



Pharmacokinetic evaluation of roxithromycin and ciprofloxacin in treating complicated avian mycoplasmosis in broiler chickens

R D SINGH¹✉, S K MODY¹, H B PATEL¹, V N SARVAIYA¹ and B R PATEL²

Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar, Gujarat 385 506 India

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ABSTRACT

The use of roxithromycin along with ciprofloxacin is having potential to be a promising antimicrobial therapy to treat complicated avian mycoplasmosis in broiler chickens. The present research was undertaken to study the influence of roxithromycin (20 mg/kg body weight) and ciprofloxacin (10 mg/kg body weight) on the oral pharmacokinetics of each other, when both drugs are concomitantly administered in eight healthy male broiler chickens (n=8) and to establish their therapeutic dosage regimens. Their plasma concentrations were assayed by validated ultra high performance liquid chromatography (UHPLC) methods using UV detector. Oral pharmacokinetic parameters were calculated from plasma concentration versus time data based on non-compartmental analysis. Statistically, plasma roxithromycin concentration was significantly higher at one time point only (0.5 h) and plasma ciprofloxacin concentration was significantly lower at the time point of 2 h only when used in combination, in comparison to their respective values obtained after their alone administrations. The pharmacokinetic parameters of roxithromycin and ciprofloxacin showed no significant effect on values of either drug when given in combination and there was a lack of pharmacokinetic interaction between the two antimicrobials. The predicted effective oral dose rate of roxithromycin was 20 mg/kg body weight, every 12 h, and that of ciprofloxacin was 10 mg/kg body weight, every 24 h, to treat complicated avian mycoplasmosis in broiler chickens.

Keywords: Broiler chickens, Ciprofloxacin, Complicated avian mycoplasmosis, Pharmacokinetic interaction, Roxithromycin

India ranks fourth in global broiler meat production after China, Brazil and the United States of America (USDA, 2018). Total broiler market size in India was estimated to be 4.2 million tonnes (carcass weight) during 2017. National Action Plan for “Egg and Poultry-2022 for doubling farmers’ income by 2022” envisages annual target production of 6.2 million tonnes of poultry meat by the year 2022–23 (DAHD, 2017). One of the major challenges posed against achieving this target is outbreak of economically devastating infectious diseases including avian mycoplasmosis (or chronic respiratory disease), caused by *Mycoplasma gallisepticum*, which generally progresses into Complicated Chronic Respiratory Disease (CCRD) due to secondary involvement of *Escherichia coli* (Ley 2003). The average mortality rate observed in the outbreaks of a single infection of *M. gallisepticum* was 7–8%, whereas *E. coli* complicated CRD outbreaks recorded almost double mortality rates. It was reported that

M. gallisepticum increases dissemination of *E. coli*, which conversely increases invasiveness of *M. gallisepticum* towards lower respiratory tract (Sivaseelan and Balasubramaniam 2013, Sivaseelan *et al.* 2013).

Macrolide antibiotics and their semi-synthetic derivatives are the most commonly used antimicrobial agents in poultry to treat avian mycoplasmosis (Landoni and Albarellos 2015). Roxithromycin is 14-C ring macrolide semi-synthetic drug derived from erythromycin, of which 9-keto group was replaced by an etheroxime side chain to prevent gastric inactivation. It is an orally administered long-acting antibacterial drug of macrolide class which shows bactericidal action at high concentrations (Gaynor and Mankin 2003). The pharmacokinetic (PK) profile of roxithromycin is characterized by high plasma and intracellular concentration, excellent tissue and body fluid penetration and a long half-life permitting an extended dosage interval (Markham and Faulds 1994). Roxithromycin has good activity against *Mycoplasma* spp. but lacks activity against *Escherichia coli*; contrary to this, ciprofloxacin has an excellent activity against *E. coli*. Ciprofloxacin, a second generation fluoroquinolone drug, is one of the low cost antimicrobial drugs, having great efficacy against aerobic Gram-negative bacteria especially the *Enterobacteriaceae*. It has lower minimal inhibitory

Present address: ¹Department of Veterinary Science and Animal Husbandry, College of Veterinary Science and Animal Husbandry, Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar, Gujarat. ²R&D Centre, Torrent Pharmaceuticals, Ahmedabad, Gujarat. ✉Corresponding author email: ratn1709@yahoo.com

concentration (MIC) values against *E. coli* as compared to other commonly used fluoroquinolones (Wimer *et al.* 1998, Papich 2018).

Thus, the combination of roxithromycin and ciprofloxacin may prove to be a promising antimicrobial therapy to treat complicated avian mycoplasmosis in broiler chickens by oral route of administration. Combination therapy using two antimicrobials is advocated in certain cases of mixed bacterial infections in which the pathogens are of diverse nature and are not showing susceptibility to a single agent (Boothe 2016). The proposed combination can be put forward for clinical application only after having the knowledge of interaction pharmacokinetics of two antimicrobials in combination. Thus, the present investigation was meant to study the pharmacokinetics (PK) of both roxithromycin (20 mg/kg body weight) and ciprofloxacin (10 mg/kg body weight) following their concomitant single dose oral administration in broiler chickens.

MATERIALS AND METHODS

Experimental birds: Twenty four male broiler chickens aging from 4 to 6 weeks and, weighing between 1.4 to 2.3 kg, procured from Department of Livestock Production and Management (LPM), College of Veterinary Science and Animal Husbandry, Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar, Gujarat, India, were kept under observation and acclimatized period for 14 days before the start of the experiment. Birds were divided into 3 groups on random basis and each group comprised of 8 birds. Group I and II birds were utilized for studying oral PK of roxithromycin and ciprofloxacin, respectively, when administered alone. Group III birds were used to investigate oral PK of both roxithromycin and ciprofloxacin following their concomitant oral administration. Feed was withheld for 12 h period prior to oral administration of the drugs for pharmacokinetic study. Approval of study protocol was taken from the Institutional Animal Ethics Committee prior to experimentation (Approval No. VetColl/IAEC/2016/11/Protocol-03).

Dosing and sample collection: Roxithromycin and ciprofloxacin hydrochloride of I.P. grade powders were obtained as a gift sample from Shantam Pharmaceuticals Pvt. Ltd., Gandhinagar, Gujarat. Fine powders of roxithromycin and ciprofloxacin hydrochloride were mixed with sterile water as a vehicle, to make the final concentration of 20 mg/ml for both the drugs for oral dosing. For PK study of roxithromycin and ciprofloxacin alone, about 0.8–1.0 ml blood was collected, whereas for combination PK study up to 1.5 ml blood was collected since analysis of two drugs in same samples required more plasma volume. Blood samples were collected in heparinized test tubes with the help of intravenous catheter (22 G, BD Venflon™) placed in the contralateral wing vein at 0 min (pre-administration), 5 (0.083 h), 15 (0.25 h) and 30 (0.5 h) min. Remaining blood samples were collected at 1, 2, 4, 8, 12, 24, 48 and 72 h using 23 G needle

(BD Precision Glide™). The harvested plasma samples were transferred to cryovials and stored at –20°C until assayed for the respective drug (roxithromycin or ciprofloxacin or both) concentrations.

UHPLC instrumentation and assay methods: The plasma concentrations of roxithromycin and ciprofloxacin were assayed by validated ultra high performance liquid chromatography (UHPLC) methods using ultraviolet (UV) detector. UHPLC apparatus (Dionex ultimate 3000®, Thermo Fisher, Germany) was comprised of ultraviolet (UV) detector (VWD-3100), gradient solvent delivery pump (LPG-3400SD) and manual injector. Data integration was performed by Chromeleon™ software (version 6.8).

Chromatographic separation of roxithromycin was performed at effluent monitoring wavelength of 210 nm using reverse phase C18 column (ODS, 25 cm × 4.6 mm ID, 4.5 µ; Purospher® Star RP-18, Merck-Millipore, Mumbai) as per method described by Singh *et al.* (2019). Mobile phase was composed of trifluoroacetic acid (0.1% TFA) and acetonitrile in the ratio of 55:45 and flow rate kept was 1 ml/min in isocratic mode. Retention times of roxithromycin and internal standard (erythromycin) were 7.1 and 4.0 min, respectively. Lower limit of detection (LOD) and limit of quantification (LOQ) of the present analytical method for roxithromycin were calculated as 0.131 and 0.398 µg/ml, respectively.

Liquid-liquid extraction using ice-cold acetonitrile was performed to extract roxithromycin from plasma samples. In brief, exactly 400 µL of plasma sample was taken into 2 ml Eppendorf® micro-centrifuge tubes, and then 40 µL of 1 M NaOH was added to it and vortexed for 10 seconds. After this alkalization, 1,200 µL ice-cold acetonitrile was added and vortexed for 3 min. Then the mixture was centrifuged at 5,000 RPM for 10 min at 4°C. Upper organic phase was transferred into evaporation vials and were completely dried under nitrogen sample evaporator using N₂ gas. Dried residues were finally reconstituted with 100 µL diluent (200 mg/ml erythromycin as an internal standard in mobile phase). The prepared sample was finally centrifuged (5,000 rpm, 5 min, 4°C) and 20 µL of the upper clear portion was manually injected into UHPLC.

Analysis of ciprofloxacin was done as per method used by Vella *et al.* (2015) with some minor modifications for broiler plasma. For ciprofloxacin analysis, a short length reverse phase C-18 column (100×4.6 mm; Chromolith® RP-18) was used. For extraction of ciprofloxacin, plasma sample (300 µL) was taken into 2 ml Eppendorf® micro-centrifuge tubes, and 20 µL strong 0.1 M phosphate buffer (pH 2.7) was added to it along with 450 µL ice-cold acetonitrile and vortexed for 3 min. Then the mixture was centrifuged at 10,000 rpm for 10 min at 4°C. Upper organic phase was transferred into evaporation vials and completely dried using N₂ evaporator. After drying, residues were reconstituted with 150 µL diluent (40 mg/ml moxifloxacin as an internal standard in mobile phase). The prepared sample was vortexed for 90 sec, and then again centrifuged at 10,000 rpm for 5 min at 4°C, and finally 20 µL clear

supernatant was injected manually into UHPLC. Flow rate of mobile phase was kept 0.5 ml/min in isocratic mode and was consisted of aqueous phosphate buffer and an organic solvent (acetonitrile) in the ratio of 80:20. Retention time of ciprofloxacin was 4.1 min whereas that of internal standard (moxifloxacin) was 7.9 min. LOD and LOQ of ciprofloxacin assay for the present validated method were calculated as 0.101 and 0.307 µg/ml, respectively.

Pharmacokinetic and statistical analysis: Non-compartmental method was used to compute PK parameters without fitting data in any compartmental model using the 'PK Solver 2.0' software (Zhang *et al.* 2010). The PK parameters for roxithromycin and ciprofloxacin derived after analysis for 'alone administration' and 'when given in combination' were statistically compared for their mean values by 'paired sample t-test' using SPSS software (version 19).

RESULTS AND DISCUSSION

Plasma disposition: Comparison of semi-logarithmic plots of plasma roxithromycin concentrations versus time following single dose oral administration of roxithromycin given alone and in combination of ciprofloxacin in broiler chickens (n=8) is shown in Fig. 1. Similar comparison for the effect of combination on plasma ciprofloxacin concentrations is shown in Fig. 2. Following concomitant administration of single oral doses of roxithromycin (20 mg/kg body weight) and ciprofloxacin (10 mg/kg body

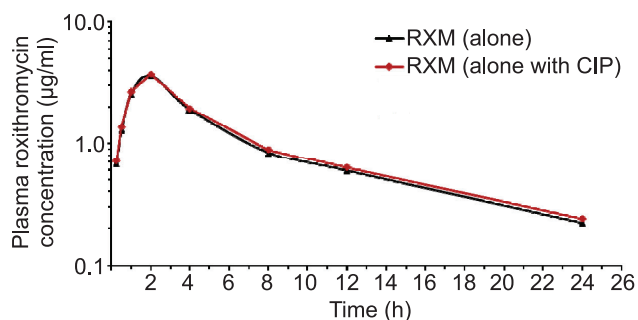


Fig. 1. Comparison of semi-logarithmic plots of plasma roxithromycin (RXM) concentrations versus time following its oral administration when given alone and along with ciprofloxacin (CIP) in broiler chickens (n=8).

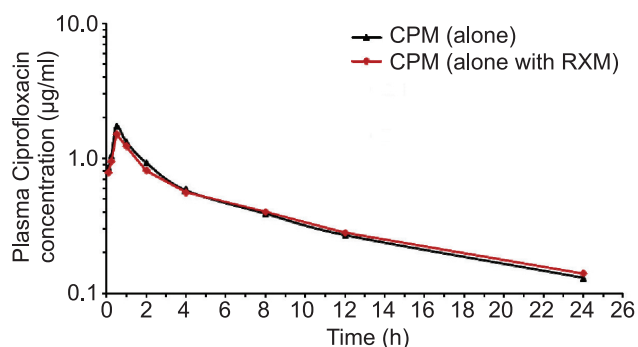


Fig. 2. Comparison of semi-logarithmic plots of plasma ciprofloxacin (CIP) concentrations versus time following its oral administration when given alone and along with roxithromycin (RXM) in broiler chickens (n=8).

weight) in broiler chickens, mean peak plasma concentrations (C_{max}) of roxithromycin and ciprofloxacin were estimated at 3.70 and 1.51 µg/ml, respectively. Similar mean C_{max} values were recorded as 3.60 and 1.75 µg/ml, respectively, when roxithromycin and ciprofloxacin were administered alone. Plasma roxithromycin concentrations were found consistently higher, but statistically non-significant (except for 0.5 h), in samples when roxithromycin was given in combination with ciprofloxacin, in comparison to alone administration of the roxithromycin. Thus, in the present study plasma concentrations of roxithromycin (macrolide drug) were increased when ciprofloxacin (fluoroquinolone drug) was administered concomitantly. Similar beneficial interaction for co-transport of macrolide and fluoroquinolone was observed *in vitro* by the mechanism of reversing P-glycoprotein efflux. Fluoroquinolones like grepafloxacin, levofloxacin and sparfloxacin significantly inhibited the P-glycoprotein mediated efflux of erythromycin (Sikri *et al.* 2004). Such interactions can modulate oral absorption and disposition of macrolide drugs when administered concomitantly with a fluoroquinolone drug.

A short lag phase of 15 min was observed in oral absorption of roxithromycin whether administered alone or along with ciprofloxacin. Kwon (2017) too reported a lag phase time of 0.25 h in the absorption of roxithromycin in humans. The lag phase of 30 min in oral absorption was also observed for clarithromycin in broiler chickens (Awadallah *et al.* 2016). Generally, delay in drug absorption is attributed to poor drug dissolution from the dosage form or incomplete wetting of drug particles owing to the hydrophobic nature of the drug (Jambhekar and Breen, 2012). Short lag phase observed in the present study appears due to the large size along with hydrophobicity of the roxithromycin molecules.

Following concurrent oral administration of both the drugs, the mean peak plasma concentrations (C_{max}) of roxithromycin (3.70 µg/ml) and ciprofloxacin (1.51 µg/ml) were achieved at 2 and 0.5 h, respectively. In fasted dogs, comparatively, lower C_{max} value (1.89 µg/ml) was achieved at 1.35 h when roxithromycin was administered orally at the dose rate of 22.5 mg/kg body weight (Lavy *et al.* 1995), whereas Lim *et al.* (2006) reported similar C_{max} value (3.34 µg/ml) attained at 1.12 h (T_{max}) in dogs after single oral administration of roxithromycin (20 mg/kg). For ciprofloxacin, higher C_{max} values in the range of 2.63–4.67 µg/ml were reported in broiler chickens by other researchers (Atta and Sharif 1997, Anadon *et al.* 2001, Ivanova *et al.* 2017). Time variation to reach peak concentration was evident in the present and other studies; this may be due to individual variations in the absorption pattern of drug, variation in samples collection time, presence and nature of gastric content and variable nature of drug formulation used in different experiments.

In the present study, drug concentrations declined to 0.63 and 0.28 µg/ml at 12 h for roxithromycin and ciprofloxacin, respectively. Post 24 h oral administration, mean plasma

concentrations were found to be 0.24 and 0.14 µg/ml respectively for roxithromycin and ciprofloxacin. None of the broiler chickens exhibited any detectable concentration of either roxithromycin or ciprofloxacin in plasma obtained from the blood samples collected at 48 and 72 h.

Pharmacokinetic parameters: Following oral administration of roxithromycin alone in broiler chickens, the mean value of elimination rate constant was 0.08 per hour and corresponding values of elimination half-life ranged from 7.21 to 9.23 h with a mean value of 8.30 h. Wide variations in elimination half-life of different macrolides is reported in broiler chickens, viz. 4.1 h for erythromycin (Goudah *et al.* 2004), 2.11 h for clarithromycin (Awadallah *et al.* 2016) and 31.5 h for azithromycin (Abo-El-Sooud *et al.* 2012) in comparison to 8.30 h for roxithromycin in present study. Thus, in broiler chickens roxithromycin showed longer elimination than erythromycin and clarithromycin but shorter than azithromycin. The roxithromycin drug molecules stayed for substantial time period in the body of broiler chickens following oral dosing as indicated by high mean resident time (MRT) observed in the present study which ranged from 8.60 to 10.63 h with a mean value of 9.57 h. The average apparent volume of distribution ($V_{d(are)}$) following alone oral administration of roxithromycin was found to be 9.39 L/kg; such higher $V_{d(are)}$ after oral administration reflecting high tissue penetration of the drug which is a basic requirement to treat poultry respiratory infections like *Mycoplasma gallisepticum*.

The value of elimination half-life ($t_{1/2\beta}$) obtained following alone oral administration of ciprofloxacin ranged from 9.34 to 11.91 h with a mean value of 10.04 h. Such long elimination half lives in poultry were also reported earlier as 11.89 h (Anadon *et al.* 2001) and 7.89 h (Ivanova *et al.* 2017). The average value of mean residence time (MRT) was 12.30 h (almost half a day) indicating that average stay of drug molecules in the broilers' body, after oral administration of ciprofloxacin, is note-worthy. The mean value of apparent volume of distribution ($V_{d(are)}$) was calculated to be 12.72 L/kg. In comparison to present study, lower mean value of $V_{d(are)}$ (7.78 L/kg) was derived in broiler chickens administered with lower dose of ciprofloxacin, i.e. 8 mg/kg body weight (Anadon *et al.* 2001). In addition to dose variation, breed/strain difference and difference in plasma protein binding capacity, hydration status of individuals may also influence the volume of distribution of a drug. Since most of the studies are performed on fasted birds with *ad lib.* access to drinking water, there is every possibility of variable water intake by the experimental birds.

Single dose pharmacokinetics of roxithromycin following its oral administration in broiler chickens was compared statistically between two groups, i.e. administered alone and along with ciprofloxacin (Table 1). Similarly, comparison of pharmacokinetics of ciprofloxacin administered alone and along with roxithromycin in broiler chickens is also presented (Table 2). Statistically, no

Table 1. Comparison of pharmacokinetic parameters of roxithromycin following its alone oral administration (20 mg/kg body weight) and along with ciprofloxacin in broiler chickens (n=8)

Pharmacokinetic Unit parameters		Values (Mean±SE)	
		Roxithromycin alone	Roxithromycin plus Ciprofloxacin
C_{max}	mg/ml	3.60±0.09	3.70±0.09
T_{max}	h	2.00±0.00	2.00±0.00
β	Per h	0.08±0.00	0.08±0.00
$t_{1/2\beta}$	h	8.30±0.25	8.49±0.30
AUC	mg·h/ml	25.59±0.85	26.91±0.72
AUMC	mg·h ² /ml	245.91±12.98	266.19±12.01
MRT	h	9.57±0.24	9.88±0.30
$V_{d(are)}/F$	L/kg	9.39±0.24	9.13±0.37
Cl_B/F	L/h/kg	0.79±0.03	0.75±0.02

No pharmacokinetic parameter showed statistical difference at $P \leq 0.05$ or $P \leq 0.01$. C_{max} , Observed peak plasma concentration; T_{max} , Time at which C_{max} was observed; β , Elimination rate constant; $t_{1/2\beta}$, Elimination half-life; $AUC_{0-\infty}$, Area under curve; AUMC, Area under first moment of the plasma drug concentration; MRT, Mean resident time; $V_{d(are)}$, Apparent volume of distribution; Cl_B , Total body clearance; F, oral bioavailability.

Table 2. Comparison of pharmacokinetic parameters of ciprofloxacin following its alone oral administration (10 mg/kg body weight) and along with roxithromycin in broiler chickens (n=8)

Pharmacokinetic Unit parameters		Values (Mean±SE)	
		Ciprofloxacin alone	Roxithromycin plus Ciprofloxacin
C_{max}	mg/ml	1.85±0.08	1.63±0.07
T_{max}	h	0.63±0.08	0.63±0.08
b	Per h	0.07±0.00	0.07±0.00
$t_{1/2b}$	h	10.04±0.32	10.40±0.48
AUC	mg·h/ml	11.52±0.52	11.49±0.25
AUMC	mg·h ² /ml	141.57±7.14	152.73±8.48
MRT	h	12.30±0.35	13.28±0.58
$V_{d(are)}/F$	L/kg	12.72±0.59	13.09±0.59
Cl_B/F	L/h/kg	0.88±0.04	0.87±0.02

No pharmacokinetic parameter showed statistical difference at $P \leq 0.05$ or $P \leq 0.01$. (C_{max} , Observed peak plasma concentration; T_{max} , Time at which C_{max} was observed; β , Elimination rate constant; $t_{1/2\beta}$, Elimination half-life; $AUC_{0-\infty}$, Area under curve; AUMC, Area under first moment of the plasma drug concentration; MRT, Mean Resident Time; $V_{d(are)}$, Apparent volume of distribution; Cl_B , Total body clearance; F, oral bioavailability.

significant difference ($P \leq 0.05$ or $P \leq 0.01$) was observed for mean values of any of the computed pharmacokinetic parameters for either roxithromycin or ciprofloxacin when compared with those obtained after oral co-administration of both the drugs. Since, roxithromycin, a weak basic drug, mainly binds to α -1-glycoprotein whereas ciprofloxacin

being a weak acidic drug, principally bounds to albumin fraction of plasma proteins, there are fewer chances of dispositional interferences for the both drugs.

Therapeutic dosage regimen: Roxithromycin, a macrolide drug, is having time-dependent activity, for which the best PK-PD integration indices to predict efficacy is 'time above the MIC ($T > MIC$)' and optimal target value of $T > MIC$ should be 50–80% of the dosage interval (Andes 2001, Toutain *et al.* 2002). The time for which the plasma drug concentrations remain above or equal to MIC value ($T > MIC$) was calculated using the following formula (Turnidge 1998).

$$\% T > MIC = \ln \frac{D}{V_{d(\text{area})} \times MIC} \times \frac{t_{1/2\beta}}{\ln(2)} \times \frac{100}{\tau}$$

where, $T > MIC$ is the time interval (in %) during which the plasma concentration is above or equal to the MIC values; $\ln$ is natural logarithm; D is the proposed dose; $V_{d(\text{area})}$ is the apparent volume of distribution; $t_{1/2\beta}$ is the elimination half-life; and τ is dose interval.

Two variables, viz. MIC and dosing interval were taken into consideration for calculation. MIC cut off value equal to 1 µg/ml was selected for roxithromycin against *Mycoplasma gallisepticum* in poultry as suggested by Hannan (2000). Calculated $T > MIC$ (%) was 80.04% for the interval of 12 h, suggesting that it would be prudent to use the oral dose of 20 mg/kg b.wt. for roxithromycin in broiler chickens to be repeated every 12 h to treat complicated avian mycoplasmosis caused by susceptible *Mycoplasma gallisepticum* having MIC less than or equal to 1 µg/ml.

AUC/MIC ratio is best and most used efficacy predictors for fluoroquinolone drugs like ciprofloxacin. Another PK/PD indices used for fluoroquinolones is C_{max}/MIC . AUC/MIC and C_{max}/MIC ratio should be generally ≥ 125 and ≥ 10 , respectively, as an indicator of effective dose of fluoroquinolones (MacGowan and Bowker 2002, Toutain and Lees 2004). For computation in the present study, MIC cut-off value of ciprofloxacin equal to 0.06 µg/ml against susceptible *E. coli* was considered (De Jong *et al.* 2012). Calculated AUC/MIC and C_{max}/MIC ratio ranged between 143–170 and 22–31, respectively, which were larger than the desired minimum value of 125 and 10, respectively. Thus, ciprofloxacin at the oral dose rate of 10 mg/kg body weight, repeated every 24 h (once daily schedule) would be sufficient to treat secondary *E. coli* infection responsible for complicating the CRD in broiler chickens. The proposed dosing interval of 24 h is also supported by the fact that good plasma ciprofloxacin concentration (≥ 0.14 µg/ml) was maintained up to 24 h after combined oral administration of both the drugs in the present study. Prolonged post antibiotic effects (PAE) of fluoroquinolones and longer oral half life of ciprofloxacin (10.04 h in present study) also favours once daily regimen of ciprofloxacin to be clinically effective.

The pharmacokinetic investigation is an indispensable tool for the appreciation of optimum dosage regimens of antimicrobials. The data set of concentration versus time is

primarily used to calculate pharmacokinetic parameters, which further act as a necessary component of pharmacokinetic-pharmacodynamic (PK-PD) integration for predicting the most optimized dosage regimens. In the present study, the pharmacokinetic parameters of roxithromycin and ciprofloxacin showed no significant effect on the values of either drug when given in combination as compared to their alone oral administrations in broiler chickens. Thus, there was a lack of pharmacokinetic interaction between the two antimicrobials and there is no need of dosage adjustment of either drug, when to be used in combination. Both the drugs exhibit high volume of distributions and long half lives; such features indicate their potential role to treat the respiratory infections like complicated avian mycoplasmosis in broiler chickens. Based on PK-PD integration, treatment approach comprising of roxithromycin (20 mg/kg body weight, twice a day) along with ciprofloxacin (10 mg/kg body weight, once a day) is a promising antimicrobial therapy for the complicated avian mycoplasmosis caused by co-infections of susceptible *Mycoplasma gallisepticum* and *Escherichia coli* in the broiler chickens.

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