Urinary antibacterial activity of fosfomycin and nitrofurantoin at registered dosages in healthy volunteers

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ABSTRACT

Given emerging uropathogen resistance to more recent antibiotics, old antibiotics used for uncomplicated urinary tract infection (UTI) warrant reexamination. We investigated the urinary antibacterial activities of fosfomycin and nitrofurantoin by determining the urinary inhibitory titer (UIT) and the bactericidal titer (UBT) against uropathogens in urine samples from female volunteers after the administration of single-dose fosfomycin (3g) or nitrofurantoin (50 mg q6h or 100 mg q8h). Urine samples were collected over 48h (fosfomycin) or 8h (nitrofurantoin) with drug levels quantified with every void. Fosfomycin concentrations ranged from <0.75 (below the limit of quantification [LOQ]) to 5729.9 mg/l, and nitrofurantoin concentrations from <4 (below LOQ) to 176.3 mg/l (50 mg q6h) or 209.4 mg/l (100 mg q8h). There is discrepancy in the response to fosfomycin between *Escherichia coli* and *Klebsiella pneumoniae* species because fosfomycin displayed strong bactericidal activity for 48h for *Escherichia coli*, and moderate bactericidal activity for 18h against *Klebsiella pneumoniae* isolates. This effect was not related to the strain's baseline minimal inhibitory concentration (MIC), but rather to the presence of a resistant subpopulation. Maximum titers of nitrofurantoin were obtained during the first 2h, but no antibacterial effect was found in most samples, regardless of the dose. In the rare samples in which antibacterial activity was detectable, titers were comparable for both species tested. These findings confirm the doubts on fosfomycin administration in UTIs caused by *Klebsiella pneumoniae* and reveal discrepancy between nitrofurantoin's measurable *ex vivo* activity and its clinical effect over multiple dosing intervals.

Keywords: fosfomycin; nitrofurantoin; urinary tract infections; pharmacokinetics; pharmacodynamics; urine; healthy volunteers

INTRODUCTION

Fosfomycin and nitrofurantoin are recommended first-line for urinary tract infection (UTI), the most common bacterial infection among otherwise healthy women (1). Though resistance among uropathogens is increasing, it remains relatively low for fosfomycin and nitrofurantoin (2–4). Despite their use over several decades, these antibiotics' pharmacokinetic (PK) and pharmacodynamic (PD) properties remain poorly defined, though such information is essential for therapy optimization and the prevention of the emergence of resistance (5, 6). While new data are beginning to emerge on the PK (7–11) and PD properties (12, 13) of both drugs, most *in vitro* PD studies have been conducted in a non-biological matrix and/or did not take into account drug-concentration changes over time *ex vivo*, thus limiting the clinical translation of these results.

A method to address these limitations is the determination of the urinary antibacterial activity of antimicrobial agents in which *ex vivo* PK data are used within a static *in vitro* model (14–16). The urinary antibacterial activity of an antimicrobial agent is described by the urinary inhibitory titer (UIT) and the urinary bactericidal titer (UBT). These are measures of antibacterial activity over time in urine, the relevant biological matrix. Thus providing *in vitro* data that more closely reflects the clinical scenario by describing antibiotic activity against the pathogen within the host's environment.

We determined the urinary antibacterial activity of fosfomycin and nitrofurantoin against common uropathogens, after administration of registered doses for the treatment of UTI, to evaluate the effectiveness of these drugs.

MATERIAL AND METHODS

Study design, subjects, drug administration and sample collection

Urine samples to determine the UIT and UBT were obtained in two previous studies evaluating the PK properties of the both fosfomycin and nitrofurantoin (7, 8). Briefly, the fosfomycin urinary PK study was a single-center study to examine the urinary PK following a single, oral 3 gram dose of fosfomycin trometamol (Monuril, Zambon Nederland B.V., Amersfoort, the Netherlands) in 40 healthy, female volunteers (7). Fosfomycin was administered under supervision of one of the researchers. Urine samples were collected in a home setting over 48 hours with every void and two times daily from 48 hours until 7 days after administration. For the present study, we used only the samples collected in the first 48 hours. There were no dietary restrictions prior to or after drug administration. Samples were kept in home freezers until handed in to investigators. The nitrofurantoin PK study was a single-center study in which macrocrystalline nitro-

furantoin was administered either 50 mg q6 hours (Furadantine MC, Mercury Pharma Ltd, Croydon UK) or 100 mg q8 hours (Furadantine® retard, Mercury Pharma Ltd, Croydon UK) in a crossover design to 12 healthy, female volunteers (8). The drug was administered with food and administration began in a home setting 24 hours prior to sample collection to achieve steady state. The last dose was administered in the hospital at the start of the 8-hour visit, during which urine samples were collected for 6 hours or 8 hours, depending on the assigned dosing interval. Volunteers were instructed to protect the nitrofurantoin samples from daylight using aluminum foil to avoid photodegradation of the drug.

Total volume, pH and time of each sample were recorded for both fosfomycin and nitrofurantoin samples prior to storage at -80°C. Stability of the samples under these conditions was confirmed during the validation of the analytical methods (17, 18). Drug levels were quantified using ultra-high performance liquid chromatography (UHPLC) with tandem mass spectrometry (MS/MS) detection for fosfomycin and ultraviolet (UV) detection for nitrofurantoin. Both methods were validated according to FDA guidelines, as described elsewhere (17, 18).

Test organisms and MICs

Isolates were obtained from clinical sources with the exception of the ATCC reference strain and were selected with a range of fosfomycin and nitrofurantoin MIC values (table 1). Fosfomycin susceptibility was determined by agar dilution using 10^4 CFU/spot of each isolate inoculated on Mueller–Hinton II agar (MHA; BD Diagnostics, Franklin Lakes, NJ, USA) containing 25 mg/L glucose-6-phosphate (G6P; Sigma, Taufkirchen, Germany) and fosfomycin (InfectoPharm, Heppenheim, Germany) following CLSI recommendations in a concentration range of 0.25 – 1024 mg/L. Isolates were tested in triplicate. Nitrofurantoin susceptibility was determined by broth microdilution according to ISO guidelines (19).

Fosfomycin containing urine samples from the volunteers were divided in two sets to allow for the limited volumes of material. Set 1 consisted of samples of the initial 20 volunteers and the second set consisted of those from the remaining 20 volunteers. Both sets were tested against two *Escherichia coli* strains, two *Klebsiella pneumoniae* strains and the ATCC strain (table 1). All strains were used for testing the nitrofurantoin samples.

Determination of UITs and UBTs

All urine samples were filtered before analysis by centrifugation (10 minutes at 13.000 rpm) using Amicon Ultra-0.5 centrifugal filter units with a 10 kDa cutoff Ultracel-10 membrane (UFC5010BK; Merck, Amsterdam, the Netherlands). The large volumes of antibiotic-free urine were filtered over 0.2 µm bottle-top vacuum filters (CLS430756;

Table 1. MICs of fosfomycin and nitrofurantoin. The MIC represents the modal value based on the results of agar dilution (fosfomycin) or micro dilution (nitrofurantoin) performed in triplicate.

Test strain	Source	MIC Fosfomycin (mg/liter)[a]	MIC Nitrofurantoin (mg/liter)
		E. coli	
ATCC 25922	Laboratory strain	1	16
51[b]	Blood	2	32
03 [b]	Urine	0.25	16
1231	Urine	16	512
4807	Rectal swab	32	16
		K. pneumoniae	
58 [b]	Urine	8	64
20 [b]	Rectal swab	32	256
31865	Blood	2	128
55	Sputum	4	256

[a] The MIC represents the modal value based on the results of agar dilution (fosfomycin) or microdilution (nitrofurantoin) performed in triplicate.
[b] Strains used for set 1 of the urine samples of volunteer 1 to 40 in the fosfomycin study.

Corning, Taufkirchen, Germany). UITs and UBTs were determined by microdilution. Urine samples underwent a serial two-fold dilution series in antibiotic-free urine from healthy volunteers such that the first well of the microtiter plate contained a 2-times diluted sample. The final bacterial inoculum within the microtiter tray was approximately 2.5×10^5 CFU/ml. Inoculated plates were incubated for 18 ± 2 hours at 35 ± 2°C. After which every well was checked visually for growth. The UIT represents the bacteriostatic activity and was defined as the highest dilution that inhibited visible growth. The UBT represents the bactericidal activity and was defined by the absence of bacterial growth following a subculture from the microtiter tray onto an antibiotic-free Tryptic Soy agar plate supplemented with 5% sheep blood (TSB, 254087, Becton Dickinson, Franklin Lakes, NJ, USA). The limit of detection was 50 cfu/ml. TSB plates were incubated for 18 ± 2 hours at 35 ± 2°C. The UBT was defined as the highest dilution of the sample that still exhibited bactericidal activity. Comparable UIT and UBT reflect antibiotic bactericidal activity, while a UIT exceeding the UBT reflects bacteriostatic activity. UITs and UBTs are presented as reciprocal values of the titers and could therefore range from <2 (no antibacterial activity observed) to 1024, with higher titers indicating greater antibacterial activity.

Determination of fosfomycin-resistant subpopulation

To determine the presence of fosfomycin low-level resistant (LLR) or high-level resistant (HLR) subpopulations, isolates were cultured overnight in both Muller-Hinton

broth (MHB) and antibiotic-free urine using a starting inoculum of 2.5 x 10^5 CFU/ml. Quantitative cultures were then performed in parallel on antibiotic-free Muller-Hinton agar (MHA) and MHA supplemented with 25 mg/liter glucose-6-phosphate together with 64 or 512 mg/liter of fosfomycin. Total bacterial density, and the comparative density of any growth on the fosfomycin-containing media, was determined by plating 20 µl from a serial 10-fold dilution of the incubated liquid media. Growth capacity and resistant-subpopulation proportions were compared between MHB and urine. MHA plates were incubated overnight at 35 ± 2°C. The limit of detection was considered to be 1.4 $\log_{10}$ CFU/ml. This additional analysis was performed for fosfomycin based on previous studies in which a resistant subpopulation was identified in susceptible *Enterobacteriales* (9, 20).

Statistical analysis

Microsoft Excel 2013, IBM SPSS Statistics 24 and Graphpad Prism (San Diego, CA, version 7.0) were used for processing the data. Fosfomycin samples were grouped in 6-hour time intervals and nitrofurantoin samples were grouped in 2-hour time intervals. The median and range of UITs and UBTs were calculated for each interval. The area under the inhibitory titer–time curve (AUIT) and the area under the bactericidal titer–time curve (AUBT) where calculated to give an indication of the inhibitory and bactericidal activity for each strain using the trapezoidal rule (14). A period of 48 hours was considered for fosfomycin and 6 and 8 hours for nitrofurantoin for the 50 mg q6 and 100 mg q8 hour dosing regimens, respectively. Titer values were compared using a two-sided Wilcoxon matched-pairs rank test ($p<0.0001$) to compare the titers of the two species and a one-sided Wilcoxon matched-pairs rank test ($p<0.0001$) was used to compare the UIT values and the UBT values per time interval. The d'Agostino-Pearson test was used to check normal distribution of data. Untransformed data were used for the statistical analysis. Titer values of <2 were transformed into 1 for statistical analysis. The UBT in the most concentrated sample was used to calculate the percentage of volunteers in whom bactericidal activity (UBT ≥2) could be measured.

RESULTS

Subjects and urine samples

Volunteers in both studies were Caucasian females with a mean age (SD) of 24.3 (± 7.9) years and 28.5 (± 7.9) years in the fosfomycin and nitrofurantoin groups, respectively. A more detailed overview of volunteer characteristics can be found in the original studies (7, 8). The number of samples collected by the volunteers varied from 6 to 19 for fosfomycin and from 3 to 9 for nitrofurantoin because they were not instructed to follow a

voiding schedule. Fosfomycin urinary concentrations ranged from <0.75 (lower limit of quantification [LOQ]) to 5729.9 mg/l and did not differ significantly between the two sets ($p<0.05$; table S1). Nitrofurantoin concentrations ranged from <4 mg/l (LOQ) to 176.3 mg/l (nitrofurantoin 50 mg q6 hours), and from <4 to 209.4 mg/l (nitrofurantoin 100 mg q8 hours; table S1). Nitrofurantoin concentrations were slightly higher for the 100 mg dose, but peak concentrations were almost equal (mean C_{max} 94.4 mg/l [± 47.8] for 50 mg q6 hours versus 94.1 mg/l [± 49.9] for 100 mg q8 hours).

(A)UITs and (A)UBTs

Fosfomycin

The high interindividual variability in urinary drug concentrations was reflected by the wide range in UITs and UBTs (7). For *E. coli*, fosfomycin UITs ranged from <2 to 256 and maximum titers were obtained during the first 12 hours after dosing (figure 1A and table S2). Likewise, UBTs ranged from <2 to 512 and were comparable to the UITs for *E. coli*. Thus, fosfomycin was bactericidal against *E. coli* (figure 1B and table 2). There was still reasonable bactericidal activity after 48 hours for *E. coli* because UBTs were ≥2 in the majority (95%) of the samples. The only exception was the *E. coli* 1231 strain (MIC 16 mg/liter) where UITs and UBTs did not exceed 2 for the full 48 hours. The $AUIT_{0\text{-}48h}$ values between the five *E. coli* strains were comparable, again with the exception of *E. coli* 1231 (table 2 and table S2). The same is true for the $AUBT_{0\text{-}48h}$ values (table 2). The difference in $AUIT_{0\text{-}48h}$ and $AUBT_{0\text{-}48h}$ values between the *E. coli* strains did not reflect their varying baseline MICs for fosfomycin (table 1).

UITs for *K. pneumoniae* ranged from <2 to 128 and maximum titers were found during the first 6-hour time period (figure 1A and table S2). UITs and UBTs were comparable, reflecting bactericidal activity of fosfomycin in *K. pneumoniae* (figure 1B and table 2). In contrast to *E. coli*, no antibacterial activity of fosfomycin in *K. pneumoniae* was observed in the majority (86%) of samples throughout the complete 48 hours. Where an antibacterial effect was detected, it was bactericidal in the majority (90%) of the samples, but it was present only during the first 18 hours after administration. UITs and UBTs declined dramatically after that 18-hour time point. UITs and UBTs for *K. pneumoniae* were significantly lower than those for *E. coli* ($p<0.0001$ for all time intervals) (figure 1A and 1B). $AUIT_{0\text{-}48h}$ and $AUBT_{0\text{-}48h}$ values ranged from 47 to 110 and were independent of strains' baseline MICs for fosfomycin (table 2 and table S2).

Nitrofurantoin

For *E. coli*, nitrofurantoin UITs ranged from <2 to 16 for the 50 mg q6 hour regimen and from <2 to 32 for the 100 mg q8 hour regimen and were generally within the same range for both dosing regimens (figure 1C and table S3). Maximum titers were

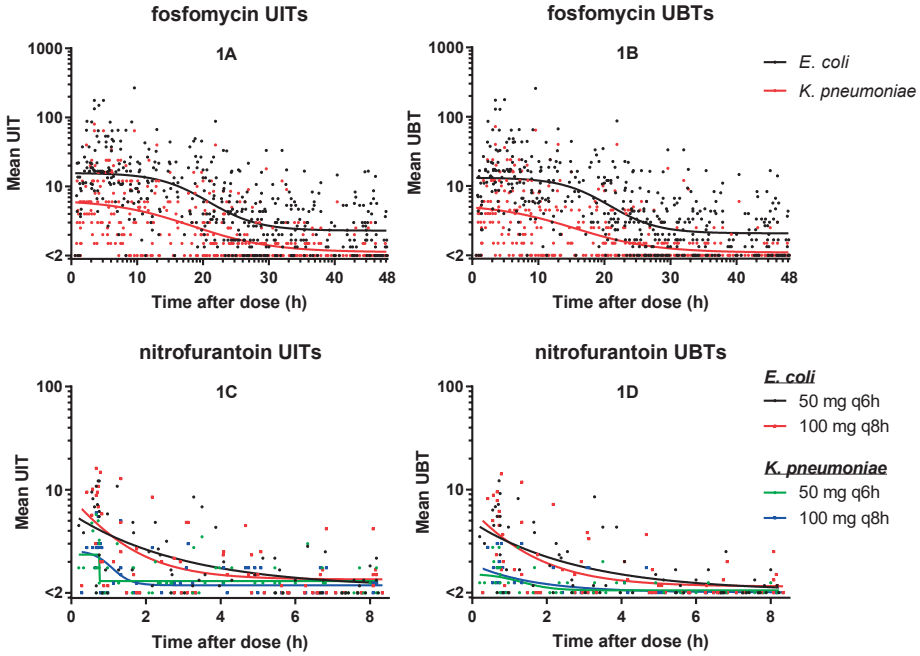

Figure 1. Fosfomycin UIT values (**1A**) and UBT values (**1B**) for *E.coli* (black) and for *K. pneumoniae* (red) for all samples. Below, nitrofurantoin UITs (**1C**) and UBTs (**1D**) for both dosing regimens for *E.coli* (black and red) and for *K. pneumoniae* (green and blue). Every dot represents the mean UIT or UBT for each sample for the *E. coli* and *K. pneumoniae* strains, respectively.

obtained within the first 2 hours after administration. UBTs for *E. coli* were comparable with the UIT values, demonstrating bactericidal activity of nitrofurantoin against *E. coli* (figure 1D and table 3). After 2 hours, no detectable antibacterial activity was found in the majority of the samples (titers of <2).

For *K. pneumoniae,* nitrofurantoin UITs ranged from <2 to 16 for both dosing regimens; maximum titers were found in the first 2 hours after administration (figure 1C and table S3). UBTs ranged from <2 to 8 and did not differ between dosing regimens (figure 1D and table 3). UITs and UBTs were comparable in these first two 2 hours, again reflecting bactericidal activity of nitrofurantoin in the few samples in which antibacterial activity was detectable.

The UITs and UBTs were higher for *E. coli* compared to those for *K. pneumoniae* for both dosing regimens (figure 1C and 1D). Similar to fosfomycin activity, the AUIT and AUBT values were found to be independent of the baseline nitrofurantoin MICs of the isolates (tables 3 and S3). This is true for both dosing regimens.

Table 2. Median (range) of UBTs and $AUBT_{0-48h}$ values for fosfomycin over time for each strain.

Strain (MIC, mg/liter)	UBT (median (range)) for the indicated time periods (h)								$AUBT_{0-48h}$
	0-6	6-12	12-18	18-24	24-30	30-36	36-42	42-48	
E. coli									
ATCC25922 (1)	16 (<2-256)	16 (<2-512)	16 (<2-128)	4 (<2-128)	4 (<2-64)	2 (<2-64)	3 (<2-32)	2 (<2-32)	152 (97-303)
51 (2)	8 (<2-64)	4 (<2-32)	4 (<2-16)	4 (<2-16)	2 (<2-16)	2 (<2-8)	2 (<2-8)	2 (<2-4)	115 (53-163)
03 (0.25)	16 (<2-64)	16 (4-64)	16 (2-32)	8 (<2-32)	3 (<2-16)	2 (<2-16)	2 (<2-8)	2 (<2-8)	143 (101-192)
1231 (16)	2 (<2-16)	2 (<2-8)	<2 (<2-8)	<2 (<2-8)	<2 (<2-4)	<2 (<2-4)	<2 (<2-4)	<2 (<2-2)	63 (46-112)
4807 (32)	16 (<2-256)	16 (<2-256)	16 (<2-64)	6 (<2-128)	3 (<2-32)	2 (<2-32)	3 (<2-16)	2 (<2-16)	162 (88-275)
K. pneumoniae									
58 (8)	2 (<2-8)	<2 (<2-16)	<2 (<2-2)	<2 (<2-4)	<2 (<2-2)	<2 (<2-2)	<2 (<2-4)	<2 (<2-2)	47 (39-102)
20 (32)	4 (<2-32)	2 (<2-32)	<2 (<2-8)	2 (<2-16)	<2 (<2-2)	<2 (<2-2)	<2 (<2-2)	<2 (<2-2)	69 (44-114)
31865 (2)	8 (<2-128)	8 (<2-64)	4 (<2-64)	2 (<2-64)	<2 (<2-32)	<2 (<2-16)	<2 (<2-8)	<2 (<2-4)	106 (74-218)
55 (4)	4 (<2-16)	2 (<2-64)	<2 (<2-16)	<2 (<2-16)	<2 (<2-4)	<2 (<2-8)	<2 (<2-4)	<2 (<2-2)	68 (44-153)

Table 3. Median (range) of UBTs and $AUBT_{0-6h}$ or $AUBT_{0-8h}$ values for nitrofurantoin over time for each strain (MIC).

Dose, strain (MIC, mg/liter), and isolate	UBT (median (range)) for the indicated time periods (h)				
	0-2	2-4	4-6	6-8	$AUBT_{0-6h}$ or $AUBT_{0-8h}$
Nitrofurantoin 50 mg q6 hours					
E. coli					
ATCC25922 (16)	8 (<2-16)	2 (<2-16)	<2 (<2-4)	<2 (<2-4)	18 (10-31)
51 (32)	4 (<2-16)	<2 (<2-8)	<2 (<2-2)	<2 (<2-2)	14 (9-25)
03 (16)	4 (<2-16)	<2 (<2-8)	<2 (<2-2)	<2 (<2-2)	15 (9-25)
1231 (512)	<2 (<2-<2)	<2 (<2-<2)	<2 (<2-<2)	<2 (<2-<2)	9 (9-22)
4807 (16)	2 (<2-8)	<2 (<2-4)	<2 (<2-<2)	<2 (<2-2)	12 (8-11)
K. pneumoniae					
58 (64)	2 (<2-8)	<2 (<2-4)	<2 (<2-8)	<2 (<2-2)	11 (7-9)
20 (256)	<2 (<2-2)	<2 (<2-2)	<2 (<2-<2)	<2 (<2-<2)	9 (9-17)
31865 (128)	<2 (<2-<2)	<2 (<2-<2)	<2 (<2-<2)	<2 (<2-<2)	9 (7-9)
55 (256)	<2 (<2-2)	<2 (<2-<2)	<2 (<2-<2)	<2 (<2-<2)	9 (7-11)
Nitrofurantoin 100 mg q8 hours					
E. coli					
ATCC25922 (16)	4 (<2-32)	2 (<2-16)	<2 (<2-8)	<2 (<2-4)	15 (10-25)
51 (32)	2 (<2-16)	<2 (<2-8)	<2 (<2-2)	<2 (<2-2)	12 (8-21)
03 (16)	2 (<2-16)	<2 (<2-8)	<2 (<2-2)	<2 (<2-4)	13 (11-24)
1231 (512)	<2 (<2-<2)	<2 (<2-<2)	<2 (<2-<2)	<2 (<2-<2)	9 (8-21)
4807 (16)	<2 (<2-8)	<2 (<2-4)	<2 (<2-2)	<2 (<2-2)	11 (7-11)
K. pneumoniae					
58 (64)	2 (<2-8)	<2 (<2-4)	<2 (<2-4)	<2 (<2-<2)	11 (7-9)
20 (256)	<2 (<2-2)	<2 (<2-<2)	<2 (<2-<2)	<2 (<2-<2)	9 (7-20)
31865 (128)	<2 (<2-2)	<2 (<2-<2)	<2 (<2-<2)	<2 (<2-<2)	9 (7-10)
55 (256)	<2 (<2-2)	<2 (<2-<2)	<2 (<2-<2)	<2 (<2-<2)	9 (7-10)

Bactericidal effect in the samples and in volunteers

To correlate with clinical antibiotic effectiveness for UTI treatment, the percentages of volunteers in which bactericidal activity was found was calculated for sequential time intervals for the five *E. coli* and four *K. pneumoniae* strains. Figure 2 demonstrates these percentages over time. A higher percentage reflects a more effective treatment.

Considering fosfomycin, the percentages for *E. coli* were higher than for *K. pneumoniae*. Bactericidal activity against *E. coli* was found in a mean percentage of 90% of the volunteers during 24 hours, but this declined to <60% thereafter. This applies to all *E. coli* strains with the exception of the *E. coli* 1231 strain, against which fosfomycin was not bactericidal in the least diluted sample in 50% of volunteers (figure 2A). This finding was supported by the detection of a resistant subpopulation in this isolate (section 3.3). Against *K. pneumoniae*, bactericidal activity of fosfomycin was found in only an average percentage of 60% of the volunteers during the first 18 hours after administration (figure 2A). Percentages declined quickly thereafter to <20% beyond 24 hours after dosing. Thus, fosfomycin remained bactericidal against *K. pneumoniae* isolates after 24 hours in a very small number of volunteers.

Nitrofurantoin was bactericidal in *E. coli* ATCC 25922, *E. coli* 51 and *E. coli* 03, regardless of the administered dose (figure 2B and 2C). However, this bactericidal activity was only found in 0% - 50% of volunteers. Percentages of >60% were found only in the first 2 hours after administration. In *K. pneumoniae*, percentages never exceeded

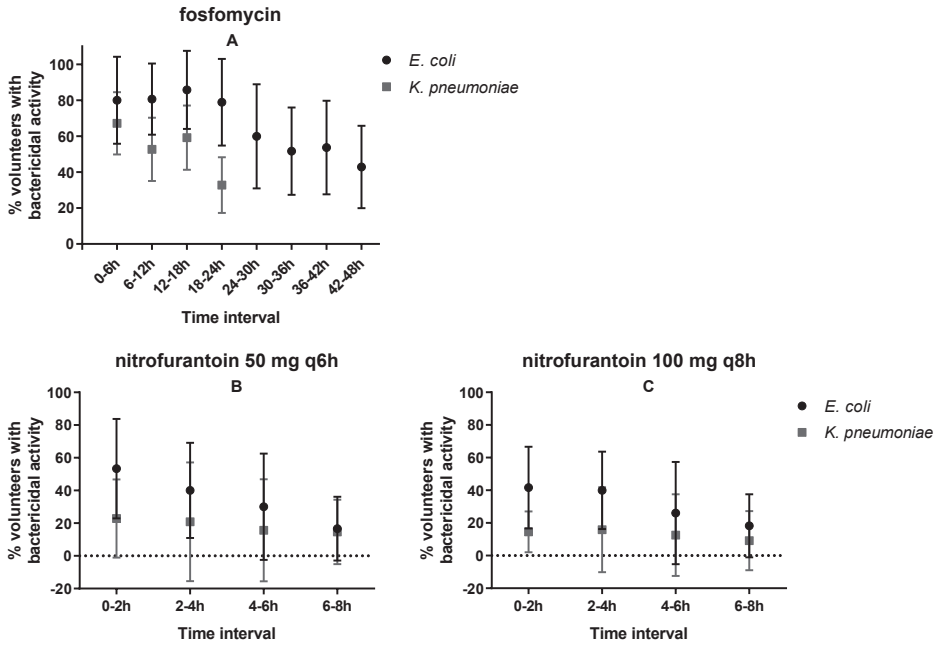

Figure 2. Percentage of volunteers where a bactericidal effect was found of fosfomycin during 48 hours (**2A**), and nitrofurantoin 50 mg q6 hours during 8 hours (**2B**) 100 mg q8 hours or (**2C**) in *E. coli* (black) and in *K. pneumoniae* (red). The numbers are expressed as the mean (± SD) percentages for both species (y axis) of the total number of volunteers that had produced urine samples in the considered time interval (x axis). Because bactericidal activity in *K. pneumoniae* was found in only a small number of volunteers (ranging from 1 to 3 volunteers) in the time intervals after 24 hours, these percentages are negligibly small and are therefore not presented in figure 2A.

17% in strains 58, 20, and 31865. This was independent of the administered dose. Only in *K. pneumoniae* 55, bactericidal activity was found in approximately 40% (100 mg q8 hours) and 60% (50 mg q6 hours) of volunteers. These percentages remained consistent over the 8-hour urine collection time period for this strain.

Fosfomycin-resistant subpopulation

Only one out of five *E. coli* isolates had a detectable fosfomycin-resistant subpopulation when grown in both standard laboratory media and human urine. Whereas, all of the *K. pneumoniae* isolates had a detected resistant subpopulation (table 4). The resistant subpopulation detected in *E. coli* 1231 and in the *K. pneumoniae* isolates had a fosfomycin MIC of >1024 mg/liter, after subculturing off the fosfomycin containing MHA onto TSA. This result is consistent with the low antibacterial activity of fosfomycin in these strains.

Table 4. Presence of a fosfomycin LLR and/or a HLR subpopulation of the strains in urine or MHB.

Strain (MIC, mg/liter)	LLR/HLR subpopulation present	
	Urine	MHB
E. coli		
ATCC25922 (1)	No	No
51 (2)	No	No
03 (0.25)	LLR	LLR
1231 (16)	HLR	HLR
4807 (32)	LLR	LLR
K. pneumoniae		
58 (8)	HLR	HLR
20 (32)	LLR	HLR
31865 (2)	HLR	HLR
55 (4)	HLR	HLR

MHB, Muller-Hinton Broth; LLR, low level resistant; HLR, high level resistant

DISCUSSION

While fosfomycin exhibited bactericidal activity for at least 48 hours against *E. coli*, no antibacterial activity was detected in the majority of *K. pneumoniae* samples. In contrast to fosfomycin, nitrofurantoin showed low antibacterial activity in both species regardless of the administered dose, though only one dose interval was examined among the many intervals intended with a course of nitrofurantoin.

In general, fosfomycin exhibited bactericidal activity as demonstrated by comparable UIT and UBT values. The duration of activity was strongly species-dependent, with ≥48 hours for *E. coli* and only 18 hours for *K. pneumoniae*. Indeed, 48-hour antibacterial activity against *K. pneumoniae* could be demonstrated in only a small subset. These findings are supported by earlier *in vitro* research demonstrating that fosfomycin was not able to reliably kill *K. pneumoniae* isolates (9, 20).

It was suggested that fosfomycin is able to kill (or at least inhibit growth of) *E. coli*, but regrowth occurs thereafter. The extent of regrowth depends on the presence of a resistant subpopulation and this is not predicted based on the baseline fosfomycin MIC for the strain (21). This is corroborated by our finding that bactericidal activity against *E. coli* over 24 hours was found in approximately 90% of volunteers, but quickly fell below 60% thereafter. This was true for all *E. coli* strains with the exception of *E. coli* 1231, the strain harboring a HLR subpopulation. For *K. pneumoniae*, we found moderate (or almost totally absent) antibacterial activity of fosfomycin in the majority of the samples, confirming other reports (9, 10, 22). All *K. pneumoniae* strains had a HLR subpopulation. This may be more a matter of intrinsic rather than acquired resistance after antibiotic exposure (9, 23). These findings suggest that a single, 3g dose may be sufficient for UTIs caused by *E. coli* without HLR subpopulations, and that fosfomycin is inappropriate for UTIs caused by *K. pneumoniae*, regardless of the MIC for the strain and the fosfomycin dose.

Maximum UITs and UBTs of nitrofurantoin were obtained in the first two-hour time interval. Titers were low but comparable for both species tested, demonstrating reasonable bactericidal activity of nitrofurantoin only in the first two hours, confirming a previous report describing early activity against extended spectrum beta lactamase (ESBL)-producing pathogens like *E. coli* and *K. pneumoniae* (12). We did not find any significant differences in antibacterial activity between the two dosing regimens; the slightly higher urinary concentrations of nitrofurantoin after 100 mg versus 50 mg did not result in more antibacterial activity in our experiment (8).

The major advantage of the method used here is that it is a*n ex-vivo* model combining patient-related pharmacokinetic properties of a drug with its PD effect. The *ex-vivo* results obtained with this method therefore may reflect the antimicrobial clinical effectiveness against uropathogens better than most other *ex-vivo/in-vitro* methods. This is important, as bacterial growth *ex-vivo/in-vitro* can be different from that in humans (24). Yet we found bactericidal activity of nitrofurantoin in less than 50% of volunteer samples, and this only for a short period of time. This contrasts with fosfomycin's bactericidal activity, which was detected in 90% of volunteer samples. These results are in conflict with what was found in a recent randomized clinical trial comparing five days of nitrofurantoin (100 mg q8 hours) to single-dose fosfomycin (3g) for acute lower UTI (25). Seventy percent of those receiving nitrofurantoin had clinical success versus

only 58% of those receiving fosfomycin. Microbiologic resolution was achieved in 74% versus 63%, respectively. There is thus discrepancy between the *ex-vivo* activity of nitrofurantoin in a single dosing interval (and also, but to a smaller extent, of fosfomycin) and its clinical efficacy.

There are several possible factors that could explain this discrepancy. Fosfomycin requires glucose-6-phosphate to enter the bacterial cell to exert its antibacterial activity so it is standard practice to add 25 mg/l glucose-6-phosphate to the laboratory media when performing in vitro experiments with fosfomycin (26). Because human urine normally does not contain glucose-6-phosphate in significant amounts, the ex vivo antibacterial activity was measured without adding glucose-6-phospate. It should be noted, however, that the baseline MICs for fosfomycin were measured in the presence of glucose-6-phosphate, as per the reference standard for fosfomycin susceptibility testing (27). This could party explain the discrepancy between the fosfomycin MICs at baseline and the urinary antibacterial activity. For nitrofurantoin, we investigated its activity during only one dosing interval of a drug intended to be administered over at least five days. It would therefore seem likely that the short period of antibacterial activity we found, would be sufficient to achieve clinical success in the majority of the patients when administered as a course of multiple oral doses. The cumulative effect of the full nitrofurantoin course after repetitive dosing has not been investigated, such that our results would underestimate the effect of the antimicrobial agent. Secondly, we considered the bactericidal activity only when calculating the percentages of bactericidal success, but whether pathogen killing is needed to achieve clinical success is questionable. Bacteriostatic activity, or a bactericidal effect during a short period of time (*e.g.*, less than 2 hours), might be sufficient to promote clinical success. In particular because of the natural urodynamics of regularly voiding episodes, during which uropathogens are flushed out together with the urine. Finally, the percentages of bactericidal success gives an underestimation of daily clinical practice since we were not able to measure the antibacterial activity in the undiluted sample due to limited sample volumes.

CONCLUSION

We found strong bactericidal activity of fosfomycin against *E. coli* over at least 48 hours after administration and moderate bactericidal activity against *K. pneumoniae* over 18 hours. High-level resistant subpopulations were found in all *K. pneumoniae* strains and in one of the *E. coli* strains, findings that further support the likelihood of intrinsic resistance of *K. pneumoniae* against fosfomycin, and highlight that MIC measurements might not be the best measure for predicting *ex-vivo* activity of fosfomycin. Titers

of nitrofurantoin were comparable for both *E. coli* and *K. pneumoniae*, demonstrating moderate bactericidal activity in the first 2 hours after dosing. In the majority of subsequent samples, however, no antibacterial activity was detected, regardless of the administered dose. This finding is in contrast to nitrofurantoin's well-observed clinical effects over multiple dosing intervals. Our findings reveal a discrepancy between nitrofurantoin's measurable *ex vivo* activity in a single dosing interval time period and its clinical effectiveness. For fosfomcyin, our findings suggest that the current single-dose approach to fosfomycin administration in UTIs caused by *E. coli* without HLR may be sufficient, but confirm the doubts of the use of fosfomycin in general in UTIs caused by *K. pneumoniae*.

Acknowledgements

The authors declare no conflicts of interest.

Funding information

This work was supported in part by the European Commission's FP7 AIDA project (Preserving old antibiotics for the future, Health-F3-2011- 278348).

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Table S1. Characteristics of the fosfomycin and nitrofurantoin samples. Median (range) of the drug concentration, number of samples and pH of the urine samples for both drugs during each time interval. Samples were grouped based on their time after dose.

Collection period	Concentration [a]		Number of samples	pH	
(h)	(mg/liter)				
Fosfomycin					
0-6	808.1	(19.9-5729.9)	77	5	(5-7)
6-12	744.4	(90-4375.9)	68	5.5	(5-7)
12-18	512.7	(145.1-1866.6)	33	6	(5-7)
18-24	348.3	(51.5-2189.1)	67	5	(5-7)
24-30	124	(<0.75-947.9)	82	5	(5-7)
30-36	78.5	(9.1-707.9)	64	5	(5-7)
36-42	80.8	(<0.75-454.8)	32	5	(5-7)
42-48	62.3	(<0.75-495.7)	61	5	(5-7)
Nitrofurantoin (50 mg q6 hours)					
0-2	53.6	(4-176.3)	23	5.6	(5-6)
2-4	26.8	(<4-106.7)	17	5.8	(5-6)
4-6	19.7	(5.3-54.4)	9	6	(6-6)
6-8	15.7	(<4-37.6)	19	5.8	(5-6)
Nitrofurantoin (100 mg q8 hours)					
0-2	32.6	(4-209.4)	25	5.7	(5-7)
2-4	21.3	(13.4-97.9)	11	5.6	(5-6)
4-6	17	(5.2-79.3)	16	5.8	(5-6)
6-8	10	(<4-56.4)	20	5.8	(5-6)

[a]The lower limit of quantification of the analytical method for fosfomycin was 0.75 mg/liter and 4 mg/liter for nitrofurantoin.

Table S2. UITs and $AUIT_{0\text{-}48h}$ values of fosfomycin. Median (range) of UITs and $AUIT_{0\text{-}48h}$ values of fosfomycin over time for each strain.

Strain (MIC, mg/liter)	UIT (median (range)) for the indicated time periods (h)								
	0-6	6-12	12-18	18-24	24-30	30-36	36-42	42-48	$AUIT_{0\text{-}48h}$
E. coli									
ATCC25922 (1)	16 (<2-256)	16 (<2-256)	16 (<2-128)	8 (<2-128)	4 (<2-64)	2 (<2-64)	4 (<2-32)	2 (<2-32)	164 (98-309)
51 (2)	16 (<2-128)	8 (2-32)	4 (<2-16)	4 (<2-16)	2 (<2-16)	2 (<2-8)	2 (<2-8)	<2 (<2-8)	117 (74-164)
03 (0.25)	16 (<2-64)	16 (4-64)	16 (2-32)	4 (<2-64)	2 (<2-16)	2 (<2-16)	2 (<2-8)	<2 (<2-8)	150 (96-199)
1231 (16)	2 (<2-16)	2 (<2-32)	<2 (<2-8)	<2 (<2-8)	<2 (<2-4)	<2 (<2-4)	<2 (<2-4)	<2 (<2-2)	69 (46-144)
4807 (32)	32 (<2-256)	16 (<2-256)	16 (<2-64)	8 (<2-128)	3 (<2-32)	4 (<2-32)	4 (<2-16)	2 (<2-16)	171 (91-279)
K. pneumoniae									
58 (8)	4 (<2-16)	2 (<2-16)	<2 (<2-8)	<2 (<2-8)	<2 (<2-2)	<2 (<2-2)	<2 (<2-<2)	<2 (<2-2)	57 (45-100)
20 (32)	4 (<2-32)	4 (<2-64)	2 (<2-8)	2 (<2-16)	<2 (<2-4)	<2 (<2-4)	<2 (<2-2)	<2 (<2-<2)	79 (41-129)
31865 (2)	8 (<2-128)	8 (<2-64)	8 (<2-64)	3 (<2-64)	<2 (<2-32)	<2 (<2-16)	<2 (<2-8)	<2 (<2-4)	110 (74-224)
55 (4)	4 (<2-64)	4 (<2-64)	4 (<2-16)	2 (<2-16)	<2 (<2-8)	<2 (<2-8)	<2 (<2-4)	<2 (<2-4)	90 (58-174)

Table S3. UITs and $AUIT_{0-6h}$ or $AUIT_{0-8h}$ values of nitrofurantoin. Median (range) of UITs and $AUIT_{0-6h}$ or $AUIT_{0-8h}$ values for nitrofurantoin over time for each strain.

Dose, strain (MIC, mg/liter), and isolate	UIT (median (range)) for the indicated time periods (h)				
	0-2	2-4	4-6	6-8	$AUIT_{0-6h}$ or $AUIT_{0-8h}$
Nitrofurantoin 50 mg q6 hours					
E. coli					
ATCC25922 (16)	8 (<2-16)	2 (<2-16)	1 (<2-4)	<2 (<2-4)	20 (12-31)
51 (32)	4 (<2-16)	2 (<2-8)	<2 (<2-4)	<2 (<2-4)	17 (10-26)
03 (16)	4 (<2-16)	2 (<2-8)	2 (<2-2)	<2 (<2-2)	18 (12-26)
1231 (512)	<2 (<2-<2)	<2 (<2-<2)	<2 (<2-<2)	<2 (<2-<2)	9 (11-32)
4807 (16)	2 (<2-8)	<2 (<2-8)	<2 (<2-<2)	<2 (<2-2)	13 (8-15)
K. pneumoniae					
58 (64)	4 (<2-16)	2 (<2-8)	2 (<2-4)	<2 (<2-8)	18 (7-9)
20 (256)	<2 (<2-4)	<2 (<2-4)	<2 (<2-<2)	<2 (<2-8)	10 (9-24)
31865 (128)	<2 (<2-2)	<2 (<2-<2)	<2 (<2-<2)	<2 (<2-<2)	9 (7-11)
55 (256)	<2 (<2-2)	<2 (<2-<2)	<2 (<2-<2)	<2 (<2-<2)	9 (7-11)
Nitrofurantoin 100 mg q8 hours					
E. coli					
ATCC25922 (16)	4 (<2-32)	2 (<2-16)	1 (<2-8)	<2 (<2-8)	18 (13-30)
51 (32)	4 (<2-16)	2 (<2-8)	2 (<2-2)	<2 (<2-4)	15 (10-23)
03 (16)	4 (<2-16)	2 (<2-8)	1 (<2-4)	<2 (<2-4)	16 (11-26)
1231 (512)	<2 (<2-<2)	<2 (<2-<2)	<2 (<2-<2)	<2 (<2-<2)	9 (11-30)
4807 (16)	<2 (<2-16)	<2 (<2-4)	<2 (<2-4)	<2 (<2-4)	12 (7-14)
K. pneumoniae					
58 (64)	4 (<2-16)	2 (<2-8)	<2 (<2-4)	<2 (<2-4)	14 (7-9)
20 (256)	<2 (<2-4)	<2 (<2-2)	<2 (<2-<2)	<2 (<2-2)	9 (7-24)
31865 (128)	<2 (<2-2)	<2 (<2-<2)	<2 (<2-<2)	<2 (<2-<2)	9 (7-11)
55 (256)	<2 (<2-2)	<2 (<2-<2)	<2 (<2-<2)	<2 (<2-<2)	9 (7-11)