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A fast and sensitive LC–MS/MS method for the quantification of fosfomycin in human urine and plasma using one sample preparation method and HILIC chromatography

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ABSTRACT

Fosfomycin is an old antibiotic that is increasingly prescribed because of emergence of the antibiotic resistance and the growing incidence of multi-drug resistant infections. Surprisingly, little is known about its pharmacokinetics (PK) and the pharmacodynamics (PD). Quantification of fosfomycin in both urine and plasma provides insight into the PK/PD characteristics of fosfomycin, which is crucial for the optimization of the therapy and the prevention of the emergence of resistance. An analytical method is therefore needed for the quantification of fosfomycin in both urine and plasma. A fast and sensitive tandem mass spectrometry method in combination with HILIC chromatography for the quantification of fosfomycin with a universal sample preparation method for urine and plasma was developed and validated according to FDA guidelines. The universal sample preparation method only requires 100 µL of a sample, the addition of the internal standard fosfomycin-$13C_3$ benzylamine and an ultrafiltration step. The method is applicable for the concentration range of 0.75–375 mg/L (R^2 of 0.9998 in both matrices) encompassing the clinically relevant concentration range based on the susceptibility of possible (uro)pathogens in the clinical setting. The validation results for urine and plasma for all QC levels, were < 2.1% and < 3.2% for accuracy, < 1.5% and < 1.7% for within day precision and < 5.0% and < 3.8% for between day precision, respectively. No matrix effects were encountered and the total recovery in urine and plasma was high (102.5% and 99.4%). Prepared samples were stable at 4 °C and 15 °C for at least 72 h and stored samples at −80 °C were stable for at least 6 months. Selectivity and sensitivity were confirmed and no carry-over was observed. The method was successfully applied in two pharmacokinetic studies in healthy volunteers and patients respectively.

1. Introduction

In an era of emerging drug resistance and lack of new antibiotics, old off-patent antibiotics are increasingly being prescribed. Oral fosfomycin has gained more attention as an alternative, or even as first line treatment, for uncomplicated urinary tract infections (UTIs) caused by extended spectrum beta lactamase (ESBL)-producing bacteria [1–3]. Oral fosfomycin is also used for the treatment of complicated urinary tract infection in some countries and has been used as prophylactic therapy in prostate resection procedures. Fosfomycin remains active against many multidrug-resistant (MDR) pathogens [4–6]. For this reason, intravenous (IV) fosfomycin is now given a more prominent role in the treatment of (critically ill) patients due to the higher prevalence of MDR pathogens [7]. The IV formulation of fosfomycin was recently approved in several countries worldwide, thereby it is expected that the use of this administration form will increase over the next few years. Fosfomycin, discovered in the late 1960s, is an old antibiotic agent, specifically suited to the treatment of UTIs [4]. The chemical structure of fosfomycin (Fig. 1a) is unique and not related to any other antibiotic drug: it is small (138 Da) and highly hydrophilic.

Urinary or plasma concentrations (PK) directly influence the kill-rate of the (uro)pathogen in vitro and hereby the effectivity of the antibiotic treatment [8]. Since resistance rates have dramatically increased over the last few years [9], it is important to investigate the

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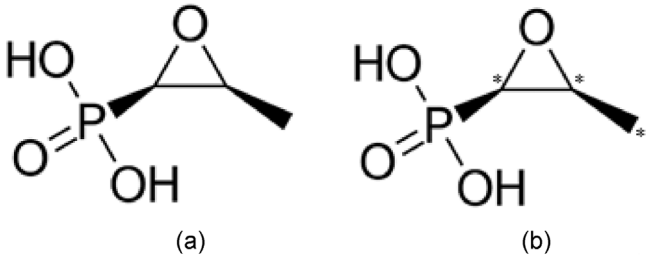

Fig. 1. (a) Chemical structure of fosfomycin and (b) the internal standard: fosfomycin-$13C_3$. Only one of the enantiomers from the racemic mixture is depicted. The three 13C atoms are marked as C*.

pharmacokinetics of fosfomycin in order to optimize the treatment response (PD), minimize the duration of treatment and minimize the risk of the development of resistant pathogens [10].

Several methods for the quantification of fosfomycin in urine and/or plasma have been developed during the last years. These vary from an older microbiological assay [11], gas chromatography methods [12,13], a flow injection spectrophotometric method [14] and ion exchange chromatography [15] to the more sophisticated method as high performance liquid chromatography tandem mass spectrometry (HPLC–MS) [16], LC–MS combined with atmospheric pressure chemical ionization [17] and Hydrophilic Interaction Liquid Chromatography (HILIC) [18]. They were all successfully validated, but lack the ability to quantitate fosfomycin in the lower range of the clinically relevant concentrations, relating to the wild-type distribution of (uro)pathogens, with a minimal inhibitory concentration (MIC) less than (or equal to) 8 mg/L [19]. The MIC is a measure for the susceptibility of the pathogen to fosfomycin. Therefore, concentrations from 1 to 256 mg/L in both urine and plasma should be able to be quantified [19]. Earlier published methods using LC–MS/MS are lacking this sensitivity [16,18]. Also, retention times of these methods are long which indicates a longer runtime (8–10 min). Only two of these methods are applicable for both urine and plasma samples [12,18], but they use different sample preparation methods for both matrices. The aim of this study was to develop a sensitive and rapid ultra performance LC–MS/MS method with HILIC chromatography for the quantification of fosfomycin in urine and plasma.

2. Experimental

2.1. Chemicals and reagents

Fosfomycin was purchased from Santa Cruz Biotechnology Inc. (Huissen, the Netherlands, purity > 98%) and racemic fosfomycin-$13C_3$ benzylamine salt, which was used as the internal standard, was purchased from Toronto Research Chemicals (North York, Canada, purity 96%). Acetonitrile and methanol were both purchased from Biosolve BV (Valkenswaard, the Netherlands) and were of LC–MS quality. Ammonium formate was purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands). The water was purified using a Milli-Q Ultrapure Water System (Merck Millipore, Darmstadt, Germany). Antibiotic free urine was donated just prior to analysis by five healthy volunteers without history of antibiotic use including fosfomycin over the past four weeks. Blank plasma was obtained from drug-free volunteers who donated blood in the blood donation center (Sanquin, Rotterdam, the Netherlands). After donation the blood was centrifuged and plasma was pooled and stored at −18 °C prior to analysis.

2.2. Solutions

A stock solution of 10000 mg/L fosfomycin disodium salt in Milli-Q Ultrapure water was used to prepare calibration standards in blank urine or plasma at eight concentration levels between 3.75 and 375 mg/L. The stock solution was stored at −20 °C. Quality control samples were prepared in the same manner as the calibration standards at concentrations of 7.5 mg/L (QC low (L)), 115 mg/L (QC medium (M)) and 335 mg/L (QC high (H)). Also, a lower limit of quantification (LLOQ) standard was prepared in a concentration of 0.75 mg/L 100 µL of each standard or quality control sample was transferred to a 1.5 mL safe-lock Eppendorf tube and stored at −80 °C until to analysis.

A stock solution of 100 mg/L racemic fosfomycin-$13C_3$ benzylamine salt was prepared in an ammonium formate/ultrapure water solution (pH 7; 4 mM).This ammonium formate solution was also used in the preparation of the mobile phase (see section 2.4). The stock solution of the internal standard was stored in a refrigerator at 2–8 °C and was brought to room temperature before use.

2.3. Instruments

The equipment used was a Dionex Ultimate UPLC system which was connected to a triple Quadrupole mass spectrometer with a Heated Electrospray Ionization-probe operating in the negative mode (Thermo Scientific, Waltham, MA). A spray voltage of 4000 kV with a capillary temperature of 250 °C and a vaporizer temperature of 400 °C were used to produce the parent ions with a mass/charge (m/z) ratio of 137.040 for fosfomycin and 137.021 for the internal standard. With nitrogen used as sheet gas and auxiliary gas, and collision gas pressure of 1.5 mTorr, the product ions with m/z = 79.170 for fosfomycin and m/z = 79.171 for the internal standard were produced. The fragmentation energies were respectively 26 eV (S-lens of 10 V) and 41 eV (S-lens of 76 V). The UPLC system consisted of a UPLC-pump, an auto sampler with flow through needle injection and a column compartment (all RS 3000 Ultimate). The software programs Chromeleon 6.80 (Dionex, Thermo Scientific), LCquan 2.6.1.32 (Thermo Scientific) and Xcalibur 2.1 (Thermo Scientific) were used for data processing.

2.4. LC–MS/MS conditions

A HILIC column (2.1 × 100 mm Acquity UPLC BEH Amide 1.7 µm, Waters, Etten-Leur, the Netherlands), operating at 40 °C was used to perform the chromatographic separation. An isocratic mobile phase containing a mixture of the previous described ammonium formate solution in ultrapure water (pH 7; 4 mM) and acetonitrile (20:80, v/v) at a flow rate of 0.4 mL/min. The retention time for both components was 1.8 min. The column was intensively preconditioned with the mobile phase for stabilization prior to the analysis.

2.5. Sample preparation

The following sample preparation method was applicable to both urine and plasma samples: 100 µL of a sample and the same volume of the internal standard solution were added together and mixed on a shaker for 10 s. The mixture was transferred to an ultrafilter tube (Amicon Ultra 0.5 mL Ultracel 10k, Millipore) and then centrifuged at 16.100g for 5 min. Ultrafiltration is a method to determine the free, protein unbound fraction of a drug. 50 µL of the filtrate was mixed with 200 µL of acetonitrile in an auto sampler insert vial (snap ring vial, 32 × 11.6 mm with integrated 0.2 mL glass micro-insert, VWR). 2 µL for urine or 4 µL for plasma was injected into the LC–MS/MS system.

2.6. Analytical validation

The following validation parameters were investigated, according to the US Food and Drug Administration guidelines for bio analytical method validations [20]:

2.6.1. Linearity

To investigate the linearity of the method, eight calibration standards (Table 1) were prepared together with two blank samples (n = 2

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