The effects of cyclosporine A or activated charcoal co-administration on the pharmacokinetics of enrofloxacin in chickens

Tsvetelina Petkova,* Aneliya Milanova ,* and Błażej Poźniak †,1

*Trakia University, Faculty of Veterinary Medicine, Department of Pharmacology, Animal Physiology, Biochemistry and Chemistry, Stara Zagora, 6000, Bulgaria; and †Wrocław University of Environmental and Life Sciences, Faculty of Veterinary Medicine, Department of Pharmacology and Toxicology 50-375 Wrocław, Poland

ABSTRACT The study aimed to investigate the possible role of efflux transporter proteins in the pharmacokinetics of enrofloxacin (**ENR**) in broilers in the model of co-administration of activated charcoal (**AC**) or cyclosporine A (**CsA**). The concentrations of enrofloxacin and its metabolite ciprofloxacin were analyzed by liquid chromatography−mass spectrometry (**LC-MS/MS**) and population approach was used for pharmacokinetic analysis. It was found that body weight has a significant effect on the volume of distribution in the central compartment and on the systemic clearance. Oral AC increased the systemic clearance of intravenously administered ENR suggesting some role of enterohepatic recirculation. For orally administered ENR, CsA increased the area under the curve which can be explained by the inhibition of efflux transporters. Metabolism of the antibacterial drug was not affected by cyclosporine. The data suggest a role of efflux transporter proteins in the pharmacokinetics of drugs in chickens and drug-drug interactions have to be considered when substrates and modulators of these transporters are co-administered.

Key words: enrofloxacin, cyclosporine A, activated charcoal, drug-drug pharmacokinetic interaction, chicken

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INTRODUCTION

The ATP-binding cassette (**ABC**) efflux transporter proteins are transmembrane proteins that can carry a wide variety of substrates across biological membranes. Their relevance for pharmacokinetics and pharmacodynamics of many drugs, often applied in veterinary patients, has been reviewed in the scientific literature (Schrickx and Gremmels, 2008). The function of ABC transporter proteins can influence important pharmacokinetic parameters such as rate of absorption after oral administration, disposition in the tissues and excretion from the body which determine the therapeutic efficacy of drugs and, thus, should be considered while designing the optimal dosage regimens (Schrickx and Gremmels, 2008). Clinical relevance of the function of P-glycoprotein (**P-gp**) and breast cancer resistance protein (**BCRP**) was proved in veterinary medicine (Mealey et al., 2012).

Fluoroquinolones are antibacterial drugs that have been widely studied and applied in veterinary practice, including in poultry. Despite the fact that significant number of studies has elaborated on their pharmacokinetics in poultry, some important aspects have not yet been explained. The published literature reveals that after oral administration there is a double peak in the serum or plasma concentrations of danofloxacin in turkeys and in geese, or of enrofloxacin (**ENR**) in chickens, which was not conclusively explained (Sumano et al., 2003; Haritova et al., 2006; Sang et al., 2016; Sartini et al., 2021). Similarly, small extra peaks have been observed in individual ENR concentration-time profiles in turkeys after intravenous drug administration (Poźniak et al., 2020a,b). Abnormalities in the shape of the pharmacokinetic profiles have also been observed after oral ENR administration in chickens with *E. coli* infection (Guo et al., 2014). The authors of this study observed that the drug was absorbed slower and to a lower extent in the infected birds which was related to the increased expression levels of ABCB1 mRNA, the gene encoding P-gp. Other studies carried out in poultry species found that danofloxacin and ENR treatments lead to the tendency for upregulation of ABCB1 and ABCG2 mRNA (Haritova et al., 2008; Pavlova et al., 2018). The effect of P-gp on ENR pharmacokinetics was further proved at functional level in broilers by co-

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[1]Corresponding author: blazej.pozniak@upwr.edu.pl

administration of verapamil, a potent and specific inhibitor of this transporter protein (Guo et al., 2014). Other studies have shown that fluoroquinolone antimicrobials may act as substrates and modulators of additional ABC-transporter proteins such as BCRP (Pulido et al., 2006; Mealey et al., 2012; Sparkes et al., 2019). Evidence for substrate overlapping for these proteins is substantial (Dei et al., 2019; Hernández-Lozano et al., 2021a). Potential inhibitory activity of several fluoroqinolones on P-gp, BCRP, and MRP2 function were tested in ex vivo model with chicken lymphocyte-subsets obtained from spleen samples (Haritova et al., 2007). Some fluoroquinolones (danofloxacin and danofloxacin mesylate) were described as stronger inhibitors for efflux transporters than others (ENR, ciprofloxacin, and marbofloxacin). The fact that fluoroquinolones are substrates and modulators of ABC efflux transporters in chickens explains their secretion in the gastrointestinal tract after parenteral administration (Haritova et al., 2007). However, the contribution of these transporter proteins to the quantitative aspects of fluoroquinolones' pharmacokinetics is not well understood.

The aim of this study was to assess whether the inhibition of multiple efflux transporter proteins by a broad-spectrum modulator and substrate such as CsA (Haritova et al., 2007) can affect the pharmacokinetics of co-administered ENR. ENR was selected as the model fluoroquinolone for the current in vivo experiment on chickens because of three reasons: 1) It is a confirmed substrate for P-gp and BCRP; 2) It is widely used in poultry industry in many countries; and 3) The specific shape of the pharmacokinetic profiles in the published literature for avian species suggests the presence of yet unidentified redistribution processes that may be related to transporter-mediated drug flux. Since this phenomenon may be based on the enterohepatic recirculation, an additional treatment with oral AC as an adsorbent was designed in order to assess the possibility of drug reabsorption from the gut. Population pharmacokinetic analysis was applied to evaluate the effect of co-administration of CsA or the adsorbent on the pharmacokinetics of ENR in broiler chickens.

MATERIALS AND METHODS

Drugs and Reagents

ENR was administered as Baytril 50 mg/mL solution for injection (Bayer Animal Health GmbH, KVP Pharma + Veterinär Produkte GmbH, Kiel, Germany) for intravenous treatment of the birds used in the experiments. Baytril 10% oral solution was used for oral treatment of the broilers (100 mg/mL ENR, Bayer Animal Health GmbH, KVP Pharma + Veterinär Produkte GmbH). Sandimmun, containing 50 mg/mL CsA (Novartis, Warszawa, Poland), was used for oral treatment of the chickens. ENR, ciprofloxacin and marbofloxacin used for analytical tests were HPLC grade (≥98%, Sigma-Aldrich, St. Louis, MO). Analysis of ENR and ciprofloxacin concentrations was performed by using the following reagents and mobile phase components: trifluoroacetic acid (99.5%) (Fisher Chemicals, Hampton, NH), acetonitrile OPTIMA, LC-MS grade (Fisher Chemicals, Hampton, NH), methanol, LC-MS grade (CHROMASOLV LC-MS, Honeywell, Charlotte, NC), formic acid for mass spectrometry ~98% (Honeywell Fluka, Seelze, Germany), and water for chromatography (LC-MS Grade LiChrosolv, Merck KGaA, Darmstadt, Germany).

Experimental Design

The experiments were carried out after obtaining ethical approval from Bulgarian Food Safety Agency (License 245/25.09.2019). Ross hybrid (Cornish ♀ × Plymouth Rock ♂) 1-day-old broilers (n = 60) were obtained from "Zhuliv" EOOD, Stara Zagora. The chickens were reared in the Biobase unit at the Faculty of Veterinary Medicine, Trakia University. They were accommodated at suitable temperature and humidity according to the requirements of the age (Ordinance No. 20/1.11.2012 on the minimum requirements for protection and welfare of experimental animals and requirements for use, rearing and delivery). The light regimen was 18 h daylight and 6 h in the dark. They were fed with feed without antibiotics and coccidiostats, dedicated to the stage of their development (Vladini Trading EOOD, Chirpan, Bulgaria). The chickens received Bioselet E (natrii selenis 0.6 mg and D, L-α-tocopheroli acetas (Vit. E) 25.0 mg in 1 mL, Biovet AD, Pestera, Bulgaria) on d 11 to 15 (0.3 mL/L water) and on d 21 to 25 (0.5 mL/L water) according to the manufacturer instructions. Feed and water were provided ad libitum. The treatment and pharmacokinetic study were performed when chickens were 30 days old (n = 60 chickens included in the experiment). Body weight (**BW**) was measured at the day before the treatment. The broilers from all the groups were feed-restricted for 12 h before the treatment. They were divided into 5 groups as described below.

The first group (total number in the group was 12 chickens, n = 6 chickens at every sampling time, BW 1.41 ± 0.13 kg) was treated intravenously (**i.v.**) with ENR at a single dose of 10 mg/kg BW. The fluroquinolone drug was administered as a bolus injection in *v. subcutanea ulnaris*. Before intravenous administration of ENR, the chickens received water twice by intraingluvial gavage (5 mL/kg BW) with an interval of 1 h. The procedure was included in the experiment in order to subject all the groups to similar manual procedures and reflected CsA or AC administration in other groups. The antibacterial drug was injected 1 h after administration of water.

The second group (total n = 12, n = 6 chickens at every sampling time, BW 1.38 ± 0.18 kg) received CsA orally (**p.o.**) via a soft tube into the crop at a dose rate of 50 mg/kg BW, divided in 2 equal consecutive doses. The chickens were treated twice with CsA with a dosing interval of 1 h. One hour after the second dose of CsA, ENR was administered i.v. at a dose rate of 10 mg/kg BW.

The third group (total n = 12, n = 6 chickens at every sampling time, BW 1.37 ± 0.11 kg) received feed mixed with AC at a dose rate of 1 g / kg BW 24 h before experiment. Next the broilers were fasted for 12 h and after this time the suspension of AC was administered twice into the crop at a dose rate of 1 g / kg BW with a dosing interval of 1 h. ENR was injected i.v. at a single dose of 10 mg / kg BW one hour after the treatment with AC.

Broiler chickens (total n = 12, n = 6 chickens at every sampling time, BW 1.39 ± 0.20 kg) from the fourth group were treated p.o. with ENR at a single dose of 20 mg/kg BW. They received water into the crop twice (5 mL/kg BW) with an interval of 1 h. The drug was administered one hour after the last intraingluvial application of water.

The fifth group (total n = 12, n = 6 chickens at every sampling time, BW of 1.51 ± 0.22 kg) was administered twice with CsA via a soft tube into the crop. The dose of 50 mg/kg BW was divided in 2 doses of 25 mg/kg BW and applied with a dosing interval of 1 h. ENR was administered into the crop at a single dose of 20 mg/kg BW, 1 h after the treatment with CsA.

Fresh water and feed were provided soon after ENR administration to the broilers. Chickens from the third group received feed mixed with AC at a dose rate of 1 g/kg BW till the end of the experiment. No adverse effects were observed after single ENR administration to chickens with or without CsA or AC during the study.

Blood samples were obtained from *v. subcutanea ulnaris* (contralateral side of the drug administration site) before treatment and thereafter at the following time intervals after intravenous ENR administration: 0, 0.033, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 3, 6, 9, 12, 14, 24, 30, 36, and 48 h. Blood samples (0.8 mL) from 6 chickens from every experimental group were taken at every sampling time. They were collected according to sparse sampling protocol and no more than 9 samples were taken from every chicken during the first 24 h. The chickens treated orally with ENR were sampled at the same time points with the only difference that the first sampling time was 0.083 h after the administration of the drug. The samples were placed in Eppendorf tubes containing heparin-sodium and centrifuged at 850 × *g* for 10 min. Plasma was immediately transferred to clean tubes and stored at −80°C until analysis.

Determination of Plasma Concentrations by LC-MS/MS Analysis

Concentrations of ENR and its pharmacologically active metabolite ciprofloxacin in plasma were analyzed by LC-MS/MS method. Extraction procedure for both fluoroquinolone drugs from plasma samples was performed according to the method described by Caldeira et al (2017) with minor modifications. Shortly, 300 μL of plasma was spiked with 10 μL of the internal standard (**IS**) marbofloxacin at a concentration of 6 μg/mL. The sample was vortexed and after that, 290 μL 0.1% formic acid in acetonitrile was added. The samples were vortexed for 1 min and shaken for 20 min. Then they were centrifuged for 15 min at 14 370 × *g* and 4°C. The supernatant was filtered through 0.22-μm syringe filters, transferred into injection vials and 5 μL were injected into LC-MS/MS system.

A chromatographic column Zorbax Eclipse Plus (2.1 × 100 mm, 1.8 μm, Agilent Technologies, Santa Clara, CA), maintained at 40°C, was used for separation of the fluroquinolone drugs. A pre-column Zorbax SB-C18 (2.1 × 5 mm, 1.8 μm, Agilent Technologies) was fixed before the chromatographic column. Mobile phase A consisted of 0.1% formic acid in LC-MS grade water and mobile phase B was 0.1% formic acid in methanol. The applied gradient program was: 0 to 1 min (98% A, 2% B), 1 to 7 min (60% A, 40% B), 7 to 11 min (100% B), 11 to 13 min (100% B), 13 to 13.1 min (98% A, 2% B), and 13.1 to 17 min (98% A, 2% B). The flow rate was 0.2 mL/min (Zhao et al., 2016). Total run time was 20 min with a post-run of 4 min. The LC-MS/MS system was assembled with 1260 Infinity II quaternary pump, 1260 Infinity II Vial Sampler and triple-quadrupole mass spectrometer Agilent 6460c with AJS technology (Agilent Technologies). Positive ion mode was applied (Agilent Jet Stream ESI+). The other conditions were as follows: gas temperature 300°C; drying gas (nitrogen) 7 L/min; nebulizer gas (nitrogen) 50 psi; sheath gas (nitrogen) 350°C; sheath flow 10 L/min; capillary voltage 3,000 V; nozzle voltage 500 V, dwell time 200 ms. The qualifying ion for ENR was 360.0 m/z and the quantifying ions were and 342.1 and 316.2 m/z. These ions for ciprofloxacin were 332.1 m/z and 314.1, and 231 m/z, respectively. Marbofloxacin was analyzed according to the qualifying ion 363.2 m/z and quantifying ion 320.1 m/z (Sun et al., 2012). The LC-MS/MS method was validated for ENR and ciprofloxacin using plasma samples from untreated chickens spiked with the following concentrations: 0, 5, 10, 50, 100, 250, 500, 750 and 1,000 ng/mL. The samples with concentrations higher than 1,000 ng/mL were diluted with same matrix − plasma from untreated chickens. The internal standard was used at the final concentration of 100 ng/mL. The standard curve was linear between 5 and 1,000 ng/mL for ENR ($R^2 = 0.999$) and between 5 and 500 ng/mL for ciprofloxacin ($R^2 = 0.999$). The calculated values of limit of detection and limit of quantification for ENR were 0.0037 and 0.011 μg/mL, and for ciprofloxacin were 0.001 and 0.004 μg/mL, respectively. The values of mean accuracy for the same compounds were 103.81 ± 9.11% and 101.42 ± 5.89%, respectively. The value of recovery for ENR was >87% and for ciprofloxacin it was >86%. The value of intra-day precision was 3.76% and interday precision was 8.85% for ENR. These values for ciprofloxacin were 3.22% and 5.34%, respectively.

Pharmacokinetic Analysis

Non-compartmental Analysis Basic pharmacokinetic parameters for ENR and its main metabolite ciprofloxacin such as area under the curve (**AUC**), maximum

plasma concentration ($\mathbf{C_{max}}$) and the time of C_{max} ($\mathbf{T_{max}}$) were calculated with non-compartmental analysis (Phoenix 8.3.1, Certara, Princeton, NJ).

Nonlinear Mixed Effects Model Building All data obtained from the 5 study groups were pooled together for nonlinear mixed−effect analysis. Changes in ENR plasma concentration over time after i.v. and p.o. administrations were analyzed simultaneously using the stochastic approximation expectation maximization (**SAEM**) algorithm as implemented in the Monolix Suite 2021R1 (Lixoft, Antony, France). Individual values of pharmacokinetic parameters were obtained post-hoc using the mean of the full posterior distribution. The model was written as described earlier by Sheiner and Ludden (1992) and adopted to veterinary settings (e.g., Pelligand et al., 2016; Wang et al., 2019):

$$y_{ij} = F(\varphi_i, t_{ij}) + G(\varphi_i, t_{ij}, \beta) \times \varepsilon_{ij}$$

$$\varepsilon_{ij} \sim N(0, \sigma^2),\ \varphi_i = h(\mu, \eta_i, \beta_i)$$

$$\varphi_i = \mu \times e^{\eta_i}, \eta_i \sim N(0, \Omega, \omega^2)$$

$$j \in \{1, \ldots, n_i\},\ i \in \{1, \ldots, N\}$$

Where y_{ij} is the observed ENR concentration measured in individual i (N is the number of all individuals) at time t_{ij}, whereas j describes the individual sample times from 1 to n_i. Function $F(\varphi_i,t_{ij})$ is the predicted drug concentration at time t_{ij} dependent on the vector of individual pharmacokinetic parameters φ_i. The term $G(\varphi_i,t_{ij},\beta) \times \varepsilon_{ij}$ is the residual error model of $F(\varphi_i,t_{ij})$ where ε_{ij} is an independent random variable distributed in a standard normal distribution with mean 0 and variance σ^2. Individual parameters belonging to the vector φ_i were modeled as a function of the mean population parameter values, μ, individual variability η_i, and individual covariates, β_i. The random variable η_i was assumed to be normally distributed with mean value 0, variance-covariance matrix Ω and variance ω^2. As a result, individual parameters φ_i are log-normally distributed. The final model was parametrized with clearance (**Cl**), volume of distribution of the central (**V1**) and peripheral (**V2**) compartments, intercompartmental clearance (**Q**), absorption rate constant (**ka**), and lag time (**Tlag**). In the case of bioavailability (**F**), the individual value estimates were limited between 0 and 1, thus, logit-normal distribution was assumed. As an additional basic pharmacokinetic parameter, AUC for enrofloxacin was calculated by integrating the individually predicted curves (different approach as compared to AUC calculation in the non-compartmental analysis). Only 7 of 537 (1.3%) concentration-time data points represented values below the limit of quantitation (**BLOQ**) therefore a separate handling of BLOQ data was not included in the model and these values were eliminated.

Parameter Correlation Estimates Scatterplots of η vs. η values for pharmacokinetic parameter estimates and the Pearson's correlation tests were used to guide the choice of correlations between the parameters. Correlation of random effects was applied when correlation coefficients were estimated to be high, met the threshold for inclusion ($P < 0.05$) and improved model performance. As recommended by earlier studies (Lavielle & Ribba, 2016; Pelligand et al., 2016), multiple samples from the posterior distribution obtained at the last SAEM iteration were preferred over the empirical Bayes estimates (**EBEs**) during the evaluation of parameter correlations. The inclusion of correlations in the final model was determined based on the selection criteria as described in the section Model evaluation.

Inclusion of Covariates First, the statistical model was built without co-variates and further it was developed by testing several scenarios and covariates. The function of automated covariate search, available in Monolix 2021R1, was also used while searching for the best covariate model. The influence of bodyweight as well as co-administration of AC and CsA on parameter estimates was evaluated using the Pearson's correlation test, Wald test and analysis of variance as implemented in the Monolix Suite 2021R1. During the covariate search, BW was evaluated as a continuous covariate and as log-normalized BW according to the formula: log-normalized BW = log[BW/weighted mean BW]). The co-administration of AC or of CsA was set as categorical covariate. The threshold of $P < 0.05$ was assumed before the evaluation for inclusion in the model. Final decision on covariate inclusion was based on the selection criteria as described in the section Model evaluation.

Model Evaluation Model quality was assessed using a set of accepted graphic and numerical tools (Pelligand et al., 2016; Nguyen et al., 2017). Convergence of the SAEM algorithm was checked by inspection of the stability of parameter search and by the precision of parameter estimates. This was measured by the relative standard error (**RSE**) of the estimate as obtained by the Fisher Information Matrix. The condition number of the eigenvalues was assessed to check for over-parameterization. Standard goodness-of-fit (**GOF**) plots were used to assess the performances of the different models: individual fits, individual predictions vs. observations, individual weighted residuals (**IWRES**), normalized prediction distribution errors (**NPDE**), and visual predictive check. Normality and independence of residuals were assessed using histograms, quantile-quantile plots, and autocorrelation of conditional weighted residuals. Normal distribution of the random effects was assessed using the Shapiro−Wilk test as well as by inspection of the full posterior distribution of random effects and residuals. For converging models with satisfactory GOF diagnostics, corrected Bayesian information criteria (**BICc**) and the precision of the model parameter estimates were used for final model selection. The BICc was selected over the Akaike Information Criterion (**AIC**) as it tends to favor more parsimonious models (Mould and Upton, 2013; Wang et al., 2019).

Statistical Analysis of the Data From Non-compartmental Analysis

Pharmacokinetic parameters, calculated by non-compartmental analysis, of ENR and its metabolite

ciprofloxacin in broilers are presented as geometric mean and range of minimum and maximum. Normal distribution of the data was confirmed with Shapiro−Wilk test. ANOVA test, followed by Bonferroni test, was applied for statistical analysis of the data after i.v. administration of ENR, alone or in combination with AC or CsA. Data for oral administration of ENR, alone or in combination with CsA were analyzed with t test. A P-value < 0.05 was considered to be significant (Statistica 10.0, Tibco, Palo Alto, CA).

RESULTS

Non-compartmental Analysis

ENR plasma concentrations after single i.v. or p.o. administration alone or with AC or CsA are presented in Figure 1. Plasma levels of the metabolite ciprofloxacin obtained in these experiments are depicted in Figure 2. The pharmacokinetic parameters AUC, C_{max} and T_{max} for the parent compound ENR and its main metabolite ciprofloxacin are presented in Table 1. Ciprofloxacin was found in plasma after intravenous or oral administration of the parent compound in broilers from all of the experimental groups. The value of AUC was significantly higher after intravenous administration of ENR alone in comparison to the co-administration of ENR (i.v.) and AC (p.o.). Oral administration of ENR in combination with CsA (p.o.) resulted in significantly higher values of AUC (increase by approx. 20%). The values of C_{max} of ciprofloxacin were lower in the group treated with the combination of ENR (i.v.) and AC (p.o.) as compared to the results for the group which received ENR (i.v.), only and for the group which received ENR (i.v.) in combination with CsA. In contrast to the AUC, no significant differences in C_{max} or T_{max} were observed when ENR was administered orally alone or with CsA pretreatment.

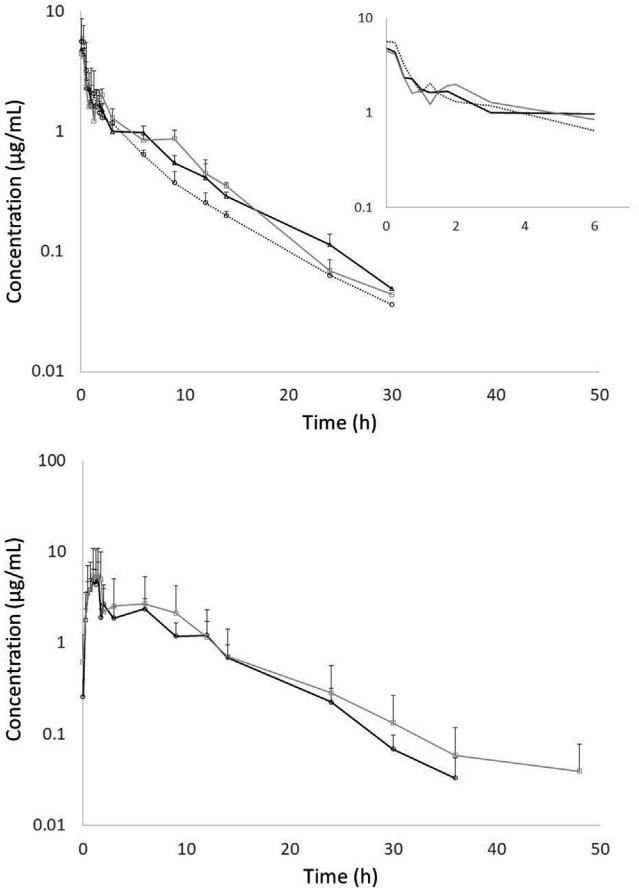

Figure 1. Upper panel: semi-logarithmic plot of plasma enrofloxacin concentrations after single i.v. administration at a dose rate of 10 mg/kg BW, alone (black line) or in combination with activated charcoal, p.o. at a dose rate of 1 g/kg BW (dashed line) or with cyclosporine A, p.o. at a dose rate of 50 mg/kg BW (gray line). Every group consists of 12 chickens. Lower panel: semi-logarithmic plot of plasma enrofloxacin concentrations after single p.o. administration at a dose rate of 20 mg/kg BW, alone (black line) or in combination with cyclosporine A, p.o. at a dose rate of 50 mg/kg BW (gray line). Every group consists of 12 chickens.

Population Pharmacokinetic Analysis

A 2-compartment model best described the pharmacokinetics of ENR in chickens. Combined 2 error model, which contains a constant term and a term proportional to the structural model, was selected to describe the residual error. The goodness of fit of the final model and its predictive power was evaluated based on the numerical criteria and the inspection of individual fits and diagnostic plots such as observations vs. predictions (Figure 3, left panel), scatter plot of the residuals (Figure 3, right panel) and visual predictive check (Figure 4). This last figure shows the data for the 10th, 50th, and 90th percentile of the population presented as empirical data as well as the confidence intervals for the model-based prediction for the respective percentiles. The final model included the correlation between the random effects for Cl, Q, and V2 (Figure 5). Final population pharmacokinetic parameters are presented in Table 2. For the typical population parameters, the RSE values were ≤20% indicating their precise estimation. Values of shrinkage were strictly <30% (between −7.9 and 1.87) suggesting that the model was not over-parameterized for the available data. This was further supported by the low eigenvalue ratio of 14.62 (correlation matrix of estimates). The values of volume of distribution in the central compartment (V_1) and in the peripheral compartment (V_2) reflect higher distribution of ENR in the peripheral compartment. Interindividual variability (omega) in V1 was not included in the model due to the low precision of the estimate. High value of oral bioavailability was calculated after limiting the upper distribution limit to 1.0 and choosing logit normal distribution. Random effects for F and k_a converged toward zero and were excluded from the model. Inclusion of Tlag improved the fit of the data at the early sampling times and the overall model performance as reflected in a decrease in BICc.

Activated charcoal co-administration and log-transformed BW were identified as significant covariates on clearance of ENR. Effect of co-administration of CsA on Cl was eliminated by fixing to 0 because of lack of statistical significance of this covariate and low precision of

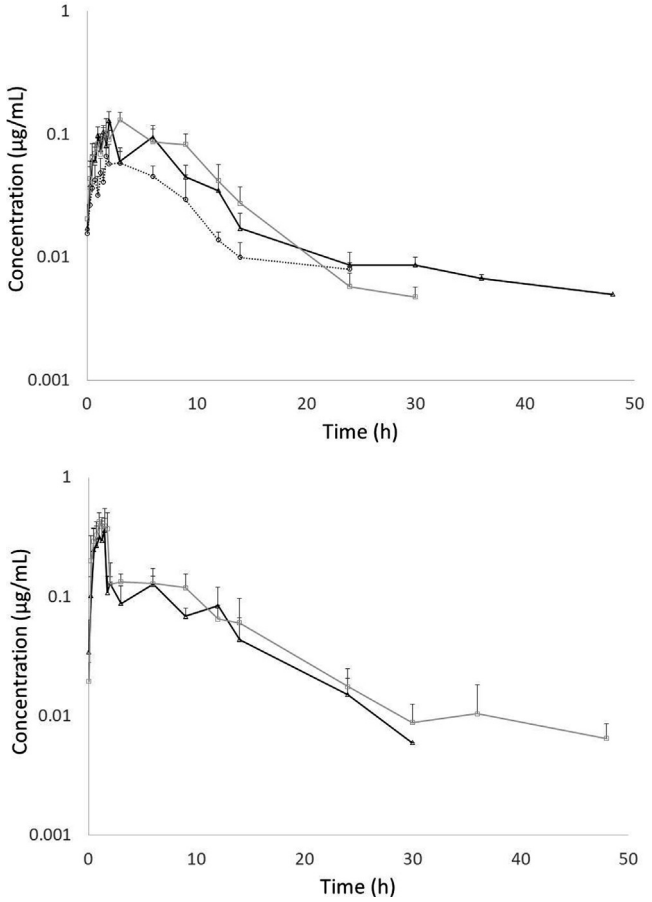

Figure 2. Upper panel: semi-logarithmic plot of plasma ciprofloxacin concentrations after single i.v. administration of enrofloxacin at a dose rate of 10 mg/kg BW, alone (black line) or in combination with activated charcoal, p.o. at a dose rate of 1 g/kg BW (dashed line) or with cyclosporine A, p.o. at a dose rate of 50 mg/kg BW (gray line). Every group consists of 12 chickens. Lower panel: semi-logarithmic plot of plasma ciprofloxacin concentrations after single p.o. enrofloxacin administration at a dose rate of 20 mg/kg BW, alone (black line) or in combination with cyclosporine A, p.o. at a dose rate of 50 mg/kg BW (gray line). Every group consists of 12 chickens.

estimate (high RSE% value). Body weight was recognized as an important covariate for V1. None of the co-administrations or the BW had significant effect on ka, V2, and Q.

DISCUSSION

ENR has been widely used in poultry husbandry and a significant body of data concerning its pharmacokinetics in broilers has been accumulated so far (Knoll et al., 1999; Sumano et al., 2003, Atef et al., 2020). Despite this scientific effort, the nature of the peculiarities seen in the ENR concentration-time profiles (i.e., double peaks after both, intravenous and oral administration) has not yet been explained. The possible involvement of enterohepatic recirculation or drug transporters and the uncertainty related to the clinical consequences of this phenomenon have given rise to this investigation. Therefore, the present study attempts to assess ENR pharmacokinetics in healthy broilers in a model of drug transporter inhibition by CsA and enterohepatic recirculation suppression by AC. The modeling was carried out by population approach and based on simultaneous analysis of data from p.o. and i.v. administration. Additionally, to facilitate comparisons and include the metabolite data, parameters such as C_{max}, T_{max}, and AUC for ENR and ciprofloxacin were derived from a classical non-compartmental analysis.

Intense sampling was performed during the first 2 h after the treatment with ENR to obtain as much information as possible for the changes in the disposition of the antibacterial drug. Published data for AUC of 15.2 ± 1.08 μg × h/mL for ENR after i.v. administration at a dose of 10 mg/kg are similar to our findings (Atef et al., 2020). The estimated population pharmacokinetic parameters were in alignment with the published data. Total body clearance of 618 mL/h/kg found by Knoll et al. (1999) was close to the typical (population) value of Cl (**tvCl**) in the current investigation. The true values of volume of distribution in the central and in the peripheral compartments are in agreement with the values estimated in the previous investigations and confirmed significant distribution of ENR in the body (Knoll et al., 1999; Temmerman et al., 2021).

The observed lower values of T_{max} in our study in comparison to the published T_{max} after oral administration of the same dose of ENR can be explained by the applied sampling schedule which allowed registering the earlier time of C_{max} (Xiao et al., 2018). Other authors reported earlier achievement of maximum plasma concentrations in 8-wk-old broilers, treated with 10 mg/kg ENR (Guo et al., 2013). These differences may be associated with different age of broilers used in the experiments, as it is known that age has a significant impact on the kinetics of drug absorption in poultry (Vermeulen & Remon, 2001; Poźniak et al., 2017). The inclusion of a lag time in the population model improved the fit of the early concentrations. As the oral formulation did not require dissolution, this parameter represents the time needed for the drug to pass the crop and the stomach in order to reach the absorption site in the intestine (3.24 min on average). Pharmacokinetic parameters C_{max} and $AUC_{0\text{-}24h}$ for the parent compound were similar to the published values of 2.86 ± 0.52 μg/mL and 30.07 ± 4.64 μg/h/mL, respectively (Xiao et al., 2018). The values of AUC in the cited study were slightly lower because they were calculated for the time interval of 0 to 24 h and not from time 0 to infinity. Population values of AUC of ENR were very close to the calculated values by non-compartmental analysis. High bioavailability calculated by population approach is typical for ENR and it was in agreement with the reported values of 77 to 89% in other studies (Knoll et al., 1999; Atef et al., 2020). Similar values of C_{max} were found for the main metabolite ciprofloxacin after oral administration of ENR at a dose of 20 mg/kg in broiler chickens (Xiao et al., 2018). Higher T_{max} value for ciprofloxacin is a consequence of the reported profile for ENR. The developed population pharmacokinetic model revealed the significant effect of the bodyweight on the volume of distribution in the central compartment and on the systemic clearance. This finding confirmed the described

Table 1. Pharmacokinetic parameters of enrofloxacin and its metabolite ciprofloxacin in broilers (geometric mean and range of minimum and maximum) computed with non-compartmental analysis. Enrofloxacin was administered intravenously (i.v., 10 mg/kg) or orally (p.o., 20 mg/kg) in healthy broiler chickens (n = 6 at every sampling time). Broilers were treated solely with enrofloxacin i.v. or p. o. ($n_{i.v.}$ = 12 and $n_{p.o.}$ = 12), with combination enrofloxacin (i.v., $n_{i.v.}$ = 12 or p.o. $n_{p.o.}$ = 12)-cyclosporine A (p.o., 50 mg/kg) and with combination enrofloxacin (i.v., $n_{i.v.}$ = 12)-activated charcoal, p.o. (1 g/kg).

Parameters	Units	Enrofloxacin	Enrofloxacin + cyclosporine A	Enrofloxacin + activated charcoal
		Intravenous administration at a dose of 10 mg/kg		
AUC	μg/h/mL	17.07 (11.42–21.78)[a]	18.65 (14.90–24.78)[a]	14.92 (10.57–24.30)[b]
		Oral administration at a dose of 20 mg/kg		
T_{max}	h	1.12 (0.50–1.50)	1.35 (1.00–1.75)	-
C_{max}	μg/mL	4.63 (2.42–8.80)	5.62 (3.87–7.68)	-
AUC	μg/h/mL	33.47 (21.09–49.18)[a]	40.37 (31.27–55.31)[b]	-
		Parameters for ciprofloxacin, the main metabolite of enrofloxacin		
		Intravenous administration of enrofloxacin at a dose of 10 mg/kg		
T_{max}	h	1.88 (1.25–6.00)	2.35 (1.75–9.00)	2.40 (0.5–9.00)
C_{max}	μg/mL	0.10 (0.06–0.20)[a]	0.12 (0.07–0.17)[a]	0.07 (0.04–0.14)[b]
AUC	μg/h/mL	1.17 (0.76–1.80)[a]	1.38 (0.90–1.76)[a]	0.75 (0.51–0.99)[b]
		Oral administration of enrofloxacin at a dose of 20 mg/kg		
T_{max}	h	1.01 (0.50–1.50)	1.30 (1.00–1.75)	-
C_{max}	μg/mL	0.30 (0.10–0.61)	0.42 (0.32–0.53)	-
AUC	μg/h/mL	1.99 (1.06–3.62)	2.53 (1.89–3.95)	

AUC – area under the curve, C_{max} – maximum concentration, T_{max} – time when C_{max} was achieved.
[ab]Values with different letters in a row differ significantly, $P < 0.05$.

allometric dependencies of systemic clearance and volume of distribution on the body weight of poultry in previously conducted experiments (Poźniak et al., 2020a).

The available literature reveals that secretion through intestinal wall is one of the mechanisms of excretion of fluoroquinolones and particularly ENR, regardless of the route of administration (Ferguson et al., 2018; De Smet et al., 2020; Erwin et al., 2020). Based on the reviewed literature (Sárközy, 2001; Alvarez et al., 2008; Ferguson et al., 2018; Erwin et al., 2020; Hernández-Lozano et al., 2021a b), AC was chosen as universal adsorbent in order to evaluate the impact of enterohepatic recirculation on the plasma levels of intravenously administered ENR. The tendency for lower AUC of ENR and significantly decreased values of C_{max} and AUC of ciprofloxacin suggest the involvement of enterohepatic recirculation (and probably intestinal secretion) in the elimination of these fluoroquinolones. The differences between ENR and ciprofloxacin secretion were explained in earlier studies with concentration-

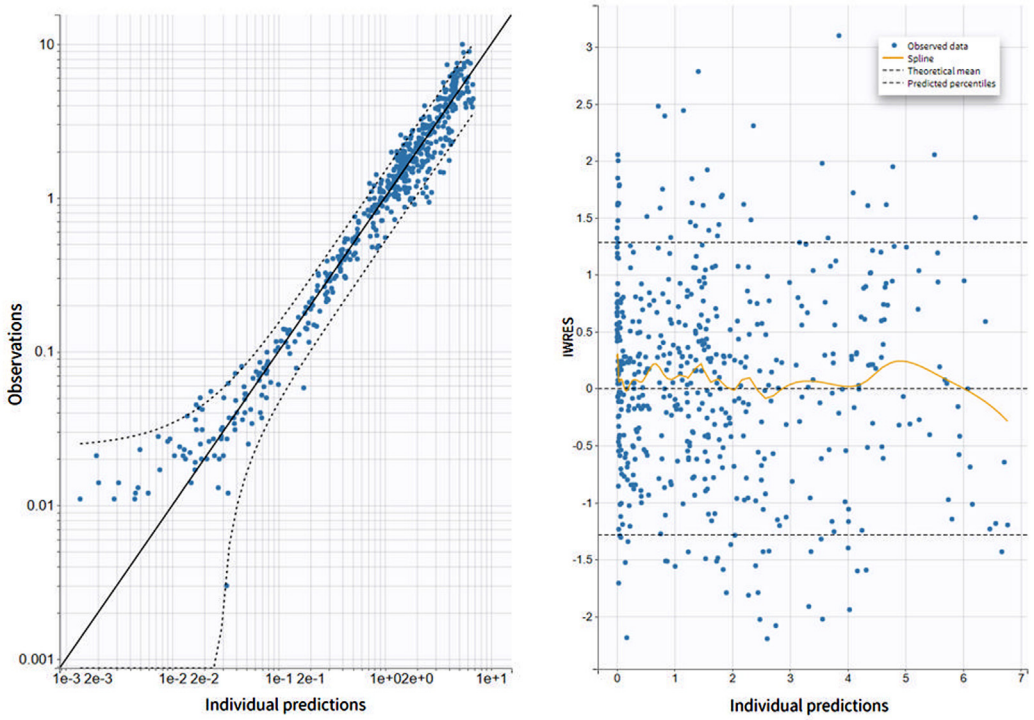

Figure 3. Left panel: plot of individual predictions vs. observations. Blue dots show observations, black line – identity line; dotted black lines presents 90% prediction interval and yellow line – spline. Outliers proportion was 7.17%. Right panel: scatter plot of individual weighted residuals versus individual predictions.

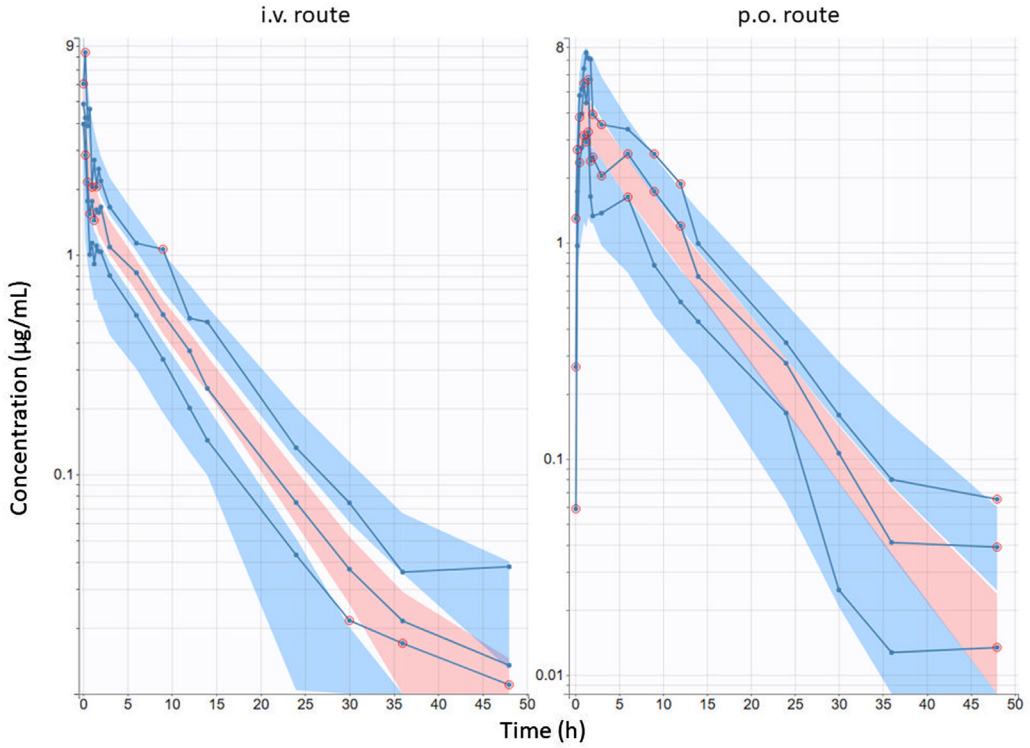

Figure 4. Visual predictive checks for enrofloxacin concentrations after i.v. and p.o. administration. The shaded areas denote the 90% confidence interval for the prediction of the median (pink) as well as the 10th and 90th percentile (blue areas). The solid lines denote the empirical data: the median as well as the 10th and 90th percentile. Red circles denote the outliers.

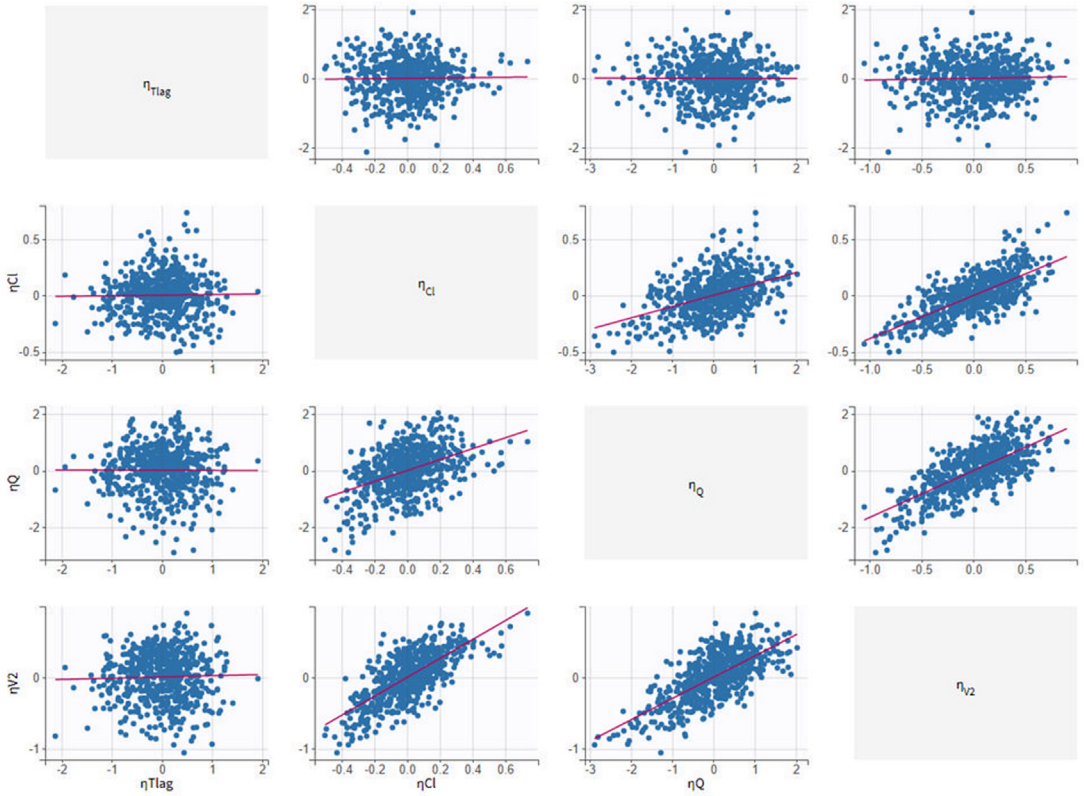

Figure 5. Correlation plot matrix of the random effects (η_i). Correlation was applied when correlation coefficients were estimated to be high and met the threshold for inclusion (Pearson's correlation test, $P < 0.05$). Therefore, they were included in the final statistical model choice. Red lines are simple linear regressions.

Table 2. Population parameters of enrofloxacin administered intravenously (i.v., 10 mg/kg) or orally (p.o., 20 mg/kg) in healthy broiler chickens (n = 6 at every sampling time). Broilers were treated solely with enrofloxacin i.v. or p.o. ($n_{i.v.}$= 12 and $n_{p.o.}$= 12), with combination enrofloxacin (i.v., $n_{i.v.}$= 12 or p.o., $n_{p.o.}$= 12)-cyclosporine A (*po*, 50 mg/kg) and with combination enrofloxacin (i.v., $n_{i.v.}$= 12)-activated charcoal, p.o. (1 g/kg).

Parameters		Units	Fixed effects	RSE (%)	Omega	RSE(%)	Shrinkage (%)
Thetas (typical value)	tvF	-	1.00	0.074	-	-	-
	tvTlag	h	0.054	19.2	0.57	24.9	1.87
	tvka	1/h	1.26	10.4	-	-	-
	tvCl	L/h	0.829	2.90	0.18	12.2	-5.47
	$\beta_{Cl\ co\text{-}adm.ACh\ .}$	-	0.204	28.6	-	-	-
	$\beta_{Cl\ BW}$	-	0.77	23.3	-	-	-
	tvV_1	L	2.11	6.52	-	-	-
	$\beta_{V1\ BW}$	-	2.11	19.5	-	-	-
	tvQ	L/h	3.82	13.4	0.77	12.6	-7.9
	tvV_2	L	4.01	5.82	0.332	15.1	-5.11
Residual error							
	a	-	0.014	15.98			
	b	-	0.29	4.41			

Additional pharmacokinetic parameters	Units	Geometricmean	Min	Max
AUCi.v. – control	μg*h/mL	15.51	12.23	17.88
AUCi.v. – activated charcoal	μg*h/mL	13.29	10.74	18.75
AUCi.v. – cyclosporine A	μg*h/mL	16.23	13.20	19.78
AUCp.o. – control	μg*h/mL	31.20	10.49	41.20
AUCp.o. – cyclosporine A	μg*h/mL	37.43	31.76	45.26

Typical value (tv) of ka - absorption rate constant.

Abbreviations: AUC, area under the curve for intravenous (i.v.) and oral (p.o.) administration; Cl, total body clearance; F, bioavailability; Q, intercompartmental clearance; RSE, residual standard error; Tlag, lag time; V_1, volume of distribution in the central compartment; V_2, volume of distribution in the peripheral compartment.

dependent active transport and biliary excretion (Ferguson et al., 2018). Additionally, lower plasma concentrations of ENR and ciprofloxacin after the sixth hour from an i.v. injection of the parent compound support the role of AC in restriction of their re-absorption. Furthermore, the population model revealed the significant effect of co-administration of AC on the systemic clearance of ENR which supported the considerable role of intestinal secretion for its elimination in broilers. However, the small additional peaks often appearing shortly after completion of the rapid distribution phase were not eliminated by the AC administration suggesting that these peaks are not related to enterohepatic recirculation. The quantitative assessment of the differences in the profiles suggests that enterohepatic recirculation may not play a significant role in the clinical use of ENR in broilers. Since in some studies on fluoroquinolones a major impact of this recirculation has been revealed (Stass et al., 2005), it seems likely that the actual relevance of this mechanism is both drug- and species-dependent.

As a further step, we selected CsA as broad spectrum inhibitor of the function of ABC efflux transporter proteins such as P-gp and BCRP to evaluate their effect on ENR pharmacokinetics (Mealey, 2012; Bakhsheshian et al., 2013; Dantzic et al., 2018; Dei et al., 2019; Anonymous, 2022). The current in vivo pharmacokinetic study in broilers has shown that CsA did not significantly affect the plasma levels of i.v. administered ENR. The applied population pharmacokinetic model confirmed absence of significant effect of co-administration of CsA on primary parameters such as volume of distribution and clearance. Although CsA can inhibit glomerular filtration rate and thus can lead to drug-drug interactions (Laskow et al., 1990), the results from the current experiment did not reveal a significant effect of CsA on ENR systemic clearance. CsA co-administration made more obvious the secondary peak in plasma concentrations of ENR between 1.5 and 2 h after its intravenous administration. These changes can be attributed to inhibitory activity on efflux transporters P-gp and BCRP (Qadir et al., 2005). These findings are in line with in vitro experiments with cell lines. There is evidence that CsA diminished secretory-directed transport of [14C]sparfloxacin and [14C]levofloxacin in Caco-2 cells by mediation of P-gp (Naruhashi et al., 2001). However, saturation of drug transporters cannot be excluded as this could decrease the influence of this transporter modulation on pharmacokinetics of the drugs (Haritova et al., 2007). Other mechanisms also contribute to fluoroquinolone elimination and, therefore, selective inhibition of efflux transporters P-gp and BCRP may not be sufficient to elicit a significant impact on ENR disposition after its i.v. administration (Guo et al., 2013). The values of C_{max} and AUC of the main metabolite in our experiment were not affected by p.o. CsA administration which confirms the lack of effect of this inhibitor on the metabolism of ENR to ciprofloxacin.

The results of oral co-administration of ENR and CsA show a clear wide second peak in plasma concentrations. This difference in the profiles contributes to the higher values of AUC in comparison to broilers that received only the fluoroquinolone drug. These findings are consistent with the results published by Guo et al. (2014) with regard to the increased values of $AUC_{0\text{-}12h}$ and C_{max}

when ENR was co-administered with verapamil, another strong P-gp inhibitor. The changes were explained by the inhibition of P-gp by verapamil which leads to a significant impact on the absorption of ENR (Guo et al., 2014). The same group found that the combination of ENR and verapamil caused significant changes in the pharmacokinetics of orally administered fluoroquinolone drug in healthy broilers at different age (Guo et al., 2013). Verapamil significantly increased the $AUC_{0-\infty}$ of ENR in broilers and oral bioavailability of the antibacterial drug was nearly doubled (Guo et al., 2013). The increase of AUC and bioavailability was logically explained by the cited authors with an effect of co-administration of P-gp inhibitor verapamil, however, they suggested lower role of biliary excretion through inhibition of P-gp in the liver. Altogether, the literature data and our results show that high exposure of intestinal ABC efflux transporters to orally administered inhibitors like CsA cause larger impact on oral pharmacokinetics of ENR than on the disposition after intravenous dosage. This suggests that in chickens the transporter role may be more important in the absorption of enrofloxacin rather than in the distribution and excretion of this drug. Similar findings were published for other fluoroquinolones. Through CsA-mediated inhibition of efflux transporters (including P-gp), it was proven that in vivo they function not only to transport grepafloxacin from blood to intestine but also to limit its intestinal absorption (Naruhashi et al., 2001). Similar effect was described for other compounds. CsA affected atorvastatin pharmacokinetics to a larger extent following oral rather than intravenous administration. This effect was mediated by P-gp/BCRP/MRP2-related efflux in addition to CYP3A-mediated metabolism (Yang et al., 2020).

There are also some limitations of the present study and all others that apply similar inhibitors as the role of other transporters, like OATP1A5, in ENR pharmacokinetics cannot easily be distinguished (Arakawa et al., 2012). There is no information about the function of OATP and its substrates in poultry and further investigations are necessary to clarify this role. Another limitation is related to the lack of organ specificity of the inhibition. Although CsA has quite low bioavailability (Bertault-Pérès et al., 1985), it is possible that it also impaired the function of efflux transporters in excretory organs such as liver and kidneys, and its impact on fluoroquinolone pharmacokinetics may be complex. What should be noted, however, is the fact that the population model did not reveal any significant effect of CsA co-administration.

In conclusion, the applied population pharmacokinetic model adequately describes pharmacokinetics of ENR after i.v. and p.o. administration in poultry. It was able to discover significant effect of the body weight on primary parameters such as volume of distribution in the central compartment and systemic clearance. The model allowed explaining the moderate but still significant effect of AC on the clearance of ENR which can be attributed to the role of intestinal efflux of the fluoroquinolone drug and enterohepatic recirculation. The results reveal only slight impact of the multispecific ABC transporter inhibitor CsA on the pharmacokinetics of intravenously administered ENR which was demonstrated by the obvious second peak in ENR plasma concentrations. The effects of CsA on ENR pharmacokinetics were more significant in case of oral administration of the antimicrobial. These effects can be attributed to the inhibition of intestinal efflux transporters which in turn modify the absorption and secretion of ENR through the intestinal wall. CsA did not cause changes in the metabolism of ENR to ciprofloxacin in broilers. Altogether, the data from the current investigation suggest a role of ABC efflux transporters in the pharmacokinetics of drugs in poultry. However, the clinical role of these interactions needs to be assessed on a drug-to-drug basis and more knowledge on the physiology of transporters involved in drug disposition in poultry is needed.

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DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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