1 **Fosfomycin therapeutic drug monitoring in real-life: development and validation of a LC-**

2 **MS/MS method on plasma samples**

3

4 Sara Baldelli ChemD[1], Matteo Cerea PhD[2], Davide Mangioni MD[3,4], Laura Alagna, MD[3], Antonio

5 Muscatello, MD[3], Alessandra Bandera MD, PhD[3,5], and Dario Cattaneo PhD[1]

6

7 *[1]Unit of Clinical Pharmacology, L. Sacco University Hospital, Milano, Italy*

8 *[2]Department of Pharmaceutical Sciences, Università degli Studi di Milano, Milan, Italy*

9 *[3] Infectious Diseases Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy*

10 *[4]Department of Medical Biotechnology and Translational Medicine, Università di Milano, Milan, Italy*

11 *[5]Department of Pathophysiology and Transplantation, Università degli Studi di Milano, Milan, Italy*

12

13

14 Correspondence to: Sara Baldelli,

15   Unit of Clinical Pharmacology

16   Luigi Sacco University Hospital

17   via GB Grassi 74, 20157 Milano, Italy

18   tel 00390250319619

19   fax 00390250319646

20   E mail: sara.baldelli@asst-fbf-sacco.it

21
22

23

24 **Short Title:** Fosfomycin concentrations in plasma patients.

25

26

Abstract:

Individualization of fosfomycin dosing based on therapeutic drug monitoring (TDM) of plasma concentrations could reduce drug-related adverse events and improve clinical outcome in complex clinical conditions.

Quantification of fosfomycin in plasma samples was performed by a rapid ultraperformance liquid chromatography mass spectrometry method. Sample preparation involved protein precipitation with $[^{13}C_3]$-fosfomycin benzylamine salt as internal standard. The calibration curve ranged from 2 to 800 mg/L. Within- and between-day precision and accuracy, sensitivity, selectivity, dilution integrity, recovery were investigated and the results met the acceptance criteria. In patients, multiple drug dosing (every 6 or 8 hours) or in continuous administration were adopted, resulting in a large interpatient variability in drug concentrations (from 7.4 mg/L and 644.6 mg/L; CV: 91.1%).

In critical care patient setting TDM can represent an important tool to identify the best fosfomycin dosing in single patients, taking into consideration clinical characteristics, infection sites and susceptibility of the treated pathogens.

1. Introduction

Nowadays, antimicrobial resistance represents a public health concern worldwide. According to the European Antimicrobial Resistance Surveillance Network (EARS-Net), in several European countries the bacterial species most frequently responsible for invasive infections display high levels of antimicrobial resistance, with a stable trend over the last years [1]. More than 670.000 infections due to bacteria resistant to antibiotics occur each year in the EU, and approximately 33.000 people die as a direct consequence of these infections [2]. The paucity of new antibiotic drugs active against drug resistant pathogens brings to a reassessment and reintroduction of already existing therapeutic options such as fosfomycin [3-7].

Discovered almost four decades ago, fosfomycin inhibits the very initial phase of bacterial cell wall biosynthesis by irreversibly inactivating the enzyme UDP-N-acetyl-glucosamine-3-o-enolpyruvyl transferase responsible for catalyzing the formation of N-acetylmuramic acid, a precursor of peptidoglycan [8]. Since both gram-positive and gram-negative bacteria require the formation of N-acetylmuramic acid for bacterial cell synthesis, fosfomycin displays activity against several genera found in clinical practice such as *Staphylococcus* spp., *Enterococcus* spp., *Enterobacterales*, and, to less extent, *Pseudomonas* spp. [3,9]. Its unique mechanism of action also prevents fosfomycin to be affected by cross-resistance to other class of antibiotics and confers a rapid bactericidal effect and a synergic effect with several antimicrobial drugs (*i.e.,* beta-lactams, colistin, daptomycin, vancomycin) [3,7,9].

Fosfomycin is a low molecular weight (138 g/mol), hydrophilic agent with negligible protein binding. It is rapidly adsorbed and penetrates well into the tissues, achieving clinically relevant concentrations in sites such as serum, soft tissues, lung, bone, cerebrospinal fluid; it is excreted unchanged in urine. Fosfomycin has also the ability to penetrate into biofilms. Experimental studies showed not only a reduction or eradication of clinically significant bacteria from biofilms, but also modifications of the biofilm structure [3].

While oral fosfomycin remains one of the first-line agents for the treatment of acute uncomplicated urinary tract infections, parenteral administration is growingly used in the treatment of serious systemic infections [10-12]. Accordingly, thanks to its rapid bacterial killing, its synergic effect with other antibiotics and its high tissue and biofilm penetration, fosfomycin is currently used for the treatment of severe bacterial infections caused by multidrug resistant (MDR) pathogens and for device-associated infections (endocarditis, implant-associated infections), usually in combination with other antibiotics. Yet parenteral fosfomycin is associated with some clinically relevant adverse events, mostly gastrointestinal disorders and electrolytic disturbances [13,14]. Hypokalemia has been reported in up to 26% of treated patients, and hypernatremia secondary to the high sodium content of fosfomycin could limit its use in patients with heart failure or those on hemodialysis [13,15].

Considerable interpatient variability in the plasma concentrations of fosfomycin has been demonstrated in critically ill patients [16-17], posing this population at risk of over- or under- drug dosing [18-19]. Indeed, it has been shown that older age and impaired renal function result in lower kidney elimination and high plasma fosfomycin concentrations [20-21]. However, these factors explain only a part of the observed pharmacokinetic variability. This scenario is further complicated by the great heterogeneity in the doses of fosfomycin adopted in different clinical settings, ranging from less than 12 up to 24 grams given 2-to-4 times daily or as continuous infusion. The issue of the adequate antibiotic dosing in intensive care units has been recently recognized by the recently updated EUCAST guidelines (www.eucast.org), which underlines the need to reach optimal PK/PD targets to ensure in vivo antibiotic efficacy, especially in the treatment of MDR and/or intensive care unit infections.

Currently the optimal pharmacokinetic/pharmacodynamics (PK/PD) index of fosfomycin microbiological activity is yet to be defined, and both concentration- and time-dependent activities have been suggested [22]. In particular, recent findings indicate that efficacy of fosfomycin might be more related to Area under the concentration-time curve for some strains, and more time-dependent for others. [23-25].

105 Therapeutic drug monitoring (TDM) of fosfomycin can, therefore, help to tailor the therapy by

106 providing effective plasma concentrations in respect to the minimum inhibitor concentration defined

107 for the pathogen, when available. The application of TDM in the routine clinical practice of hospital

108 laboratories requires, however, the availability of robust and reliable analytical methods.

109 Here, we aimed to develop and validate a rapid ultraperformance liquid chromatography (UPLC)

110 mass spectrometry method for the determination of fosfomycin according to the principles established

111 in the Food and Drug Administration Guideline on Bioanalytical Method Validation [26-27], and to

112 test its clinical application in plasma patients undergoing fosfomycin therapy.

113

2. Materials and Methods

2.1 Chemicals and reagents

Fosfomycin sodium was purchased by Sigma Aldrich (Milan, Italy, purity: 98.3%) and its isotopically labeled internal standard $[^{13}C_3]$-fosfomycin benzylamine salt (fosfomycin IS; purity 96%) from Toronto Research Chemicals (North York, Canada).

Because of high hygroscopicity of fosfomycin powder, weighing resulted very challenging. For this reason, the whole content (100 mg) of the glass vial as received by the supplier was dissolved in 10 mL volume of water:methanol mixture (70:30) for obtaining the concentration of 10 mg/mL, and working solutions at the concentration of 10 mg/L, 100 mg/L and 1000 mg/L were prepared by dilution with the same solution of water:methanol (70/30). To be sure of the procedure, fosfomycin disodium powder (20 mg) was purchased by another supplier (Divbio Science Italia, Verona, Italy) and used to prepare solutions for quality control samples. All the solutions were stored at −20˚C.

Internal standard powder was dissolved in the appropriate volume of water:methanol (70/30) to obtain a concentration of 1 mg/mL in $[^{13}C_3]$-fosfomycin. Working solution was obtained per dilution 1 to 10 with of water:methanol (70/30).

Acetonitrile and formic acid (VWR, Milan, Italy) were mass spectrometry (LC-MS) grade. Ammonium formate, analytical grade, was received from Sigma Aldrich (Milan, Italy). Milli-Q type water obtained from a Milli-Q water purification system (Millipore, Milan, Italy) was used for the preparation of all the solutions.

Drug-free blank plasma used for the preparation of calibration and control samples was obtained from healthy volunteers participating in local blood donation programs after written informed consent collection.

2.2 Instruments

Chromatographic separation was performed with a Waters Acquity H Class UPLC (Waters, Italy) on an Acquity BEH HILIC column (1.7μm 2.1 x 50mm, Waters, Milan, Italy) set at 30 °C.

Final selected mobile phase consisted of HCOONH$_4$ 50 mM pH 4.2 (solvent A) and CH$_3$CN / HCOONH$_4$ 50mM (90/10) 0.1% formic acid (solvent B) delivered at 0.65 mL/min and regularly prepared prior to each analytical series.

A gradient elution programme from 100% to 50% of B in 3.70 min and a re-equilibration step to the initial solvent composition up to 5 min was applied. The autosampler was maintained at 4 °C.

Ionization and detection of analyte and IS were carried out on a TQS micro MS/MS system (Waters, Milan, Italy) operating in a negative electrospray ionization (ESI) mode. For quantification, multiple-reaction monitoring (MRM) mode was applied. The precursor ions m/z 136.85 for fosfomycin and m/z 139.85 for IS fosfomycin were filtered in the first quadrupole (Q1), and submitted to collision induced fragmentation in the second quadrupole (Q2). The corresponding product ions m/z 78.87 (collision energy 20 eV, for fosfomycin quantification and for fosfomycin IS) and 62.83 (collision energy 12 eV, for fosfomycin confirmation) were monitored via the third quadrupole (Q3) under the optimized identification and quantification parameters. The measurements were made under conditions of a source and capillary temperatures of 125 and 350 °C, respectively, while the capillary voltage was set at 2.6 kV. Desolvation gas was nitrogen while high-purity argon was the collision gas. MassLynx software was used for instrument control and data acquisition.

2.3 Calibration curve and quality control samples

A seven-point standard calibration curve on plasma was prepared over the concentration range of 2 to 800 mg/L (2, 10, 20, 40, 100, 200, 800 mg/L), in addition to a blank calibrator, using least square weighted (1/x) linear regression of the peak area ratio of the analyte to IS versus the respective analyte nominal concentration.

Quality control (QC) samples were prepared as calibrators from independent solutions to obtain four concentrations within the linear concentration range at lower limit of quantification (2 mg/L) low (6 mg/L), medium (30 mg/L), and high (600 mg/L) levels. 50 μL QC samples were stored frozen at −20 °C until analysis.

166

2.4 Sample preparation

Plasma samples were obtained after centrifugation of blood samples collected in EDTA tubes at $3000 \times g$ for 10 min at the temperature of 4°C and then stored at $-80°C$. Before analysis, the plasma sample was thawed to room temperature and 50 µL volume of calibration, QCs and patient plasma samples, were pipetted into labelled disposable polypropylene Eppendorf tubes and added with 10 µL of IS solution. The tubes were vortex-mixed for 20 s, and then diluted with 0.1 mL of water. For protein precipitation, 0.4 mL of CH_3CN was added. The tubes were vortex-mixed for further 30 seconds and left 15 minutes at room temperature. Sample were then centrifuged at $13000 \times g$ for 12 min at 4°C. 100 µL were transferred to glass vials and 5 µL were injected into the UPLC -MS/MS for analysis.

2.5 Method validation

Method validation was performed according to the principles established in the Food and Drug Administration Guideline on Bioanalytical Method Validation [26]. The validation parameters included specificity, linearity, intra and inter-assay precision and accuracy, recovery and process efficiency and stability.

Matrix effect analysis was performed to determine the possible ionization enhancement or suppression by sample matrices. Six different drug-free blank plasma were processed according to the sample preparation procedure described above and then spiked with the antibiotic and with the IS after extraction at low (6 mg/L) and high concentrations (600 mg/L). The ratio between the peak area in presence of matrix (spiked blank plasma) to the peak area in absence of matrix (standard solution at the same concentration) was quantified (MF). A value close to 1 indicate the absence of any direct or indirect alteration or interference in response because of the presence of unintended analytes or other interfering substances in the sample. Acceptance criteria require the coefficient of variation (CV) % of matrix factors to be less than 15%.

192 Per FDA recommendations, studies have to be performed to ensure the dilution integrity of specimens

193 that may either be sample limited or contain analyte levels above the upper limit of quantification.

194 The dilution integrity of high and medium QC plasma samples was tested by performing 2-fold

195 dilution of these samples with blank plasma (n=6). The calculated concentrations were compared to

196 the nominal concentration. Accuracy and precision should be within ±15%.

197 The stability of the analytes was investigated on QC samples after storage at room temperature for

198 24 hours, at +4 °C for 36 hours, and after three freeze-thaw cycles from -20°C to room temperature.

199 Post-preparative stability in the autosampler was also assessed by leaving the extracted sample at +4

200 °C for 36 hours and 72 hours. Finally, the long term stability was also assessed by keeping one set of

201 aliquots at −20 °C for 28 weeks. For all experiments, percent difference in analyte concentrations was

202 determined by comparison to the nominal levels. The relative standard deviations of the set of 3

203 samples were calculated.

204 Incurred sample reanalysis was performed on patient samples at different concentrations (n=20).

205 Samples were analysed in two different analytical runs, the bias of repeat [(result 2 − result 1)/result

206 1) × 100] was calculated and had to be within 20% in at least 67% of the repeats as requested [26].

207 The acceptability of re-analysed samples was also investigated using the method proposed by M.

208 Rocci et al [28].

209

210 *2.6 Clinical application*

211 The present method was successfully applied to samples collected for clinical purposes of patients in

212 therapy with fosfomycin from the IRCCS Ca' Grande Ospedale Maggiore Policlinico of Milan.

213 Treatment initiation and follow up were managed by Infectious Diseases Consultants as per clinical

214 practice.

215 All patients provided informed consent for the medical procedures used for the purpose of routine

216 treatment.

217 Blood samples were collected into EDTA plasma sampling tubes at different time intervals. Samples

218 were promptly centrifuged after sampling, sent to the laboratory on ice and frozen at −20 °C until

219 analysis.

3. Results

3.1 In vitro method validation

Under the chromatographic conditions described, the total run time was 4.5 min; retention times for fosfomycin and fosfomycin IS were 1.16 ± 0.02 and 1.15 ± 0.01, respectively.

Representative chromatograms of extracts from human drug-free whole-blood samples, control human plasma sample at the lower limit of quantification for fosfomycin, and a patient sample are shown in Figure 1. No detectable interfering peaks from other possible co-eluting compounds of the matrix were found with retention times of the analytes considered. Similarly, no interferences were detected due to drugs co-administered to the patients (most common antifungal, antihypertensive, hypolipidaemic and antiepileptic drugs as well as other antibiotics) (see Supplementary table).

The mean correlation coefficient for regression equation was 0.9997 (SD $\pm$ 0.0002; range 0.9995–1.0000, n=6). The percentage deviations from nominal values determined for mean back calculated concentrations for each calibrator, ranged from −10.1 to +9.3, indicating a satisfactory fit of the concentration/response data to the weighted linear regression equation.

The within- and between-day precision, expressed as percent relative standard deviation (% RSD) at the mean measured concentration, determined in one day and on five different days, respectively (n = 5) at all QCs concentration levels was below 7.5% (acceptance criteria <15%).

Accuracy, expressed as the percentage ratio of nominal concentration to mean measured concentration, was between 86.5-110% (acceptance criteria 85-115%) (Table 1).

Minimal ion enhancement was observed and was consistent at low and high concentrations: mean MF 1.10 ± 0.04 (CV:3.7%) and 1.12 ± 0.05 (CV:4.3%), respectively.

Mean drug recovery determined by comparing the mean area response of extracted QCs to extracts of blank plasma samples spiked with pure standard solutions of fosfomycin post-extraction at the same concentrations, was 70.9% (SD $\pm$ 2.6%) and was reproducible.

245 The total process efficiency, calculated as the ratio between mean area response of extracted QCs

246 (n=3 for every concentration) to area of pure standard solution at the same concentrations, was 78.2%

247 (SD, ± 1.98) and demonstrated no concentration dependency (see Table 1).

248 No significant carryover was observed: the ratio between the area of the peaks in the blank sample

249 after the injection of the highest calibrator and the area of the first calibrator was 2.4% of the signal

250 measured with the lowest calibrator, (n=10, carry-over effect = 0.096 mg/L).

251 The dilution integrity of high and medium QC plasma samples was tested by performing 2-fold

252 dilution of these samples with blank plasma (n=6) (Table1).

253 Stability studies on QCs showed that fosfomycin is stable at 4 °C, over a 24-hour and 72-hours storage

254 period (mean concentration variation 2.3%). The variation of drug concentrations after three freeze–

255 thaw cycles revealed no significant loss for fosfomycin (mean deviation from fresh QCs -3.6%). No

256 evidence of decomposition was found during plasma samples storage in the freezer at −20 °C for 28

257 weeks (maximum deviation for low QCs of -6.7%). Autosampler stability at 4°C of extracted samples

258 confirmed the stability of samples (mean variation: -2.1%). These data are in accordance with

259 previous ones reported in literature [29].

260 *3.2 In vivo method validation*

261 In-vivo validation of the proposed method was carried out by analyzing plasma sample from patients

262 on therapy with fosfomycin.

263 Sample reanalysis was performed on 20 samples with concentrations between 2 mg/L and 700 mg/L.

264 The percentage difference in results between the concentration of repeat and the original sample was

265 within the threshold limit of 20% in 19 samples (0.0 ± 7.7 %; highest difference +21.5%). Considering

266 the more conservative approach by Rocci et al [28], the mean ratio obtained for fosfomycin was 1.00

267 with ratio limits of 0.97-1.04 and limits of agreement 0.93-1.08 that contain all the determinations.

268 Paired t- test revealed that, statistically, the mean difference between the paired observations the in

269 the two samples is zero (p = 0.5513).

270 *3.3 Therapeutic drug monitoring of fosfomycin*

Eighteen patients undergoing the routine TDM of fosfomycin were given fosfomycin in combination with other antibiotics (Table 2). Daily fosfomycin doses ranged from 2 g to 24 g, depending on patient's weight (pediatric dose) or renal function and minimum inhibitory concentration (MIC) of the pathogen involved (adult dose). Fosfomycin was administered either as intermittent or as continuous intravenous infusion (Table 2).

Measured fosfomycin plasma concentrations in patient samples ranged from 7.4 mg/L and 644.6 mg/L; fosfomycin trough samples had mean trough concentrations (Cmin) of 181.1 (CV: 91.1%). All TDM assessments felt within the calibration range established for the present method. No dilution steps were required and no patient samples resulted below the lower limit of quantification of the method.

Of note, 4/18 (22.2%) patients had more than one drug evaluation to assess plasmatic levels after significant alteration of renal function (Table 2). In particular, in one patient with augmented renal clearance (patient #6) fosfomycin concentration was below the MIC value at the first TDM. Total dose was maintained at 24g/die but the infusion was modified from fractioned to "in continuous" and a second plasma concentration reached a higher value (from Cmin 7.36 to average concentration (Cavg) 142.6 mg/L). On the contrary, patients #9, #17 and #18 obtained a second drug plasmatic evaluation because of high fosfomycin concentration at the first TDM. In patients #9 and #18 the dose was reduced with a consequent decrease in plasmatic levels (from Cmin 644.6 to Cmin 69.9 mg/L and from Cavg 209.3 to Cavg 61 mg/L, respectively). In patient #17, drug administration was not changed and plasmatic levels decreased after an improvement of renal function (from Cavg 411.3 to Cavg 198.5 mg/L).

4. Discussion and conclusions

Reversed phase chromatography is the most employed technique in bioanalysis due to its versatility to retain and resolve numerous compounds. However, it is not particularly suitable for polar hydrophilic compounds such as fosfomycin, which has an octanol/water partition coefficient of -1.6. Actually, using an Acquity UPLC BEH C18, fosfomycin elute in the void volume of the column, unretained (retention time (tr): 0.26 minutes).

Hydrophilic interaction chromatography (HILIC) has been used to successfully improve the retention of this polar compound while enhancing ESI response through the use of high organic mobile phase (acetonitrile) and the direct compatibility with sample protein precipitation solvent.

Different columns were initially tested in particular an Acquity UPLC HSS T3 column, an Acquity UPLC BEH Amide and an Acquity UPLC BEH C18 HILIC with different solvents, but best results in terms of retention time and peak shape were obtained with the last one. Peak distortion was mitigated by maintaining final composition of the extract sample as the starting mobile phase conditions.

To achieve good reproducibility, the use of a buffered mobile phase resulted necessary: in particular, the addition of ammonium formate due to its solubility as well as its volatility was the better solution. Despite the gradient elution program, with the re-equilibration step, that was found to be essential for stability in the retention time of the peaks, the use of the UPLC allowed a great reduction of the chromatographic times with a significant improvement in the turnaround times.

This method permits high throughput analysis of samples and can be easily applied for TDM of fosfomycin in different clinical settings.

In recent years, fosfomycin has gained renewed interest as combination partner in treating MDR pathogens or other difficult-to-treat infections, due to its rapid bacterial killing, high synergistic and/or cumulative antibacterial effect and high tissue penetration [3,7,9,30]. This is evident also from our study: since the majority of infections were caused by MDR gram negative bacteria, fosfomycin was intravenously administered in combination regimens associated mostly with carbapenems,

cephalosporins and tigecycline. Therefore, the actual use of fosfomycin goes beyond the treatment of urinary tract infections, being expanded to the treatment of bacteremias especially in severe settings, including critically ill patients [12, 20, 31]. The wide variety of fosfomycin dosages employed in our study was chosen considering PK/PD parameters, namely renal function and pathogen MIC (Table 2).

The excellent distribution in different body sites due to its ability to penetrate in most tissue and reach sufficient concentrations at the site of infection, the safety and tolerability profile, make in clinical practice fosfomycin a therapeutic option worth considering in difficult-to-treat infections also in pediatric population. This is confirmed in our population in which sites of infection were mostly lung for which it has been demonstrated a concentration half of that of plasma [4], but also blood and bile and in one case cerebrospinal fluid.

Several doses and strategies of administration have been proposed. Fosfomycin was initially categorized as a time dependent antibiotic and T>MIC was indicated as the appropriate pharmacokinetic/pharmacodynamic index. Therefore, multiple drug dosing (every 6 or 8 hours) or in continuous administration are adopted, resulting in a large interpatient variability in drug concentrations as demonstrated in our population. In fact, the observed interindividual variability of fosfomycin trough and steady state concentrations in our patients was marked, with a nearly ten-fold distribution of the daily drug doses adopted. Taken together, these evidences underline the importance of TDM as tool to identify the best fosfomycin dosing in single patients, taking into consideration peculiar clinical characteristics, infection sites and sensibility of the treated pathogens.

In the settings of critically ill patients and severe infections due to MDR bacteria, TDM of fosfomycin could offer great advantages in terms of both optimization of therapeutic target attainment and reduction of dose-related adverse events. This was evident also in the case of our small population with the use TDM to guide dose adjustment: in particular, the determination of fosfomycin concentrations revealed underexposure in one patient and large overexposure in others. This could be

faced trough dosage modification and subsequent evaluations of drug concentrations, in order to reduce adverse events and eventually maximize clinical outcomes.

Compared to the few analytical methods proposed so far [32-37], the present method offers an important innovation for the TDM of fosfomycin particularly in consideration of the wide range of linearity, which corresponds to the actual MIC values and to concentrations available in real clinical applications. Furthermore, the use of UPLC associated with a great reduction in chromatographic times and the very simple and fast extraction procedures allow a significant improvement in the turnaround time.

Disclosure of conflicts of interest

The authors declare no conflict of interest.

References:

357

358 1. European Centre for Disease Prevention and Control. Antimicrobial resistance in EU/EEA (EARS-

359 Net) – Annual Epidemiological Report 2019. Stockholm: ECDC; 2020.

360 2.Cassini A, Högberg LD, Plachouras D, Quattrocchi A, Hoxha A, Simonsen GS, et al. Attributable

361 deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the

362 EU and the European Economic Area in 2015: a population-level modelling analysis. Lancet Infect

363 Dis. 2019;19:56-66. doi: 10.1016/S1473-3099(18)30605-4.

364 3. Falagas ME, Vouloumanou EK, Samonis G, Vardakas KZ. Fosfomycin. Clin Microbiol Rev.

365 2016;29:321-47. doi: 10.1128/CMR.00068-15.

366 4.Popovic M, Steinort D, Pillai S, Joukhadar C. Fosfomycin: an old, new friend? Eur J Clin Microbiol

367 Infect Dis. 2010;29:127–42. doi: 10.1007/s10096-009-0833-2.

368 5. Michalopoulos AS, Livaditis IG, Gougoutas V. The revival of fosfomycin. Int J Infect Dis.

369 2011;15:e732-739. doi: 10.1016/j.ijid.2011.07.007

370 6. Falagas ME, Grammatikos AP, Michalopoulos A. Potential of old-generation antibiotics to address

371 current need for new antibiotics. Expert Rev Anti Infect Ther. 2008;6:593-600. doi:

372 10.1586/14787210.6.5.593.

373 7. Avery LM, Sutherland CA, Nicolau DP. In vitro investigation of synergy among fosfomycin and

374 parenteral antimicrobials against carbapenemase-producing Enterobacteriaceae. Diagn Microbiol

375 Infect Dis. 2019;95:216-20. doi: 10.1016/j.diagmicrobio.2019.05.014.

376 8. Kahan FM, Kahan JS, Cassidy PJ, Kropp H. The mechanism of action of fosfomycin

377 (phosphonomycin). Ann N Y Acad Sci. 1974;235:364–86. doi: 10.1111/j.1749-6632.1974.tb43277.x.

378 9. Falagas ME, Athanasaki F, Voulgaris GL, Triarides NA, Vardakas KZ. Resistance to fosfomycin:

379 Mechanisms, Frequency and Clinical Consequences. Int J Antimicrob Agents. 2019;53:22-8. doi:

380 10.1016/j.ijantimicag.2018.09.013.

381 10. Falagas ME, Giannopoulou KP, Kokolakis GN, Rafailidis PI. Fosfomycin: use beyond urinary

382 tract and gastrointestinal infections. Clin Infect Dis. 2008;46:1069-77. doi: 10.1086/527442.

383   11. Tsegka KG, Voulgaris GL, Kyriakidou M, Falagas ME. Intravenous fosfomycin for the treatment

384   of patients with central nervous system infections: evaluation of the published evidence. Expert Rev

385   Anti Infect Ther. 2020:18:657-68. doi: 10.1080/14787210.2020.1754193.

386   12. Bassetti M, Graziano E, Berruti M, Giacobbe DR. The role of fosfomycin for multidrug-resistant

387   gram-negative   infections.   Curr   Opin   Infect   Dis.   2019;32:617-25.   doi:

388   10.1097/QCO.0000000000000597.

389   13. Iarikov D, Wassel R, Farley J, Nambiar S. Adverse Events Associated with Fosfomycin Use:

390   Review of the Literature and Analyses of the FDA Adverse Event Reporting System Database. Infect

391   Dis Ther. 2015;4:433-58. doi: 10.1007/s40121-015-0092-8.

392   14. Putensen C, Ellger B, Sakka SG, Weyland A, Schmidt K, Zoller M, er al. Current clinical use of

393   intravenous fosfomycin in ICU patients in two European countries. Infection. 2019;47:827-36. doi:

394   10.1007/s15010-019-01323-4.

395   15. Florent A, Chichmanian RM, Cua E, Pulcini C. Adverse events associated with intravenous

396   fosfomycin. Int J Antimicrob Agents. 2011;37:82-3. doi: 10.1016/j.ijantimicag.2010.09.002.

397   16. Matzi V, Lindenmann J, Porubsky C, Kugler SA, Maier A, Dittrich P, et al. Extracellular

398   concentrations of fosfomycin in lung tissue of septic patients. J Antimicrob Chemother. 2010;65: 995-

399   8. doi: 10.1093/jac/dkq070

400   17. Parker S, Lipman J, Koulenti D, Dimopoulos G, Roberts JA. What is the relevance of fosfomycin

401   pharmacokinetics in the treatment of serious infections in critically ill patients? A systematic review.

402   Int J Antimicrob Agents. 2013;42:289-93. doi: 10.1016/j.ijantimicag.2013.05.018.

403   18. Pontikis K, Karaiskos I, Bastani S, Dimopoulos G, Kalogirou M, Katsiari M, et al. Outcomes of

404   critically ill intensive care unit patients treated with fosfomycin for infections due to pandrug-resistant

405   and extensively drug-resistant carbapenemase-producing Gram-negative bacteria. Int J Antimicrob

406   Agents. 2014;43:52-9. doi: 10.1016/j.ijantimicag.2013.09.010.

407   19. Dorn C, Petroff D, Neumann N, Kratzer A, El-Najjar N, Dietrich A, et al. Plasma and tissue

408   pharmacokinetics of fosfomycin in morbidly obese and non-obese surgical patients: a controlled

409   clinical trial. J Antimicrob Chemother. 2019;74:2335-40. doi: 10.1093/jac/dkz203.

410   20. Duez JM , Mousson C , Siebor E , Péchinot A , Freysz M , Sixt N, et al. Fosfomycin and its

411   application in the treatment of multidrug-resistant Enterobacteriaceae infections. Clin Med Rev Ther.

412   2011;3:123–42. doi: 10.4137/CMRT.S5102

413   21. Borsa F, Leroy A, Fillastre JP, Godin M, Moulin B. Comparative pharmacokinetics of

414   tromethamine fosfomycin and calcium fosfomycin in young and elderly adults. Antimicrob Agents

415   Chemother. 1988;32:938-41. doi: 10.1128/aac.32.6.938.

416   22. Roussos N, Karageorgopoulos DE, Samonis G, Falagas ME. Clinical significance of the

417   pharmacokinetic and pharmacodynamic characteristics of fosfomycin for the treatment of patients

418   with   systemic   infections.   Int   J   Antimicrob   Agents.   2009;34:506–15.   doi:

419   10.1016/j.ijantimicag.2009.08.013.

420   23. Lepak AJ, Zhao M, VanScoy B, Taylor DS, Ellis-Grosse E, Ambrose PG, et al. In vivo

421   pharmacokinetics and pharmacodynamics of ZTI-01 (Fosfomycin for Injection) in the neutropenic

422   murine thigh infection model against Escherichia coli, Klebsiella pneumoniae, and Pseudomonas

423   aeruginosa. Antimicrob Agents Chemother. 2017;61:476–517. doi: 10.1128/AAC.00476-17

424   24. Fransen F, Melchers MJ, Meletiadis J, Mouton JW. Pharmacodynamics and differential activity

425   of nitrofurantoin against ESBL-positive pathogens involved in urinary tract infections. J Antimicrob

426   Chemother. 2016;71:2883-9. doi: 10.1093/jac/dkw212.

427   25. Fransen F, Hermans K, Melchers MJB, Lagarde CCM, Meletiadis J, Mouton JW.

428   Pharmacodynamics   of   fosfomycin   against   ESBL-   and/or   carbapenemase-producing

429   Enterobacteriaceae. J Antimicrob Chemother. 2017;72:3374-81. doi: 10.1093/jac/dkx328.

430   26. Food and Drug Administration. Guideline on bioanalytical method validation, 2018.

431   https://www.fda.gov/media/70858/download (Last accessed January 2021).

432 27. Baldelli S, Marrubini G, Cattaneo D, Clementi E, Cerea M. Application of Quality by Design

433 Approach to Bioanalysis: Development of a Method for Elvitegravir Quantification in Human

434 Plasma. Ther Drug Monit. 2017;39:531-42. doi: 10.1097/FTD.0000000000000428.

435 28. Rocci ML Jr, Devanarayan V, Haughey DB, Jardieu P. Confirmatory reanalysis of incurred

436 bioanalytical samples. AAPS J. 2007;9:E336-343. doi: 10.1208/aapsj0903040.

437 29. Wijma RA, Bahmany S, Wilms EB, van Gelder T, Mouton JW, Koch BCP. A fast and sensitive

438 LC-MS/MS method for the quantification of fosfomycin in human urine and plasma using one sample

439 preparation method and HILIC chromatography. J Chromatogr B Analyt Technol Biomed Life Sci.

440 2017;1061-2263-9. doi: 10.1016/j.jchromb.2017.07.036.

441 30. Kastoris AC, Rafailidis PI, Vouloumanou EK, Gkegkes ID, Falagas ME. 2010. Synergy of

442 fosfomycin with other antibiotics for Gram-positive and Gram-negative bacteria. Eur J Clin

443 Pharmacol. 2010:66:359–68. doi:10.1007/s00228-010-0794-5

444 31. Michalopoulos A, Virtzili S, Rafailidis P, Chalevelakis G, Damala M, Falagas ME. Intravenous

445 fosfomycin for the treatment of nosocomial infections caused by carbapenem-resistant Klebsiella

446 pneumoniae in critically ill patients: a prospective evaluation. Clin Microbiol Infect. 2010;16:184-6.

447 doi: 10.1111/j.1469-0691.2009.02921.x.

448 32. Li L, Chen X, Dai X, Chen H, Zhong D. Rapid and selective liquid chromatographic/tandem mass

449 spectrometric method for the determination of fosfomycin in human plasma. J Chromatogr B Analyt

450 Technol Biomed Life Sci. 2007:856:171-7. doi: 10.1016/j.jchromb.2007.05.037.

451 33. Papakondyli TA, Gremilogianni AM, Megoulas NC, Koupparis MA. A novel derivatization

452 method for the determination of Fosfomycin in human plasma by liquid chromatography coupled

453 with atmospheric pressure chemical ionization mass spectrometric detection via phase transfer

454 catalyzed derivatization. J Chromatogr A. 2014;1332:1-7. doi: 10.1016/j.chroma.2014.01.041.

455 34. Parker SL, Lipman J, Roberts JA, Wallis SC. A simple LC-MS/MS method using HILIC

456 chromatography for the determination of fosfomycin in plasma and urine: application to a pilot

457   pharmacokinetic study in humans. J Pharm Biomed Anal. 2015;105:39-45. doi:

458   10.1016/j.jpba.2014.11.042.

459   35. El-Najjar N, Jantsch J, Gessner A. A rapid liquid chromatography-tandem mass spectrometry for

460   the quantification of Fosfomycin in plasma, urine, and aqueous fluids. J Chromatogr B Analyt

461   Technol Biomed Life Sci. 2017:1061-2:57-64. doi: 10.1016/j.jchromb.2017.06.046.

462   36. Martens-Lobenhoffer J, Bode-Böger SM. A validated method for the quantification of fosfomycin

463   in human plasma by liquid chromatography-tandem mass spectrometry. J Chromatogr B Analyt

464   Technol Biomed Life Sci. 2015;990:164-8. doi: 10.1016/j.jchromb.2015.03.029.

465

466 **Table 1.:** Results of the in-vitro validation of the proposed method

Fosfomycin concentrations		Results
Within day		
LLQC (2 mg/L)	Mean ± SD (mg/L)	1.76 ± 0.07
	CV (%)	3.8
	Accuracy (%)	88
Low (6 mg/L)	Mean ± SD (mg/L)	5.42 ± 0.27
	CV (%)	4.9
	Accuracy (%)	90.3
Medium (30 mg/L)	Mean ± SD (mg/L)	30.5 ± 0.7
	CV (%)	2.6
	Accuracy (%)	95.1
High (600 mg/L)	Mean ± SD (mg/L)	612 ± 12
	CV (%)	2.0
	Accuracy (%)	102
Between day		
LLQC (2 mg/L)	Mean ± SD (mg/L)	1.73 ± 0.04
	CV (%)	2.3
	Accuracy (%)	86.5
Low (6 mg/L)	Mean ± SD (mg/L)	6.0 ± 0.45
	CV (%)	7.5
	Accuracy (%)	100.3
Medium (30 mg/L)	Mean ± SD (mg/L)	31.1 ± 1.5
	CV (%)	4.9
	Accuracy (%)	103.6
High (600 mg/L)	Mean ± SD (mg/L)	662 ± 45
	CV (%)	6.8
	Accuracy (%)	110.3
Dilution Integrity		
Medium (30 mg/L)	Mean ± SD (mg/L)	29.3 ± 4.1
	CV (%)	13.9
	Accuracy (%)	97.6
High (600 mg/L)	Mean ± SD (mg/L)	664 ± 86
	CV (%)	13.0
	Accuracy (%)	110.6
Matrix effect		
Low (6 mg/L)	Mean ± SD (mg/L)	1.10 ± 0.04
	CV (%)	3.7
High (600 mg/L)	Mean ± SD (mg/L)	1.12 ± 0.05

	CV (%)	4.3
Extraction efficiency		
Low (6 mg/L)	Mean (%)	78.1
Medium (30 mg/L)	Mean (%)	78.2
High (600 mg/L)	Mean (%)	78.4

469   Table 2. Main clinical features of patients undergoing TDM of fosfomycin

470

Patient #	Dyalisis	eGFR°	Site of infection	Pathogen	MIC	Dose/frequency of the intravenous injection	Fosfomycin (mg/L)	Concomitant antibiotic therapy
1	HD	7	lung	NA	NA	2g (infusion over 2h)	80.9	meropenem
2*		79	CSF	*S. epidermidis*	NA	7.2 g: 1.8g q6h (infusion over 30 min)	7.6 (Cmin)	vancomycin
							110.8 (Cmax)	vancomycin
3		194	lung	*S. aureus /P. aeruginosa*	NA/32	24 g: 8g q8h (infusion over 1h)	52.2	ceftobiprole ceftolozane/tazobactam
4		199	lung	*P. aeruginosa*	<= 16	24g: CI	132.9 (Cavg)	ceftolozane/tazobactam tobramycin
5		25	lung	*P. aeruginosa*	64	16g:4g q6h (infusion over 3h)	625.6	meropemem
6		170	bile/ blood	*K. pneumoniae*	32	24g:8g q8h (infusion over 3h)	7.4	ceftazidime/avibactam metronidazole
		217				24g: CI	142.6 (Cavg)	meropenem/vaborbactam
7		75	lung	*P. aeruginosa*	32	16g: CI	148.4 (Cavg)	imipenem
8	CVVHDF	31	lung	*P. aeruginosa*	<= 16	24g:8g q8h (infusion over 3h)	248.8 (Cmin)	ceftolozane/tazobactam
							382,7 (Cmax)	ceftolozane/tazobactam
9		29	bile/ blood	*K. pneumoniae*	64	20g:8g/4g/8g q8h (infusion over 4h)	644.6	meropenem/vaborbactamt igecycline
		84				12g:4g q8h (infusion over 1h)	69.4	meropenem/vaborbactam tigecycline
10		61	lung	*P. aeruginosa*	64	10g:4g/2g/2g/2g q6h (infusion over 4h)	65.9	ceftolozane/tazobactam
11		80	lung	*P. aeruginosa*	32	16g: 4g q6h (infusion over 3h)	87.4	imipenem
						16g: 4g q6h (infusion over 3h)	174.2	imipenem
12	HD	7	lung	NA	NA	2g (infusion over 2h)	173.8	meropenem
13		90	blood	*S. aureus*	NA	24g:6g q6h (infusion over 2h)	237.6	daptomycin
						16g:4g q6h (infusion over 4h)	71.5	daptomycin
14		128	lung	*P. aeruginosa*	64	16g:4g q6h (infusion over 4h)	301	meropenem
15		200	lung	*K. aerogenes*	<= 16	24g: CI	100 (Cavg)	cefepime

								daptomycin
16		118	lung	*P. aeruginosa*	32	24g: CI	164.5 (Cavg)	cefepime
17		43	lung	*P. aeruginosa*	<= 16	16g:CI	411.3 (Cavg)	meropenem ciprofloxacin
		79				16g:CI	198.5 (Cavg)	cefiderocol
18	CVVHD	75	blood	*K. pneumoniae*	<= 16	20g: CI	209.3 (Cavg)	cefotaxime
		66				16g: CI	61 (Cavg)	cefotaxime

CI: continuous infusion; CSF: cerebrospinal fluid; Cmin: trough concentration; Cmax: peak concentration; Cavg: average concentration; eGFR: estimated glomerular filtration rate; NA: not available.

HD: hemodialysis; CVVHD: continuous veno-venous hemodialysis; CVVHDF: continuous veno-venous hemodiafiltration

°eGFR was estimated with Cockcroft-Gault formula
*pediatric

471

Legend to the Figures

473

474 *Figure 1*

475 Extracted Ion Current Chromatograms. A: Negative MRM (136.85>78.87) for detection of

476 fosfomycin in human drug-free plasma sample (solid black line); control human plasma sample

477 spiked with known amounts of drug (dashed black line), lower limit of quantification 2 mg/L) and in

478 a patient plasma in therapy with fosfomycin. (dashed grey line, 102 mg/L). B: Negative MRM

479 (139.85>78.87) for detection of fosfomycin IS.

480