



# Ex Vivo Urinary Bactericidal Activity and Urinary Pharmacodynamics of Fosfomycin after Two Repeated Dosing Regimens of Oral Fosfomycin Tromethamine in Healthy Adult Subjects

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**ABSTRACT** The *ex vivo* bactericidal activity and pharmacodynamics of fosfomycin in urine were evaluated in 18 healthy subjects. Subjects received 3 g every other day (QOD) for 3 doses and then every day (QD) for 7 doses or vice versa. Serial urine samples were collected before and up to 24 h after dosing on days 1 and 5. Eight bacterial strains with various genotypic and phenotypic susceptibilities to fosfomycin were used for all experiments (5 *Escherichia coli*, 2 *Klebsiella pneumoniae*, and 1 *Proteus mirabilis*). MICs were performed via agar dilution. Urinary bactericidal titers (UBTs) were performed via modified Schlichter test using participant's drug-free urine as the diluent. Urinary time-kill analyses were performed on pooled 24-h urine aliquots from days 1 and 5. All experiments were performed in triplicate with and without the addition of 25 mg/liter of glucose-6-phosphate (G6P). Mean 24-h urine concentrations of fosfomycin ranged from 324.7 to 434.6 mg/liter regardless of study day or dosing regimen. The urinary antibacterial activity of fosfomycin was also similar across study days and dosing regimens. UBT values did not correlate with MICs determined in the presence of G6P. Fosfomycin was reliably bactericidal in urine only against the 5 *E. coli* strains, regardless of genotype or MIC value. Together, these data do not support the use of oral fosfomycin tromethamine for pathogens other than *E. coli* or at a dosing frequency higher than QOD. Fosfomycin MICs determined in the presence of G6P may not accurately reflect the *in vivo* activity given the lack of G6P in human urine. (This study has been registered at ClinicalTrials.gov under identifier NCT02570074.)

**KEYWORDS** fosfomycin, urinary bactericidal titers, pharmacodynamics, urine, urinary tract infection

Oral fosfomycin tromethamine is considered a first-line agent for the treatment of uncomplicated urinary tract infections (UTIs) (1) and is approved in the United States as a one-time 3-g oral dose for uncomplicated cystitis (2). Despite this narrow indication, fosfomycin maintains broad *in vitro* activity against many Gram-positive and -negative pathogens, including multidrug-resistant phenotypes (3). Consequently, the use of oral fosfomycin has increased dramatically in response to the rise in bacterial resistance and increase in more complicated UTIs (4–7). Its purported utility for these difficult-to-treat infections has also prompted the use of more aggressive dosing regimens, ranging from 3 g every other day (QOD) to 3 g daily (QD) for weeks to months (8–11). Despite being commercialized since 1971, there is a paucity of modern

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robust pharmacodynamic (PD), pharmacokinetic (PK), or safety data to support these clinical practices. As such, there is an urgent need to establish reliable PD, PK, and safety data to optimize the use of oral fosfomycin tromethamine.

Our recent work evaluated the PK and safety of oral fosfomycin tromethamine after repeated doses in healthy adults and failed to find a clear PK advantage to QD dosing compared to QOD dosing, while QD dosing resulted in significantly more diarrhea (12). As fosfomycin tromethamine is used clinically only for the treatment of UTIs, it is crucial to understand the antibacterial activity and PD of fosfomycin in human urine. Human urine is a complex and constantly dynamic medium that can alter bacterial growth and antimicrobial activity markedly compared to blood or plasma. Additionally, fosfomycin is unique in that it requires uptake into the bacterial cell via the  $\text{L}$ -alpha-glycerolphosphate (GlpT) or hexose-6-phosphate transport (UhpT) system, which are induced by their substrates glycerol-3-phosphate (G3P) and glucose-6-phosphate (G6P), respectively (3). Therefore, it is recommended to supplement *in vitro* fosfomycin susceptibility assays with G6P to mimic the physiologic environment *in vivo* and counteract excess phosphate present in laboratory media (13, 14). Human urine is naturally devoid of G6P, and it is unknown how this affects the antibacterial activity of fosfomycin in the target matrix. Finally, it is unclear if QD dosing provides a PD advantage over QOD dosing, and there is an overall dearth of published data regarding the activity of fosfomycin against *Enterobacteriaceae* spp. other than *E. coli* in human urine and after multiple repeated oral doses. Therefore, the purpose of this study was to determine the *ex vivo* urinary bactericidal activity and urinary pharmacodynamics of fosfomycin after two dosing regimens of oral fosfomycin tromethamine in a randomized, two-period crossover study in healthy subjects.

(This work was presented in part at the 2018 ECCMID meeting in Madrid, Spain.)

## RESULTS

A total of 19 healthy adult subjects were enrolled in the study. Of the 19 subjects enrolled, 18 received both dosing regimens and were included in the PD analysis, while one subject completed only the QOD regimen due to scheduling conflicts. The mean ( $\pm$  standard deviation [SD]) baseline demographics of these subjects included age of  $28 \pm 7$  years, body mass index (BMI) of  $24.9 \pm 2.5$  kg/m<sup>2</sup>, and measured creatinine clearance (via 24-h urine collection) of  $109 \pm 30$  ml/min. Urine fosfomycin concentrations peaked through the first 8 h of collection, and approximately 35 to 40% of the administered dose was excreted in the urine by 24 h postdose, regardless of study day or dosing regimen. Urine concentrations of fosfomycin were highly variable but similar between study days 1 and 5 and between the QOD and QD dosing regimen (see Table S1 in the supplemental material). The mean ( $\pm$ SD) pooled 24 urine concentration for all 18 subjects on days 1 and 5 of the QOD regimen were  $361.7 \pm 254.2$  mg/liter and  $434.6 \pm 343.4$  mg/liter, respectively, compared to  $342.4 \pm 324.7$  mg/liter and  $387.9 \pm 224.8$  mg/liter during the QD regimen. The mean ( $\pm$ SD) pooled 24 urine concentration for the 3 subjects used for G6P experiments on day 5 of the QOD and QD regimen were  $313.5 \pm 184.2$  mg/liter and  $381.9 \pm 229.5$  mg/liter, respectively. The mean ( $\pm$ SD) urine pH across all collection intervals, study days, and dosing regimens was  $5.96 \pm 0.8$ . No G6P was detected in any tested urine sample.

**Susceptibility.** Genotypic and phenotypic susceptibilities and the interpretive category of each isolate against fosfomycin are displayed in Table 1. In the presence of 25 mg/liter of G6P, all 5 *E. coli* strains were susceptible to fosfomycin (15). Three of five *E. coli* strains were wild type (WT) (MIC, 2 to 32 mg/liter), while both strains (UIC45 and UIC46) with MIC values at the CLSI susceptibility breakpoint (64 mg/liter) harbored mutations in genes associated with the G3P/G6P uptake transport system. Despite possessing MICs of  $\geq 256$  mg/liter, *P. mirabilis* was wild type, as it intrinsically does not utilize the UhpT transport system, while both *K. pneumoniae* strains harbored multiple mutations in the uptake transport system, the target site *murA*, and/or possessed the fosfomycin-modifying enzyme *fosA*. When tested without G6P, a 16-fold increase in MIC was observed for both wild-type ATCC *E. coli* strains. MICs for the 3 clinical *E. coli* strains

**TABLE 1** Genotypic and phenotypic susceptibilities obtained against isolates included in the study

Organism	Resistance mechanism(s)	MIC in mg/liter (MIC interpretation <sup>a</sup> ) in presence or absence of G6P <sup>b</sup>	
		Presence	Absence
<i>E. coli</i> ATCC 25922	WT	4 (S)	64 (S)
<i>E. coli</i> BAA-2326	WT	2 (S)	32 (S)
<i>E. coli</i> UIC44	WT	32 (S)	128 (I)
<i>E. coli</i> UIC45	<i>uhpB</i> , <i>uhpC</i> , <i>cyaA</i> <sup>c</sup>	64 (S)	128 (I)
<i>E. coli</i> UIC46	$\Delta$ <i>uhpABC</i> <sup>d</sup>	64 (S)	128 (I)
<i>P. mirabilis</i> ATCC 35659	WT	256 (NA)	256 (NA)
<i>K. pneumoniae</i> ATCC 33495	<i>fosA</i>	256 (NA)	≥512 (NA)
<i>K. pneumoniae</i> ATCC 700603	<i>fosA</i> , <i>uhpA</i> , <i>uhpC</i> , <i>uhpT</i> , <i>glpT</i> , <i>ptsI</i> , <i>murA</i> <sup>e</sup>	512 (NA)	≥512 (NA)

<sup>a</sup>According to CLSI M100-S29 (16). NA, CLSI breakpoints not available; S, susceptible; I, intermediate; R, resistant.

<sup>b</sup>Reference broth microdilution method includes 25 mg/liter G6P. MIC results obtained in the absence of G6P were interpreted for comparison purposes only.

<sup>c</sup>The following genes and respective alterations were observed in isolate UIC45 compared with wild-type ATCC 25922: *uhpB* (P84S, S374T, Q441H, G459D, Q463H, H482T), *uhpC* (T72P, A177S, S417A, A435T), and *cyaA* (S222G, A349E, S356K, G359E, E362D, D837E, T840A).

<sup>d</sup>The transport system regulator UhpABC was not detected.

<sup>e</sup>The following genes and respective alterations were observed in the ATCC 700603 compared with wild-type ATCC 43816: *uhpA* (T3I, A25T, T41A, E64A, I87V, S183N), *uhpC* (E55D, A185T, C192G, M236L, S237T, A240E, V415A, T438A), *uhpT* (V434I), *glpT* (G196A, K234E, E237Q, I260V, V337I, P344A, I429V), *ptsI* (S241N), and *murA* (S148N, T206S, S210T).

increased only 1 to 2 log<sub>2</sub> dilutions without G6P, and no appreciable change in MIC was observed for the *P. mirabilis* and *K. pneumoniae* strains.

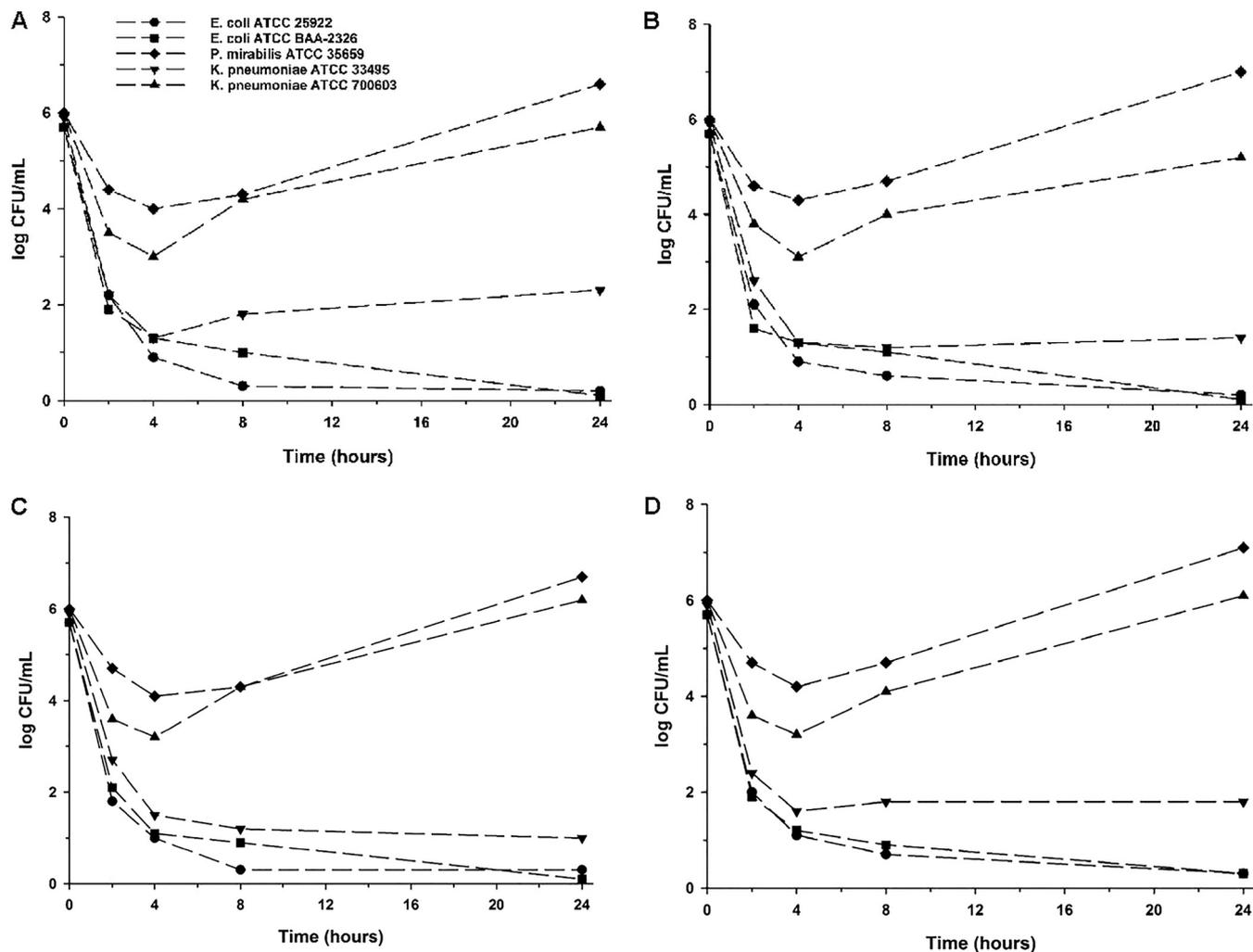
**Urinary titers.** The urinary inhibitory titer (UIT) was the same as the urinary bactericidal titer (UBT) in all cases, so only UBT values are reported. The median reciprocal fosfomycin UBT against the initial 5 isolates tested from each collection interval on days 1 and 5 of both dosing regimens are displayed in Table 2, along with area under the 24-h UBT-time curve (AUBT<sub>24</sub>) values. Analogous to the urine concentrations, UBTs were similar between dosing regimens and study days and peaked during the 0- to 8-h collection interval postdose. UBTs and AUBTs were the highest for the two *E. coli* isolates with the lowest fosfomycin MICs. UBTs were consistently less than 2 and AUBTs less than 20 for both *Klebsiella* strains and less than 4 and 50, respectively, for *P. mirabilis*. Although the observed UBTs and corresponding AUBTs were correlative to the respective isolate’s fosfomycin MIC performed in the presence of 25 mg/liter of G6P, both were significantly lower than expected based on the known urine fosfomycin concentrations (>300 mg/liter over 24 h), likely due to the lack of G6P in human urine. To investigate this, UBTs were repeated with and without 25 mg/liter G6P and with the 3 additional clinical *E. coli* strains on 3 representative subjects’ urine from day 5 of each dosing regimen. Results of these experiments are displayed in Table 3. The addition of 25 mg/liter G6P increased the UBT value just 1- to 3-fold for the

**TABLE 2** Median reciprocal fosfomycin UBT and AUBT<sub>24</sub> for all 18 subjects against the first 5 isolates tested

Collection interval (h)	Median (IQR) value by strain and dosing regimen									
	<i>E. coli</i> ATCC 25922		<i>E. coli</i> BAA-2326		<i>P. mirabilis</i> ATCC 35659		<i>K. pneumoniae</i> ATCC 33495		<i>K. pneumoniae</i> ATCC 700603	
	QOD	QD	QOD	QD	QOD	QD	QOD	QD	QOD	QD
Day 1										
0–4	4 (15)	8 (16)	4 (15)	8 (8)	4 (7)	2.5 (8)	1 (2)	0 (4)	1 (2)	0 (2)
4–8	8 (30)	4.5 (16)	8 (14)	2.5 (16)	4 (7)	0.5 (8)	2 (2)	0 (1)	1 (2)	0 (0)
8–12	4 (8)	3 (7)	4 (8)	3 (7)	1 (4)	1 (4)	0 (0)	0 (0)	0 (1)	0 (0)
12–24	4 (7)	6 (6)	2 (3)	4 (2)	1 (3)	2 (3)	0 (1)	1 (2)	0 (1)	0 (0)
AUBT <sub>24</sub>	92 (246)	86 (224)	72 (182)	81 (138)	40 (124)	27 (64)	10 (20)	8 (12)	10 (12)	1 (8)
Day 5										
0–4	8 (28)	4 (15)	16 (28)	4 (6)	4 (7)	4 (8)	2 (4)	1.5 (2)	1 (2)	1 (2)
4–8	16 (32)	12 (31)	8 (32)	16 (28)	2 (8)	4 (15)	1 (4)	2 (4)	1 (2)	1 (2)
8–12	4 (8)	6 (15)	4 (8)	4 (7)	2 (8)	1 (4)	0 (1)	0 (0)	0 (1)	0 (0)
12–24	4 (7)	4 (7)	4 (3)	4 (6)	1 (4)	2 (2)	0 (1)	1 (1)	1 (2)	0 (0)
AUBT <sub>24</sub>	124 (350)	104 (340)	108 (288)	104 (226)	28 (100)	46 (78)	18 (34)	12 (20)	12 (14)	8 (8)

**TABLE 3** Median reciprocal fosfomycin UBT and AUBT<sub>24</sub> for 5 representative subjects against all 8 isolates tested on study day 5 with and without 25 mg/liter G6P

Collection interval (h)	Median (IQR) value by strain and dosing regimen															
	<i>E. coli</i> ATCC 25922		<i>E. coli</i> BAA-2326		<i>E. coli</i> UIC44		<i>E. coli</i> UIC45		<i>E. coli</i> UIC46		<i>P. mirabilis</i> ATCC 35659		<i>K. pneumoniae</i> ATCC 33495		<i>K. pneumoniae</i> ATCC 700603	
	QOD	QD	QOD	QD	QOD	QD	QOD	QD	QOD	QD	QOD	QD	QOD	QD	QOD	QD
No G6P																
0-4	6 (22)	12 (7)	6 (10)	8 (3)	2 (1.5)	3 (1.5)	3 (1)	4 (1.3)	3 (3.3)	3 (1)	8 (19.5)	10 (9)	0.5 (3)	2 (0.8)	0.5 (2.5)	2 (1.5)
4-8	16 (10)	12 (6)	12 (10)	10 (6)	2 (3.5)	2 (2)	3 (5)	6 (1.5)	4 (3.5)	4 (4.5)	16 (34)	24 (29)	3 (2)	2.5 (1.8)	2.5 (1)	1 (0.5)
8-12	8 (3.5)	8 (6)	6 (7.5)	12 (14.5)	1.5 (0.5)	2 (1.3)	1.5 (1.5)	5 (6.5)	1.5 (1.3)	4 (3)	6 (8)	9 (4)	0 (0.8)	0 (0.5)	0 (0.5)	0.5 (0.5)
12-24	6 (2)	4 (3)	3 (0.5)	3 (7.8)	2 (1.3)	1.5 (0.5)	2 (5.5)	3 (3.5)	4 (1)	1.5 (0.8)	8 (3.3)	3 (2.3)	0.5 (0.3)	1 (0.5)	0.5 (0.3)	0.5 (0.5)
AUBT <sub>24</sub>	112 (95)	156 (48)	88 (93)	128 (142)	44 (14.5)	46 (10.5)	67 (33)	66 (57.5)	63 (24)	74 (18)	148 (236)	190 (162)	15 (18)	18 (13.5)	13 (10)	14 (3.5)
G6P																
0-4	96 (16)	80 (64)	64 (256)	48 (8)	8 (6.5)	8 (6.5)	2 (7)	6 (2)	4 (3.3)	4 (4.5)	32 (31)	16 (43)	1.5 (2.5)	1.5 (0.8)	1.5 (2.5)	1.5 (1.8)
4-8	640 (488)	192 (128)	96 (80)	192 (224)	16 (7)	12 (10)	6 (3.3)	6 (4.5)	4 (2)	4 (7)	32 (40)	64 (54)	2 (0.8)	4 (3.3)	2 (0.8)	3 (3.3)
8-12	48 (20)	128 (80)	72 (24)	64 (52)	4 (1)	8 (4)	2 (2.3)	8 (3)	2 (0.5)	3 (1.5)	16 (4)	20 (8)	1 (0.5)	1 (0.5)	1 (0.5)	1 (3.8)
12-24	48 (40)	128 (80)	48 (148)	64 (8)	6 (4)	6 (2)	2 (2)	4 (3.5)	2.5 (1)	4 (3.5)	16 (8)	32 (8)	1 (0.8)	0.5 (1)	1 (0.8)	2 (1.8)
AUBT <sub>24</sub>	3280 (1968)	3,200 (944)	2,144 (968)	1,920 (744)	128 (63)	184 (61)	45 (47)	128 (40)	54 (17.5)	70 (54)	384 (230)	608 (322)	19 (9)	30 (12)	44 (42)	27 (45)

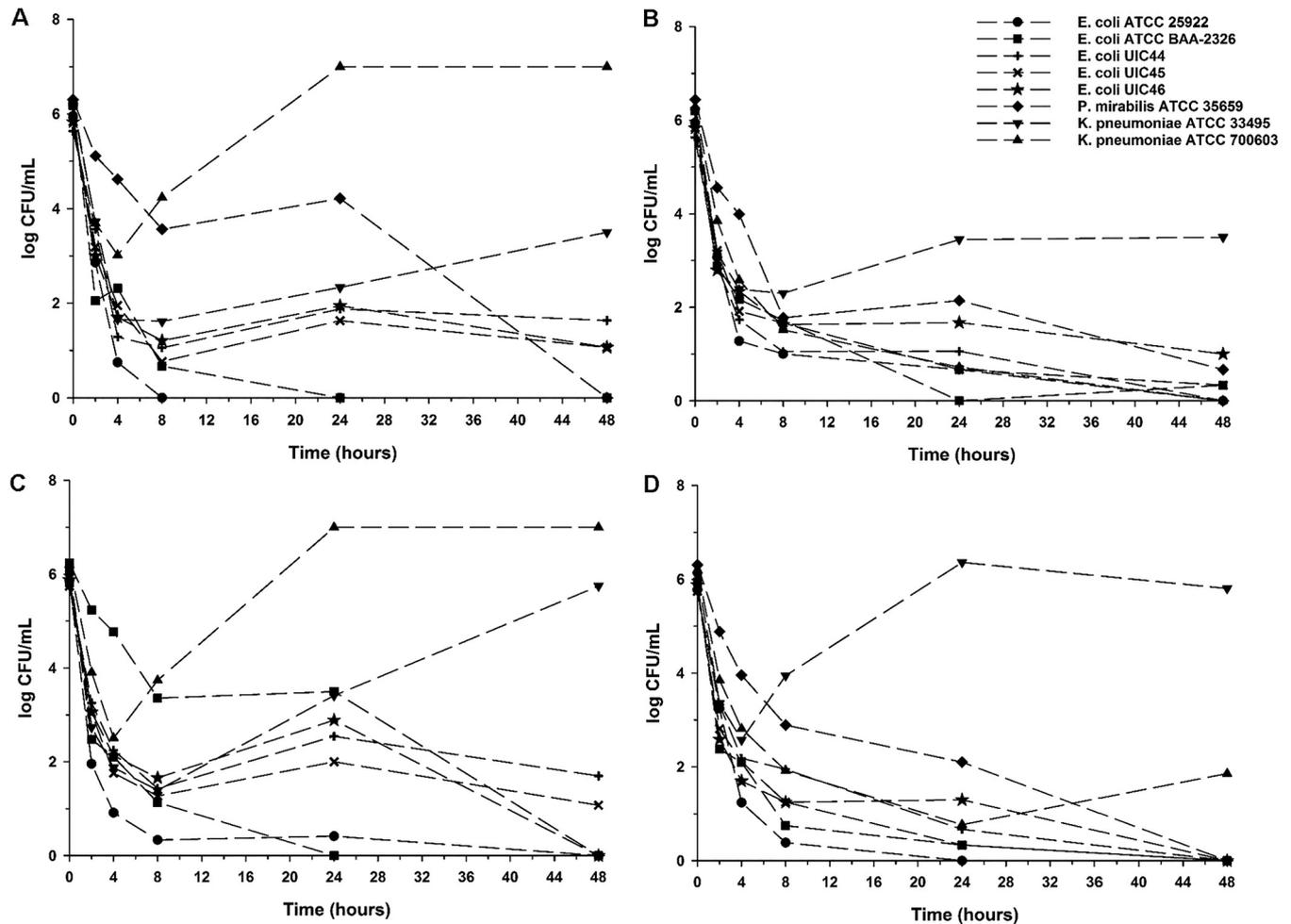


**FIG 1** Mean log<sub>10</sub> CFU/ml versus time profile for each isolate against fosfomycin-laden urine collected from subjects during the QOD dosing regimen on day 1 (A) and day 5 (B) and during the QD dosing regimen on day 1 (C) and day 5 (D). Curves represent average concentrations for triplicate experiments. The y axis is in the log scale.

5 isolates with known intrinsic or acquired mutations in the G3P/G6P uptake transport system. Conversely, for the *E. coli* strains without known mutations, the addition of G6P increases the AUBT<sub>24</sub> values up to 30-fold. Notably, for the two wild-type *E. coli* strains exquisitely susceptible to fosfomycin (*E. coli* ATCC 25922 and BAA-2326), the difference between the UBT values with and without G6P (Table 3) was virtually identical to the difference in MIC with and without G6P (~16-fold) (Table 1).

**Urinary time-kill analyses.** Results of time-kill experiments from pooled urine collected on days 1 and 5 of each dosing regimen against the initial 5 isolates tested are displayed in Fig. 1. Regardless of dosing regimen or study day, fosfomycin-laden urine was rapidly bactericidal in less than 2 h against both ATCC *E. coli* strains and *K. pneumoniae* ATCC 33495 and maintained bactericidal activity through 24 h. An initial ~2-log<sub>10</sub> CFU/ml inoculum reduction was observed against *P. mirabilis* ATCC 35659 and *K. pneumoniae* ATCC 700603 within the first 4 h, follow by rapid regrowth and 8- to 16-fold increases in MIC by 24 h (data not shown).

In order to assess the impact of G6P and any potential PD advantage of QD dosing, additional time-kill experiments were performed with and without 25 mg/liter G6P on a representative group of 3 subjects' urine from day 5 of the QD dosing regimen and extended to 48 h (Fig. 2). No regrowth of any strain was observed between 24 and 48 h, while the addition of G6P improved the rate and extent of the bactericidal activity of



**FIG 2** Mean  $\log_{10}$  CFU/ml versus time profile for each isolate against fosfomycin-laden urine collected from subjects during the QOD dosing regimen on day 5 without G6P (A) and with 25 mg/liter G6P (B) and during the QD dosing regimen on day 5 without G6P (C) and with 25 mg/liter G6P (D). Curves represent average concentrations for triplicate experiments. The y axis is in the log scale.

the fosfomycin-laden urine against all 8 isolates tested except for the *fosA*-positive *K. pneumoniae* ATCC 33495 strain.

## DISCUSSION

To our knowledge, this is the first study to evaluate the *ex vivo* urinary antibacterial activity and urinary PD of fosfomycin after repeated oral doses of fosfomycin tromethamine. There are several notable and clinically relevant findings from this study. First, fosfomycin MICs obtained *in vitro* in the presence of 25 mg/liter G6P as recommended (16) may not accurately reflect the antibacterial activity of fosfomycin *in vivo*. This study demonstrates that the antibacterial activity and PD of fosfomycin is significantly impaired in human urine due to the lack of G6P. As previously mentioned, the UIT and UBT values of fosfomycin were identical in all cases. This is expected given fosfomycin's bactericidal activity and is consistent with previous studies (17). Therefore, barring any influence of urine on bacterial growth and/or antimicrobial activity, the UBT should be roughly equal to the corresponding urine drug concentration divided by the pathogen's MIC for bactericidal drugs (18–21). In the present study, the average urine concentrations of fosfomycin during the 4- to 8-h collection interval were >400 mg/liter, and the MICs against the two wild-type *E. coli* strains were 4 and 2 mg/liter, respectively, which should translate into UBT values of  $\geq 100$  to 200. In fact, measured median UBT values without G6P peaked at 8 during the same 4- to 8-h interval. When G6P was added, UBT values increased into the range predicted. Importantly, the

discordance in UBTs with and without G6P was almost identical to the discordance observed in MICs with and without G6P (~16-fold) against *E. coli* isolates without mutations in the G6P transport system (Table 1). These data suggest that the properties of the urine itself, other than lacking G6P, do not attenuate the activity of fosfomycin. These results are supported by a recent study evaluating the *ex vivo* UBTs of fosfomycin in 40 healthy females given a single 3-g dose of oral fosfomycin (17). Wijma et al. also found that UBTs were not related to the strain's MIC tested in the presence of G6P, although no further analyses were done to quantify the magnitude of this discrepancy.

Second, the antibacterial activity of fosfomycin was poor against *Enterobacteriaceae* isolates other than *E. coli* tested in this study, particularly *K. pneumoniae*. UBT values against *K. pneumoniae* were rarely above 2 at any time point, and the addition of G6P did not improve the activity of fosfomycin against these strains given the numerous resistance mechanisms present in both strains. This suboptimal activity against *K. pneumoniae* has been previously demonstrated in an *in vitro* dynamic bladder infection model where, despite MICs of  $\leq 16$  mg/liter and simulated peak urine fosfomycin concentrations of 1,053 to 4,415 mg/liter, 15/16 *K. pneumoniae* isolates tested demonstrated rapid regrowth and development of fosfomycin resistance (22). The aforementioned *ex vivo* fosfomycin UBT study was also unable to detect any antibacterial activity against the majority of *K. pneumoniae* isolates included (17). Together, these data support the FDA indication and the CLSI recommendation for susceptibility testing only against *E. coli* (16) and caution against the clinical use of oral fosfomycin for the treatment of UTIs not due to *E. coli*.

Third, given that multiple-dose regimens of oral fosfomycin are often employed clinically, it was important to evaluate the PK, PD, and safety of commonly used repeated-dose regimens. We observed similar urine concentrations of fosfomycin and correspondingly similar urinary antibacterial activity across dosing regimens (QOD and QD) and study days (1 and 5). Additionally, extending our time-kill analyses from 24 to 48 h did not demonstrate bacterial regrowth during that period. Collectively these PK/PD data, along with the higher rate of diarrhea observed in the QD dosing arm (12), do not support increasing the dosing interval of oral fosfomycin above QOD in an attempt to treat more complicated or resistant UTIs.

The data generated from this study are particularly opportune given the pending approval of intravenous (i.v.) fosfomycin disodium in the United States. We have previously demonstrated that a single 8-g i.v. dose of fosfomycin disodium produces a urinary maximum concentration ( $C_{max}$ ) of approximately 15,580.7 mg/liter in healthy volunteers, roughly 7-fold higher than that after a single 3-g dose of oral fosfomycin tromethamine (23). These results were used to inform the dose of 6 g every 8 h (q8h) i.v. utilized in the recently completed phase 2/3 trial in patients with complicated UTI or acute pyelonephritis, which demonstrated noninferiority compared to piperacillin-tazobactam (24). Importantly, clinical cure and microbiological eradication rates against *E. coli* were similar to those of other Gram-negative pathogens, including *Klebsiella* and *Proteus* spp., suggesting that i.v. fosfomycin disodium is preferred over oral fosfomycin tromethamine for the treatment of complicated UTIs due to less susceptible *E. coli* or other *Enterobacteriaceae* species.

Added strengths of the present study include use of an *ex vivo* model, which allowed us to evaluate the antibacterial activity and PD of fosfomycin in human urine based on real-world obtainable urine concentrations, and the use of whole-genome sequencing to select pathogens with a range of genotypic and phenotypic susceptibilities to fosfomycin. Limitations include the number of strains tested, the static nature of UBT and PD experiments, and the lack of a clear association between UBTs and clinical outcomes, although some thresholds for other bactericidal drugs have been suggested (25).

In summary, the significant variability in urine concentrations and corresponding urinary antibacterial activity may help to explain the suboptimal efficacy rates observed for oral fosfomycin in the treatment of uncomplicated UTIs (26, 27). Further, fosfomycin MICs determined *in vitro* in the presence of G6P may not accurately reflect the *in vivo*

activity, given the lack of G6P in human urine observed in this study and others. Finally, our PK, PD, and safety results do not support the use of oral fosfomycin for pathogens other than *E. coli* or at a dosing frequency higher than QOD. Confirmation of these findings in controlled clinical trials is warranted.

## MATERIALS AND METHODS

**Study design and subjects.** This was an *ex vivo* annex to a phase I, randomized, open-label, two-period-crossover, multiple-dose study of oral fosfomycin tromethamine (Monurol; Forest Pharmaceuticals, Inc., St. Louis, MO) in healthy adult subjects (12). This study was approved by the University of Illinois at Chicago (UIC) Office for the Protection of Research Subjects Institutional Review Board and conducted in accordance with good clinical practices at the UIC Clinical Research Center. Written informed consent was obtained from each subject prior to the conduct of any study-related procedures.

Subjects were enrolled in study drug administration sequences in parallel so that each subject received both dosing regimens in a randomized, crossover fashion. The two regimens were 3 g QOD for 3 doses followed by 3 g QD for 7 doses or vice versa. Fosfomycin was delivered as a powder sachet mixed in 3 to 4 oz water under fasted conditions. Each administration sequence was separated by a 5- to 14-day washout period. Subjects' fluid intake was allowed *ad libitum*.

Full details regarding eligibility criteria, plasma sampling, bioanalytical procedures, PK analysis, and safety assessments can be found in Wenzler et al. (12).

**Urine sample collection.** Urine samples for PD analyses were collected before and at intervals of 0 to 4, 4 to 8, 8 to 12, and 12 to 24 h postdose on days 1 and 5. Single predose urine samples were also collected on days 3 and 7. Urine samples were stored at  $\leq 4^{\circ}\text{C}$  during collection intervals. After completion of the collection interval, aliquots of urine were extracted, frozen, and stored at  $-80^{\circ}\text{C}$  until analyzed. Urine was thawed and filter sterilized prior to all PD experiments. Urine pH was measured and recorded prior to all experiments but was not adjusted. Urine G6P content was measured via G6P assay kit (Sigma-Aldrich, St. Louis, MO).

**Bacteria and susceptibility testing.** Initially, five bacterial strains representing typical uropathogens were evaluated: *E. coli* ATCC 25922, *E. coli* ATCC BAA-2326 (CTX-M-15 producing), *Proteus mirabilis* ATCC 35659, *Klebsiella pneumoniae* ATCC 33495, and *K. pneumoniae* ATCC 700603 (sulphydryl variable-18 [SMV-18] producing). Subsequently, three clinical *E. coli* strains from patients with UTIs (UIC44, UIC45, and UIC46) were added in select experiments to elucidate the antibacterial activity of fosfomycin on clinical strains with higher fosfomycin MICs and underlying genotypic mutations. Isolates were maintained at  $-80^{\circ}\text{C}$  in cation-adjusted Mueller-Hinton broth (CAMHB) with 20% glycerol and subcultured on tryptic soy agar with 5% sheep's blood twice prior to use. Analytical-grade fosfomycin was purchased commercially (Sigma-Aldrich, St. Louis, MO). Fosfomycin MICs were performed in triplicate on the same day via agar dilution at standard inoculum, according to CLSI guidelines, with and without the addition of 25 mg/liter of G6P (28). Modal MIC values are reported. *E. coli* ATCC 25922 was included as a quality control, and susceptibilities to fosfomycin were interpreted according to CLSI interpretive criteria (16).

**Determination of fosfomycin resistance mechanisms.** Genomic DNA was extracted and used for library preparation (Nextera XT). Paired-end genome sequencing was performed on an Illumina MiSeq (Illumina, San Diego, CA) in a  $2\times 150$ -bp configuration (JMI Laboratories, North Liberty, IA). Each raw sequencing data set was quality assured, error corrected, and assembled *de novo* using the SPAdes genome assembler (29). FASTQ format sequencing files for each sample set were applied to a JMI Laboratories-designed software workflow to align against a curated database containing known fosfomycin resistance genes, including *fomA*, *fomB*, *fosA*, *fosA2*, *fosA4*, *fosA5*, *fosB*, *fosB1*, *fosB2*, *fosC*, *fosD*, *fosE*, *fosF*, *fosG*, *fosK*, and *fosX* (30, 31). In addition, intrinsic genes associated with the fosfomycin binding site (MurA) and transport system regulators (*glpT*, *uhpT*, *uhpABC*, *cyaA*, *crp*, and *ptsI*) were extracted and amino acid sequences compared with the respective wild-type reference sequence.

**Urinary titers.** Urinary inhibitory titers (UIT) and bactericidal titers (UBTs) were performed as previously described (18) via a modified Schlichter test (32), utilizing the participant's drug-free urine as the diluent. Aliquots of urine from each subject obtained during each urine collection interval on study days 1 and 5 of both dosing regimens were inoculated with the test pathogen and then serially diluted with the same subject's drug-free urine in a 96-well microtiter plate. Plates were then incubated at  $35^{\circ}\text{C}$  in ambient air for 24 h before being read. The UIT was defined as the highest dilution that inhibited visible bacterial growth. For UBTs, a  $50\text{-}\mu\text{l}$  aliquot of subcultured urine from the first visibly clear well was then plated and incubated at  $35^{\circ}\text{C}$  for 24 h prior to enumeration. UBT was defined as the greatest urinary dilution that achieved a  $\geq 3\text{-log}_{10}$  reduction in CFU/ml compared to the starting inoculum of  $10^6$  CFU/ml, ranging from 1:1,024. Reciprocal UIT and UBT values are reported. The area under the 24-h UBT-time curve (AUBT<sub>24</sub>) was calculated via the trapezoidal rule using reciprocal UBT values. In order to assess the impact of G6P, UBTs were repeated with the addition of 25 mg/liter of G6P on 3 representative subjects' urine samples from each dosing regimen on study day 5.

**Urinary time-kill analyses.** Urinary time-kill analyses were performed in triplicate according to CLSI guidelines (33), modified utilizing urine as the milieu in a total volume of 2 ml in deep-well plates. A direct suspension of 3 to 4 isolated colonies selected from a pure overnight culture was incubated at  $35^{\circ}\text{C}$  with shaking to ensure log-phase growth. Suspensions were then adjusted to a 0.5 McFarland standard in saline and further diluted to a starting inoculum of approximately  $10^6$  CFU/ml in CAMHB. Colony counts were performed to ensure starting inoculum densities.

An aliquot of each subject's urine from each collection interval on days 1 and 5 of both dosing regimens was pooled in order to represent the activity of fosfomycin in the urine over 24 h and added

to the bacterial suspension. A growth control without any fosfomycin-laden urine was included with each experiment. At the specified time points of 0, 2, 4, 8, and 24 h, aliquots of 20  $\mu$ l were removed from the suspensions and serially diluted in  $\log_{10}$  dilutions. A 100- $\mu$ l aliquot was then plated on Mueller-Hinton agar and incubated at 35°C for at least 24 h prior to enumeration. Cystine-lactose-electrolyte-deficient (CLED) agar was used for experiments containing *P. mirabilis* to abate swarming. Time-kill curves were generated by plotting the average  $\log_{10}$  CFU/ml from triplicate experiments versus time. Bactericidal activity was defined as a  $\geq 3$ - $\log_{10}$  CFU/ml reduction in bacterial density compared to the starting inoculum. In order to assess the impact of G6P, time-kill analyses were repeated with the addition of 25 mg/liter of G6P on 3 representative subjects' urine from each dosing regimen on study day 5.

**Statistical analysis.** For each bacterial isolate, the time-specific reciprocal UBT and  $AUBT_{24}$  values are summarized separately for each dosing regimen and study day. Summary median and interquartile ranges (IQR) are reported. An artificial value of 0 indicates no bactericidal activity of undiluted urine.

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.01 MB.

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E.W. serves on the scientific advisory board for GenMark Diagnostics and Shionogi and on the speaker's bureau for Melinta Therapeutics and Astellas Pharma. We have no relevant conflicts of interest to report.

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