DOI: 10.2478/cipms-2023-0037 Curr. Issues Pharm. Med. Sci., Vol. 36, No. 4, Pages 217-220

Current Issues in Pharmacy and Medical Sciences
Formerly ANNALES UNIVERSITATIS MARIAE CURIE-SKLODOWSKA, SECTIO DDD, PHARMACIA

journal homepage: http://www.curipms.umlub.pl/

Fosfomycin activity *in vitro* against *Escherichia coli* strains isolated from urine specimens

Magdalena Zdzieblo[1], Anna Biernasiuk[2*], Pawel Helon[1], Anna Malm[2]

[1] Branch in Sandomierz, Jan Kochanowski University in Kielce, Poland
[2] Department of Pharmaceutical Microbiology, Medical University in Lublin, Poland

ARTICLE INFO

Received 21 June 2023
Accepted 12 November 2023

Keywords:
Escherichia coli,
urinary tract infections,
minimum inhibitory concentration,
fosfomycin-resistance,
fosfomycin-sensitive strains,
in vitro re-growth.

ABSTRACT

Urinary tract infections are caused mostly by uropathogenic *Escherichia coli* strains. The aim of this paper was to assess the activity of fosfomycin *in vitro* against 74 *E. coli* strains isolated from urine samples of outpatients with acute cystitis. Minimum inhibitory concentration (MIC) was determined by the recommended method of serial drug dilution in Mueller-Hinton agar supplemented with glucose-6-phosphate (25 mg/L). The estimated MIC values were in the range of 1-32 mg/L. The prevalence of fosfomycin-sensitive strains was 45.9%, while that of fosfomycin-resistant strains – 54.1%. The time-kill assay was performed for a chosen clinical strain (MIC = 1 mg/L) in Mueller-Hinton broth also supplemented with glucose-6-phosphate in the presence of various concentrations of fosfomycin (0.5-64 mg/L). The bacterial population density was expressed as log CFU (colony forming units)/mL. The decrease of the bacterial population viability after 6h incubation was found with Δlog CFU/mL = 1.05-5.02, depending on fosfomycin concentration. However, bactericidal effect of this antibiotic (Δlog CFU/mL >3) was observed only at the highest concentrations (32-64 mg/L). Moreover, after prolonged incubation (24 h), the re-growth of bacterial population revealed as the increase of its viability (Δlog CFU/mL = 1.03-3.03) was observed at fosfomycin concentration in the range 0.5-32 mg/L, but not at 64 mg/L. This phenomenon may be due the presence of subpopulations of spontaneous mutants sensitive only to higher concentrations of this antibiotic comparing to MIC. The presented data confirm a need to monitor the sensitivity of uropathogenic *E. coli* strains to fosfomycin.

INTRODUCTION

Urinary tract infections (UTIs) are amongst the most frequent infections found both in hospitalized patients and outpatients. Totally, 80% of all UTIs are classified as uncomplicated infections. Uncomplicated acute cystitis is the most prevalent form of UTIs in women and is most commonly caused by uropathogenic *Escherichia coli* (UPEC) [1-3]. These strains possess several virulence factors, including those responsible for the colonization of the lower urinary tract and persistence despite the effectively functioning host defense mechanisms [3].

The current guidelines recommend the employment of antibiotics as the first choice treatment for uncomplicated acute cystitis, including fosfomycin trometamol as first-line therapy [2,4]. This antibiotic is usually used as single 3 g dose taken orally as fosfomycin trometamol (tromethamine). Its therapeutic concentration in urine, lasting for 1-2 days, allows for elimination of the majority of uropathogens from the urinary tract [5,6]. The peak of urinary fosfomycin concentration was reported to be about 4000 μg/mL and fosfomycin remained at concentrations >100 μg/mL for 48 hrs after a single 3 g oral dose [7]. Fosfomycin also appears to be effective for treatment of recurrent UTIs [4].

Fosfomycin is a phosphonic acid derivative, possessing a broad spectrum of activity against both Gram-positive and Gram-negative bacteria that are the main bacterial species of uropathogens, such as *E. coli*. This antibiotic is an inhibitor of the first step of peptidoglycan synthesis, as it inactivates cytosolic *N*-acetylglucosamine enolpyruvyl transferase (MurA). In *E. coli*, two carrier-dependent systems can actively transport fosfomycin: the α-glycerophosphate permease and the hexose phosphate uptake system [8]. Fosfomycin resistance in Gram-negative bacteria can be conferred by 1. defects in the transmembrane transporters, 2. amino acid substitution in the MurA active site which decreases fosfomycin binding affinity, and 3. production

* **Corresponding author**
e-mail: anna.biernasiuk@umlub.pl

of the fosfomycin-inactivating enzyme FosA (Mn^{2+}- and K^+-dependent glutathione *S*-transferase) [9].

Antimicrobial resistance is one of the important current global public health problems. This problem is especially urgent regarding antibiotic resistance in bacteria, including uropathogens [10]. The overuse, miss-use or inappropriate use of antibiotics contribute to the emergence of resistance [11]. The large number of antibiotic prescriptions due to UTIs are known to be a major cause of the spread of antimicrobial resistance [3]. Therefore, there is a need to monitor the antibiotic sensitivity of clinical isolates of bacterial species causing the most common infections such as UTIs.

AIM

The aim of this paper was to assess the activity of fosfomycin *in vitro* against *E. coli* strains isolated from urine samples of outpatients (women) with acute cystitis by determination of its minimum inhibitory concentration (MIC). The time-kill assay was also performed so as to gain understanding the interactions between an antibacterial agent and bacterial cells over time.

MATERIALS AND METHODS

The 74 *E. coli* clinical strains included in this study were from the collection of clinical strains of Department of Pharmaceutical Microbiology, Medical University in Lublin (Poland). These strains were primarily isolated from urine samples of female patients with acute cystitis according to the consent of the Bioethics Committee (no. KE-0254/75/2011). The reference strain *E. coli* ATCC 25922 was also used. All the strains were stored at -75°C in vials containing bacterial cultures in tryptic soy broth with addition of 10% glycerol (v/v) as cryoprotectant. In order to revive and propagate the bacterial strains, a portion of frozen culture was thawed and transferred on tryptic soy agar plate, followed by incubation under standard conditions at 35°C for 24 hrs. Through the application of reduction sieving, the fresh bacterial cultures obtained after incubation at the mentioned above conditions were used subsequently for experiments and were stored on tryptic soy agar plates at 4°C.

The activity of fosfomycin was estimated on the basis of minimum inhibitory concentration (MIC) values determined by the method of serial antibiotic dilution in Mueller-Hinton agar (MHA) supplemented with glucose-6-phosphate (25 mg/L) recommended by European Committee for Antimicrobial Sensitivity Testing (EUCAST) [12]. The fosfomycin concentrations in the plates with this solid medium were in the range of 0.5-128 mg/L. Bacterial suspensions were prepared in sterile saline to obtain an optical density equal to 0.5 McFarland standard, corresponding to 1.5 × 108 colony forming units (CFU)/mL. Next, 10 µl of individual bacterial suspension was applied on the medium surface of plates containing a defined fosfomycin concentration. After the absorption of suspension at room temperature, the inoculated media were incubated under standard conditions at 35°C for 18-24 hrs. Bacterial propagation was assessed visually by comparison with the growth of bacterial strains on MHA plus glucose-6-phosphate without addition of fosfomycin (positive control). The negative control was only the growth medium. The lowest concentration of fosfomycin with no visible bacterial growth was read as MIC. Each experiment was performed in triplicate. The most common values (mode) were presented.

Population parameters such as MIC_{50} and MIC_{90} were then calculated. The MIC_{50} represents the MIC value at which ≥50% of the isolates in a tested population are inhibited, while MIC_{90} is defined as the MIC value at which ≥90% of the strains within a tested population are inhibited [12,13].

The time-kill assay was performed for a selected clinical *E. coli* strain showing MIC for fosfomycin equal to 1 mg/L. Bacterial suspension of optical density of 0.5 McFarland standard was prepared in sterile saline, and then it was diluted ten-fold in Mueller-Hinton broth (MHB) with glucose-6-phosphate. Next, a serious of fosfomycin concentrations (0.5 mg/L, 1 mg/L, 2 mg/L, 4 mg/L, 8 mg/L, 16 mg/L, 32 mg/L, 64 mg/L) were prepared in 100 mL flasks in MHB with glucose-6-phosphate. Each flask containing 19 mL of growth medium with a defined fosfomycin concentrations was inoculated by addition of 1 mL of the diluted bacterial suspension. The inoculated media were incubated under standard conditions at 35°C with shaking (200 cycles/min). The positive control contained the inoculated MHB plus glucose-6-phosphate without fosfomycin, while the negative control held only the growth medium. To obtain the time-kill curve, the bacterial growth rate was counted at different time intervals, i.e. 0, 0.5, 1, 2, 4, 6, 24 hrs by collecting 100 µL samples. Each sample was plated on MHA using easySpiral Dilute® (Interscience, France). After incubation (standard conditions, 35°C, 24 hrs), the number of colonies was counted. The density of bacterial population was assessed using the number colonies and a dilution factor, and expressed as CFU/mL or log of CFU/mL. Each experiment was performed in triplicate. Mean values ± standard deviations (SD) were presented.

RESULTS

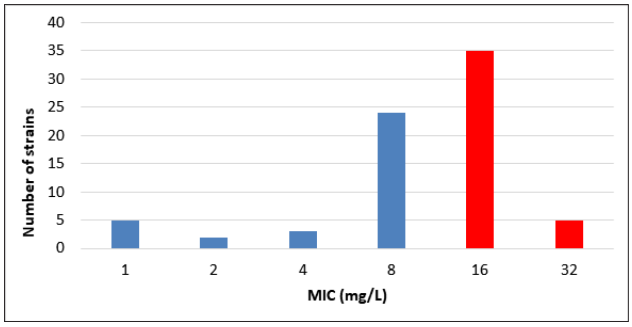
Using the recommended method of serial antibiotic dilution in MHA supplemented with glucose-6-phosphate, it was found that the clinical *E. coli* strains studied were inhibited by fosfomycin with MIC = 1-32 mg/L (Figure 1). According to the current EUCAST clinical breakpoint for oral fosfomycin, being ≤8 mg/L [12], 34 (45.9%) of the strains studied were sensitive to fosfomycin, while the remaining 40 (54.1%) were fosfomycin-resistant. The population sensitivity parameters MIC_{50}= 16 mg/L and MIC90 = 16 mg/L were higher than the clinical breakpoint. Moreover, MIC for the reference strain *E. coli* ATCC 25922 was in the range 4-8 mg/L fosfomycin, while mode was 4 mg/L.

The time-kill assay was performed for a chosen clinical *E. coli* strain (MIC = 1.0 mg/L) in MHB supplemented with glucose-6-phosphate in the presence of various fosfomycin concentrations (0.5-64 mg/L). The bacterial population density was expressed as log CFU/mL. The decrease of the bacterial population density by fosfomycin after 6 hrs incubation (Δlog = 1.05-5.02) was found to be concentration-dependent fosfomycin concentration in the range 0.5-64 mg/L

(Figure 2, Table 1). This inhibitory effect was only temporary because the phenomenon of re-growth of *E. coli* population (Δlog = 1.03-3.03) was observed after 24 hrs incubation at fosfomycin concentration in the range 0.5-32 mg/L (Figure 2, Table 1). The treatment of bacterial population with higher fosfomycin concentration resulted in lower intensity of its re-growth. This phenomenon was not found at 64 mg/L fosfomycin (Figure 1).

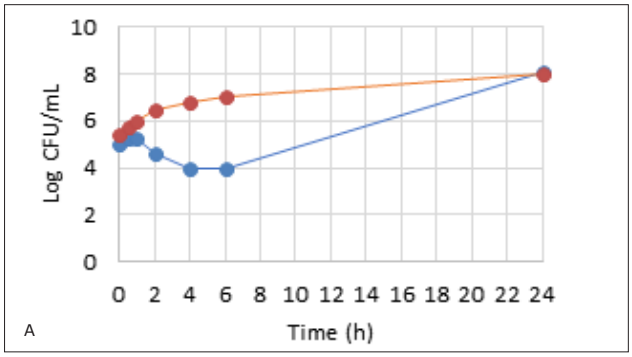
Table 1. The changes in the population density (Δlog CFU/mL) of clinical *E. coli* strain (MIC of fosfomycin = 1 mg/L) in the presence of various fosfomycin concentrations after 6 and 24 hrs incubation

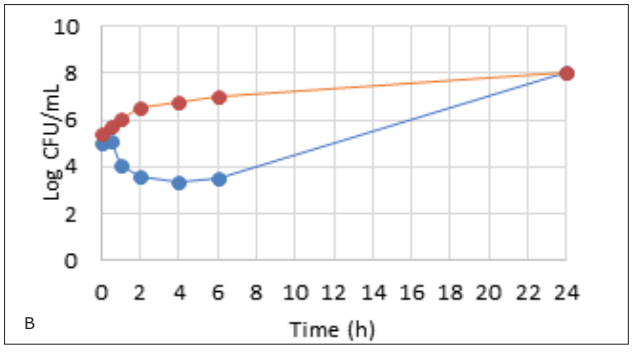
Fosfomycin concentration (mg/L)	Δlog CFU/mL after 6 hrs	Δlog CFU/mL after 24 hrs
0 (control)	↑1.6	↑2.64
0.5	↓-1.05	↑3.03
1	↓-1.53	↑3.02
4	↓-1.73	↑2.76
8	↓-2.21	↑2.64
32	↓-5.02	↑1.03
64	↓-5.02	↓-5.02

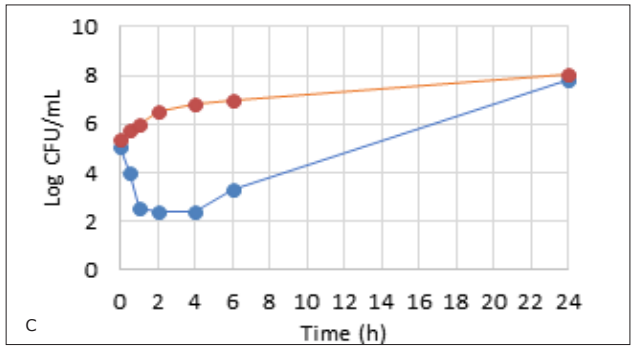


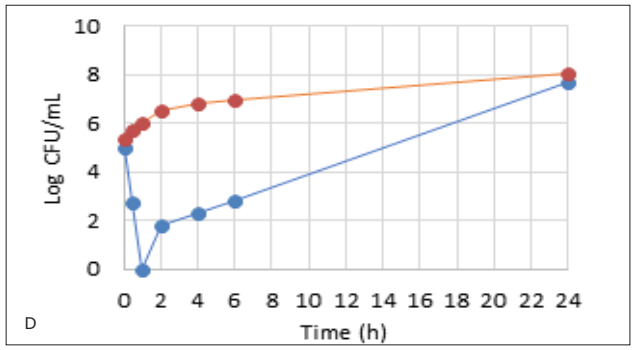
(blue) sensitive strains; (red) resistant strains

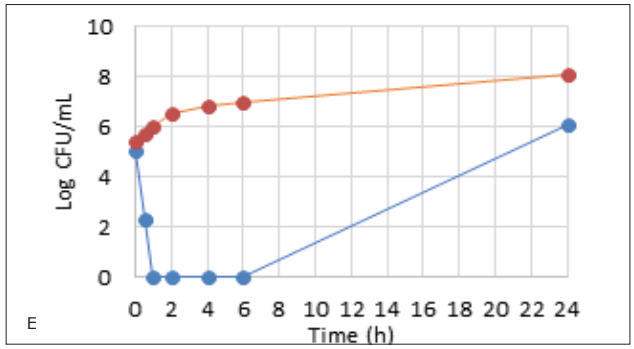
Figure 1. MIC distribution among clinical *E. coli* strains. The most common (mode) values were presented











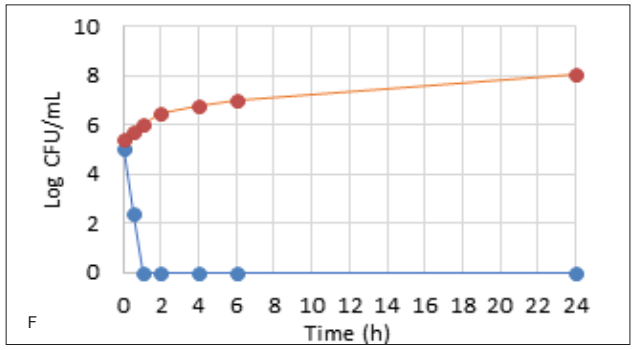


Figure 2. Time-kill kinetics for clinical *E. coli* strain (MIC of fosfomycin = 1 mg/L) in the presence of various fosfomycin concentrations: A. 0.5 mg/L, B. 1 mg/L, C. 4 mg/L, D. 8 mg/L, E. 32 mg/L, F. 64 mg/L (blue symbols); control growth curve in the absence of fosfomycin (red symbols)

DISCUSSION

The MIC can be currently regarded as the best available parameter for describing the effectiveness of a given antibiotic against bacterial strains. However, the determined MIC values must be compared with the clinical breakpoints to assess whether the strain is susceptible or resistant to a given antibiotic. The clinical breakpoints are currently published by two organizations: the European Committee

on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI) [13]. According to the EUCAST, the clinical breakpoint for oral fosfomycin up to 2020 was ≤32 mg/L for the sensitive strains belonging to *Enterobacterales*, including *E. coli,* isolated from patients with uncomplicated UTIs [12,14]. In light of these data, all of the *E. coli* strains studied should be categorized as sensitive to fosfomycin. Still, in 2020 EUCAST revised breakpoints for oral fosfomycin. This resulted in *E. coli* becoming the only target species for uncomplicated UTIs therapy, as well as a decrease in the breakpoint from 32 to 8 mg/L [14,15]. This revision was driven by both clinical data and PK/PD data that were not previously available [14]. Taking into account the revised clinical breakpoint currently applied [12], only 34 (45.9%) of all *E. coli* strains studied were sensitive to fosfomycin, while the remaining 40 (54.1%) strains were categorized to be resistant. However, this observation is in contrary to the literature data indicating the relatively low prevalence of fosfomycin resistance in *E. coli* strains isolated from urine in patients with symptomatic bacteriuria that purportedly was reaching <20% [7,16,17]. These differences may be due to the mentioned above revision of oral fosfomycin clinical breakpoint. It is, however, worth noting an increasing trend of resistance observed in countries where fosfomycin trometamol is extensively used [14]. The presented data and those from the literature confirm a need to monitor the sensitivity of uropathogenic *E. coli* strains to fosfomycin.

The phenomenon of *E. coli* re-growth exposed *in vitro* to the inhibitory concentrations of fosfomycin was described by other authors [18,19]. This can be explained by the mutant selection window (MSW) hypothesis or the heteroresistance phenotype [20]. According to the MSW hypothesis, antibiotic concentrations inside the MSW are expected to allow the acquisition of resistance concurrently with eradication of susceptible pathogen populations. Heterogeneous resistance phenotype can be partly explained by a high mutation rate, together with the presence of mutations in genes associated with fosfomycin resistance [20]. Of note, the effects described above were not observed in animal models most probably due to the host immune response. Moreover, fosfomycin susceptibility and its antibacterial activity were found to be greatly affected by the urine environment [18]. The clinical significance of these results requires further studies.

FUNDING

This research was funded by the Medical University of Lublin (DS 30).

ORCID iDs

Magdalena Zdziebło https://orcid.org/0000-0001-8531-5615
Anna Biernasiuk https://orcid.org/0000-0002-2844-9876
Paweł Helon https://orcid.org/0000-0002-5519-1890
Anna Malm https://orcid.org/0000-0003-1503-7634

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