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A validated method for the quantification of fosfomycin in human plasma by liquid chromatography–tandem mass spectrometry

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ABSTRACT

Fosfomycin is a small, hydrophilic antibiotic drug with activity against Gram-positive as well as Gram-negative pathogens. It is in increasing use in intensive care units as a last line antibiotic since it shares no cross-resistance with other antibiotics. It is not metabolized and plasma levels are dependent on renal excretion rate and renal replacement therapy such as hemofiltration or hemodialysis. Measurement of fosfomycin plasma concentrations is therefore highly desirable in order to optimize dosing. We have developed a method for the quantification of fosfomycin in human plasma using HILIC chromatography on a silica stationary phase and tandem mass spectrometric detection. Sample preparation consisted only of protein precipitation without derivatization. Propylphosphonic acid was used as internal standard. Two calibration ranges from 15 to 150 μg/ml and 100 to 750 μg/ml were necessary to cover the whole range of plasma concentrations expected from intensive care patients. Intraday precision ranged from 4.0% to 6.4%, depending on the concentration level, with accuracies ranging from −1.1% to 11.5%. The corresponding interday precisions and accuracies were 2.0–11.0% and 0.6–7.8%, respectively. Fosfomycin was stable in human plasma under all storing conditions relevant for clinical samples. First experiences with this method in clinical routine use confirmed the applicability and ruggedness of the analytical procedure.

1. Introduction

Fosfomycin is a broad-spectrum antibiotic drug with activity against Gram-negative as well as Gram-positive bacteria. It is a small and highly hydrophilic phosphonic acid derivative (see Fig. 1), which is not significantly metabolized and excreted mostly unchanged via the kidneys [1]. It is structurally unrelated to other antibiotic substances and shares no cross-resistance with them. Owing to the growing incidence of infections by multidrug resistant bacteria strains, therapy with fosfomycin is applied in increasing rates in intensive care units as a last line antibiotic [2]. Daily doses of up to 24 g fosfomycin divided into 3–4 intravenous applications per day were administered to counteract sensitive pathogens with MIC ≤ 32 μg/ml [3,4], leading to very high maximal plasma concentrations of more than 300 μg/ml. However, studies evaluating the effectiveness and safety of fosfomycin in intensive care settings remain scarce [2]. Patients in critical conditions may exhibit rapidly changing renal excretion rates, making predictions about the pharmacokinetic parameters of fosfomycin very difficult. Furthermore, these patients often undergo renal replacement therapy by hemofiltration or hemodialysis, making a proper dosing estimation even more difficult [5,6]. Monitoring of the plasma concentrations of fosfomycin in intensive care unit patients can be helpful in adjusting individual dosage regimes and may increase therapeutic efficiency and prevent induction of drug resistance. Thus, a fast and rugged method for the determination of fosfomycin in human plasma optimized for this patient group is highly desirable.

The extremely acidic and polar nature of fosfomycin and the lack of chromophores in the molecule make its analytical separation and detection quite difficult. Because of these properties, gas chromatographic separation is only possible after adequate derivatization, as it was described previously for conventional [7,8] or mass spectrometric detection [9]. On the other hand, capillary electrophoresis was utilized to analyze fosfomycin in its underivatized state [10], but the unselective UV-detection led to difficult to interpret chromatograms. Applying LC–MS/MS, fosfomycin can be analyzed unambiguously in its underivatized state [11,12], but the chromatographic retention was marginal despite the highly aqueous mobile phases and the cyano- or polar-modified stationary phases. A retention on a reversed phase stationary phase was

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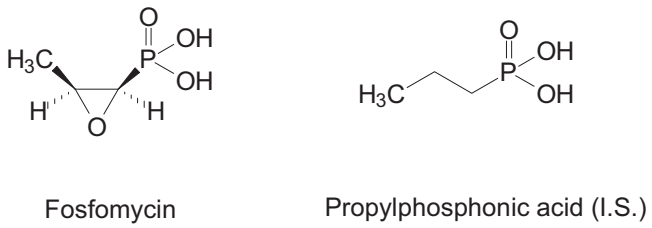

Fig. 1. Molecular structures of fosfomycin and the I.S. propylphosphonic acid.

only possible after derivatization [13]. Yet, derivatization reactions make an analytical method tedious, time consuming and potentially error prone and are therefore less appropriate for the sample preparation of urgent clinical samples. Recently, a LC–MS/MS method applying the HILIC separation technique on a zwitterionic (sulfobetaine) modified column for the retention of underivatized fosfomycin was reported [14]. However, this method experienced some problems in terms of distorted peak shapes with long tailing and instable retention times. Thus, the aim of this study was to develop an easy and rugged LC–MS/MS method providing reasonable chromatographic retention without derivatization using a HILIC separation on a bare silica column for the precise and accurate detection of fosfomycin in plasma samples of intensive care unit patients.

2. Materials and methods

2.1. Chemicals

Fosfomycin was purchased as its disodium salt (purity > 99%) from Sigma–Aldrich (Munich, Germany). The internal standard (I.S.) propylphosphonic acid (purity 99.6%) was also obtained from Sigma–Aldrich. All other chemicals were of analytical grade or better. Pooled drug free human plasma was obtained from the blood bank of the Otto-von-Guericke University (Magdeburg, Germany).

2.2. Instrumentation

The HPLC part of the analytical apparatus consisted of an Agilent 1100 system (Waldbronn, Germany) comprising a binary pump, an autosampler and a thermostatted column compartment. The chromatographic separation took place on an Atlantis™ HILIC silica column with 5 μm particle size and with the dimensions 150 mm × 2.1 mm (Waters, Eschborn, Germany), protected by a SecurityGuard system (Phenomenex, Aschaffenburg, Germany) equipped with a 4 mm × 2 mm silica filter insert. The analytes were detected by a Thermo Fisher Scientific (Waltham, MA, USA) TSQ Discovery Max triple quadrupole mass spectrometer, equipped with an electrospray ionization (ESI) ion source. System control and data handling were carried out by the Thermo Electron Xcalibur software, version 1.2.

2.3. Calibration and quality control samples

For both fosfomycin and the I.S. propylphosphonic acid, 10 mg/ml stock solutions in water were prepared. From the fosfomycin stock solution, after appropriate dilution, calibration samples were prepared in blank human plasma. The calibration range was divided into 2 sub-ranges containing each 5 calibration levels, one covering the concentrations from 15 to 150 μg/ml, the other from 100 to 750 μg/ml. Batches of quality control (QC) samples were prepared at 15 μg/ml (low), 150 μg/ml (medium) and 750 μg/ml (high) from blank human plasma. The QC samples were stored at −20 °C until analysis.

2.4. Sample preparation

Unknown human plasma samples underwent 2 distinct sample preparation procedures, differentiated by the amount of I.S. added. In both cases, to 10 μl plasma 10 μl of I.S. solution (20 μg/ml in water for the low calibration range or 200 μg/ml in water for the high calibration range) was added. Subsequently, 20 μl of buffer solution (2% ammonium formate and 1% formic acid in water) and 360 μl acetonitrile were added. Precipitated proteins were separated by centrifugation at 10,000 × *g* for 5 min and about 100 μl of the clear supernatant was transferred to autosampler vials with microliter inserts.

2.5. Chromatographic separation

From the prepared samples, 10 μl were injected into the HPLC-system in case of the low range calibration and 1 μl in case of the high range calibration. In order to clean the autosampler tubing and switching valve from residual sample content, 40 μl of a purging mixture consisting in equal parts of mobile phase A and B (see below) was injected after a runtime of 8 min. On the analytical column, fosfomycin was chromatographically separated by the HILIC methodology. Mobile phase A consisted of 0.1% ammonium formate plus 0.05% formic acid in water and mobile phase B was prepared from 90% acetonitrile containing 0.1% ammonium formate plus 0.05% formic acid and 10% mobile phase A. The stationary phase was pure silica (see Section 2.2). The mobile phase composition started at 100% B, evolved linearly to 45% A and 55% B in 4 min and was held constant until 8.5 min. The flow rate was 0.35 ml/min and the column temperature was set to 35 °C. A post run equilibration time of 4 min was necessary. Under the described conditions, fosfomycin eluted at 7.2 min and the I.S. slightly earlier at 7.1 min.

2.6. Mass spectrometric analysis

The mobile phase flow from the HPLC-system was directed without splitting into the ESI ion source of the mass spectrometer. The ion source was operated in the negative mode, applying an ionization voltage of −3.5 kV and a capillary temperature of 350 °C, with the sheath gas and auxiliary gas (both nitrogen) set to 20 and 15 units, respectively. Under these conditions, the quasi-molecular $[M-H]^{-1}$ ions with the mass-charge ratios $m/z = 137$ for fosfomycin and $m/z = 123$ for the I.S. were produced. These ions underwent collision induced fragmentation using argon as collision gas at a pressure of 1.5 mTorr. For fosfomycin, the fragment ions $m/z = 137 \rightarrow 79$ at 20 eV fragmentation energy and $m/z = 137 \rightarrow 63$ at 14 eV fragmentation energy were produced. The former fragment ion was recorded for quantification and the latter one for qualification. For the I.S., only the fragment ion $m/z = 123 \rightarrow 79$ was produced at 22 eV fragmentation energy.

2.7. Validation

The extraction yield was tested by comparing spiked and extracted plasma samples with samples spiked after the sample extraction ($n = 5$). Ion suppression was examined by comparing the results of spiked and extracted plasma samples with spiked and extracted aqueous samples ($n = 5$). The intra- and inter-day precision and accuracy of the method was tested using the QC-samples. The QC-low and QC-high samples were used in the low and the high calibration ranges, respectively, whereas the QC-medium samples applied to both calibration ranges. For the evaluation of the stability of fosfomycin in prepared samples, QC-samples were injected in their freshly prepared state and again after standing at room temperature for 24 h in the autosampler tray. Furthermore, the