FULL PAPER

Internal Medicine

Pharmacokinetics in plasma and alveolar regions of healthy calves subcutaneously administered a single dose of enrofloxacin

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ABSTRACT. This study aimed to analyze the pharmacokinetics of enrofloxacin (ERFX) and its metabolite ciprofloxacin (CPFX) in plasma, as well as their migration to, and retention in, the epithelial lining fluid (ELF) and alveolar cells within the bronchoalveolar fluid (BALF). Four healthy calves were subcutaneously administered a single dose of ERFX (5 mg/kg). ERFX and CPFX dynamics post-administration were analyzed via a non-compartment model, including the absorption phase. The Cmax of plasma ERFX was 1.6 ± 0.4 μg/ml at 2.3 ± 0.5 hr post-administration and gradually decreased to 0.14 ± 0.03 μg/ml at 24 hr following administration. The mean residence time between 0 and 24 hr (MRT_{0-24}) in plasma was 6.9 ± 1.0 hr. ERFX concentrations in ELF and alveolar cells peaked at 3.0 ± 2.0 hr and 4.0 ± 2.3 hr following administration, respectively, and gradually decreased to 0.9 ± 0.8 μg/ml and 0.8 ± 0.5 μg/ml thereafter. The plasma half-life (t1/2) of ERFX was 6.5 ± 0.7 hr, while that in ELF and alveolar cells was 6.5 ± 3.6 and 7.4 ± 4.3 hr, respectively. The Cmax and the area under the concentration-time curve for 0–24 hr for ERFX were significantly higher in alveolar cells than in plasma ($P<0.05$). These results suggest that ERFX is distributed at high concentrations in ELF and is retained at high concentrations in alveolar cells after 24 hr in the BALF region; hence, ERFX may be an effective therapeutic agent against pneumonia.

KEY WORDS: bronchoalveolar lavage fluid, calf, ciprofloxacin, enrofloxacin, pharmacokinetics

Mycoplasma spp. and other bacterial pathogens can infect the respiratory system in calves, causing potentially severe, adverse effects to their development and life expectancy [1], thus, presenting a major challenge in veterinary medicine [2]. Numerous cases of calf respiratory diseases have been classified as alveolar pneumonia [2]. Accordingly, the treatment of alveolar pneumonia requires an antibacterial agent with adequate distribution to the intrapulmonary region, including alveolar or BALF fluid. Enrofloxacin (ERFX) is a quinolone-based agent with broad-spectrum antimicrobial activity against pathogens commonly associated with bovine respiratory diseases [5, 19, 22, 29]. However, to determine if ERFX is an effective treatment for alveolar pneumonia, its pharmacokinetics and intrapulmonary distribution must first be characterized. Furthermore, an intrapulmonary ERFX concentration reaching the upper limit of the minimum inhibitory concentration (MIC) for the target pathogen should be achieved [22, 29].

We previously reported the distribution of ERFX in plasma, pulmonary epithelial lining fluid (ELF), and cells in the bronchoalveolar fluid (BALF; alveolar cells) through analysis of BALF samples following subcutaneous administration to healthy calves [25]. However, we previously investigated only short-term ERFX distribution and did not clarify the dynamics of the ERFX concentration in detail. Accordingly, this study aimed to elucidate the chronological dynamics in ERFX concentration in plasma, ELF, and alveolar cells by analyzing blood and BALF samples obtained from calves. For pharmakokinetic (PK) analysis, we targeted ERFX and its metabolite ciprofloxacin (CPFX) in each matrix to investigate their distribution from plasma to ELF and

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alveolar cells.

MATERIALS AND METHODS

Four clinically healthy Holstein bull calves with body weights of 63.8 ± 5.2 kg (mean ± SD) were used herein. Sample collections were conducted five-weeks-old calves. This study was approved by the ethics committee of the Joint Faculty of Veterinary Medicine, Kagoshima University, and the animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals of the Joint Faculty of Veterinary Medicine, Kagoshima University.

A single dose (5 mg/kg) of commercial ERFX (Baytril 5% injectable solution, Bayer, Tokyo, Japan) was subcutaneously administered to the neck of each calf. Thereafter, their body temperatures, heart rates, and respiratory rates were measured, and visual examinations were carried out at 0 (before administration), 2, 6, 10, and 24 hr post-administration.

Peripheral blood samples were collected from the jugular vein at 0 (before administration), 1, 2, 3, 6, 10 and 24 hr post-administration, using heparinized Vacutainer tubes (VP-H050K, Terumo, Tokyo, Japan), and Vacutainer tubes (VP-NA052K, Terumo) treated with dipotassium ethylenediaminetetraacetic acid (EDTA-2AK). Blood collected in heparinized tubes was centrifuged to obtain plasma, which was stored at −80°C until analysis. Blood collected in EDTA-2AK-treated tubes was analyzed for white blood cells (WBCs), red blood cells (RBCs), hemoglobin (Hb) and hematocrit (Ht) using an automated cell counter (Poch-100iv, Sysmex, Kobe, Japan) within 1 hr of collection. Blood collection time was determined according to that reported in a previous method [25]. Additionally, the 3, 6, 10, and 24 hr time points were included to accurately assess the PK parameter in blood.

BALF was collected at 0 (before administration), 2, 6, 10, and 24 hr post-administration, using a flexible electronic endoscope (VQ TYPE 5112B, Olympus, Tokyo, Japan). The lower airway of each animal was prepared for bronchoscopy with 2% lidocaine. A flexible electronic endoscope was inserted into a sub-segment of each lobe. Two 30 m*l* aliquots of sterile 0.9% normal saline solution were injected into the lobe and immediately aspirated. BALF obtained during the second aspiration was pooled with that obtained from the first.

BALF was collected from three sites in both the left and the right lobes, totaling six sites per calf (right middle lobe, third bronchiole of the right caudal lobe, fifth bronchiole of the right caudal lobe, second bronchiole of the left caudal lobe, third bronchiole of the left caudal lobe, and fifth bronchiole of the left caudal lobe). BALF was promptly transported to a laboratory for analysis. After the cell count was determined, a 1.5 m*l* aliquot of each BALF sample was centrifuged at 400 × *g* for 5 min. The supernatant and cell pellets were separated and frozen at −80°C until use.

ERFX concentrations and CPFX concentrations were measured via high-performance liquid chromatography with tandem mass spectrometry (LC/MS/MS), using a previously reported method [6, 25]. Plasma samples (100 μl) were diluted 10-fold with distilled water. Each BALF cell pellet was mixed with 0.5 m*l* of 1 mol sodium hydroxide for cell lysis and then mixed with 1.0 m*l* of 3% formic acid. Three-hundred microliters of each sample (diluted plasma, BALF supernatant, and lysed BALF cell pellet) was mixed with 60 μl of the internal standard (Lomefloxacin, Sigma-Aldrich, Tokyo, Japan; 300 *ng*/m*l* in 1% formic acid/methanol (4:1)) and 60 μl of methanol, using a previously reported method [6, 25]. Each sample (350 μl) was loaded into a solid-phase extraction column (Oasis HLB, Waters, Tokyo, Japan). The residue was dissolved in 250 μl of the mobile phase, and an aliquot (10 μl) of the extract was injected into the LC/MS/MS system (Prominence, Shimadzu, Kyoto, Japan; 4000 QTRAP, AB, Sciex, Tokyo, Japan). The detection limits for ERFX and CPFX were 0.001 μg/m*l*.

Once data for BALF analysis were obtained, ERFX concentrations were determined for ELF and alveolar cells using previously reported methods [12, 13]. The ERFX concentrations in ELF were calculated on the basis of the ERFX concentrations in BALF, as well as the plasma and BALF urea concentrations, using a previously reported method [25]. The alveolar cells ERFX concentration was determined on the basis of that in the alveolar cell pellet and the mean total alveolar cells obtained from each calf. In total, 1.28 $\mu l/10^6$ alveolar cells were used on the basis of previous reports [12, 13]. To confirm the BALF cells fractions, prepare a cytospin smear (Cytospin4, Thermo Fisher scientific, Waltham, MA, USA) and follow the standard method, and counted 200 white blood cells.

PK parameters were determined for each animal after subcutaneous administration of ERFX at 5 mg/kg on the basis of ERFX and CPFX concentrations in plasma, ELF, and alveolar cells. ERFX and CPFX levels were determined using a non-compartment model (including the absorption phase). The area under the concentration-time curve between 0 and 24 hr (AUC_{0-24}), and the area under the first moment curve between 0 and 24 hr ($AUMC_{0-24}$) were determined using a linear trapezoidal method; meanwhile, the mean residence time between 0 and 24 hr (MRT_{0-24}) was determined as AUMC/AUC. The terminal phase half-life (t1/2) was determined as 0.693/terminal slope.

Statistical analyses of data were conducted using analysis of variance (one-way ANOVA) followed by the Tukey-Kramer multiple comparison test to determine the differences in ERFX among three types of samples at the same sampling time point. Furthermore, ANOVA was used to determine the differences in ERFX among three types of PK parameters and AUC/MIC ratios. All statistical analyses were performed using the IBM SPSS Statistics 24 software (IBM, Tokyo, Japan), and *P*<0.05 was considered statistically significant. Data are shown as the mean ± SD.

RESULTS

The body temperature, heart rates, respiratory rates, as well as WBC, RBC, Hb, and Ht levels of each calf did not fluctuate significantly, and abnormal clinical findings were not recognized by visual inspection throughout the study.
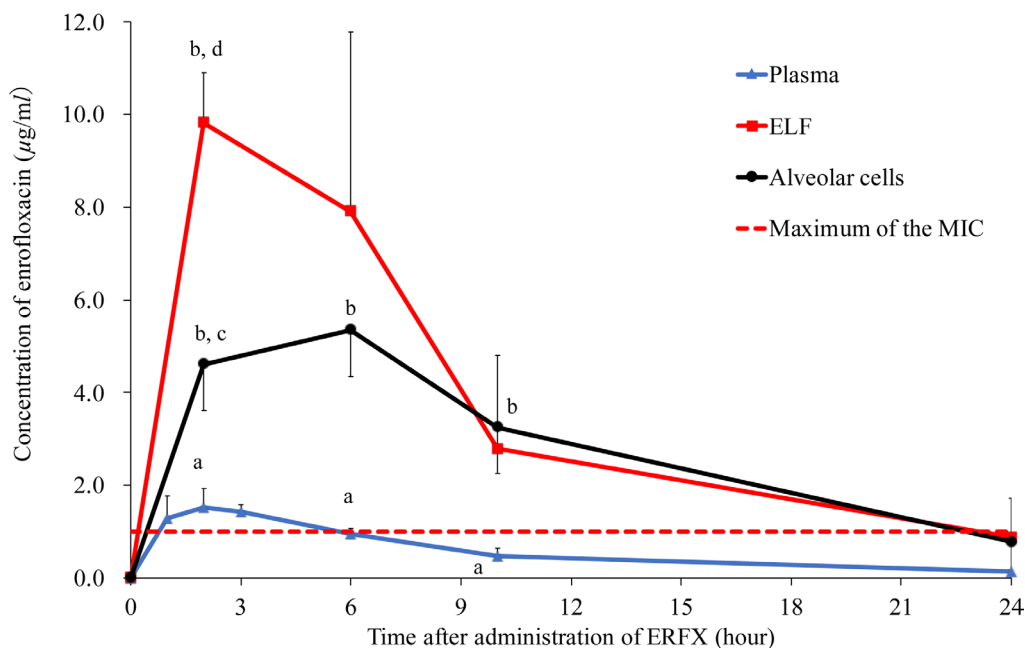
Fig. 1. Concentrations of enrofloxacin in plasma, epithelial lining fluid (ELF), and alveolar cells 0 to 24 hr after a single subcutaneous dose of enrofloxacin (5 mg/kg) in calves. Data are shown as the mean ± SD. Values with the different letters indicate significant differences among each region at the same sampling time (a–b, c–d: $P<0.05$). Maximum of the minimum inhibitory concentration (MIC), MIC of *Mycoplasma bovis* (Harada *et al.* [15]).

Table 1. Pharmacokinetics parameters of enrofloxacin (ERFX) in plasma, epithelial lining fluid (ELF), and bronchoalveolar fluid (BALF) cells following single subcutaneous dose (5 mg/kg) of ERFX (5% formulation) in calves
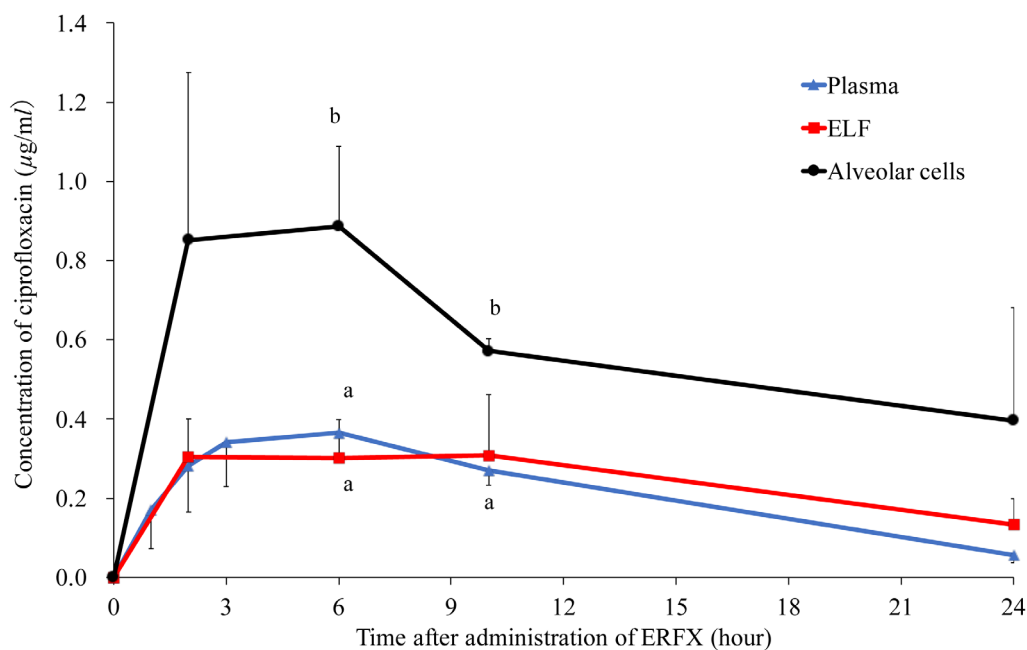
PK Parameters	Plasma	ELF	Alveolar cells
Cmax (μg/ml)	1.6 ± 0.4[a)]	10.4 ± 2.0[b,c)]	5.9 ± 2.1[b,d)]
Tmax (hr)	2.3 ± 0.5	3.0 ± 2.0	4.0 ± 2.3
AUC$_{0-24}$ (μg·hr^2/ml)	14.2 ± 1.1[a)]	92.3 ± 34.0[b)]	70.0 ± 25.2[b)]
AUMC$_{0-24}$ (μg·hr^2/ml)	99.6 ± 21.1[a)]	646.9 ± 278.6[b)]	580.0 ± 212.3[b)]
MRT$_{0-24}$ (hr)	6.9 ± 1.0	6.9 ± 1.5	8.3 ± 0.9
t1/2 (hr)	6.5 ± 0.7	6.5 ± 3.6	7.4 ± 4.3

Data are shown as the mean ± SD. AUC: area under the concentration-time curve; AUMC: area under the first moment curve; Cmax: peak concentration; Tmax: time to Cmax; t1/2: half-life; MRT: mean residence time. Values with the different letters indicate significant differences among each region in PK parameters (a–b, c–d: $P<0.05$).

The BALF recovery rates, before administration, and at 2, 6, 10, 24 hr post-administration were 43.3 ± 8.2, 44.4 ± 8.3, 47.9 ± 6.9, 42.9 ± 4.8 and 41.7 ± 5.8%, respectively. Hence, the BALF recovery rate was essentially unchanged. Similarly, the recovery rate of alveolar cells did not significantly change before administration, and at 2, 6, 10, 24 hr post-administration with values of 38.7 ± 12.8, 36.6 ± 10.3, 37.1 ± 12.9, 38.2 ± 11.1, and 37.8 ± 13.2 (×10^5/ml) detected, respectively. In all specimens of the BALF cell smear, as for the compartmentation, 95% or more were macrophages.

ERFX concentrations in plasma, ELF, and alveolar cells are shown in Fig. 1. The corresponding PK parameters are shown in Table 1. Plasma ERFX concentrations peaked (Cmax ; 1.6 ± 0.4 μg/ml) at 2.3 ± 0.5 hr after subcutaneous administration and decreased to 0.14 ± 0.03 μg/ml gradually thereafter. The ERFX distribution was in accordance with a non-compartment model, including the absorption phase. ELF and alveolar cells EFRX concentrations peaked at 3.0 ± 2.0 hr (Cmax: 10.4 ± 2.0 μg/ml) and 4.0 ± 2.3 hr (Cmax: 5.9 ± 2.1 μg/ml) after administration, respectively, and decreased to 0.9 ± 0.8 and 0.8 ± 0.5 μg/ml, respectively, gradually thereafter (Fig. 1).

Additionally, the Cmax in ELF and alveolar cells was significantly higher than that in the plasma ($P<0.05$), and that in ELF was significantly higher than in alveolar cells ($P<0.05$). Moreover, the Tmax in plasma, ELF, and alveolar cells were 2.3 ± 0.5, 3.0 ± 2.0, and 4.0 ± 2.3 hr (Table 1), respectively. The ERFX concentration in ELF and alveolar cells at 2 hr was significantly higher than that in the plasma ($P<0.05$), and the ERFX concentration in alveolar cells was significantly higher than that in the ELF ($P<0.05$). Similarly, the ERFX concentration in ELF at 6 hr was significantly higher than that in the plasma ($P<0.05$), and that in alveolar cells at 24 hr was significantly higher than in the plasma ($P<0.05$). Furthermore, the AUC$_{0-24}$ in plasma, ELF, and alveolar

Fig. 2. Concentrations of ciprofloxacin (as metabolite) in plasma, epithelial lining fluid (ELF), and alveolar cells 0 to 24 hr after a single subcutaneous dose of enrofloxacin (5 mg/kg) in calves. See Fig. 1 for key.

Table 2. Pharmacokinetics parameters of ciprofloxacin (CPFX) in plasma, epithelial lining fluid (ELF), and bronchoalveolar fluid (BALF) cells following a single subcutaneous dose (5 mg/kg) of enrofloxacin (ERFX) (5% formulation) in calves

PK parameters	Plasma	ELF	Alveolar cells
Cmax (μg/ml)	$0.4 \pm 0.09^{a)}$	$0.4 \pm 0.06^{a)}$	$1.1 \pm 0.2^{b)}$
Tmax (hr)	5.3 ± 1.5	5.0 ± 3.8	4.0 ± 2.3
AUC_{0-24} (μg·hr^2/ml)	$5.2 \pm 0.6^{a)}$	$5.1 \pm 0.4^{a)}$	$14.1 \pm 3.1^{b)}$
$AUMC_{0-24}$ (μg·hr^2/ml)	43.9 ± 4.1	52.5 ± 10.5	144.5 ± 49.8
MRT_{0-24} (hr)	8.5 ± 1.0	10.2 ± 1.9	10.1 ± 1.3
t1/2 (hr)	$6.1 \pm 1.5^{a)}$	$10.6 \pm 1.5^{b)}$	16.9 ± 11.1

AUC: area under the concentration-time curve; AUMC: area under the first moment curve; Cmax: peak concentration; Tmax: time to Cmax; t1/2: half-life; MRT: mean residence time. Values with the different letters indicate significant differences among each region in PK parameters (a–b: $P<0.05$).

cells were 14.2 ± 1.1, 92.3 ± 34.0, and 70.0 ± 25.2 μg·hr/ml (Table 1), respectively, with that in ELF and alveolar cells significantly higher than that in the plasma ($P<0.05$). The AUMC in plasma, ELF, and alveolar cells were 99.6 ± 21.1, 646.9 ± 278.6, and 580.0 ± 212.3 μg·hr^2/ml (Table 1), respectively, with that in ELF and alveolar cells significantly higher than that in the plasma ($P<0.05$). The MRT_{0-24} in plasma, ELF, and alveolar cells were 6.9 ± 1.0, 6.9 ± 1.5, and 8.3 ± 0.9 hr, respectively; however, no statistical differences were observed. The t1/2 in plasma, ELF, and alveolar cells were 6.5 ± 0.7, 6.5 ± 3.6, and 7.4 ± 4.3 hr, respectively; however, no statistical differences were observed.

CPFX concentrations in plasma, ELF, and alveolar cells are shown in Fig. 2. The corresponding PK parameters in plasma, ELF, and Alveolar cells are shown in Table 2. Results show that plasma CPFX levels peaked (Cmax: 0.4 ± 0.09 μg/ml) at 5.3 ± 1.5 hr post-administration and gradually decreased to 0.06 ± 0.02 μg/ml thereafter. The CPFX distribution formed a non-compartment model, including the absorption phase. CPFX concentrations peaked in ELF and alveolar cells at 5.0 ± 3.8 hr (Cmax: 0.4 ± 0.06 μg/ml) and 4.0 ± 2.3 hr (Cmax: 1.1 ± 0.2 μg/ml), respectively, post-administration and gradually decreased to 0.1 ± 0.07 and 0.4 ± 0.3 μg/ml, respectively, thereafter (Fig. 2).

The Cmax and Tmax in alveolar cells were longer than those in the plasma and ELF. Moreover, the CPFX concentration in alveolar cells at 2 hr was significantly higher than that in the plasma and ELF ($P<0.05$). Meanwhile, the CPFX concentration in alveolar cells at 6 hr was significantly higher than that in the plasma ($P<0.05$). AUC_{0-24} in plasma, ELF, and alveolar cells were 5.2 ± 0.6, 5.1 ± 0.4, and 14.1 ± 3.1 μg·hr/ml (Table 2), respectively, with that in alveolar cells significantly higher than in the plasma and ELF ($P<0.05$). Similarly, the AUMC in plasma, ELF, and Alveolar cells were 43.9 ± 4.1, 52.5 ± 10.5, and 144.5 ± 49.8 μg·hr^2/ml (Table 1), respectively. The MRT_{0-24} in plasma, ELF, and alveolar cells were 8.5 ± 1.0, 10.2 ± 1.9, and 10.1 ± 1.9 hr, respectively; however, no statistical differences were observed. The t1/2 in plasma, ELF, and alveolar cells were 6.1 ± 1.5, 10.6 ± 1.5, and 16.9 ± 11.1 hr (Table 2), respectively, with that t1/2 in ELF significantly longer than that in plasma ($P<0.05$).

DISCUSSION

Herein, PK analysis revealed a higher Cmax and longer Tmax for both ERFX and CPFX in plasma than previously reported by de Lucas *et al*. [8]. Furthermore, the AUC_{0-24} for both ERFX and CPFX in plasma was higher, and the t1/2 and MRT_{0-24} in plasma for both ERFX were longer than that previously reported [8]. However, although Shoaf *et al*. [31] reported changes in metabolic enzyme activity, such as cytochrome P-450, during the early developmental period, they used 2–3-month-old calves, while we used 5-week-old calves for our study. Therefore, differences in the pk parameters of ERFX and CPFX could be due to age differences associated with metabolic capacity.

The ERFX distribution in the alveolar region was evaluated by comparing parameters between ELF, alveolar cells, and plasma. The AUC_{0-24} and Cmax in the ELF and alveolar cells were significantly higher than in plasma. Accordingly, we considered that ERFX administration to calves would potentially result in very high drug distribution to the alveolar region. Moreover, since the t1/2 in plasma, ELF, and alveolar differed, that of plasma, ELF, and alveolar cells did not achieve steady-state values.

Next, the CPFX distribution in the alveolar region was similarly evaluated, and results show that AUC_{0-24} and Cmax in alveolar cells were statistically more significant than that in plasma and ELF. These results suggest that only relatively small quantities of CPFX are distributed to the alveolar region following subcutaneous ERFX administration to calves. Moreover, since the t1/2 was significantly longer in ELF compared to in plasma, that for ELF achieved steady-state values.

Conversely, new quinolone-based antimicrobial agents are rapidly taken up by macrophages, with the relevant drug concentrations reportedly higher in the cellular fractions than in the tissue [7, 16, 27]. Furthermore, the neutrophil extracellular trap phenomenon is enhanced by ERFX. Such drugs are incorporated into neutrophils, and its bactericidal activity is strengthened [17, 20]. Our results demonstrated that ERFX and CPFX concentrations were higher in alveolar cells than in plasma, suggesting a strong antibacterial effect against invading microbes, a possibility supported by the high ERFX and CPFX concentrations in phagocytic cells collected from sites of inflammation.

Treatment of infectious diseases requires identification of the affected organs and tissues, and the relevant pathogen and therapeutic strategies are decided on the basis of these findings. In cases of pneumonia, the prediction of the antimicrobial effect of the agent is required, which is based on both effective PK and phamacodynamic (PD) analyses. Such combined PK/PD approaches have been widely used in clinical practice [3]. Blood levels of antimicrobial agents at the level of the MIC may deter bacterial proliferation; however, they are not necessarily sufficient for therapy. For instance, if the host immune system is compromised, resistant bacteria may emerge even though most bacteria are killed at drug levels similar to the MIC, implying the requirement for a higher MIC. An increase in the MIC also requires the administration of additional doses of the antimicrobial agent, potentially yielding further strains of resistant bacteria. This vicious cycle ultimately renders antimicrobial agents ineffective. In human medicine, microbial agents should thus be administered at a higher concentration, the mutation prevention concentration (MPC), to eliminate bacteria, including resistant bacteria generated by mutations due to drug administration at doses approaching the MIC [9]. The same approach is being adopted in veterinary medicine [14, 23, 26, 28].

Basic parameters for predicting the effects of clinical treatment for PK/PD are Cmax/MIC and AUC/MIC ratios, and the duration beyond the MIC (% T>MIC) [3, 26, 28]. Aminoglycosides and quinolone-based drugs (concentration-dependent systems) reportedly display a correlation between antimicrobial effects and the Cmax/MIC or AUC/MIC ratio, while β-lactam drugs reportedly exhibit a correlation between antibacterial effects and the % T>MIC period prior to the dosing interval [3]. Since new quinolone-based drugs have concentration-dependent effects, parameters should be selected in accordance with the treatment effect [4, 24, 32], prevention of the acquisition of bacterial drug resistance [10, 18, 33], and cost-effectiveness [30]. Accordingly, the Cmax/MIC ratio and AUC/MIC ratio are useful indicators in PK/PD analysis. AUC/MIC ratios for gram-negative bacteria in human medicine range from 100–105, and the corresponding Cmax/MIC ratio ranges from 8–10 [4, 11, 24, 32]. However, McKellar *et al*. [23] recommend a Cmax/MIC ratio of at least 10, and an AUC/MIC ratio of at least 125 for concentration-dependent antimicrobial agents in veterinary medicine.

The PK parameters used herein include the AUC_{0-24}. The MICs of ERFX for the three target pneumonia-inducing bacteria *Pasteurella multocida* (*P. multocida*), *Mannheimia haemolytica* (*M. haemolytica*) [15], and *Mycoplasmabovis* (*M. bovis*) [34] were obtained from health research on cattle in Japan as PD parameters. The MICs of *P. multocida*, *M. haemolytica,* and *M. bovis* were 0.12, 0.5, and 1.0 μg/m*l*, respectively. The ratios of MIC to AUC_{0-24} (AUC/MIC) in each region of each bacterial species are shown in Table 3. The ratios of MIC to AUC_{0-24} (AUC/MIC) were evaluated, and the therapeutic effect against each bacterial

Table 3. Area under the concentration-time curve between 0 and 24 hr/minimum inhibitory concentration $(MIC)_{90}$ ratio for enrofloxacin (ERFX) following a single subcutaneous dose of ERFX, using MIC_{90} values of 0.12, 0.5 and 1.0 μg/m*l* for *Pasteurella multocida* (*P. multocida*), *Mannheimia haemolytica* (*M. haemolytica*), and *Mycoplasmabovis* (*M. bovis*), respectively

Bacteria name	Plasma	ELF	Alveolar cells	References
P. multocida	117.1 ± 7.4[a)]	842.8 ± 273.3[b)]	603.6 ± 294.1[b)]	[15]
M. haemolytica	28.1 ± 1.8[a)]	202.3 ± 65.6[b)]	144.9 ± 70.6[b)]	[15]
M. bovis	14.1 ± 0.9[a)]	101.1 ± 32.8[b)]	72.4 ± 35.3[b)]	[34]

ELF, epithelial lining fluid. Values with the different letters indicate significant differences among each region in each bacterial species (a–b: $P<0.05$).

species was assessed. The AUC_{0-24}/MIC ratios of these bacteria in plasma were below 125. The AUC_{0-24}/MIC ratios of these bacteria in plasma was significantly lower than that in the ELF and alveolar cells ($P<0.05$). However, the AUC_{0-24}/MIC ratio of *P. multocida* and *M. haemolytica* were beyond 125 for ELF and alveolar cells, with the ERFX exceeding the MPC in both ELF and alveolar cells. Therefore, ERFX was considered to have excellent antibacterial activity against *P. multocida* and *M. haemolytica*. Alternatively, the AUC_{0-24}/MIC ratio for *M. bovis* was below 125 for ELF and alveolar cells. Moreover, ERFX was not found to exceed the MPC against *M. bovis* in ELF and alveolar cells. However, ERFX concentrations against *M. bovis* for ELF and alveolar cells at 24 hr post-administration were 0.9 ± 0.8 and 0.8 ± 0.5 μg/ml, respectively, suggesting that they exceeded the MIC approximately 24 hr after administration (Fig. 1). Since ERFX has a post-antibiotic effect (PAE) [21, 35], it seems that its antibacterial activity is maintained at 1.0 μg/ml or less. This result suggested that ERFX exceeds MIC at 24 hr in ELF and alveolar cells. Therefore, ERFX antimicrobial agents must exceed the MIC required for specific bacteria to exhibit antimicrobial activity. Specifically, antibacterial activity against *M. bovis* was achieved at concentrations higher than the MIC and lower than the MPC, which is a range referred to as the MSW (Mutant Selection Window) [14, 23, 26, 28], in which resistant bacteria are readily selected for and induced.

In PK/PD analysis, the AUC/MIC ratios in the alveolar region indicated that this regimen displayed therapeutic effects against *P. multocida*, *M. haemolytica*, and *M. bovis*. However, the elimination half-life in ELF and alveolar cells in this study was 6.5 ± 3.6 hr and 7.4 ± 4.3. Moreover, although distribution to the alveolar region was adequate, a steady-state was not achievable with a single daily dose of ERFX at 5 mg/kg. Therefore, multiple doses should be administered. Future field measurements of MIC are needed for further investigation of treatment methods. In clinical practice, an antibiogram is generated for each farm, and a clinically therapeutic effect against these bacteria may be expected when its MIC is less than 1 μg/ml.

In this study, we measured the concentration of ERFX and CPFX (as metabolites) in plasma, ELF, and alveolar cells after subcutaneous administration of ERFX (5 mg/kg). PK parameter analysis suggested that this treatment was effective against various bacterial species. However, additional study is needed to clarify aspects such as the migration of ERFX in calves with pneumonia prior to the practical application of this treatment.

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