine.

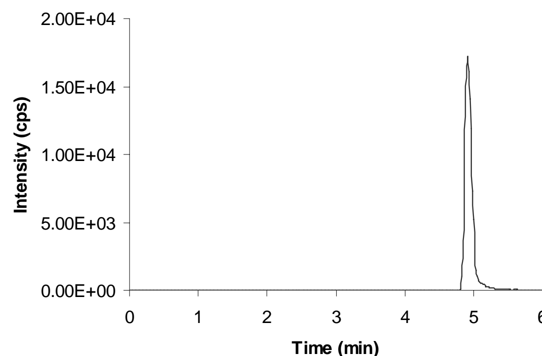
3.2. Chromatography

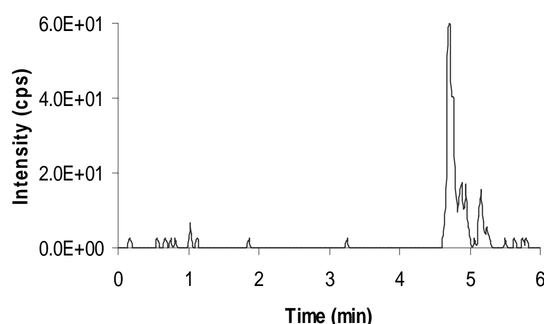
To our knowledge, no other assay has been described so far for the simultaneous determination of EO9, EO5a and EO9-Cl in human urine using HPLC coupled to the tandem MS. The chromatography was described earlier by us for determination of EO9 and EO5a in human plasma [16]. Representative chromatograms of EO9, EO5a and the internal standards at their LLOQ levels in stabilized human urine are depicted in Fig. (**2**). Peak shapes were excellent with the asymmetry factors of 1.0 for EO9, EO9-Cl and EO9-d3 and 1.2 for EO5a and EO5a-d4 and the capacity factors (k') for all analytes of approximately 3. Overall LC run time was only 6 min.

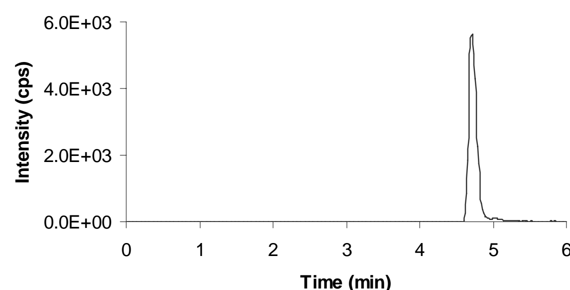
3.3. Mass Spectrometry

In Fig. (**3**), Q1 mass spectrum EO9-Cl is presented. Apart from the protonated species at m/z 325 for EO9-Cl, sodium adducts are also observed at m/z 347, and the characteristic Cl isotope abundances are visible. Figs. (**4-6**), show MS/MS product ion scans (Q3 scan) of EO9, EO5a and EO9-Cl, respectively, across the range of m/z 100-400. An ion corresponding to the elimination of water (m/z 271) from the molecular ion of EO9 was chosen for the parent mass transition









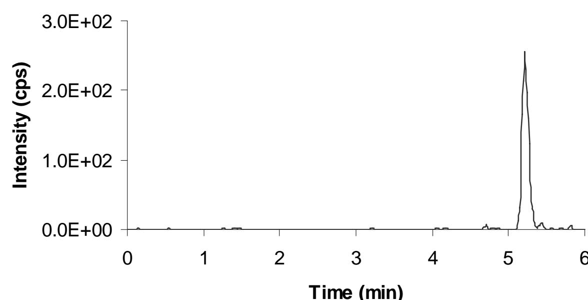


Fig. (2). Representative HPLC-MS/MS chromatograms of an LLOQ sample for EO9 (0.1 μg/mL), EO9-d3 (1,000 ng/mL), EO5a (0.2 μg/mL), EO5a-d4 (1,000 ng/mL) and EO9-Cl (0.1 μg/mL) from control human urine.

of EO9, because it was the most prominent, with its elimination occuring immediately in the ion source. The most abundant fragments observed in the fragmentation of the *m/z* of 271 (EO9; Fig. **4**), 307 (EO5a; Fig. **5**) and 325 (EO9-Cl; Fig. **6**) were product ions at *m/z* of 241, 231 and 241, respectively, corresponding to the loss of the CH_2O from the allylic alcohol (*m/z* 241), the cleavage of the amine moiety and the elimination of water (*m/z* 231) and the loss of the CH_2O from the allylic alcohol and the elimination of the Cl atom (*m/z* 241), respectively. As expected, no typical Cl pattern is seen at the *m/z* of 241. Consequently, multiple reaction monitoring (MRM) parameters were optimized on the *m/z* of 271/241, 307/231 and 325/241 transitions for EO9, EO5a and EO9-Cl [19, 20]. For EO9-d3 and EO5a-d4 similar transitions as for EO9 and EO5a, respectively, were selected and optimized.

3.4. Validation

3.4.1. Linearity

The assay was linear over a concentration range of 0.1 μg/mL to 50 μg/mL for EO9, 0.2 μg/mL to 50 μg/mL for EO5a and 0.1 μg/mL to 4 μg/mL for EO9-Cl in stabilized human and dog urine. This range proved to be sufficient when measuring the samples from the (pre)-clinical studies with EO9. Interestingly, the linear range of EO9-Cl was much

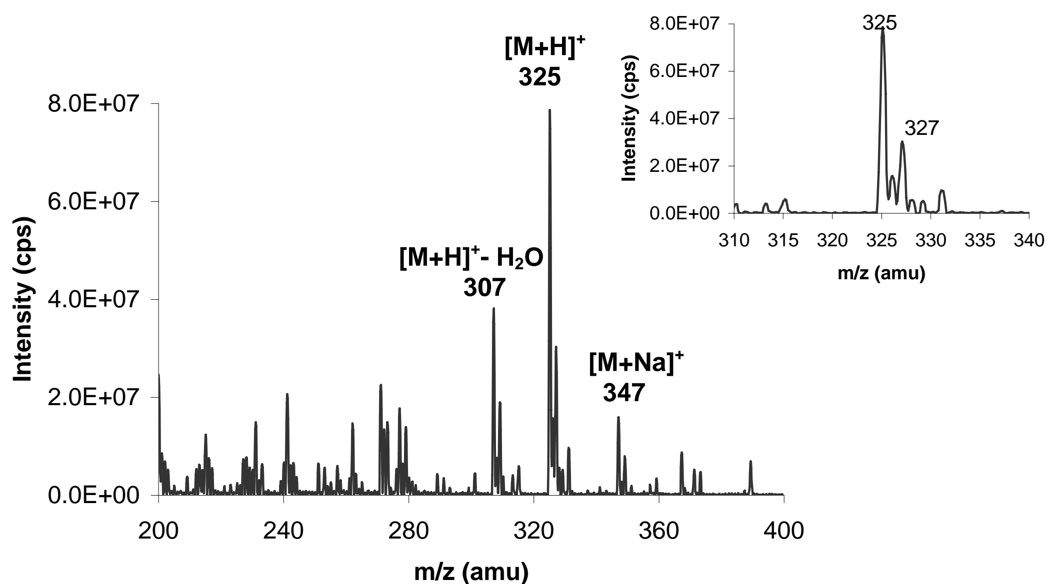


Fig. (3). Q1 (*m/z* 200-400) mass spectrum of EO9-Cl.

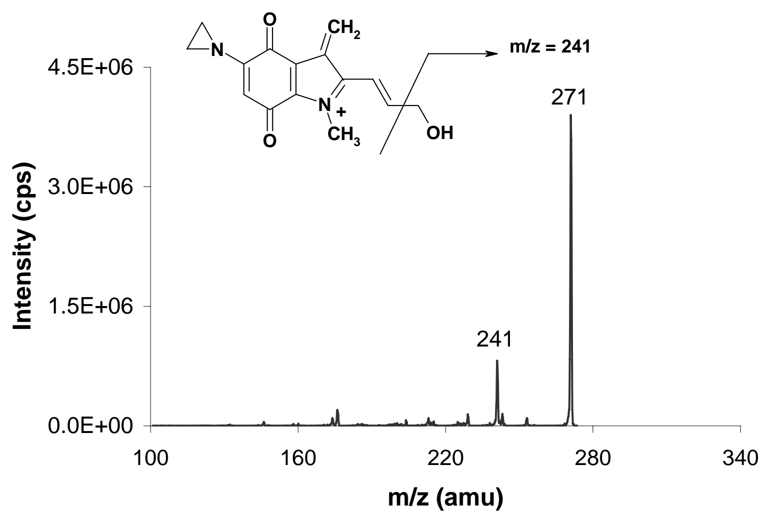


Fig. (4). MS/MS product ion scan of EO9 (precursor ion m/z 271).

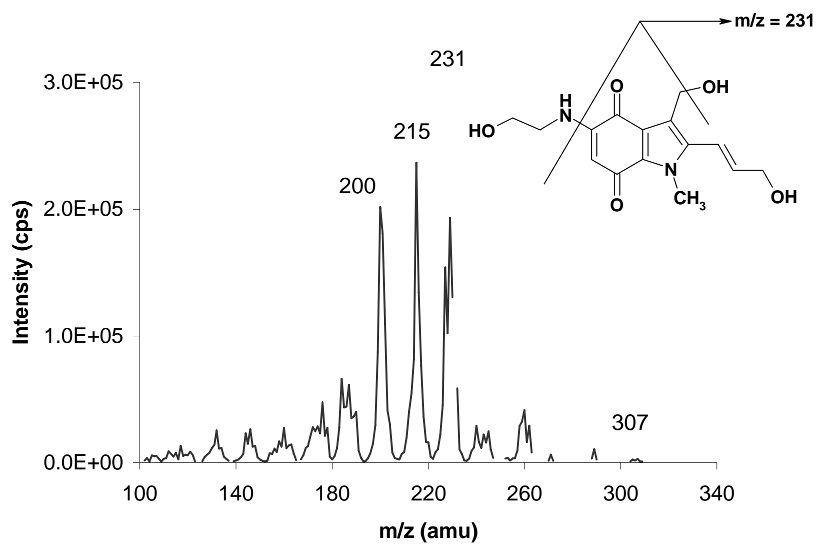


Fig. (5). MS/MS product ion scan of EO5a (precursor ion m/z 307).

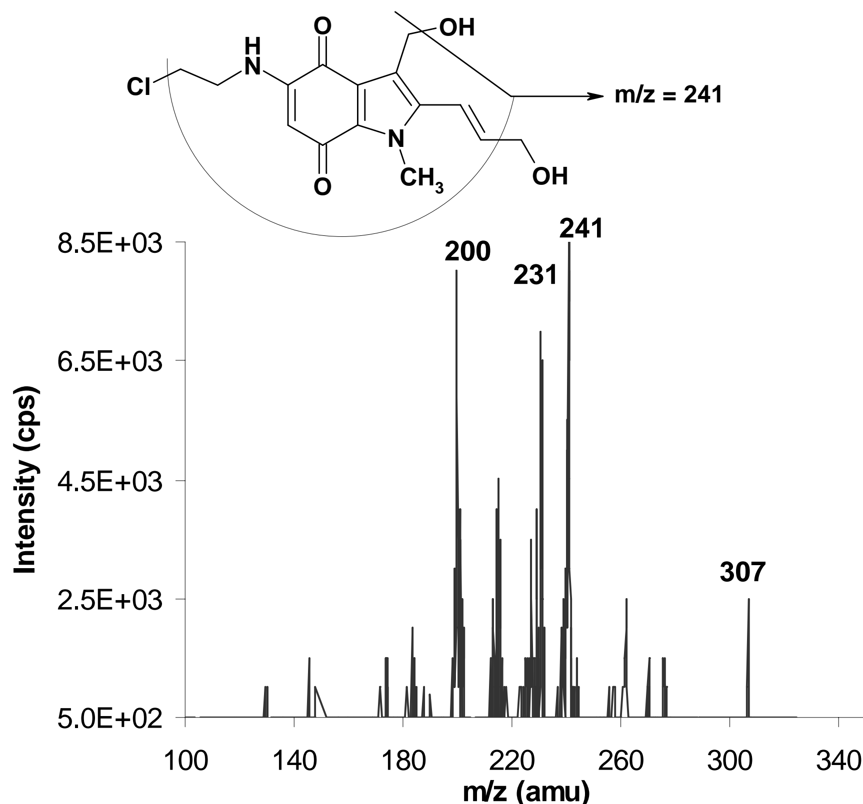


Fig. (6). MS/MS product ion scan of EO9-Cl (precursor ion *m/z* 325).

smaller than for EO5a and EO9. This may be due to the different ionization properties of EO9-Cl compared to EO9 and EO5a.

Namely, it is believed that signal suppression occurs when matrix components compete with the analyte ions for access to the droplet surface for gas phase emission. This is described in a widely accepted model, proposed by Iribarne and Thomson [19], which explains the formation of gas phase ions by direct emission from the surface of highly charged spray droplets. Therefore, when contaminants, from samples processing or analyte extraction from physiological matrices, are ionized at the same time as the compound of interest (EO9-Cl), matrix signal ionization tend to occur, leading to the signal suppression of the analyte.

Another model, which may explain the limited linear range of EO9-Cl, is based on the hypothesis that the ion evaporation rate from the droplet is proportional to the ion concentration in the droplet [21]. The basis of this model was the assumption that the Iribarne and Thomson (as described above) ion evaporation model was the principal mechanism for the ion transfer to the gas phase and involves the competition among ions for the limited number of excess charge sites on the generated droplet during ESI. This model was proposed and supported by the observations of Enke *et al.* [22], that at low concentrations of analyte, the response curves were linear and indifferent of other present low concentration analytes. However, at higher analyte concentrations, the response became independent of analyte concentration, but highly affected by the presence of other analytes [23, 24].

Because EO9-Cl has different chemical properties (less hydrophilic) than EO9 and EO5a, the above described models might contribute to the explanation of the non-linear behavior of EO9-Cl.

Using linear regression and $1/x^2$ weighing, the lowest total bias and the most constant bias across the range were obtained. Correlation coefficients of the calibration curves in human urine were better than 0.9945 for EO9, better than 0.9933 for EO5a and better than 0.9914 for EO9-Cl. Correlation coefficients of the calibration curve in dog urine were 0.9984 for EO9, 0.9974 for EO5a and 0.9948 for EO9-Cl. At all concentration levels, deviation of measured concentrations from nominal concentration were between -8.9% and 6.8% with C.V. values less than 11.7% for human urine and between -8.4% and 12.4% with C.V. values less than 16.3% for dog urine.

3.4.2. Accuracy and Precision

Assay performance (inter-assay accuracies and precisions) data for EO9, EO5a and EO9-Cl is summarized in Table **1**. The intra-assay accuracies (% bias) in human urine were within ± 12.4% for EO9, within ± 10.7% for EO5a and within ± 13.6% for EO9-Cl for all concentrations and found to be acceptable (data not shown). The intra-assay precisions for EO9 in human urine were less than 7.79%, for EO5a less than 12.8% and for EO9-Cl less than 5.42% for all concentrations and found to be acceptable. The intra-assay accuracies (% bias) in dog urine were within ± 4.3% for EO9, within ± 9.0% for EO5a and within ± 9.0% for EO9-Cl for all concentrations and found to be acceptable (data not shown). The intra-assay precisions in dog urine for EO9

were less than 7.62%, for EO5a less than 10.6% and for EO9-Cl less than 12.7% for all concentrations and found to be acceptable. As defined by the lower and upper validation sample concentrations possessing acceptable accuracy and precision, the validated range for EO9, based on 20 µL of stabilized human/dog urine is from 0.1 µg/mL to 50 µg/mL, for EO5a is from 0.2 µg/mL to 50 µg/mL and for EO9-Cl is from 0.1 µg/mL to 4 µg/mL, respectively.

3.4.3. Limit of Detection

The limit of detection is the concentration of EO9, EO5a and EO9-Cl in which the response of the analyte is 2 times higher than the response of the blank, and is established in three analytical runs in human urine. The mean LOD concentration is estimated at 6.7 ng/mL for EO9, 31.4 ng/mL for EO5a and 3.3 ng/mL for EO9-Cl.

3.4.4. Specificity and Selectivity

The MRM chromatograms of six batches of stabilized control drug-free human and dog urine contained no co-eluting peaks > 20% of the EO9, EO5a and EO9-Cl area at the LLOQ level, and no co-eluting peaks > 5% of the area of internal standards. Deviations from the nominal concentrations at the LLOQ level in human urine were between -14.0 and 18.8% for EO9, between -18.5 and 3.8% for EO5a and between -19.6 and -5.7% for EO9-Cl and found to be acceptable. Deviations from the nominal concentrations at the LLOQ level in dog urine were between -18.2 and 5.8% for EO9, between -4.3 and 10.9% for EO5a and between -18.2 and -7.0% for EO9-Cl and found to be acceptable.

3.4.5. Ion Suppression

The mean ion-suppressions for EO9 and its internal standard EO9-d3 in human urine were 22.4 ± 7.5% and

Table 1. Assay Performance Data for EO9, EO5a and EO9-Cl in Stabilized Human and Dog Urine

Compound	Matrix (Urine)	Nominal Concentration (µg/mL)	Measured Concentration (µg/mL)	Overall Accuracy (%)	Intra-Assay Precision (%)	Inter-Assay Precision (%)	Number of Replicates
EO9	human	0.103	0.102	99.0	7.79	7.42	15
	human	0.309	0.312	101	4.60	9.12	15
	human	10.3	10.3	99.2	4.26	8.39	15
	human	41.1	42.2	102	3.23	2.32	15
	dog	0.103	0.101	97.8	7.62	-	5
	dog	0.309	0.319	103	6.47	-	5
	dog	10.3	10.7	104	2.84	-	5
	dog	41.1	41.7	101	4.77	-	5
EO5a	human	0.211	0.207	97.9	12.8	12.0	15
	human	0.632	0.600	94.9	10.1	8.97	15
	human	10.5	9.89	94.2	5.27	5.60	15
	human	42.1	41.3	98.1	5.43	5.57	15
	dog	0.211	0.201	95.1	9.07	-	5
	dog	0.632	0.625	98.8	10.6	-	5
	dog	10.5	10.3	98.3	3.99	-	5
	dog	42.1	38.3	91.0	3.93	-	5
EO9-Cl	human	0.101	0.105	104	4.91	7.83	15
	human	0.304	0.306	101	5.32	8.11	15
	human	2.02	2.07	102	5.42	4.69	15
	human	3.04	3.36	110	3.22	3.40	15
	dog	0.101	0.102	101	12.7	-	5
	dog	0.304	0.298	97.9	8.43	-	5
	dog	2.02	2.05	102	10.4	-	5
	dog	3.04	3.31	109	2.96	-	5

22.7 ± 11.4%, respectively. The mean ion-suppressions for EO5a and its internal standard EO5a-d4 were 29.3 ± 4.9% and 30.0 ± 9.5%, respectively. The mean ion-suppression for EO9-Cl was 24.9 ± 6.7%. The ion-suppression was much higher in human urine in comparison to human plasma (± 5%). The high ion-suppression in urine is probably due to the salts present, which compete with the analytes for access to the droplet surface for gas phase emission.

3.4.6. Carry-Over

No responses in the first blank matrix at the retention times of EO9, EO5a and EO9-Cl were measured, indicating that no carry-over takes place.

3.4.7. Stability

The stability data for EO9, EO5a and EO9-Cl are summarized in Tables **2-4**, respectively. EO9, EO5a and EO9-Cl are stable in blank formulation in human urine for up to 1 hour at an ice/water bath. EO9, EO5a and EO9-Cl are stable in human and dog urine for at least three freeze (-70 ^{o}C) / thaw cycles, and are also stable in human and dog urine stored at nominally ambient temperatures for up to 6 hours. Furthermore, EO9, EO5a and EO9-Cl are stable up to 10 days in the final extract (from human urine) at ambient temperatures. Re-injection reproducibility was established and the analytical run can be re-injected after at least 24 hours of storage in the autosampler.

Table 2. Stability Data of EO9

Compound	Conditions	Matrix	Initial Conc. (µg/mL)	Found Conc. (µg/mL)	Dev. (%)	C.V. (%)	Number of Replicates
EO9	ice/water batch, 1h	Blank formulation – urine	0.236	0.227	-3.79	5.51	3
		(1:2, v/v)	40.8	41.1	0.731	1.33	3
EO9	3 freeze (-70ºC)/ thaw	human urine	0.312	0.309	-0.744	4.51	3
	cycles		39.8	36.1	-9.16	3.74	3
EO9	3 freeze (-70ºC)/ thaw	dog urine	0.349	0.366	4.88	3.73	3
	cycles		43.2	40.0	-7.54	0.250	3
EO9	Ambient, 6h	human urine	0.312	0.296	-4.99	2.35	3
			39.8	40.1	0.666	5.09	3
EO9	Ambient, 6h	dog urine	0.349	0.311	-10.8	4.74	3
			43.2	45.0	4.25	3.45	3
EO9	4ºC, 15 days	final extract	0.312	0.301	-3.61	11.1	3
			39.8	38.4	-3.58	0.259	3
EO9	Autosampler, 10ºC, 24h	final extract (human urine)	0.280	0.288	2.96	5.12	3
			9.82	10.0	2.12	4.06	3
			41.6	44.3	6.45	1.53	3
EO9	Autosampler, 10ºC, 24h	final extract (dog urine)	0.320	0.329	2.71	1.68	3
			10.7	10.4	-2.80	2.88	3
			41.6	41.6	0.00	5.54	3
EO9	-20ºC, 6 months	working solutions in ethanol	1.00	0.951	-5.01	7.09	3
			2.02	1.86	-7.88	22.0	3
			10.0	10.4	4.56	1.87	3
			20.2	21.5	6.59	8.00	3
			100	94.6	-5.34	5.67	3
			202	196	-3.52	2.38	3
			403	369	-8.37	2.00	3
			503	551	9.71	3.72	3
EO9	-70ºC, 6 months	stabilized human urine	0.315	0.320	1.60	12.4	3
			39.8	41.9	5.30	6.07	3
EO9	-70ºC, 3 months	stabilized dog urine	0.349	0.319	-8.60	19.6	3
			43.2	44.4	2.93	5.61	3

Notes: C.V. = coefficient of variation; Dev = deviation from time zero.

Table 3. Stability Data of EO5a

Compound	Conditions	Matrix	Initial Conc. (µg/mL)	Found Conc. (µg/mL)	Dev. (%)	C.V. (%)	Number of Replicates
EO5a	ice/water batch, 1h	Blank formulation - urine	0.603	0.581	-3.65	10.8	3
		(1:2, v/v)	40.4	39.1	-3.22	7.13	3
EO5a	3 freeze (-70ºC)/ thaw	human urine	0.600	0.602	0.333	7.26	3
	cycles		38.9	37.0	-4.80	3.32	3
EO5a	3 freeze (-70ºC)/ thaw	dog urine	0.611	0.589	-3.65	10.5	3
	cycles		39.1	39.5	1.02	2.16	3
EO5a	Ambient, 6h	human urine	0.600	0.593	-1.11	9.02	3
			38.9	37.7	-2.92	3.77	3
EO5a	Ambient, 6h	dog urine	0.611	0.601	-1.64	4.52	3
			39.1	41	4.86	6.88	3
EO5a	4ºC, 15 days	final extract	0.600	0.566	-5.67	3.93	3
			38.9	36.8	-5.32	3.55	3
EO5a	Autosampler, 10ºC, 24h	final extract (human urine)	0.591	0.652	10.2	6.39	3
			9.76	10.2	4.17	8.31	3
			38.8	40.4	4.21	5.85	3
EO5a	Autosampler, 10ºC, 24h	final extract (dog urine)	0.619	0.653	5.44	1.53	3
			10.4	9.80	-5.50	6.62	3
			39.0	38.9	-0.342	1.71	3
EO5a	-20ºC, 6 months	working solutions in ethanol	2.04	2.05	0.404	7.35	3
			5.10	4.77	-6.46	11.7	3
			10.2	9.85	-3.52	5.73	3
			20.4	20.5	0.290	11.9	3
			102	101	-1.02	7.29	3
			204	197	-3.33	8.02	3
			408	388	-4.65	2.07	3
			510	518	1.69	6.48	3
EO5a	-70ºC, 6 months	stabilized human urine	0.600	0.612	2.00	15.1	3
			38.9	39.3	1.03	7.36	3
EO5a	-70ºC, 3 months	stabilized dog urine	0.611	0.592	-3.22	3.13	3
			39.1	41.0	4.86	4.03	3

Notes: C.V. = coefficient of variation; Dev = deviation from time zero.

Finally, EO9, EO5a and EO9-Cl are stable in the stock solutions for up to 6 months of storage at –20°C [16]. EO9, EO5a and EO9-Cl are stable in the working solutions in ethanol and in the stabilized human urine for up to 6 months and in the stabilized dog urine for up to 3 months at nominally -70°C.

4. CONCLUSIONS

An accurate, simple, reproducible, and selective LC-MS/MS assay has been developed for the quantification of EO9 and its conversion products EO5a and EO9-Cl in human and dog urine. Using 20 µL stabilized human and dog urine aliquots, the assay quantifies a range of 0.1 µg/mL to 50 µg/mL for EO9, 0.2 µg/mL to 50 µg/mL for EO5a, and of 0.1 µg/mL to 4 µg/mL for EO9-Cl. Validation results demonstrate that the EO9, EO5a and EO9-Cl concentrations can be accurately and precisely quantified in human and dog urine. This assay is used to support (pre)-clinical pharmacologic studies with EO9.

ABBREVIATIONS

API	=	Atmospheric pressure ionisation
CE	=	Collision energy
C.V.	=	Coefficient of variation
DEV	=	Deviation from time zero
DP	=	Declustering potential

Table 4. Stability Data of EO9-Cl

Compound	Conditions	Matrix	Initial Conc. (µg/mL)	Found Conc. (µg/mL)	Dev. (%)	C.V. (%)	Number of Replicates
EO9-Cl	Ice/water batch, 1h	Blank formulation - urine	0.293	0.304	3.76	4.76	3
		(1:2, v/v)	2.59	2.69	3.73	3.36	3
EO9-Cl	3 freeze (-70ºC)/ thaw	human urine	0.305	0.275	-9.85	6.52	3
	cycles		2.84	2.61	-8.09	2.24	3
EO9-Cl	3 freeze (-70ºC)/ thaw	dog urine	0.328	0.362	10.3	3.61	3
	cycles		2.67	2.78	4.38	4.98	3
EO9-Cl	Ambient, 6h	human urine	0.305	0.275	-9.74	2.62	3
			2.84	2.63	-7.50	5.93	3
EO9-Cl	Ambient, 6h	dog urine	0.328	0.287	-12.6	11.5	3
			2.67	2.69	0.875	6.72	3
EO9-Cl	4ºC, 10 days	final extract	0.305	0.329	7.88	0.63	3
			2.84	3.16	11.1	4.66	3
EO9-Cl	Autosampler, 10ºC, 24h	final extract (human urine)	0.297	0.274	-7.64	0.73	3
			2.06	1.99	-3.55	4.52	3
			3.31	3.38	2.01	2.52	3
EO9-Cl	Autosampler, 10ºC, 24h	final extract (dog urine)	0.302	0.309	2.32	3.79	3
			2.00	2.19	9.68	8.03	3
			3.27	3.12	-4.69	9.05	3
EO9-Cl	-20ºC, 6 months	stock solution in ethanol	1000	956	-4.44	11.1	3
EO9-Cl	-20ºC, 6 months	working solutions in ethanol	2.00	1.91	-4.64	6.73	3
			10.0	9.20	-8.00	1.56	3
			20.0	21.1	5.57	3.89	3
			30.0	27.4	-8.67	4.33	3
EO9-Cl	-70ºC, 6 months	stabilized human urine	0.305	0.260	-14.7	3.81	3
			2.84	2.83	-0.362	4.77	3
EO9-Cl	-70ºC, 3 months	stabilized dog urine	0.328	0.324	-1.32	14.9	3
			2.67	2.58	-3.25	2.54	3

EDTA = Ethylenediaminetetraacetic acid

ESI = Electrospray ionisation

FDA = Food and Drug Administration

FP = Focusing potential

LLOQ = Lower limit of quantitation

LOD = Limit of detection

MRM = Multiple reaction monitoring

SD = Standard deviation

SWFI = Sterile water for injection

TRIS = Tris(hydroxymethyl)aminomethane

ULOQ = Upper limit of quantitation

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