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Original Article

Ex vivo antibacterial activity of levofloxacin against *Escherichia coli* and its pharmacokinetic profile following intravenous and oral administrations in broilers

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Abstract

The use of antibiotics is necessary to treat bacterial diseases. Determination of optimal dosage in the target animals is increasingly being recognized as vital for maximizing efficacy and minimizing the risk of resistance, so this study aimed to evaluate the pharmacokinetics/pharmacodynamics (PK/PD) of levofloxacin in broilers. Using a parallel study design, each group of animals (n=20) received 5 mg/kg of levofloxacin intravenously (IV) and orally (PO). Plasma, serum and tissues were collected for PK and PD studies. Plasma concentrations of levofloxacin were determined by HPLC. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined against *E. coli*, isolated in clinical broilers. *Ex vivo* antibacterial activity was evaluated using the time killing method. Mean values of terminal half-life for IV and PO groups were 6.93 and 8.09 h, respectively. Following oral administration, the peak plasma concentration was achieved at 0.88 h (T_{max}). Mean value of oral bioavailability was 123.25%. Levofloxacin residues were found in all the tissues tested (muscle, liver, kidney and lung). Plasma concentration above 8 x MIC lead to eradication of *E. coli* (incubation period of 24h). The results of *ex vivo* growth inhibition curves were consistent with the *in vitro* time-kill study. Levofloxacin showed dependent plasma concentration antibacterial activity against a clinical isolate of *E. coli*. According to the assessment of PK/PD relationship, administration of 5 mg/kg of levofloxacin seems to be effective in killing *E. coli*. Also, simulated optimal dose based on the *ex vivo* PK/PD approach was 2.9 mg/kg/day (bactericidal) to 4.3 mg/kg/day (eradication) PO against *E. coli* (MIC = 0.125 µg/ml).

Keywords: Levofloxacin; Pharmacokinetics; Pharmacodynamics; Broilers; *Escherichia coli*

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Introduction

The poultry industry has burgeoned in the last few decades (Landoni and Albarellos, 2015). It produces meat and eggs, which are two of the most important food sources for humans and for dogs and cats. Farms are organized in cluster breed system, an intensive breeding technique that requires specific prevention and treatment strategies to prevent and manage infectious diseases. Effective prevention of infectious diseases is a critical goal for veterinarians, farm owners and poultry foodstuff consumers.

Fluoroquinolones are widely used for the treatment of bacterial infections in both human and veterinary fields. Drugs belonging to this class are effective against a broad range of important poultry pathogens, including *Escherichia coli*, *Salmonella* spp. *Mycoplasma*, and *Pasteurella* spp. (Wolfson and Hooper, 1989; Janecko et al., 2016).

Recently, the development of resistance to fluoroquinolones has been reported and become a debated issue among microbiologists, physicians and veterinarians (Brown, 1996; Nakamura, 1997; EFSA, 2012; WHO, 2012). The spread of bacterial resistance is mainly due to overuse and/or improper use of antibiotics: inappropriate dosage regimens or extra-label use can contribute to the development of bacterial resistance in animals and in humans. Thus, it has been suggested that the optimal dosing regimen should be determined to maximize the therapeutic efficacy and to minimize the risk of resistance emerging during treatments (Ambrose et al., 2007; Toutain and Lees, 2004). To achieve these goals, the optimal dosage of an antimicrobial drug must be evaluated using pharmacokinetic/pharmacodynamic (PK/PD) integration.

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For antimicrobial drugs, there are three major PK/PD indices: maximal plasma concentration correlated to minimal inhibitory concentration (C_{max}/MIC), area under the curve correlated to minimal inhibitory concentration (AUC/MIC), and the time over minimal inhibitory concentration (T > MIC), these are used as a basis for determining optimal dosage (Craig, 1998, Lees and Aliabadi, 2002). These parameters, especially C_{max}/MIC and AUC/MIC, are the most significant predictors of the efficacy of fluoroquinolones and these indices have been extensively investigated in the veterinary field (McKellar et al., 2004). According to Thomas et al. (1998), a AUC_{24h}/MIC ratio of 100 or greater is correlated to reduced selection for resistance.

Levofloxacin is a third generation fluoroquinolone labelled for use in humans, and it has excellent anti-bacterial activity against gram-negative, gram-positive and anaerobic bacteria (North et al., 1998). Moreover, it is efficient against *Streptococcus pneumoniae*, most Enterococci, *Enterobacteriaceae*, *Escherichia coli*, *Klebsiella*, *Proteus*, *Pseudomonas*, *Bacteroides*, *Clostridium*, *Haemophilus*, *Moraxella*, *Legionella*,and *Mycoplasma* (Langtry and Lamb, 1998). Levofloxacin is extensively used in human medicine because has enhanced effects if compared to ciprofloxacin and ofloxacin.

Although this antibacterial is not registered in veterinary medicine, several PK studies recently demonstrated that it could be successfully used in calves, goats and poultry (Dumka, 2007; Goudah et al., 2009; Kumar et al., 2009; Ram et al., 2008; Varia et al., 2009). However, a PK/PD study of levofloxacin in broilers is lacking. Considering all these facts, this study aimed to: (a) evaluate PK and tissue disposition of levofloxacin after intravenous (IV) and oral (PO) administrations at the dose of 5 mg/kg in broilers, (b) to evaluate the drug disposition in different tissues, (c) to calculate the MIC and MBC of levofloxacin against *E.*

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coli in two matrices (serum and broth), (d) to assess *in vivo* and *ex vivo* antibacterial effects of levofloxacin against *E. coli* using time kill experiment, (e) to predict the optimal doses corresponding to bacteriostatic, bactericidal and eradication effects based on AUC_{24h}/MIC *ex vivo* parameter for PK/PD modelling.

Material and methods

Chemical and reagents

Pure levofloxacin and enrofloxacin (used as internal standard –IS-) with a standard purity >99.0%, were purchased from Sigma-Aldrich (Milan, Italy). HPLC-grade acetonitrile (ACN), methanol (MeOH), trichloromethane ($CHCl_3$) and isopropanol (C_3H_8O) were purchased from Merck. Tetraethylamine was obtained from Sigma-Aldrich (Milan, Italy). Orthophosphoric acid, disodium hydrogenophosphate, sodium dihydrogen phosphate and potassium dihydrogenophosphate were purchased from Carlo Erba Reagents (Milan, Italy). Deionized water was produced by a Milli-Q Millipore Water System (Millipore, Milan, Italy). All the other reagents and materials were of analytical grade and were supplied by commercial sources. The aqueous and organic components of the mobile phase, degassed under pressure, were mixed by the HPLC. The LC mobile phases were filtered through 0.2 µm cellulose acetate membrane filters (Sartorius Stedim Biotech, Goettingen, Germany) with a solvent filtration apparatus.

Animal experiment

Sixty-five healthy broiler chickens (Ross 308), 35 days old, with an average weight of 2.2 ± 0.3 kg were supplied by a local farm and checked by a veterinarian (H.K.) who certificated the good health status, and the absence of recent drug treatment and parasites. Animals were acclimated for a 2-week period prior to beginning the study. During this period,

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chickens were housed in a communal indoor area on the floor. Regular veterinary checks, based on daily physical examination through observation of behavior and appetite, assessed that animals were in good health. Animal care and handling were performed according to the EC council Directive 2010/63 and also according to Institutional Animal Care and Use directives issued by the Animal Welfare Committee of Latvia (authorization n° 025564), which approved the study design. The day before the commencement of the experiment, animals were randomly divided (65 slips of paper marked with the numbers 1 to 65 selected blinded from a box) into 4 groups: groups A, B and C with 20 subjects and group D with 5 subjects. A ring with an identification code was applied to the right leg of each animal. Animals were then moved to cages (4 chickens/cage) until the end of the experiment. Water was provided ad libitum. Standard management practices were followed to keep animals free from stress.

Groups A, B and C received levofloxacin at the dose rate of 5 mg/kg of body weight (Levofoxacin Kabi 5 mg/ml, Fresenius Kabi, Verona, Italy) according to a randomized, parallel study design. Group D received saline solution at the same volume as the drug. After overnight fasting, groups A and B received levofloxacin orally directly into the chickens' crop using an 18-gauge metal ball-tipped gavage connected to a 2.5 ml syringe. Group C received the drug intravenously through the vein of the right wing using a 2.5 ml syringe and 26- gauge needle. Although poultry are not usually medicated via the IV route, this was necessary to calculate key PK parameters such as absolute CL and absolute oral bioavailability, these values allow for calculation of optimal dose. Group D received saline solution by oral administration via the same method as groups A and B. Concerning blood collections, samples (0.7 ml) were collected from groups A and C at time 0 (prior drug administration) 5, 30 min, and 1, 4, 6, 8, 10, 24, 48 h using an IV catheter (24Gx25mm, BD

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Venflon, Milan, Italy) fixed into the left wing vein. After centrifugation at 400x*g* for 10 min, plasma and serum were separated and split into two aliquots, for PK and PD analysis, respectively, and stored at -20 °C until use within 30 days of collection. Animals in group B were divided into 5 sub-groups (n = 4) and euthanized through cervical dislocation at 1, 6, 10, 24 and 48 h after drug administration for organ collection. Pectoralis muscle, liver, lungs and kidney were separated from each carcass, promptly washed in saline and stored at -80 °C until analysis. To provide control blood and organs for analytical purposes, animals in group D (control group) were bled and organs collected at 1, 6, 10, 24 and 48 h (one animal for each time point).

Preparation of solutions

Singular stock solutions of levofloxacin and IS in MeOH were prepared using volumetric flasks at the concentration of 1000 µg/ml and were stored at -20 °C. To reach a final concentration of 100 µg/ml, appropriate dilutions of stock standard solutions were prepared by diluting 1 ml of each solution to 10 ml. Levofloxacin solution was diluted in glass tubes (10 ml) to reach final concentrations of 5, 2 and 1 µg/ml and were stored at 4 °C. These last concentrations were then diluted to prepare a 6-point calibration curve at the following concentrations 5, 1, 0.500, 0.250, 0.100, 0.050 µg/ml and 2, 1, 0.500, 0.250, 0.100, 0.050 µg/ml, of levofloxacin in plasma and organs respectively. The analyte was stable for at least 10 weeks if stored at 4 °C.

Instrumentation and chromatographic conditions

The HPLC system was an LC system (Jasco Inc., Easton, Maryland, USA) consisting of a high-pressure mixer pump (model PU 980 Plus), spectrofluorometric detector (model 2020 Plus), and a loop of 50 µl. Data were processed using Borwin software (Jasco Inc.,

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Easton, Maryland, USA). Chromatographic separation assay, based on a method previously described by Giorgi et al. (2013) with slight modifications, was performed using a Gemini C18 analytical column (250 x 4.6-mm inner diameter, 5-µm particle size, Phenomenex, Torrance, California, USA) maintained at 25 °C. The mobile phase consisted of acetonitrile: aqueous solution (20:80, v/v%) at a flow rate of 1ml/min.

The aqueous solution consisted of potassium dihydrogen phosphate (0.02 M), and 860 µl of tetraethylamine (0.012 M) in water adjusted to pH 4 with orthophosphoric acid (0.006 M). Excitation and emission wavelengths were set at 295 and 490 nm, respectively.

Sample preparation

The procedure of levofloxacin extraction from plasma and organs was based on that previously reported by Giorgi et al. (2013) with slight modifications.

Plasma extraction

Aliquots (0.2 ml) of plasma were added to 0.1 ml of IS (10 µg/ml) diluted with 800 ml of 0.1M phosphate buffer at pH 7.1. After adding 4 ml of a mixture of trichloromethane: isopropanol (5:1 v/v), the samples were shaken at 200 oscillations/min for 10 min and centrifuged at 4000 x *g* for 5 min. Three ml of the organic layer was transferred into a clean tube and dried at 40 ° C under nitrogen stream. The residue was dissolved in 0.2 ml MeOH, vortexed, and an aliquot was injected onto the chromatographic system.

Organs extractions

Liver, kidney, lungs and muscle were thawed and immediately dissected into small pieces. A total of 1 g per sample was placed into 10 ml glass tubes and was added to 3 ml of

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homogenization reagent consisting of 0.1M phosphate buffer at pH 7.1. The suspension was homogenized using an Ultra Turrax for 30 sec. Internal standard aliquots of 0.1 ml of IS (10 µg/ml) was added to 500 µl of suspension and vortexed for 1 min. Four ml of a mixture of trichloromethane: isopropanol (5:1 v/v) was added and the samples were shaken at 200 oscillations/min for 10 min and centrifuged at 4000 x g for 5 min. Three ml of the organic layer was transferred into a clean tube and dried at 40°C under nitrogen stream. The residue was dissolved in 0.2 ml MeOH, vortexed, and an aliquot was injected onto the chromatographic system.

Quantification

Standard curves were constructed with standard levofloxacin concentrations *vs.* ratio of levofloxacin/internal standard peak areas. Linearity of the regression curves in the range 50-5000 ng/ml and 50-2000 ng/ml for plasma and organs respectively were assessed on the basis of the residual plot, the fit test and the back calculation (within 20% of known amount). Limit of detection (LOD) and limit of quantitation (LOQ) were determined as analyte concentrations giving signal-to-noise ratios of 3 and 10, respectively. The quantitative HPLC method for plasma and each organ were validated by examining the measurement of consistency of results (within-run and between-run), correlation (coefficient of determination of the standard curve), and extraction efficiency of the assay. The within-run precision was calculated from similar responses from six repeats of three control samples (10, 500, and 1000 ng/ml) in one run. The between-run precision was determined by comparing the calculated response (in ng/ml back-fit of the standard curve) of the low (10 ng/ml), middle (500 ng/ml), and high (1000 ng/ml) control samples over three consecutive daily runs (total of six runs). The assay accuracy for within-run and between-run was established by determining the ratio of calculated response to expected response for low (10 ng/ml), middle

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(500 ng/ml), and high (1000 ng/ml) control samples over six runs. The extraction efficiency was determined by comparing the response (in area) of low, middle, and high standards, and the internal standard, spiked into blank plasma eluent before evaporation, to the response from equivalent extracted standards.

Pharmacokinetic analysis

PK parameters for intravenous and oral administration were determined individually as a non-compartmental model using WinNonlin 5.3.1 software (Pharsight, Princeton, New Jersey, USA). The maximal concentration (C_{max}) and time to reach maximal concentration (T_{max}) were determined from the raw data. The elimination rate constant (λz) was estimated by semi-log lineal regression of the terminal slope, and elimination half-life (HLλz) was estimated by ln2/λz. Area under the plasma vs time curve (AUC) and area under the first moment curve (AUMC) were calculated from 0 to the last quantifiable concentration using a linear trapezoidal method. Mean residence time (MRT) was calculated as AUMC/AUC. For intravenous administration, total plasma clearance (Cl) and volume of distribution during the terminal phase (Vd) were determined by the equations:

$$\text{Cl} = (Dose\ (IV))/(AUC\ (IV))$$

$$\text{Vd} = Cl/HL\lambda\text{z}$$

The oral bioavailability (F) was calculated as:

$$\text{F} = (AUC\ (PO))/(AUC\ (IV))$$

The differences between the $AUC_{0\text{-last}}$ and $AUC_{0\text{-}\infty}$ were always lower than 20%

Serum protein binding

The ultrafiltration method was used according to Whitlam and Kenneth (1981). Five ranges of levofloxacin concentrations (10, 50, 100, 500, 1000 ng/ml) were used to determine

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the percentage of binding. Each experiment was run in triplicate. The EMIT free level filter system (Syva Co., Palo Alto, California, USA) was used for the ultrafiltration. One ml of serum spiked with various concentrations of levofloxacin was placed in the filter. The samples were centrifuged for 10 min at 2000 x g, 37 °C. The obtained ultra-filtrate containing free levofloxacin underwent HPLC analysis.

Isolation of Escherichia coli

Two field *Escherichia coli* isolates were collected from cloacal swabs of healthy broiler chickens. Animals were housed in a poultry farm near Turin (Italy) and no pharmacological treatments had been administered in the previous two months. The samples were cultured on Mac Conkey agar (Oxoid, Milan, Italy) and incubated at 37 °C overnight. Lactose-fermenting, indole-positive colonies were evaluated using the BBL Crystal test (Becton Dickinson, Milan, Italy). Then, the identified *E. coli* colonies were stored in Luria-Bertani broth (Oxoid, Milan, Italy) containing 15% glycerol at -80 °C until further testing. American Type Culture Collection (ATCC, Virginia, USA) strain of *E. coli* 25922 was also used in this study as reference strain.

Determination of MIC and MBC values in broth and in serum

Minimal inhibitory concentration (MIC) was determined in broth and in serum obtained from healthy broiler chickens using the microdilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2009). Briefly, *E. coli* strains were seeded on Trypticase Soy Agar (TSA) (Oxoid, Milan, Italy). Colonies following overnight growth were directly suspended in Mueller-Hinton broth (MHB) (Oxoid, Milan, Italy) to obtain a turbidity comparable to the McFarland turbidity standard of 0.5. Cultures were diluted 1:100 with broth to reach a final concentration of 10^6 colony forming units

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(CFU)/ml. Levofloxacin solution (final concentration of 64 μg/ml) was added either to MHB or to serum obtained from the control broiler chickens. Serial dilutions from this solution were prepared in broth and in serum to reach concentrations ranging from 32 μg/ml to 0.008 μg/ml, and in presence of approximately 5×10^5 CFU/ml. Plates were incubated at 37 °C for 18 h and read at 600 nm, using the Ultraspec 2000 Spectrophotometer (Pharmacia Biotech, Milan, Italy). The MIC was reported as the lowest concentration of tested drug able to inhibit bacterial growth. From the wells showing no visible sign of growth/turbidity during MIC determination, bacteria were transferred into TSA plates by the streak plate method. The plates were then incubated at 37 °C for 24 h. The smallest concentration showing no growth of tested organisms was considered as the minimum bactericidal concentration (MBC). MIC and MBC of levofloxacin against *E. coli* ATCC 25922 were only determined in MHB, whereas the same parameters were evaluated for the sensitive field strains in both MHB and serum. All experiments were performed in triplicate.

In vitro bacterial killing curves

Twenty blood samples were obtained from untreated healthy broiler chickens. The animal samples were collected from a poultry farm near Turin (Italy). Samples were collected prior to slaughter. Blood samples were kept at room temperature for 60 min and then centrifuged at 2000 x *g* for 10 min, and immediately frozen at −20 °C until further analysis.

Eight colonies from 24 h TSA culture of the sensitive field *E. coli* strains were suspended in 9 ml MHB and incubated for 20 h at 37 °C. Solutions of levofloxacin in serum (1 ml) were prepared at the following concentrations: 0.0 μg/ml (control), 0.03 μg/ml, 0.06 μg/ml, 0.125 μg/ml, 0.25 μg/ml, 0.5 μg/ml, and 1 μg/ml. These solutions were added to 10 μl broth culture in order to reach the final concentration of approximately 2×10^7 CFU/ml. To

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determinate colony forming units, serial dilutions from 10^{-2} to 10^{-6} in sterile saline were prepared (control = 10^{-8}) and were incubated for 3, 6 and 24 h at 37 °C. Then, 10 µl of each dilution were inoculated on TSA and colony-forming units were counted after 16 h. The countable dilution is the dilution that gives 3 to 30 colonies per 10 µl drop of sample dispensed (Herigstad et al., 2001). All experiments were performed in triplicate.

Ex vivo bacterial killing curves

Eight to ten colonies from overnight growth of *E. coli* in TSA (as described above) were used to inoculate 9 ml of MHB, and then incubated overnight at 37 °C. To each 0.5 ml serum sample from treated animals, 5 µl of the stationary-phase bacterial culture was added to give a final concentration of approximately 3×10^{7} CFU/ml. To determine the numbers of CFU, serial dilutions (ranging from 10^{-2} to 10^{-6}) were prepared with sterile saline and incubated at 37 °C for 3, 6, and 24 h. Thereafter, aliquots of 10 µl were plotted on TSA plates and the numbers of CFU were counted after 16 h incubations. The countable dilution is the dilution that gives 3 to 30 colonies per 10 µl drop of sample dispensed (Herigstad et al., 2001). All experiments were performed in triplicate.

PK/PD integration and optimal dose determination

For PK/PD integration, the indices, *in vivo* C_{max}/MIC and AUC_{24h}/MIC, were calculated by linking PK data with PD index (MIC) for serum after oral administration of levofloxacin in clinically isolated *E. coli*.

For PK/PD modeling, *ex vivo* AUC_{24h}/MIC were obtained after 24h incubation from *ex vivo* time killing curve and fitted using the sigmoidal inhibitory E_{max} model and the following equation:

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$$E = E_max - ((E_max - E_0) \times C_e\text{^}N)/(\ 〖EC〗_50\text{^}N + C_e\text{^}N\ )$$

where E_{max} is the $\log_{10}$ difference in bacterial counts in the test sample containing levofloxacin after 24 h of incubation when the limit of detection is reached; E_0 is the $\log_{10}$ difference in bacterial counts in the control sample (without treatment) after 24 h incubation compared to the initial inocula $\log_{10}$ count in samples incubated between time 0 and 24 h; Ce is the *ex vivo* AUC_{24h}/MIC in the effect compartment; and N is the Hill coefficient which describes the steepness of the *ex vivo* AUC_{24h}/MIC-effect curve. These PD indices were calculated using a non-linear regression software (WinNonlin, Princeton, New Jersey, USA).

The antibacterial effect of levofloxacin was quantified from the sigmoidal inhibitory E_{max} model for three levels of growth inhibition: bacteriostatic (no change in bacterial count from control zero time count), bactericidal effect (99.9% reduction in bacterial count) and bacterial eradication (the lowest *ex vivo* AUC_{24h}/MIC that produced a reduction in bacterial counts to the limit of detection). Thus, the values of *ex vivo* AUC_{24h}/MIC for bacteriostatic and bactericidal effect were the values which produce E = 0 and -3, respectively.

Using PK/PD modelling, the optimal oral dose in broilers was predicted and calculated using the following equation (McKellar et al., 2004):

$$Dose\ per\ day = (Cl \times ex\ vivo\ \ 〖AUC〗_24h/MIC \times MIC)/(fu \times F \times 24h)$$

where Cl is the clearance; *ex vivo* AUC_{24h}/MIC is the *ex vivo* AUC_{24h}/MIC ratio for optimal efficacy; F is the bioavailability; MIC is the minimum inhibitory concentration; *fu* is the free fraction of drug in plasma.

Statistical analysis

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PK and PD data were expressed as mean ± SD, except for PK/PD indices which were presented as mean ± SE. Statistical comparison of PK parameters was determined with Student's t-test and performed by Excel program (Microsoft, Redmond, Washington, USA).

Results

MIC and MBC determination

The results from the determination of MIC in MHB showed that the two field *E. coli* isolates were sensitive to levofloxacin. MIC and MBC values are summarized in Table 1. The MICs of levofloxacin in MHB and serum were 0.03 μg/ml and 0.06 μg/ml, respectively. MBCs in MHB and serum were 0.03 and 0.125 μg/ml, respectively.

Pharmacokinetics of levofloxacin

No adverse effects were observed during the experiment. Following IV and PO administration of levofloxacin at 5 mg/kg, the plasma concentration *vs.* time curve is illustrated in Fig 1. Levofloxacin concentration following IV and PO administration were detectable in the plasma up to 24. PK parameters of levofloxacin were presented in Table 2. After a PO administration, levofloxacin showed rapid absorption with the mean T_{max} value of 0.88 h. The mean values of $HL_{\lambda Z}$ for IV and PO were 6.93 and 8.09 h, respectively, but this difference was not statistically significant. MRT was also consistent between the two groups. The bioavailability for PO administration was complete with a mean value of 123.25%.

Disposition of levofloxacin in tissues

Levofloxacin residues were found in the investigated tissues up to 48 h following oral administration at 5 mg/kg (Fig 2). At 1 h after oral administration, tissue drug concentrations in most organs were high and then decreased over time except in muscle. Maximum

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concentration in muscle was reached at 6 h after administration. The highest levels of levofloxacin (6576 ± 1741 ng/ml) were observed in the liver. The concentrations of levofloxacin in kidney, lungs and muscle were the next highest. The levels of levofloxacin were similar in liver and kidney, and higher than those in muscle and lung.

Serum protein bound

Levofloxacin is approximately 20 to 30% bound to serum proteins. The average value was 24 ± 5%.

In vitro bacterial killing curves

In vitro bacterial activities of levofloxacin against *E. coli* is presented in Fig3A. The number of bacteria in the control sample increased by ~5 $\log_{10}$ CFU/ml after 24 h incubation. For 1/2 MIC and MIC treatment, the growth of bacteria was reduced up to 6 h, after that a slight increase in growth was visible. At 2 MIC, the number of bacteria decreased in count to >3 $\log_{10}$ CFU/ml after 3 h exposure, it then reduced below the limit of detection. Concentrations ranging from 4 to16 MIC showed bacterial eradication after 3 h of exposure.

Ex vivo bacterial killing curves

The *ex vivo* antibacterial activity of levofloxacin against sensitive field *E. coli* was determined using serum samples collected at different time points (10, 30 min, 1, 4, 6, 10, 24 and 48 h), these corresponded to mean levofloxacin concentrations of 841, 1591, 2135, 981, 651, 329, 137 and 27 ng/ml, respectively. The *ex vivo* time killing curves are presented in Fig. 3B. *Ex vivo* antibacterial effect of levofloxacin was similar to the *in vitro* time killing curve, this indicated that a levofloxacin concentration above 2MIC lead to eradication of *E. coli* after 24 h of incubation. In the lowest plasma concentration sample (48 h), the number of

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bacteria remained stable at the 3 h incubation time point, then, the level of bacteria increased in amount so it was similar to that of the control sample after 6 h. All the other tested samples, ranging from 10 min to 10 h, demonstrated a high bactericidal effect, that started after 3 h of incubation and was maintained for the entire experiment. The 24 h samples demonstrated efficiency after 6 h of incubation till the end of the experiment.

PK/PD integration

The mean values of *in vivo* C_{max}/MIC, AUC_{24h}/MIC, C_{max}/MBC and AUC_{24h}/MBC against *E. coli* are represented in Table 3. After oral administration of levofloxacin, *in vivo* C_{max}/MIC and AUC_{24h}/MIC obtained against *E. coli* were 32.48 and 261.59 h, respectively. Also, the mean values for *in vivo* C_{max}/MBC and AUC_{24h}/MBC were 15.59 and 125.56 h, respectively.

PK/PD modelling and optimal dose determination

The PK/PD indices obtained from the E_{max} model are presented in Table 4. The mean values of *ex vivo* AUC_{24h}/MIC corresponding to bacteriostatic, bactericidal and eradication effects were 18.77, 24.02 and 36.27 h, respectively. The slope of E_{max} model was steep with mean N value of 5.21.

Based on the obtained *ex vivo* AUC_{24h}/MIC by PK-PD modelling, the daily optimal dose of levofloxacin corresponding to bacteriostatic, bactericidal and eradication effects was determined. The MIC_{90} values (range from 0.06 to 0.125 µg/ml) of levofloxacin against *E. coli* were sourced from the literature for the determination of optimal dose (Hansen and Blondeau, 2005). Optimal doses for the expected antibacterial effects are presented in Table 5.

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Discussion

It has been widely recognized that inappropriate usage, including over- and misuse of antibiotics, is a risk factor for both emergence of resistance and therapeutic failure. Thus, the appropriate dosage should be determined, not only to maximize clinical efficacy, but also to minimize the emergence of resistance for target animal species (Schentag, 2000; Dagan et al., 2001; Toutain et al., 2002). The PK/PD approach is considered a proven tool in the design of optimal dosage strategies. In addition, PK/PD modelling of time–kill curves has the advantage of describing the PD effect by parameters, which represent the potency and efficacy of drug. Data on the PK/PD of levofloxacin in poultry are limited, and, to the authors' knowledge, this is the first complete study that considers PK/PD of levofloxacin in broilers.

After IV and PO administration of levofloxacin to broilers, the mean half-life values for IV and PO groups were 6.93 and 8.09 h, respectively, which are longer than those reported in sheep (Patel et al., 2012), camels (Goudah, 2009), goats (Goudah et al., 2009) and calves (Kumar et al., 2012), but shorter than those in cats (Albarellos et al., 2005). These data indicate that levofloxacin shows some difference in elimination rate among animal species. However, these findings are not consistent with data reported in the study of Varia et al (2009) in which levofloxacin was administered at 10 mg/kg in broilers. These differences may potentially be explained by experimental discrepancies of dosage, age, breed and number of animals between the two studies.

The apparent volume of distribution at steady state (Vss) was 2881 ml/kg in broilers indicating a relatively wide distribution and it was higher than that reported in sheep (Patel et al., 2012), camels (Goudah, 2009), goats (Goudah et al., 2009) and cats (Albarellos et al., 2005). Also it was consistent with that reported in broilers (Varia et al., 2009). Levofloxacin

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is eliminated primarily by the kidney with the involvement of both glomerular filtration and tubular secretion (Fish and Chow, 1997). The CL (mean value; 381.17 ml/hr/kg) in broilers was similar to those observed in calves (Kumar et al., 2012), sheep (Patel et al., 2012) and camels (Goudah, 2009).

The absolute bioavailability of levofloxacin in broilers after PO administration was almost complete with mean %F value (123.25%) indicating the excellent absorption of levofloxacin in broilers. However, variation in bioavailability has been found among animal species. This value was similar to that measured in camels (94%, Goudah, 2009), but higher than those observed in calves (62%, Kumar et al., 2012; 57%, Dumka and Srivastava, 2006; 2007), and cats (71%, Albarellos et al., 2005).

Following PO administration of levofloxacin at the dose of 5 mg/kg, the drug concentration in liver was 6.57±1.74 μg/g after 1 h and 0.14±0.01 μg/g after 48 h respectively, whereas in muscles the concentration was 0.94±0.07 μg/g after 1 h and 0.04±0.01 μg/g after 48 h respectively. This persistence of drug in tissue supports the wide value for Vss found in this investigation. The levels of levofloxacin in liver and kidney were higher (4-5 folds) than those measured in muscle and lungs 1 h after administration. This might suggest a specific levofloxacin affinity for these two clearing organs.

The PK/PD approach allows determination of the optimal dosage on the basis of the microbiological susceptibility and the variation in disposition kinetics (Hyatt et al., 1995; Sanchez- Recio et al., 2000; Toutain and Bousquet-Melou, 2006). In defining PK/PD relationships the key parameters of PKs are the area under the plasma concentration curve (AUC), the maximum plasma concentration (C_{max}) and the duration of plasma concentration

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exceeding the MIC (T>MIC) (Hyatt et al., 1995). The key PD parameter is the minimum inhibitory concentration (MIC) (Hyatt et al., 1995). Although the methods for MIC are standardized and reproducible (CLSI, 2009), the results of this method may be influenced by many technical factors (inoculum size, incubation times, medium content, etc.) (Hyatt et al., 1995). Significant differences in MIC or MBC values both in broth and serum have been found in many investigations (Aliabadi and Lees, 2001; Hyatt et al., 1995). In the present investigation, MIC and MBC of levofloxacin against *E. coli* were determined in broth and serum of broilers to overcome this limitation. MIC (0.03_{MHB}–0.06_{serum} μg/ml) and MBC (0.03_{MHB} –0.125_{serum} μg/ml) were significantly different in both matrices. This result indicates that the serum inhibitory activity was reduced and that corresponds with previous data demonstrating the decreased antimicrobial effects of most fluoroquinolones in serum (two- to four-fold higher MICs) (Aliabadi et al., 2003a; Aliabadi et al., 2003b; Haritova et al., 2004). This result may be accounted for by the moderate protein binding of levofloxacin reported in humans (38%, Fish and Chow, 1997) and in buffalo (19%, Ram et al., 2008). The protein binding of levofloxacin in broilers determined in this investigation was in between those previously reported, but still in the same range.

Data from the time killing curve indicates that levofloxacin showed concentration-dependent killing pattern against *E. coli*. For *E. coli*, levofloxacin produced an *in vitro* antibacterial activity with low multiples (2- and 4-) of MIC in serum. The bactericidal activity rapidly manifested within 3-6 h of incubation, followed by eradication with 4 MIC at 3 h. The elimination of bacteria was maintained up to 24 h of incubation (where drug concentration > 2 MIC). *Ex vivo* antibacterial activity was consistent with *in vitro* time killing curve data that indicated levofloxacin concentrations ≥2 MIC lead to eradication of *E. coli*. To the author's knowledge, this is the first paper reporting this comparison.

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According to Hyatt et al. (1995), in quantitative terms the PK/PD parameters which have been most extensively investigated, and for which the most robust information is currently available, are AUC_{24}/MIC, Cmax/MIC and T > MIC. AUC_{24h}/MIC and C_{max}/MIC are the best predictor of efficacy correlating with concentration-dependent antibiotics (Craig, 1993; McKellar et al., 2004). It has been widely proposed that dosage regimens which produce high PK/PD parameters such as AUC_{24h}/MIC > 125 and C_{max}/MIC > 10, are more likely to be successful and less likely to be associated with emergence of resistance (Forrest et al., 1993; Schentag, 2000). However, more recent studies suggested that these values might be overestimated and should be lowered in veterinary medicine (Papich, 2014). The calculated mean C_{max}/MIC and AUC_{24h}/MIC for levofloxacin in this study were 32 and 262, respectively. According to the concept outlined above, the dose of 5mg/kg/day PO in broilers seems to be effective for the treatment of colibacillosis.

In addition, *in vitro* MIC method using artificial growth media are limited by the lack of various host factors such as their immune function. To address this limitation, integration of PD to PK in an *ex vivo* model was attempted in this study. The *ex vivo* AUC_{24}/MIC parameters were utilised for this integration using the sigmoid E_{max} model with the reduction in bacterial numbers after 24 h of incubation. The lowest effective *ex vivo* AUC_{24}/MICs, corresponding to bacteriostatic, bactericidal activity and eradication of the bacteria, were determined for serum. The mean values of *ex vivo* AUC_{24}/MIC corresponding to bacteriostatic, bactericidal and eradication effects were 18.77, 24.02 and 36.27 h, respectively. The optimal dosages corresponding to bacteriostatic, bactericidal activity and eradication of the bacteria were predicted by using the *ex vivo* AUC_{24}/MIC. The calculated optimal doses for bacteriostatic, bactericidal activity and eradication were 1.1, 1.4 and 2.1 mg/kg,

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respectively, considering MIC_{90} of 0.06 µg/ml. However, using recently reported MIC_{90} (0.125 µg/ml) of *E. coli* the predicted doses were 2.2, 2.9 and 4.3 mg/kg/day PO, respectively.

Conclusion

This is the first study to assess the PK/PD relationship of levofloxacin in broilers. PK/PD indices against *E. coli* were investigated in an *ex vivo* model. The data presented would suggest optimal doses of 2.9 to 4.3 mg/kg/day PO for levofloxacin against *E. coli* if the MIC is 0.125 µg/ml. Thus, further studies are needed to determine the efficacy in naturally diseased animals or disease models.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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Table 1

Minimal inhibitory concentrations (MICs), minimal bactericidal concentrations (MBCs) for field *Escherichia coli* isolates and the reference *E. coli* ATCC 25922 strain.

***E. coli* strain**	**MIC µg/ml**		**MBC µg/ml**	
	MHB	**Serum**	**MHB**	**Serum**
Sensitive *E. coli* A	0.03	0.06	0.03	0.125
Sensitive *E. coli* B	0.03	0.06	0.03	0.125
E. coli ATCC 25922	0.015	-	0.03	-

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Table 2

Pharmacokinetic parameters of levofloxacin after IV and PO administration at 5 mg/kg in broilers.

Parameters	**Units**	IV			PO		
		Mean		**SD**	**Mean**		**SD**
R^2		0.99	±	0.02	0.97	±	0.04
λ_Z	1/h	0.12	±	0.04	0.09	±	0.02
$HL_{\lambda Z}$	h	6.93	±	2.94	8.09	±	1.71
T_{max}	h		±	/	0.88	±	0.23
C_{max}	ng/ml		±	/	1949	±	382
C_0	ng/ml	3543	±	2278	/	±	/
AUC_{0-24}	h*ng/ml	12734	±	3772	15695	±	1464
Vss	ml/kg	2881	±	1071	/		/
$V^{\S}$	ml/kg	3599	±	1335	3288	±	659
$CL^{\S}$	ml/h/kg	381.17	±	90.10	282.69	±	26.42
AUMC	h*h*ng/ml	68569[a]	±	27012	108156	±	9980
MRT	h	5.37	±	1.31	6.90	±	0.37
F	%	/	±	/	123.25	±	NA

λ_Z, first-order rate constant; $HL_{\lambda Z}$, half-life of the terminal portion of the curve; T_{max}, time at the maximum drug concentration; C_{max}, maximum plasma drug concentration; C_0, concentration at time 0; AUC_{0-24}, area under the curve from 0 to the last; Vss, volume of distribution at steady state; V/F, volume of distribution during the elimination phase; CL/F, body clearance during the elimination phase; AUMC, area under the first moment curve from 0 to the last; MRT, mean residence time; F, absolute bioavailability. NA, not assessable.

[§],this value is divided for its F% in the PO group.

[a], significant difference between each treatment group ($P<0.05$).

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Table 3

Indices of PK/PD integration after PO administration of levofloxacin at 5 mg/kg in broilers.

Parameters	Units	Mean ± SD
C_{max}/MIC	-	32.48 ± 6.37
AUC_{24h}/MIC	h	261.59 ± 24.41
C_{max}/MBC	-	15.59 ± 3.06
AUC_{24h}/MBC	h	125.56 ± 11.71

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Table 4

Parameters of PK/PD modelling from *ex vivo* experiments after oral administration of levofloxacin at 5 mg/kg in broilers.

Parameters	Units	**Mean ± SD**
Log_{10} E_{max}	CFU/ml	-6.65 ± 0.18
Log_{10} E_0	CFU/ml	3.35 ± 0.08
Log_{10} E_{max}-E_0	CFU/ml	-10.01 ± 0.21
EC_{50}	h	21.46 ± 6.94
AUC_{24h}/MIC for bacteriostatic effect	h	18.77 ± 0.12
AUC_{24h}/MIC for bactericidal effect	h	24.02 ± 0.28
AUC_{24h}/MIC for bacterial eradication	h	36.27 ± 0.88
Slope (N)	-	5.21 ± 0.83

E_0, difference in bacterial count in control sample between time 0 and 24 h; E_{max}, difference in bacterial count in sample incubated with levofloxacin between time 0 and 24 h, when the detection limit is reached; EC_{50}, AUC_{24h}/MIC of drug producing 50% of the maximum antibacterial effect; N, slope of the AUC_{24h}/MIC – response curve.

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Table 5

Predicted optimal daily dose of levofloxacin for PO administration in broilers.

Expected effects	**[a]MIC_{90}: 0.06 µg/ml**	**[a]MIC_{90}: 0.125 µg/ml**
Dose for bacteriostatic effect	1.1 mg/kg	2.2 mg/kg
Dose for bactericidal effect	1.4 mg/kg	2.9 mg/kg
Dose for eradication	2.1 mg/kg	4.3 mg/kg

[a] MIC_{90} values from a literature (Hansen and Blondeau, 2005).

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Figure legends

Fig. 1. Mean plasma concentration vs. time curve of levofloxacin after IV (—○—) and PO (--□--) administrations at 5 mg/kg in broilers. Bars represent the SD.

Fig. 2. Tissue disposition of levofloxacin after PO administration at 5 mg/kg in broilers. Bars represent the SD.

Fig. 3. *In vitro* (A) and *ex vivo* (B) antibacterial effects of levofloxacin in serum of broilers against *E. coli* after PO administration at 5 mg/kg.

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Figure 1

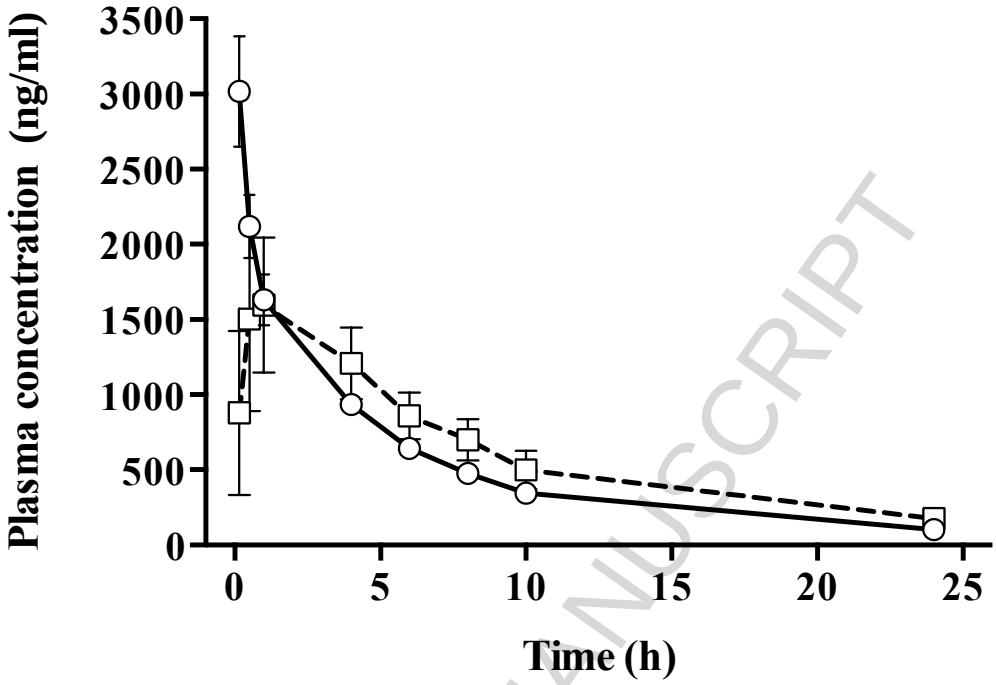

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Figure 2

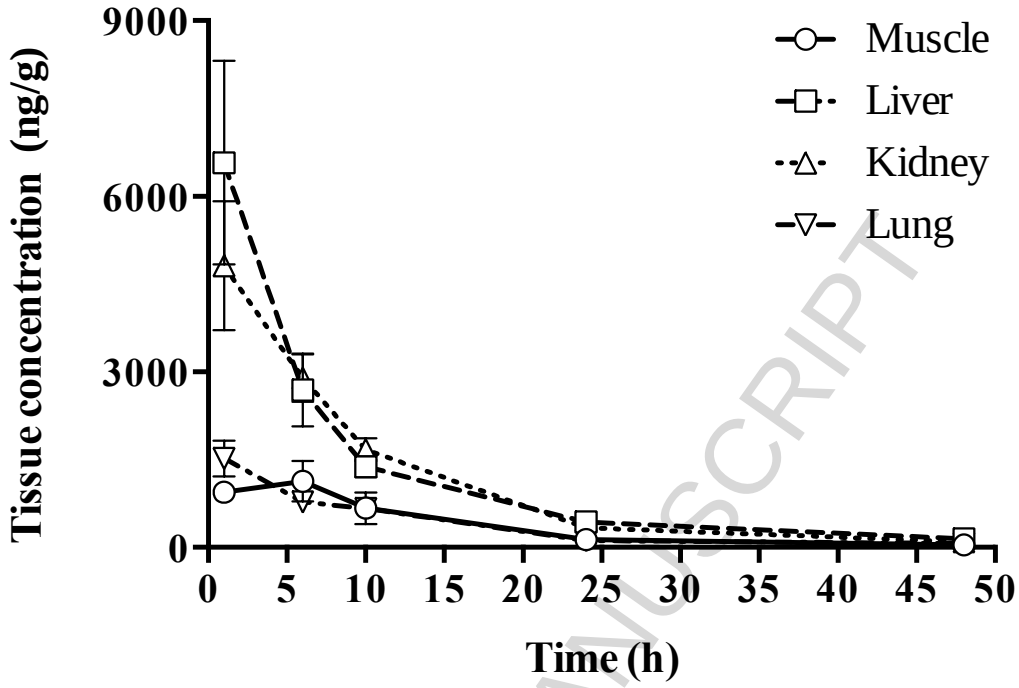

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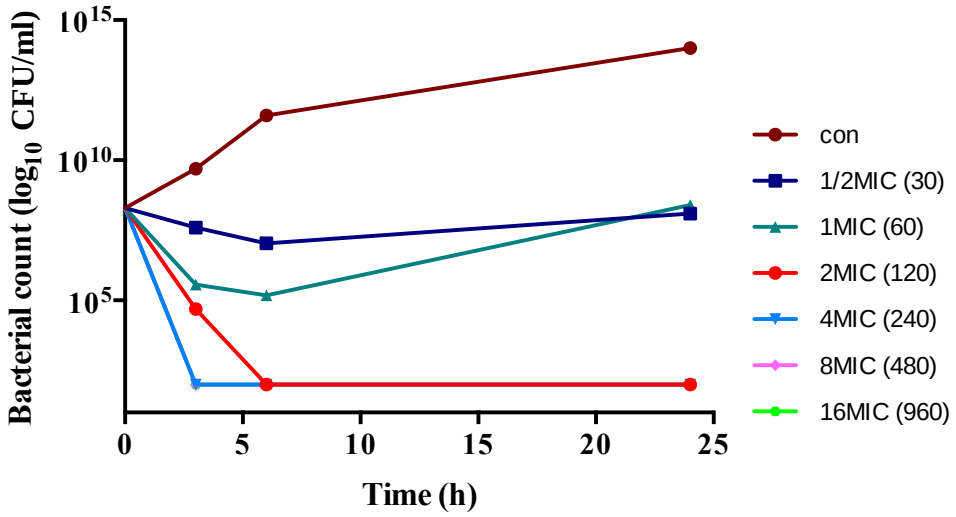
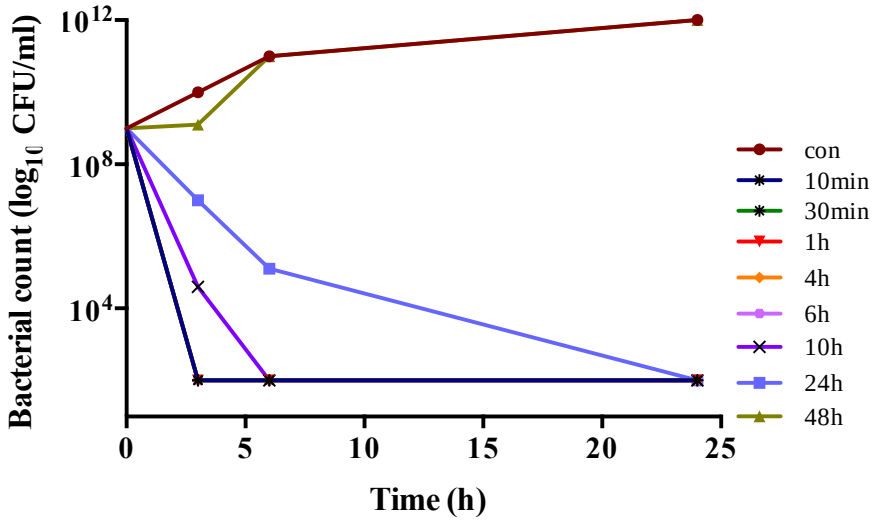
Figure 3

A)



B)



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Highlights

- MIC of levofloxacin in wild *E.coli*: 0.03 µg/ml and 0.06 µg/ml in MHB and serum.
- MBC in MHB and serum: 0.03 and 0.125 µg/ml, respectively.
- Mean values of terminal half-life for IV and PO groups: 6.93 and 8.09 h.
- PO administration: the peak plasma concentration was achieved at 0.88 h (T_{max}).
- Mean value of oral bioavailability was 123.25%.
- Levofloxacin was found in tissues up to 48 h following PO administration at 5 mg/kg.
- PK/PD integrated results suggest that 5 mg/kg is an effective dose to kill *E.Coli*.
- Levofloxacin was deposited in liver and kidney with high concentration.
- Predicted maximum antibacterial effect (E_{max}): $10^{-6.65}$ CFU/ml.

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