



Pharmacokinetics of Marbofloxacin Following Oral Administration in Lactic Acid Pretreated Broiler Chickens

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10.18805/ajdfr.DR-2046

ABSTRACT

Background: It is hypothesized that feeding lactic acid as a feed additive has the potential to alter the pharmacokinetics of many antimicrobial drugs being used for the treatment of infectious disease in general and marbofloxacin in particular; leading to either increased or decreased efficiency. Hence, the present study was planned to explore the effects of lactic acid pre-treatment on the pharmacokinetics of marbofloxacin after oral administration in broiler chickens.

Methods: The pharmacokinetics of marbofloxacin was investigated following single dose oral administration (5 mg/kg) in lactic acid pre-treated (8 gm/l each, oral, 10 days) broiler chickens. The plasma concentration of marbofloxacin was determined by ultra-high-performance liquid chromatography to workout pharmacokinetic profile of marbofloxacin using non compartment model.

Result: The AUC (area under curve) of marbofloxacin declined from 14.70 ig·h/mL to 6.16 ig·h/mL after pre-treatment of lactic acid. Similarly, the average values of maximum plasma concentration (C_{max}) of drug decreased from 2.11 ig/mL to 0.98 ig/mL and mean body clearance (Cl_b) increased from 0.47 L/h/kg to 1.03 L/h/kg. The average elimination half-lives ($t_{1/2\beta}$) of marbofloxacin before and after pre-treatment with lactic acid were 4.89 h and 2.81 h, respectively. Furthermore, the investigation revealed alteration of pharmacokinetic parameters evident in lactic acid pre-treated broiler chickens (as compared to non pre-treated birds) requiring adjustment of dosage regimens.

Key words: Broiler chickens, Lactic acid, Marbofloxacin, Oral pharmacokinetics, Pre-treatment.

INTRODUCTION

For decades, organic acids and their salts have been utilized in poultry feeds and drinking water as feed additives, as they appear to improve growth performance. Commonly used organic acids in poultry include short-chain fatty acids such as formic (C_1), acetic (C_2), propionic (C_3), and butyric acid (C_4), as well as other carboxylic acids such as lactic, malic, tartaric, fumaric and citric acid (Dibner and Buttin, 2002). Lactic acid is a short-chain carboxylic acid and chemically known as 2-hydroxy propanoic acid. A large number of studies have revealed that lactic acid possesses antimicrobial activities similar to those of antibiotics. Lactic acid enters in the cell wall of dangerous microorganisms, disrupting normal cell function and resulting in the death of the microbes because the undissociated form of acid is more lipophilic, it can pass readily through the semi-permeable membrane of bacteria to the cytoplasm having neutral pH, where it dissociates and release protons (H^+) and lowering the pH inside the cell. As a result, the microorganism's enzymatic reactions of glycolysis signal transductions and nutrition transport are hampered, causing energy depletion in their efforts to return the pH to normal (Mroz *et al.*, 2006). Regular use of lactic acid as a feed additive provides control of infections caused by *Salmonella*, *Campylobacter* and *Escherichia coli* (Van Immerseel *et al.*, 2009; Gharib *et al.*, 2012). Adding 0.5% organic acid (lactic acid, acetic acid, or formic acid) to drinking water during pre-transport feed withdrawal with beneficial effects on the health of birds was also evident, where it was able to minimize *Salmonella* and

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How to cite this article: Patel, A., Patel, H.B., Sarvaiya, V.N., Singh, R.D., Patel, H.A., Vaghela, S., Tukra, S. and Mody, S.K. (2023). Pharmacokinetics of Marbofloxacin Following Oral Administration in Lactic Acid Pretreated Broiler Chickens. Asian Journal of Dairy and Food Research. doi:10.18805/ajdfr.DR-2046

Submitted: 17-11-2022 **Accepted:** 25-03-2023 **Online:** 11-05-2023

Campylobacter contamination of crop and broiler carcasses during processing (Byrd *et al.*, 2001). Because of these advantages, lactic acid is commonly used as a feed additive in poultry industry.

Marbofloxacin is a third-generation, fluorinated quinolone compound, exclusively developed for use in animals (Walker, 2000). Marbofloxacin produces its action via inhibition of topoisomerase II (DNA gyrase) and topoisomerase IV enzymes, which are responsible for the supercoiling of bacterial DNA (Paradis *et al.*, 2001). It has a broad spectrum of antimicrobial activity against Gram-negative and Gram-positive bacteria (Shan *et al.*, 2014). Its action is bactericidal and kills the most sensitive pathogens by a concentration-dependent mechanism (AliAbadi and

Lees, 2002). As with the other fluoroquinolones, marbofloxacin is a highly lipid-soluble drug with good tissue penetration. Because of its low MIC, broader spectrum of action and favorable pharmacokinetic profile, it is extensively used in poultry for the control and treatment of infectious diseases caused by susceptible microorganisms.

It is hypothesized that feeding lactic acid as a feed additive has potential to alter the pharmacokinetics of many antimicrobial drugs being used for the treatment of infectious disease in general and marbofloxacin in particular; leading to either increased or decreased efficiency. The literature on the pharmacokinetic behaviour of marbofloxacin in broilers pre-treated with lactic acid is not available to support this hypothesis, therefore the objectives of the present study was to explore the absorption and elimination characteristics of marbofloxacin in broiler chickens after lactic acid pre-treatment and to evaluate the effect of lactic acid (acidifiers) on the pharmacokinetics of marbofloxacin.

MATERIALS AND METHODS

Approval of project by ethics committee

The study was prior recommended and approved by Institutional Animal Ethics Committee (IAEC) of College of Veterinary Science and Animal Husbandry, Kamdhenu University, Sardarkrushinagar (734/GO/Re/SL/03/CPCSEA/2003) vide proposal approval no. VETCOLL/IAEC/2022/19/PROTOCOL-13 dated 09-01-2022.

Experimental animals

Sixteen (n=16) healthy male broiler chickens (Vencobb strain) of 1 to 1.5 weeks of age and weighing more than 1.0 kg, were used in the present study. The study was conducted during March-April, 2022 when the ambient temperature ranged from 30°C to 35°C at Laboratory Animal House, College of Veterinary Science and Animal Husbandry, Sardarkrushinagar. Sardarkrushinagar (Dist: Banashkantha, Gujarat, India) is located on the latitude of 24°19'34.3" North and a longitude of 72°19'02.1" East. It is at an altitude of 154.52 meter above mean sea level and falls under a tropical and semi-arid climatic zone of North Gujarat, India. Birds were housed in cages having water and feeding trough facilities. Adequate feed and *ad libitum* water were provided to experimental birds throughout the experimental period as per the standard schedule of CPCSEA guidelines for poultry/birds facility-2020 (CPCSEA, 2020).

Chemicals and reagents

Marbofloxacin powder of I.P. grade was procured from Nexia Enterprise, Mumbai, India. Water, methanol, formic acid, and acetonitrile of HPLC grade were purchased from S.D. Fine Chemicals Ltd., Mumbai. Lactic acid was procured from S. D. Fine Chemicals Ltd., Mumbai. Perchloric acid (70-72%) was procured from the same manufacturer. All the chemicals were stored adequately and used prior to the expiry date.

Experimental design

A total sixteen birds were selected for the pharmacokinetic study of marbofloxacin. The birds were divided into two groups of eight birds each, randomly *i.e.* group 1 and group 2. In group 1 marbofloxacin alone was administered orally @ 5 mg/kg in experimental birds (served as a control group). In group 2, marbofloxacin was administered orally at the same dose in birds pre-treated with lactic acid (8.0 gm/L drinking water, each, PO, for 10 days). Broiler chickens were fasted for 12 h before oral administration of marbofloxacin. Oral administration was done using an oral gavage needle (16 G×38 mm, curved needle). Periodical blood samples were collected from the wing veins of the birds in a sterilized pre-heparinized test tube of 5.0 ml capacity at 0 (before drug administration), 5 (0.083 h), 15 (0.25 h), 30 (0.5 h) min., 1 hour, 2 hours, 4 hours, 8 hours, 12 hours, 24 hours, 36 hours and 48 hours after administration (for both groups). Approximately 0.5 ml of blood was withdrawn at each collection point. The plasma was separated after centrifugation of blood samples at 4000 revolutions per minute (RPM) for 10 minutes using a refrigerated centrifuge at 4°C. The plasma samples were transferred to cryo-vials (2 ml capacity) and then stored at -20°C until assayed for marbofloxacin concentration using Ultra High Performance Liquid Chromatography (UHPLC) procedure.

HPLC analysis of marbofloxacin concentration

The plasma samples were analyzed for marbofloxacin concentration with minor modifications in method described by Carpenter *et al.*, (2006). UHPLC apparatus (Thermo Fisher, Germany) consisting of UV detector (Dionex ultimate 3000), gradient solvent delivery pump (Dionex ultimate 3000) and manual injector was used for quantification of marbofloxacin from collected plasma samples. Chromatographic separation was performed by using reverse phase C₁₈ analytical column (GL Science Inc., Japan, ODS-3V; 5 µm, 250×4.6 mm) at room temperature. The mobile phase consisted of mixture of 0.01 M formic acid (prepared in HPLC water) and acetonitrile (82:18), which was filtered by 0.45 µm size filter (Millipore®, Merck Life Science Pvt. Ltd., Bangalore) and degassed by ultrasonication (Frontline Ultrasonic Cleaner, Ahmedabad, India). The mobile phase was pumped into column at a flow rate of 1.0 ml/min. Effluents were monitored at 297 nm wavelength in UV detector.

Extraction of plasma samples

Deproteinization of each plasma sample (150 µl) was done by precipitation with addition of 150 µl of perchloric acid (20%). Mixture was vortexed for 1 minute followed by centrifugation at 10,000 rpm for 10 min at 4°C. Clear supernatant was collected in 2 ml ependroff tubes. An aliquot of the resulting supernatant (50 µl) was injected in to UHPLC system through manual injector. The data integration was performed by "Chromeleon" software version 6.8.

Method validation

For standardization, initially stock solution of marbofloxacin was prepared by dissolving 10 mg of pure drug powder in 5.0 ml of blank plasma. The stock solution were used to prepare working standard solution of marbofloxacin (in drug free plasma of broiler chickens) having concentrations of 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.07 and 0.03 $\mu\text{g/mL}$. The standard calibration curve of marbofloxacin was linear for concentrations ranging from 0.03 to 10 $\mu\text{g/mL}$. Quantification of marbofloxacin in plasma samples was done by reference to the resultant standard curve. The assay was sensitive and reproducible and liner with mean correlation coefficient (R^2) >0.998. The marbofloxacin was detected from plasma at the retention time of 5.6 ± 0.4 min as depicted in Fig 1. Accuracy and precision were studied at three different concentrations i.e. 0.1, 1 and 10 $\mu\text{g/mL}$ in triplicates. The respective mean values of accuracy for three different concentrations were calculated to be 86.13% (for 0.1 $\mu\text{g/mL}$), 89.29% (for 1 $\mu\text{g/mL}$) and 97.78% (for 10 $\mu\text{g/mL}$). The values of intra-day and intraday precision varied from 1.01 to 5.37% and 0.44% to 5.10%, respectively. The extraction recovery for marbofloxacin from plasma as compared to water at three different concentration (of 0.1, 1 and 10 $\mu\text{g/mL}$) were observed as 99.04%, 95.51% and 96.30%.

Pharmacokinetic analysis

The plasma concentration-time curves of individual birds were subjected to non-compartmental analysis (NCA) for working out the targeted pharmacokinetic parameters of marbofloxacin. Values of PK parameters were presented as a mean \pm standard error (SE). The plasma concentration-time curves of individual broilers were analyzed for obtaining PK parameters with the software "PK Solver 2.0", a freely available menu-driven add-in program for Microsoft Excel

written in Visual Basic for Application (VBA) in solving basic problems in pharmacokinetic data analysis developed by Zhang *et al.*, (2010) at Department of Pharmaceutical University, China. The software was sourced from www.boomer.org/software/pksolver.zip.

Statistical analysis

All the data including plasma concentrations of marbofloxacin and pharmacokinetic parameters have been presented as mean values along with standard error (Mean \pm SE). The effects of lactic acid pre-treatment on marbofloxacin concentrations and pharmacokinetic parameters between birds of group-1 (without pre-treatment) and group-2 (with pre-treatment of lactic acid) were compared and tested for statistical significance using a "t- test" by software IBM SPSS (version 20), where $p \leq 0.05$ was considered statistically "significant" and $P \leq 0.01$ was considered statistically "highly significant".

RESULTS AND DISCUSSION

The pharmacokinetic profile of marbofloxacin has been extensively studied and investigated in different species of birds viz., in broiler chickens (Atef *et al.*, 2017; Patel *et al.*, 2018), Japanese quails (Aboubakr and Abdelazem, 2015), bilgorajka geese (*Anser anser domesticus*) (Sartini *et al.*, 2020); ostriches (De Lucas *et al.*, 2005) and blue-gold macaws (Carpenter *et al.*, 2006). The effects of lactic acid pre-treatment on pharmacokinetics of enrofloxacin in Chinese mitten crab (*Eriocheir sinensis*) (Su *et al.*, 2019) and effects of *Lactobacillus acidophilus* on pharmacokinetics of marbofloxacin in rats (Birhanu *et al.*, 2017) were reported. However, no similar studies involving lactic acid pre-treatment

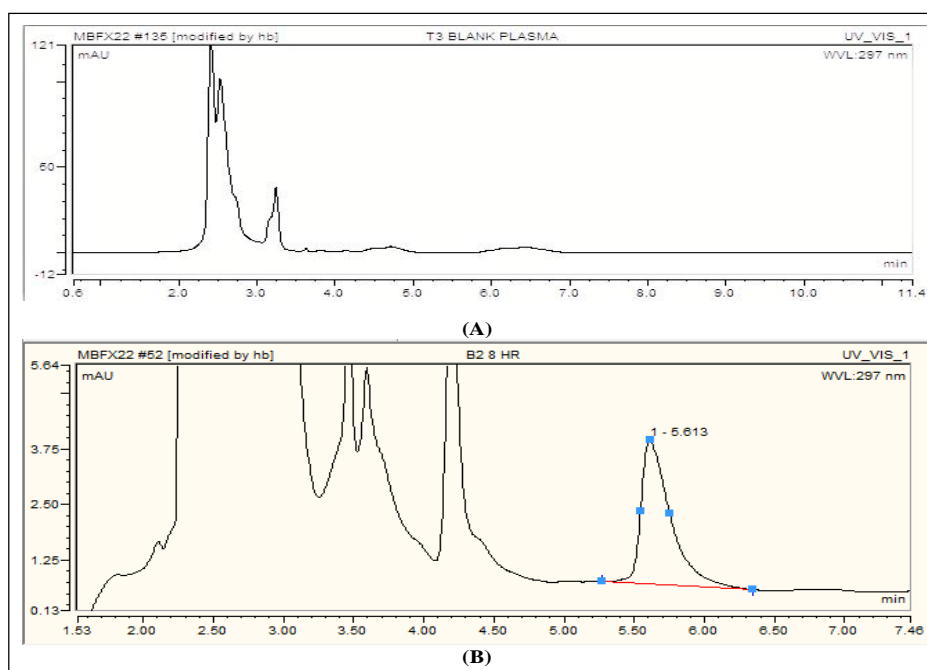


Fig 1: Chromatogram of control (A) and spiked (B) plasma with marbofloxacin.

in broiler chickens and its impact on pharmacokinetics of marbofloxacin have been reported.

The present study on effects of feeding lactic acid on pharmacokinetics of marbofloxacin in broiler chickens stands unique with respect to explore the paucity of scientific data reflecting alteration of pharmacokinetics of marbofloxacin by lactic acid pre-treatment in broilers. The outcome of the present investigation provides a great insight to clinician while treating diseases in lactic acid pre-treated poultry using marbofloxacin.

The semi-logarithmic plots of marbofloxacin plasma concentrations following single dose (5 mg/kg body weight) oral administration in without pre-treated lactic acid and with pre-treated lactic acid (8.0 mg/kg each, PO, for 10 days) broiler chickens are depicted in Fig 2.

The values of plasma concentrations of marbofloxacin at each time point were statistically analysed using a student's t-test for the test of significant difference between groups 1 and 2 of birds (with or without lactic acid pre-treatment). The lactic acid pre-treatment caused significant differences in the values of plasma concentrations of marbofloxacin at 0.5 and 1 h. Decreased concentrations of marbofloxacin in the plasma of pre-treated birds (0.65 ± 0.06 at 0.5 h and 0.85 ± 0.05 at 1 h) were observed as compared to those observed in the plasma of birds of the control group *i.e.*, without pre-treatment (1.09 ± 0.09 at 0.5 h and 2.11 ± 0.37 at 1 h) (Table 1).

Highly significant differences ($p \leq 0.01$) between group of birds without pre-treatment and with pre-treatment of lactic acid were found in the concentrations of time points of 0.5 h (1.09 ± 0.09 v/s 0.65 ± 0.06) and 1 h (2.11 ± 0.37 v/s 0.85 ± 0.05), while a non-significant difference ($p \geq 0.05$) was found in the concentrations of the remaining time points (0.25 h, 2 h, 4 h, 8 h and 12 h) (Table 2). The values of PK parameters that showed a significant difference ($p \leq 0.05$) between with pre-treatment of lactic acid and without a pre-treatment of lactic acid group were elimination half-life, area under curve (AUC), area under moment curve (AUMC) and mean residence time (MRT) whereas, highly significant difference ($p \leq 0.01$) was

seen in the values of maximum concentration (C_{max}) and the remaining PK parameters showed non-significant differences ($p \geq 0.05$) statistically (Table 2).

The mean value of elimination half-life ($t_{1/2\beta}$) after single dose oral administration of marbofloxacin (5 mg/kg) in pre-treated broiler chickens was found as 2.81 h in present study. This value was found to be significantly lower ($p \leq 0.05$) than the value reported in birds without pre-treatment after single oral administration of marbofloxacin (4.89 h). Similar scenario in the values of elimination half-life was also observed by Birhanu *et al.*, (2017) in a study conducted in rats, where the elimination half-lives of marbofloxacin before and after treatment with *L. acidophilus* in rats were also reported as 1.19 h and 0.69 h, respectively. The mean value of AUC was 6.16 ± 1.34 $\mu\text{g}\cdot\text{h}/\text{mL}$ after single dose oral administration of marbofloxacin (5 mg/kg) in pre-treated broiler chickens (Group-2), which was significantly lower ($p \leq 0.05$) than the value of AUC (14.70 ± 2.94 $\mu\text{g}\cdot\text{h}/\text{mL}$) observed in birds without pre-treatment (Group-1). This showed low exposure of the body to the drug, when lactic acid was used as pre-treatment in broiler chickens.

Table 1: Comparison of marbofloxacin concentration (mean \pm SE) between group-1 (without pre-treatment of lactic acid) and group-2 (with pre-treatment of lactic acid).

Time (hour)	Without pre-treatment of lactic acid (Group-1)	With pre-treatment of lactic acid (Group-2)
0.083	0.30 ± 0.06	ND
0.25	0.61 ± 0.13	0.33 ± 0.05
0.5	1.09 ± 0.09	$0.65 \pm 0.06^{**}$
1	2.11 ± 0.37	$0.85 \pm 0.05^{**}$
2	1.61 ± 0.30	0.98 ± 0.08
4	1.12 ± 0.26	0.60 ± 0.13
8	0.67 ± 0.15	0.24 ± 0.09
12	0.41 ± 0.10	0.12 ± 0.06
24	ND	ND

* = $p \leq 0.05$; ** = $p \leq 0.01$ (significantly differed while comparison).

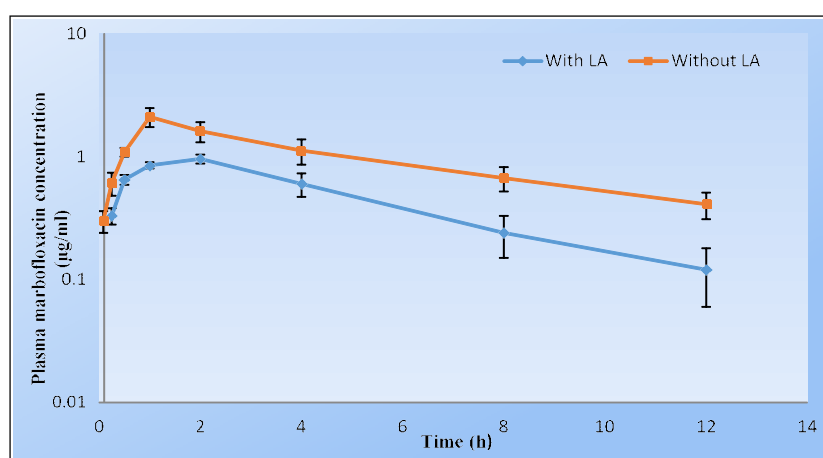


Fig 2: Semi-logarithmic plot of concentrations of marbofloxacin in plasma versus time after oral administration (5 mg/kg) without pre-treated lactic acid and with pre-treated lactic acid in broiler chickens.

Table 2: Comparative pharmacokinetics parameters of marbofloxacin (Mean±SE) between group-1 (without pre-treatment of lactic acid) and group-2 (with pre-treatment of lactic acid).

Pharmacokinetic parameters	Unit	Without pre-treatment of lactic acid (group-1)	With pre-treatment of lactic acid (group-2)
β	h^{-1}	0.17±0.03	0.31±0.06
$t_{1/2\beta}$	h	4.89±0.65	2.81±0.52*
C_{\max}	$\mu\text{g/mL}$	2.11±0.37	0.98±0.08**
$\text{AUC}_{0-\infty}$	$\mu\text{g}\cdot\text{h/mL}$	14.70±2.94	6.16±1.34*
AUMC	$\mu\text{g}\cdot\text{h}^2/\text{mL}$	117.25±25.88	36.28±15.05*
MRT	h	7.48±0.94	4.73±0.80*
$V_{d(\text{area})}$	L/kg	2.80±0.40	3.50±0.37
Cl_B	L/h/kg	0.47±0.11	1.03±0.16*

* $p \leq 0.05$; ** $p \leq 0.01$ (significantly differed while comparison).

(Notations used: μ : Elimination rate constant; $t_{1/2\beta}$: Elimination half-life; $\text{AUC}_{0-\infty}$: Area under curve; AUMC: Area under first moment of the plasma drug concentration; MRT: Mean resident time; $V_{d(\text{area})}$: Apparent volume of distribution; $V_{d(\text{ss})}$: Volume of distribution at steady state; Cl_B : Total body clearance).

Significantly higher ($p \leq 0.05$) value of Cl_B was observed in birds of lactic acid pre-treatment group (1.03 L/h/kg; Group-2) as compared to the value observed in birds without pre-treatment (0.47 L/h/kg; Group-1). Present findings suggest that lactic acid accelerates clearance rate of marbofloxacin from body of broiler chickens.

In present study, the elimination rate constant (β) calculated after single dose oral administration of marbofloxacin (5 mg/kg) in pre-treated broiler chickens (Group-2) was 0.31 h^{-1} , which was significantly higher ($p \leq 0.05$) than value of β (0.17 h^{-1}) observed in birds without pre-treatment (Group-1). The decreased values of elimination half-life and increased value of total body clearance in pre-treated birds indicates comparatively faster elimination from body, which is evident with comparatively lower values of MRT (4.73 v/s 7.48 h) in pre-treated birds. This may be due to changes in physiological environment of body organs involved in processing of drugs viz., liver, kidney, gastrointestinal tract. The lactic acid acts as an acidifier and is likely to bring significant changes in the ADME of marbofloxacin. Marbofloxacin metabolism is a typical CYP450 reaction involving the oxidation of NADPH, it can be speculated that lactic acid may directly influence the TCA cycle to generate sufficient metabolic enzyme (CYP450 enzyme and GST) to influence the pharmacokinetics of marbofloxacin as observed for enrofloxacin (Su *et al.*, 2019). This is similar to the view that lactic acid is a primary circulating TCA substrate in most mammalian tissues (Hui *et al.*, 2017). In short, lactic acid may influence the rate of marbofloxacin elimination by enhancing the biotransformation activity of the hepato-pancreas and induce the activity of microsomal enzymes.

The activity of drug metabolizing enzymes including Phase I and Phase II of biotransformation is key factor determining the pharmacokinetic profiles of the drug. The CYP2 and CYP3 subfamilies have important roles in xenobiotic metabolism (Ren *et al.*, 2017). The possible pharmacokinetic alterations due to enzyme induction depend

on the localisation of the enzyme. These alteration occur via lowered or no bioavailability for orally administered drugs, increased hepatic metabolism or accelerated formation of reactive metabolites (Fuhr, 2000).

The reports of impact of lactic acid on pharmacokinetics of marbofloxacin in broilers are not available, however Su *et al.*, (2019) studied the effects of LA on drug residues and elimination of oral another fluoroquinolone (enrofloxacin) in Chinese mitten crab (*Eriocheir sinensis*) with gene expression levels of drug-metabolizing enzymes in the hepatopancreas. Significant differences were observed in the pharmacokinetic profile of enrofloxacin along with no effects on the expression of CYP2A (phase I) and significant up regulation of CYP3 (phase I) and GST (phase II). The results provided evidences that LA contributed to comparatively faster elimination of drug, and thus, enhances hepatopancreas and biotransformation.

The mean apparent volume of distribution ($V_{d(\text{area})}$) following single dose oral administration of marbofloxacin (5 mg/kg) in pre-treated broiler chickens was calculated to be 3.50 L/kg. The value was more than the value reported for marbofloxacin alone after single oral administration (2.80 L/kg). There was no statistical significance observed for difference. The time required for an intact drug molecule to transit through body is termed as mean residence time (MRT). Thus, MRT becomes an important parameter to describe the length of drug persistence in the body. Statistically significant lower ($p \leq 0.05$) value of MRT was observed in lactic acid pre-treatment (4.73 h; Group-2) as compared to without pre-treatment (7.48 h; Group -1).

CONCLUSION

In conclusion, lactic acid may influence the rate of marbofloxacin elimination by enhancing the biotransformation activity of the hepatopancreas and induce the activity of microsomal enzymes. Following oral administration of marbofloxacin in lactic acid pre-treated broiler chickens, the drug absorption from gastro intestinal tract is delayed, but

plasma concentration was maintained above therapeutic level for up to 12 hours. Lactic acid also accelerates clearance rate of marbofloxacin from broiler chickens body. Further detailed pharmacokinetics and pharmacogenomics interaction studies are required to design appropriate therapeutic dosage regimens of lactic acid as feed additive with routinely used antibiotics in broiler chickens.

Conflict of interest: None.

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