Pharmacokinetic/pharmacodynamic evaluation of the efficacy of flumequine in treating colibacillosis in turkeys

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ABSTRACT Flumequine (FLU) is used in the treatment of systemic bacterial infections in poultry, including colibacillosis, which is a common disease in turkeys. The pharmacokinetic (PK) behavior of FLU administered to 32 healthy turkeys as an oral bolus via gavage or as 10-h pulsed administration in drinking water were compared, using the authorized dose of 15 mg/kg and the double dose of 30 mg/kg. The minimum inhibitory concentrations (MIC) of 235 Escherichia coli field strains isolated from poultry were determined for pharmacodynamics (PD) to develop a PK/PD model. Blood samples were collected at established times over 24 h, and the obtained plasma was analyzed using a liquid chromatography tandem mass spectrometry method that was validated in-house. A monocompartmental model and a noncompartmental model were applied to the data to obtain the PK results. For both types of administration and both dosages, the ratios of the maximum concentration (Cmax)/MIC50 and the area under the plasma concentration-time curve (AUC)/MIC50 achieved were considerably lower than the fluoroquinolone breakpoints usually adopted for efficacy. The Cmax/MIC50 and AUC0–24/MIC50 ratios were, respectively, 0.67 ± 0.09 and 4.76 ± 0.48 and 1.18 ± 0.35 and 7.05 ± 2.40 for the 15 and 30 mg/kg bolus doses, respectively. After 10-h pulsed administration of 15 mg/kg, values of Cmax/MIC50, 0.19 ± 0.02 on d 1 and 0.30 ± 0.08 on d 5 of therapy were obtained, the AUC/MIC50 ratios were 2.09 ± 0.29 and 3.22 ± 0.93 on d 1 and 5, respectively. Higher values were obtained with the doubled dose of 30 mg/kg: the Cmax/MIC50 ratios were 0.49 ± 0.11 on d 1 and 0.69 ± 0.18 on d 5; the AUC/MIC50 ratios were 5.15 ± 1.15 and 6.57 ± 1.92 on d 1 and 5, respectively. Based on these results, FLU administration should be adopted when specific diagnostic findings indicate its efficacy, and revising the dosage scheme to comply with the prudent and responsible use of antimicrobials in veterinary medicine is advisable.

Key words: flumequine, pharmacokinetics/pharmacodynamics, turkey, colibacillosis

INTRODUCTION

The turkey (Meleagris gallopavo), although generally considered a minor species, is an extremely important livestock in northern Italy. Scarce data exist about the usage of antimicrobial drugs in this species, and even less is known about their efficacy in this species (Sanders, 2001; Bywater, 2005).
spite the availability of newer FQ, FLU is still employed because of its relatively low cost and good tolerability, especially in minor species such as turkey, for which the cost of therapy is relevant. In avian species, drinking water is the most common route of administering mass medication; the treatments can be conducted following 2 schemes: continuous administration during the entire light period or pulsed administration for a limited period between 4 and 10–12 h (Charleston et al., 1998). Individual therapy is reserved for valuable animals and breeders for practical reasons, although it represents a prudent use of antimicrobials for the limitation of antibiotic resistance in bacteria. Mass therapy is reportedly one of the main causes of the development of microbial resistance in veterinary species (EMA, 2006; Löhren et al., 2008); an increase in the number of FQ-resistant strains of E. coli, Campylobacter, and Salmonella spp. has been frequently reported in recent years (Walsh and Fanning, 2008; EFSA, 2010).

Several scientific and health institutions have expressed serious concern over the emergence of FQ resistance, manifesting the need for risk management intervention regarding the use of FQ in humans and animals. Due to the development of FQ resistance in Campylobacter strains of poultry origin, the Food and Drug Administration has banned the use of FQ for the treatment of poultry infections since 2005 (FDA, 2005). In the European Union (EU), risk evaluation is ongoing, and according to the guidelines for risk management, FQ should be reserved for the treatment of clinical conditions that have responded poorly to other classes of antimicrobials (EMA, 2006). In addition, better dosage regimens should be determined based on pharmacokinetic/pharmacodynamic (PK/PD) integration (Martinez et al., 2006).

The available data on FLU have mainly concerned species other than turkeys and generally involved intravenous, intramuscular, or oral-bolus administration (Mevius et al., 1990; Villa et al., 2005). Therefore, pharmacokinetic parameters specific for turkeys given medicated water are still lacking, which may result in frequent improper dosages.

To improve the availability of PK data about FLU in turkeys, the present study first aimed to evaluate 2 different oral treatments (a single oral gavage and 5 d of repeated 10-h pulsed water medication) and 2 different doses of FLU (the EU authorized dose, 15 mg/kg, and double the EU authorized dose, 30 mg/kg). Oral administration by gavage allows precise control of the predetermined dose intake, and 10-h pulsed water medication is a frequent dosage scheme in avian clinical practice that is easily handled by farmers.

Finally, to evaluate the effectiveness of FLU against E. coli, the most common zoonotic avian pathogen, a PK/PD approach was implemented, correlating the PK results after gavage or 10-h pulsed administration with the MIC determined for the 235 E. coli strains isolated from poultry in Italy.

**MATERIALS AND METHODS**

**Birds**

Thirty-two female turkeys (breed B.U.T. 6) that were 63 to 79 d old, weighed approximately 4 to 6 kg, and were determined to be healthy by a thorough physical examination were selected from a farm belonging to an industrial group. The birds were housed according to the requirements of the European Union (Council of Europe, 2007) and were kept in 4 groups of 8 individuals, housed in 4 boxes of 5 m² on wood shavings at 20°C and 65% RH and receiving 16 h of light/day. Commercial diets and water were provided ad libitum. Before the experiments, the birds did not receive any pharmacological treatment.

After an acclimatization period of 8 d, the birds were weighed and individually marked for identification.

The study was conducted according to Italian law (D.L. 116/1992) and received ethical approval by the Italian Health Ministry (Animal Welfare Unit, 2009R4KM4F_002).

**Experimental Design**

Flumequine was orally administered to turkeys via gavage as a single bolus or via 10-h pulsed medicated water for 5 consecutive days at the target dose of 15 mg/kg of BW and at the doubled dose of 30 mg/kg of BW.

Food and water were withdrawn 8 h before administration to reduce any variability in the absorption due to drug-feed interaction and overdilution of the drug.

The turkeys were randomly assigned to 4 groups of 8 animals each, indicated as groups 1, 2, 3, and 4.

Groups 1 and 2 received FLU (Flumechina 40% DOXAL, Sulbiate MB, Italy) as a single oral dosage via gavage at the doses of 15 and 30 mg/kg of BW, respectively, whereas groups 3 and 4 were treated via drinking water at the same respective doses. The water intake over a period of 10 h was measured for 3 d before the treatment. The FLU was added to the water based on the birds’ mean weight and mean daily water intake.

The medicated water was provided in a pulsed scheme for 10 h/d, from 8.00 to 18.00 for 5 d, and was then replaced with fresh water. The daily water consumption was measured at the end of the pulse period of each day to calculate the mean antibiotic intake.

For groups 1 and 2 (oral gavage), blood samples were collected from ulnar or metatarsal veins in heparinized tubes before treatment and at 0.25, 0.5, 1, 2, 4, 8, and 12 h and 24 h posttreatment. For groups 3 and 4 (10-h pulsed medicated water given for 5 consecutive days), blood samples were collected on d 1 and 5 of treatment. Samples were taken immediately before treatment, at 1, 3, 6, and 9 h during the 10-h treatment, and then at 1, 2, 4, 8, and 14 h after the withdrawal of medicated water, which was h 11, 12, 14, 18, and 24 after the onset
of the treatments. Plasma was separated by centrifugation at 1,500 × g for 10 min at 20°C and was stored at −20°C, pending analysis.

Liquid Chromatography–Mass Spectrometry Analysis

The plasma sample purification was performed as reported by Samanidou et al. (2005) with slight modifications: 200 µL of plasma was spiked with 10 µL of the internal standard (IS) Norfloxacin (3 µg/mL) to have a final concentration of 0.15 µg/mL; afterward, 3 mL of acetonitrile was added. The tubes were briefly vortexed and centrifuged at 3,082 × g for 10 min. The supernatant was transferred to another tube and then evaporated to dryness at 50°C. The residue was dissolved in 200 µL of the mobile phase and filtered through a 0.22-µm pore-size membrane, and 10 µL was injected into the HPLC system after an appropriate dilution.

An Accela 600 HPLC pump with a CTC automatic injector was used (Thermo Fischer Scientific, San Jose, CA). Chromatographic separation was achieved using a C-18 Kinetex column (100 × 2.1 mm, 2.6 µm, Phenomenex, Torrance, CA) with guard column. The mobile phase consisted of (A) ammonium acetate solution (10 mM, pH 2.5) and (B) 0.1% formic acid in methanol. The mobile phase composition (A:B, vol/vol) was: 80:20 at 0 min, 50:50 at 10 min, 10:90 at 13 min and unchanged until 14 min, 0:100 from 14.50 to 16 min, 80:20 from 17 to 20 min to re-equilibrate the system. The sample tray was maintained at 4°C.

Mass spectrometric analysis (Garcés et al., 2006) was performed using a LTQ XL ion trap (Thermo Fisher Scientific, San Jose, CA) equipped with a heated electrospray ionization probe (HESI-II) operating in the positive-ion mode under the following conditions: sheath and auxiliary gas flow: 40 and 5 arbitrary units, respectively; ion spray voltage: 3.5 kV; capillary temperature: 300°C; capillary voltage: 26 V; and tube lens: 80 V. The collision energies that were determined to be necessary for fragmentation in MS² and MS³ of the molecules of interest, precursor ions, product ions, and collision energies are shown in Table 1. The Xcalibur (version 2.1) data acquisition software from Thermo Fisher Scientific was used.

Calibration curves were constructed using pooled turkey plasma obtained from untreated animals. The blank plasma was spiked with 10 µL of IS and with FLU to obtain a concentration range of 2.5 to 200 ng/mL. Quantification was based on the ratios of the peak areas of the analyte to that of IS, and a least-squares linear regression analysis was performed to calculate the calibration curves. Flumequine (>99% pure) and norfloxacin (>99% pure) were purchased from Sigma Aldrich (Steinheim, Germany). Other reagents and solvents were purchased from Carlo Erba-Reagenti (Milano, Italy).

Prior to being routinely applied, the method was validated in-house using a set of parameters (linearity, within-run and between-run accuracy and precision, limit of quantification (LOQ), limit of detection (LOD), and selectivity) that were in compliance with the recommendations defined by the European Community (Commission Decision 2002/657/EC, 2002) and with the reference guidelines defined in other EU and FDA documents (VICH GL 49, 2011). The calibration curves were constructed using matrix-matched calibrator samples (concentration range: 2.5–200 ng/mL), and the correlation coefficient was always r > 0.99 for 6 replicates.

The within-day precision (repeatability) and accuracy were determined by analyzing blank samples that were spiked with 2.5 (n = 6), 10 (n = 6), or 50 (n = 6) ng/mL on the same day. The between-day precision and accuracy were determined by analyzing quality control samples [concentration levels: 2.5 (n = 18), 10 (n = 18), and 50 (n = 18) ng/mL] with each batch of analytical samples on 3 different days. The following mean values were obtained: within-run accuracy 2.10 ± 0.12, 9.8 ± 0.6, and 52.5 ± 2.6 ng/mL and between-run accuracy 2.5, 10.2, and 50.5 ng/mL.

The results fell within the accepted ranges for precision (within-run precision: 5.9, 6.03, and 4.9% for 2.5, 10, and 50 ng/mL, respectively; between-run precision: 12.2, 5.9, and 6.2% for 2.5, 10, and 50 ng/mL, respectively).

An LOQ value of 2.5 ng/mL was obtained. None of the values below the LOQ were included in the plasma concentration-time curves or in the pharmacokinetic analysis. The LOD was defined as the concentration corresponding to a signal-to-noise ratio of 3 and was found to be 0.5 ng/mL. The specificity of the method was demonstrated because no interference from endogenous compounds was observed in the 20 blank samples tested.

Antimicrobial Susceptibility Testing

The minimum inhibitory concentrations (MIC) of FLU for 235 E. coli strains isolated from poultry in

Table 1. Characteristics obtained using mass spectrometry (MS) analysis

<table>
<thead>
<tr>
<th>Compound</th>
<th>Precursor ion [M-H]+ (m/z)</th>
<th>Collision energy MS² (%)</th>
<th>Precursor ion MS² (m/z)</th>
<th>Collision energy MS³ (%)</th>
<th>Product ion MS³ (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flumequine</td>
<td>202</td>
<td>40</td>
<td>244</td>
<td>25</td>
<td>202</td>
</tr>
<tr>
<td>Norfloxacin (internal standard)</td>
<td>320</td>
<td>36</td>
<td>276</td>
<td>30</td>
<td>256, 233</td>
</tr>
</tbody>
</table>
Italy were determined using the broth microdilution method according to CLSI (2008) guidelines.

Pharmacokinetics and Statistical Analysis

The pharmacokinetic parameters were deduced from the plasma concentration-time data using the WinNonLin 6.1 software (Pharsight Corporation, Mountain View, CA), which allows both compartmental and non-compartmental analyses of experimental data. Minimum information criterion estimation (Yamaoka et al., 1978) was used to choose the best-fitting model for the data. All of the data points were weighted by the inverse square of the fitted value. The plasma concentrations after a single oral bolus were fitted to a standard monocompartmental model, and also noncompartmental analyses were conducted. The kinetics after the 10-h pulsed administrations was determined at d 1 and 5 using a noncompartmental analysis (Gibaldi and Perrier, 1982).

The peak concentrations, \( C_{\text{max}} \), and the time to peak, \( T_{\text{max}} \), were obtained from the experimentally observed data. The elimination half-life was calculated as \( \ln 2/\lambda_{\text{u}} \), and the mean residence time (MRT) was determined using the following equation: MRT = AUMC/AUC, where AUMC is the area under the moment curve and AUC is the area under plasma concentration-time curve.

The pharmacokinetic parameters are reported as the mean values (±SD). The harmonic mean values and pseudo-SD were calculated for the half-lives using a jackknife technique (Lam et al., 1985). The normality of the kinetics data was assessed using the Kolmogorov–Smirnov test. The differences between the 2 bolus gavage doses (group 1 vs. 2), the two 10-h pulsed doses (group 3 vs. 4) and the 2 administrations methods (groups 1 vs. 3 and 2 vs. 4) were compared using a 2-tailed unpaired \( t \)-test; \( P < 0.05 \) was considered statistically significant (GraphPad Prism version 4.00 software, San Diego, CA).

The following PK/PD indices were calculated as predictors of the success or failure of the therapy: the \( C_{\text{max}}/\text{MIC} \) and \( \text{AUC}/\text{MIC} \) ratios (Toutain et al., 2002; McKeelar et al., 2004). The breakpoint values of \( C_{\text{max}}/\text{MIC}_{50} = 8–10 \) and \( \text{AUC}/\text{MIC}_{50} = 100 \) h were considered representative of the therapeutic efficacy of this antimicrobial class to prevent the development of resistant bacterial strains in poultry (Anadón et al., 2001; Dimitrova et al., 2007; Ozawa et al., 2010).

RESULTS

Single Bolus Gavage Administration (Groups 1 and 2)

The mean plasma concentrations ± SD of FLU at the various sampling times after oral gavage administration of both doses adopted are shown in Figure 1. The individual plasma concentration-time profiles were similar, and a low interindividual variability was observed, particularly in group 1, whereas a larger interindividual variability was observed when the double dose was administered (group 2). The maximum concentrations were reached at approximately 2 h in both
groups; the drug concentrations rapidly decreased, but the drug was still detectable at 24 h after administration, with mean concentrations of 0.26 ± 0.11 µg/mL (group 1) and 0.72 ± 0.74 µg/mL (group 2).

The pharmacokinetic parameters obtained from the mono- and noncompartmental analyses are presented in Table 2.

10-h Medicated Water Administration for 5 Consecutive Days (Groups 3 and 4)

In groups 3 and 4, the medicated water concentration was adjusted daily based on water intake; nevertheless, the daily measurements of water intake showed that the mean dose received by the turkeys was lower than the targeted doses of 15 and 30 mg/kg of BW (Table 3). The mean FLU concentration-time profiles following repeated oral administrations of medicated water at the 2 dosages are shown in Figures 2 and 3, respectively; the data refer to d 1 and 5 of therapy. In both trials, the highest concentrations were achieved on d 5 of treatment.

The pharmacokinetic parameters obtained in the noncompartmental analysis are summarized in Table 2.

MIC Determination and PK/PD Integration

The range of FLU MIC for the 235 poultry-derived E. coli isolates was 0.016 to >256 µg/mL. Only 29.8% of the strains were susceptible (n = 70) to FLU, whereas 70.2% were classified as resistant (n = 165). The MIC50, defined as the minimum inhibitory concentration at which 50% of the isolates were inhibited, was 16 µg/mL.

Based on the PK parameters and the MIC50 value, the PK/PD integrations were calculated for the different forms of administration; the values are presented in Table 2. Significant differences in the Cmax/MIC50 and AUC/MIC50 ratios were observed when the dose was doubled for the gavage and 10-h pulsed administrations and between those at d 1 and 5 of 10-h pulsed administration (Table 2).

DISCUSSION

When given by oral gavage, FLU was very rapidly absorbed, presenting a Tmax of approximately 2 h, in contrast to those reported for other FQ, such as enrofloxacin and danofloxacin, for which higher values have been recorded (Tmax: 6.33 ± 2.5 h for enrofloxacin and 6.0 ± 3.29 h for danofloxacin; Haritova et al., 2006; Dimitrova et al., 2007). In our birds, the half-life proved to be rather short (t1/2 elim: 4.01 ± 0.57 and 4.25 ± 1.60 h), and as expected, was not dependent on the dose given. After gavage administration of the 2 different doses of FLU, the increase in the Cmax and AUC values was related to the increase in the dose, whereas the concentration-time profiles were similar (Figure 1).
Oral administration by gavage is not easily practicable in intensive turkey farming due to the high population density in the sheds, the need for trained farmers to individually handle the birds, and the occurrence of bird stress, despite the higher $C_{\text{max}}$ and AUC$_{0-24}$ values obtained compared with those obtained using medicated water, for which a prolonged intake of lower drug concentrations occurs (Table 2).

The pulsed administration trials showed that the AUC$_{0-24}$ was increased from d 1 to d 5 in group 3 (AUC$_{0-24}$: $33.46 \pm 4.65$ to $51.54 \pm 14.87$ h$^*$µg/mL); and that the $C_{\text{max}}$ in group 4 likewise increased ($C_{\text{max}}$: $7.89 \pm 1.7$ to $10.84 \pm 2.92$ µg/mL). This slight increase can be explained by the different intakes of medicated water and do not support an accumulation of the drug. Lower intake of the drug was observed at the first day of the trial, and medicated water concentrations were thus adjusted based on the water intake of the previous administrations. Most likely due to the poor palatability of the medicinal veterinary product, the targeted doses were never reached using a 10-h pulsed administration. It is likely that the availability of unmedicated water during the remaining 6 h of the light period affected the animals’ behavior and that the birds drank less medicated water while waiting for the unmedicated water. It is known that increasing the concentration of a drug can affect the intake of an adequate amount of the drug due to its limited solubility in water and alteration of the water’s palatability; moreover, the photoperiod is also a very important parameter that can have repercussions for the water uptake and thus on the amount of drug intake (Vermeulen et al., 2002).

Administering the 2 different doses as medicated water allowed a significant increase in the $C_{\text{max}}$, AUC$_{0-24}$, and AUMC$_{0-24}$, but not in the T$_{\text{max}}$ and MRT with the higher dosage; thus a correlation with the increased dosage was observed, although the targeted dose of 30 mg/kg was not reached.

In agreement with many previous studies on $E.\ coli$ resistance to FQ (EFSA, 2010; Ozawa et al., 2010; Russo et al., 2012), a very high percentage of the isolated strains proved to be resistant to FLU. According to our MIC results, 70.2% of the $E.\ coli$ strains tested were resistant. This result suggests that FQ should be used in turkeys only when a susceptibility test clearly indicates the efficacy of the drug, as proscribed by EMA (2006) and WHO (2011) in the last sets of guidelines on the prudent use of antimicrobials.

### Table 3. Mean values ± SD of doses in turkeys after 10-h pulsed water administration

<table>
<thead>
<tr>
<th>Item</th>
<th>Actual dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target dose: 15 mg/kg of BW</td>
<td></td>
</tr>
<tr>
<td>d 1</td>
<td>12.53</td>
</tr>
<tr>
<td>d 2</td>
<td>7.56</td>
</tr>
<tr>
<td>d 3</td>
<td>9.94</td>
</tr>
<tr>
<td>d 4</td>
<td>14.25</td>
</tr>
<tr>
<td>d 5</td>
<td>15.34</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>11.92 ± 3.18</td>
</tr>
<tr>
<td>Target dose: 30 mg/kg of BW</td>
<td></td>
</tr>
<tr>
<td>d 1</td>
<td>15.24</td>
</tr>
<tr>
<td>d 2</td>
<td>14.76</td>
</tr>
<tr>
<td>d 3</td>
<td>20.48</td>
</tr>
<tr>
<td>d 4</td>
<td>26.50</td>
</tr>
<tr>
<td>d 5</td>
<td>23.68</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>20.13 ± 5.15</td>
</tr>
</tbody>
</table>

**Figure 2.** Mean values ± SD in the plasma concentration-time profiles of flumequine in group 3 at d 1 (filled triangles) and 5 (filled squares) following 10-h oral pulsed administration of an average dosage of 11.92 mg/kg of BW for 5 d.
Although specific breakpoints have not been defined for avian colibacillosis, several studies on FQ in poultry (Anadón et al., 2001; Dimitrova et al., 2007; Ozawa et al., 2010) adopted a $C_{\text{max}}/\text{MIC}_{50}$ ratio of 8 or 10 and an $AUC_{0-24}/\text{MIC}_{50}$ ratio of 100 as the minimal dose required to prevent the selection of resistant bacteria. As reported in Table 2, after gavage administration, the mean $C_{\text{max}}/\text{MIC}_{50}$ and $AUC_{0-24}/\text{MIC}_{50}$ ratios were, respectively, $0.67 \pm 0.09$ and $4.76 \pm 0.48$ and $1.18 \pm 0.35$ and $7.05 \pm 2.40$ for the 15 and 30 mg/kg doses, respectively. After the administration of 10-h pulsed medicated water with the dosage regimen of 15 mg/kg, lower values of $C_{\text{max}}/\text{MIC}_{50}$, $0.19 \pm 0.02$ on d 1 and $0.30 \pm 0.08$ on d 5 of therapy were obtained; the $AUC_{0-24}/\text{MIC}_{50}$ ratios were $2.09 \pm 0.29$ and $3.22 \pm 0.93$ on d 1 and 5, respectively. The following slightly higher values were obtained with the doubled dose: the $C_{\text{max}}/\text{MIC}_{50}$ ratios were $0.49 \pm 0.11$ on d 1 and $0.69 \pm 0.18$ on d 5; the $AUC_{0-24}/\text{MIC}_{50}$ ratios were $5.15 \pm 1.15$ and $6.57 \pm 1.92$ on d 1 and 5, respectively. The breakpoints values were not reached with either type of administration or dosages, and the PK/PD correlation yielded very unsatisfactory results. These findings are discouraging but not completely surprising, as FLU was licensed for use in food animals at the beginning of the 1980s and has been widely used for the mass medication of poultry and because the adoption of 10-h pulsed medication is conflicting with its rapid clearance from the blood.

Based on these results, the EU-authorized dosage of 15 mg/kg may be ineffective to achieve adequate drug plasma concentrations. Furthermore, the 10-h pulsed doses of medicated water did not allow reaching plasma concentrations that were efficacious in controlling $E. \text{coli}$, due to the long periods with unmedicated water. Medicated water should always be provided continuously, as reported in the leaflets of the commercial products.

To improve treatment efficacy and comply with the prudent and responsible use of antimicrobials in food-producing species, our results suggest that FLU administration should be adopted when specific diagnostic results indicate its efficacy and that a revision of the dosage scheme is advisable.

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