Assessment of urinary pharmacokinetics and pharmacodynamics of orbifloxacin in healthy dogs with ex vivo modelling

Takae Shimizu, ¹, ² Kazuki Harada, ¹, ², ⋆ Saki Manabe, ² Taku Tsukamoto, ³ Norihiko Ito ² and Yoshiaki Hikasa ¹, ²

INTRODUCTION

Urinary tract infections (UTIs) are common bacterial infections in dogs, occurring in approximately 14% of dogs in their lifetime, with variable age of onset [1]. Escherichia coli is the most common infecting bacteria, but various Gram-negative and Gram-positive bacteria can cause UTIs in dogs [2, 3].

Antimicrobial treatment is required for dogs with UTI [4]. Successful antimicrobial treatment is based on site-specific pharmacokinetic/pharmacodynamic (PK/PD) principles [5]. In dogs with UTIs, most bacteria are present in the urine within the urinary tract, including the kidneys, ureters, urinary bladder and urethra [4]. Therefore, drug concentrations and antimicrobial activity in the urine (urinary PK/PD) can indicate the treatment efficacy of antimicrobial drugs [5, 6]. An ex vivo model has been established in humans to determine urinary bactericidal titres (UBTs), which can serve as a PK/PD assessment of antimicrobial agents in the urine [6]. However, the UBT model has not yet been applied to dogs.

Orbifloxacin (OBFX) is a fluoroquinolone drug that is indicated for the treatment of bacterial UTIs in companion animals. The drug exhibits bactericidal activity against numerous Gram-negative and Gram-positive bacteria [7]. The aim of the present study was to use liquid chromatography–tandem mass spectrometry (LC–MS/MS) to investigate the urinary PK of OBFX in dogs. A further aim was to measure UBTs and related parameters of OBFX against the common UTI pathogens of dogs.

METHODS

Sampling of urine from dogs administered OBFX

Animal experiments were conducted under an ethics committee-approved protocol in accordance with the...
Tottori University Animal Use Committee (approval number: 14-T-18). Six beagle dogs (five males and one female; mean weight, 11.0±1.4 kg) were purchased from Kitayama Labes, Nagano, Japan. Three dogs were 10–13 years of age; the remaining three dogs were 2 years old (Table 1). Prior to this study, all dogs were confirmed to be clinically healthy based on physical examination, complete blood count, biochemical blood testing and urinalysis. A balloon catheter was placed in the urinary bladder of each dog to allow urine collection. Dogs were orally administered OBFX (Victas, provided by DS Pharma Animal Health, Osaka, Japan) at a dose of 5 mg kg⁻¹ body weight, which is the approved dose in the treatment of canine UTIs in Japan. Whole urine was obtained via the catheter at 4, 8, 12, 16, 20 and 24 h after administration; samples were sterilized by filtration and stored at −80 °C until analysis.

**Measurement of urine OBFX concentration with LC–MS/MS**

Reference standard OBFX and lomefloxacin (LMFX) as the internal standard were separately dissolved in acetonitrile and then diluted with ultra-pure water. LC–MS/MS was carried out using a Nexera ultra-high-performance liquid chromatograph equipped with an LCMS-8050 triple-quadrupole mass spectrometer (Shimadzu, Kyoto, Japan). An electro-spray ionization source interface operating in positive-ion mode was applied for multiple reaction monitoring. The precursor ions of OBFX and LMFX were represented by peaks at m/z 396.10 and m/z 352.10, respectively. The product-ion mass spectra of OBFX were m/z 352.10 and m/z 295.15, whereas those of LMFX were m/z 265.10 and m/z 307.90. The interface settings were as follows: nebulizing gas flow, 31 min⁻¹; desolvation line temperature, 250 °C; heat block temperature, 400 °C; and drying gas flow, 101 min⁻¹. The compounds were separated on a 2 mm internal diameter, 50 mm length, 3 µm analytical column operated at 40 °C (Mastro C18; Shimadzu GLC, Tokyo, Japan). The mobile phase comprised 0.1% formic acid aqueous solution and acetonitrile, and the flow rate was 0.3 ml min⁻¹. The injection volume was 0.1 µl. Standard samples for creation of a calibration curve were prepared with blank urine matrix spiked with four concentrations of OBFX (1, 10, 50 and 500 µg ml⁻¹). Standard and canine urine samples (50 µl) were mixed with 100 µg ml⁻¹ of LMFX (50 µl) for the internal standard and methanol (400 µl). After centrifugation at 13 000 r.p.m. for 5 min, the supernatants were harvested and then diluted 100-fold with ultra-pure water for analysis. The validity of the LS-MS/MS assay was verified according to guidelines from the US Food and Drug Administration [8]. The area under the time–urine concentration curve during the first 24 h after administration (urinary AUC₀–24) was calculated with the trapezoidal rule. The half-life time (T₁/₂) in urine was calculated by linear regression of the semi-logarithmic plot of urine concentration versus the midpoint of the urine collection time.

**Test organisms**

The following 14 bacterial strains from the urine of dogs with UTI were used in this study: *E. coli* (strains EC1–EC6), *Pseudomonas aeruginosa* (strains PA1 and PA2), *Klebsiella pneumoniae* (strains KP1 and KP2), *Proteus mirabilis* (strains PM1 and PM2) and *Staphylococcus pseudintermedius* (strains SP1 and SP2). Strains of *K. pneumoniae* and *S. pseudintermedius* were kindly provided by Mr Y. Tsuyuki (Sanritsu Zelkova Veterinary Laboratory); the other strains were selected from our collection [9–11].

**Determination of MIC and minimum bactericidal concentration (MBC)**

MICs of OBFX in cation-adjusted Mueller–Hinton broth (CAMHB) against all strains were determined using the broth-dilution method, according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [12]. Based on MICs, all strains were categorized as susceptible, intermediate or resistant, according to the MIC breakpoint established by the CLSI [13]. For quality control, *E. coli* ATCC 25922, *S. aureus* ATCC 29213 and *P. aeruginosa* ATCC 27853 were used. The MBCs were also determined after MIC determination, and were defined as the minimum concentration of drug needed to kill ≥99.9% of viable organisms after incubation for 24 h, according to CLSI guidelines [14].

**Table 1. Urine concentration, area under the time–urine concentration curve (urinary AUC₀–24) and half-life time (T₁/₂) of OBFX after a single oral dose of 5 mg kg⁻¹ in six healthy dogs**

<table>
<thead>
<tr>
<th>Animal</th>
<th>Age</th>
<th>Gender</th>
<th>Urine concentration (µg ml⁻¹) for the following collection period (h)*</th>
<th>Urinary AUC₀–24 (h µg ml⁻¹)</th>
<th>T₁/₂ (h) in urine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog 1</td>
<td>11</td>
<td>Male</td>
<td>278.58</td>
<td>3348.92</td>
<td>6.95</td>
</tr>
<tr>
<td>Dog 2</td>
<td>13</td>
<td>Male</td>
<td>357.07</td>
<td>4646.29</td>
<td>6.29</td>
</tr>
<tr>
<td>Dog 3</td>
<td>10</td>
<td>Male</td>
<td>431.17</td>
<td>5686.88</td>
<td>6.26</td>
</tr>
<tr>
<td>Dog 4</td>
<td>2</td>
<td>Female</td>
<td>611.33</td>
<td>5345.67</td>
<td>6.04</td>
</tr>
<tr>
<td>Dog 5</td>
<td>2</td>
<td>Male</td>
<td>107.45</td>
<td>1982.20</td>
<td>9.06</td>
</tr>
<tr>
<td>Dog 6</td>
<td>2</td>
<td>Male</td>
<td>16.18</td>
<td>5758.58</td>
<td>6.31</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td>300.39</td>
<td>4461.42</td>
<td>6.82</td>
</tr>
</tbody>
</table>

*The maximum urine concentration is indicated in bold type.
Determination of UBT, the area under the UBT–time curve (AUBT) and minimum urinary bactericidal concentration (MUBC)

UBTs corresponding to the maximal-dilution titre of urine allowing bactericidal activity were determined as described previously [15]. A logarithmic serial twofold dilution was prepared using a 1 : 1 mixture of the urine sample obtained at 4 h intervals after administration (see section 'Sampling of urine from dogs administered OBFX') and the subject's individual antimicrobial-free urine obtained prior to drug administration. UBTs were determined using a microdilution test system. Each microplate well contained 100µl of the prepared dilution. The final inoculum was about 5×10^{5} c.f.u.ml^{-1}. The plates were incubated at 35°C for 18 h. The subcultured urine was then transferred to antimicrobial-free agar. The plates were incubated at 35°C overnight. The number of colonies subsequently grown was used to determine the bactericidal endpoint. The UBT was defined as ≥99.9% reduction of the initially inoculated colony counts. A UBT of 0 was defined as no bactericidal activity and a UBT of 1 was assigned when only undiluted urine displayed bactericidal activity. UBTs were transformed into ordinal data and described with reciprocal numbers [6].

The AUBT was calculated as the sum of the products of the reciprocal UBT values and the respective time (h) intervals considering the time intervals of 4 h and the nonlinear kinetics in urine [6].

The MUBC for each strain was determined by dividing the maximum antimicrobial concentration in a urine sample by the corresponding UBT [15].

Statistical analysis

The median values of UBT, AUBT and MUBC among the six dogs were calculated from the average value of the two middle elements. Spearman's rank correlation coefficient (ρ) was calculated between parameters (i.e., urine concentration versus median UBT, MIC versus median AUBT, and MBC versus median MUBC). A P-value of <0.05 was considered significant for all analyses.

RESULTS

Urine concentration

In this study, the LC–MS/MS assay showed a lower limit of quantitation at 1 ng ml\(^{-1}\) for OBFX in dog urine. The temporal changes in urine OBFX concentration in each dog are shown in Table 1. The maximum concentration periods were 0–4 h \((n=2)\), 4–8 h \((n=3)\) and 8–12 h \((n=1)\). The maximum urinary concentration \((U_{\text{max}})\) and urinary AUC\(_0\)\(_{24}\) ranged from 118.82–611.33 µg ml\(^{-1}\) (383±171 µg ml\(^{-1}\)) and from 1982.20–5758.58 µg ml\(^{-1}\) (4461±1509 h µg ml\(^{-1}\)), respectively. The \(T_{1/2}\) in urine ranged from 6.04 to 9.06 h (6.82±1.14 h).

MICS and MBCs

Table 2 shows that the MICs determined using the broth-microdilution method ranged from 0.03–128 µg ml\(^{-1}\). Three strains of \(E.\ coli\) were categorized as susceptible, one as intermediate and two as resistant. One strain of \(P.\ aeruginosa\) was categorized as susceptible and one as intermediate. Two strains of each \(K.\ pneumoniae\) and \(P.\ mirabilis\) were categorized as susceptible. In addition, one strain of \(S.\ pseudintermedius\) was categorized as susceptible and one as intermediate. The MBCs ranged from 0.06 to 128 µg ml\(^{-1}\), one to four times the corresponding MIC for each strain.

### Table 2. The MIC, MUBC and AUBT of OBFX for the 14 bacterial strains tested in this study

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>MIC (µg ml(^{-1}))</th>
<th>Category*</th>
<th>MBC (µg ml(^{-1}))</th>
<th>MUBC (µg ml(^{-1}))</th>
<th>MUBC/MBC</th>
<th>AUBT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Median</td>
<td>Range</td>
<td></td>
<td></td>
<td>Median</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Range</td>
</tr>
<tr>
<td>(E.\ coli)</td>
<td>EC1</td>
<td>0.03</td>
<td>S</td>
<td>0.06</td>
<td>0.25</td>
<td>0.1–0.7</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>EC2</td>
<td>0.06</td>
<td>S</td>
<td>0.06</td>
<td>0.6</td>
<td>0.1–2.7</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>EC3</td>
<td>1</td>
<td>S</td>
<td>2</td>
<td>15.2</td>
<td>0.1–23.7</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>EC4</td>
<td>2</td>
<td>I</td>
<td>2</td>
<td>14.4</td>
<td>2.2–26.2</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td>EC5</td>
<td>32</td>
<td>R</td>
<td>32</td>
<td>ND(\uparrow)</td>
<td>ND(\uparrow)</td>
<td>ND(\uparrow)</td>
</tr>
<tr>
<td></td>
<td>EC6</td>
<td>128</td>
<td>R</td>
<td>128</td>
<td>ND(\uparrow)</td>
<td>ND(\uparrow)</td>
<td>ND(\uparrow)</td>
</tr>
<tr>
<td>(P.\ aeruginosa)</td>
<td>PA1</td>
<td>1</td>
<td>S</td>
<td>4</td>
<td>8.7</td>
<td>1.3–10.9</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>PA2</td>
<td>4</td>
<td>I</td>
<td>8</td>
<td>38.6</td>
<td>22.1–80.7</td>
<td>4.8</td>
</tr>
<tr>
<td>(K.\ pneumoniae)</td>
<td>KP1</td>
<td>0.125</td>
<td>S</td>
<td>0.125</td>
<td>1.4</td>
<td>0.5–3.3</td>
<td>10.9</td>
</tr>
<tr>
<td></td>
<td>KP2</td>
<td>0.25</td>
<td>S</td>
<td>0.25</td>
<td>2.9</td>
<td>1.0–11.8</td>
<td>11.4</td>
</tr>
<tr>
<td>(P.\ mirabilis)</td>
<td>PM1</td>
<td>1</td>
<td>S</td>
<td>2</td>
<td>8.0</td>
<td>3.5–11.8</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>PM2</td>
<td>1</td>
<td>S</td>
<td>2</td>
<td>6.8</td>
<td>3.2–11.8</td>
<td>3.4</td>
</tr>
<tr>
<td>(S.\ pseudintermedius)</td>
<td>SP1</td>
<td>0.5</td>
<td>S</td>
<td>1</td>
<td>3.4</td>
<td>2.1–29.6</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>SP2</td>
<td>2</td>
<td>I</td>
<td>4</td>
<td>59.8</td>
<td>27.7–133.7</td>
<td>15.0</td>
</tr>
</tbody>
</table>

*Strains were categorized as susceptible (S), intermediate (I) or resistant (R), based on CLSI breakpoints for OBFX for UTI in dogs [13].

\(\uparrow\)Not determined because of extremely low UBT (median 0).
UBTs and AUBTs

The temporal changes of median UBTs for each strain are shown in Fig. 1. The median UBTs of OBFX peaked at 0–4 or 4–8 h after administration and then gradually decreased for all strains, except for two resistant strains of *E. coli*, in which most median UBTs were consistently 0 during the 24 h after administration. The highest median UBTs (range) of OBFX for strains EC1, EC2, EC3 and EC4 were 1536 (256–2048), 768 (4–1024), 16 (8–64) and 24 (1–64), respectively. In strains other than *E. coli*, the highest median UBTs (range) for strains PA1, PA2, KP1, KP2, PM1, PM2, SP1 and SP2 were 64 (16–256), 12 (4–32), 160 (4–1024), 96 (16–256), 48 (16–128), 64 (16–64), 80 (1–128) and 3 (1–16), respectively.

Of the tested strains, the highly susceptible (MIC <1 µg ml\(^{-1}\)) strains (\(n=5\)) had the highest median AUBTs (1045–16 768), followed by less susceptible (MIC 1 µg ml\(^{-1}\)) strains (\(n=4\), 225–652), intermediate (MIC 2–4 µg ml\(^{-1}\)) strains (\(n=3\), 53–229) and resistant (MIC >4 µg ml\(^{-1}\)) strains (\(n=2\), 2–5). The Spearman’s rank correlation coefficient between MICs and median AUBTs was −0.968 (\(P<0.01\)).

MUBCs

The median values (ranges) of MUBCs in tested strains are shown in Table 2. In OBFX-susceptible and intermediate strains, the median MUBCs ranged from 0.25 to 59.8 µg ml\(^{-1}\). Ratios of median MUBC to corresponding MBC ranged from 2.2 to 15.0, and median MUBCs significantly correlated with MBCs (\(P=0.925, P<0.01\)). In contrast, median MUBCs of OBFX-resistant strains could not be determined because of extremely low UBTs.

DISCUSSION

To date, many antimicrobials, including OBFX, have been approved for treatment of UTIs in dogs. However, little is known about the urinary PK/PD of veterinary antimicrobials. Accumulation of knowledge and better understanding of PK/PD principles is essential to promote evidence-based veterinary medicine. To our knowledge, this is the first report to investigate the urinary PK/PD of veterinary drugs in dogs by using LC–MS/MS and an *ex vivo* model established in humans.

Unlike the maximum drug concentration time (\(T_{\text{max}}\)) in blood, there has been little investigation of the urinary \(T_{\text{max}}\) of veterinary antimicrobials in dogs. This study showed that the urine concentration of OBFX peaked at 0–4, 4–8 or 8–12 h after oral administration, whereas the serum \(T_{\text{max}}\) value was previously reported to be 2.42 and 1.70 h after oral administration at doses of 2.5 and 5.0 mg kg\(^{-1}\) body weight, respectively [16, 17]. In addition, the values of \(T_{1/2}\) in urine were comparable with those in serum after oral administration at doses of

![Fig. 1. Reciprocal UBTs of OBFX (5 mg kg\(^{-1}\)) for the 14 bacterial strains tested in this study.](image-url)
2.5 and 5.0 mg kg\(^{-1}\) (7.14 and 6.51 h, respectively) [16, 17].

Our study also clarified the extremely high \(U_{\text{max}}\) of OBFX, which was over 100 times higher than the maximum drug concentration in plasma (\(C_{\text{max}}\) 3.29 \(\mu\)g ml\(^{-1}\)) after oral administration at the same dose [16]. Similarly, in the dogs orally administered with enrofloxacin, the \(U_{\text{max}}\) of enrofloxacin plus ciprofloxacin reached over 100 times \(C_{\text{max}}\) [18]. These findings are likely explained by the fact that both drugs are eliminated mainly by renal excretion [19]. Such urinary PK may elucidate that once-daily administration of OBFX, as well as enrofloxacin, maintains high concentrations in canine urine up to 24 h.

In the present study, the temporal UBTs and AUBTs of OBFX for 12 bacterial strains were determined for six dogs. In all strains except EC6, the period of maximum UBTs was 0–4 and/or 4–8 h after administration, similar to the urinary \(T_{\text{max}}\). In addition, the median UBTs in susceptible and intermediate strains fluctuated closely with the urine concentration during the same period. Thus, it is likely that the temporal UBTs of OBFX strongly reflect its urinary PK in dogs. In contrast, AUBTs, which reflect the overall UBT values, greatly depended on the respective MIC of each strain. A similar finding was confirmed in a study on the urinary PK/PD of fluoroquinolone drugs in humans [20]. Therefore, UBTs and AUBTs can be parameters used to estimate urinary PK/PD of antimicrobials in both dogs and humans.

Breakpoints are usually established on the basis that they are relevant at all sites of infection. However, this assumption vastly increases the complexity of breakpoint setting, especially in infections where drug concentrations are substantially different, such as urinary tract infections [21]. The CLSI [13] has defined the MIC breakpoint of OBFX for UTI in dogs as \(\geq 8 \mu\)g ml\(^{-1}\). The reasonableness of this breakpoint is supported by our finding that OBFX concentration in canine urine maintained bactericidal activity against the susceptible and intermediate strains up to 24 h after oral administration, but not against the resistant strains.

We found broad interindividual variability in the median UBTs and AUBTs for the same strain, although significant differences in these parameters between ages were not confirmed. Likewise, an approximately 22-fold variation in UBT and AUBT values was confirmed among seven different human patients with UTIs [6]. In this study, the interindividual variation in these values might be explained by the differences in maximum urinary concentration and peak concentration period among individuals. Further studies would be needed to clarify whether such variable urinary PK of OBFX can cause inter-individual differences in clinical efficacy.

It is known that fluoroquinolones exhibit lower antimicrobial activity in human urine than in standard microbiological media such as CAMHB [15, 22]. In the present study, we calculated MUBCs for each test strain based on urine concentration and UBTs to assess the activity of OBFX in dog urine. As a result, the median MUBCs were approximately two- to 15-fold higher than the corresponding MBCs. These findings indicate that the antimicrobial activity of OBFX against the bacterial species tested in this study decreases in dog urine, and can explain the reason why OBFX in dog urine had no antibacterial activity against the resistant strains in spite of the high drug concentration in urine. In humans, the activity of fluoroquinolones in urine depends on the urine pH, osmotic pressure and the concentrations of various solutes, mainly cations [23–25]. These factors might similarly affect the activity of fluoroquinolones in canine urine; however, verification of this speculation needs further study. At least, such a decrease in antimicrobial activity in dog urine implies that the urine concentration of antimicrobials does not fully indicate the PD of the drug in urine.

In conclusion, we assessed the urinary PK/PD of OBFX in dogs with LC–MS/MS and an ex vivo model. The fluctuation of UBTs closely correlated with that of urine concentration, and UBT values depended on the susceptibility of the bacterial strains to OBFX. The present data support the reasonableness of the CLSI breakpoint for OBFX for UTIs in dogs when administered at 5 mg kg\(^{-1}\) once daily. We strongly believe that the UBTs and the related parameters are important indicators of urinary PK/PD of antimicrobials indicated for UTIs in both dogs and humans.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References


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