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Fluoroquinolone Antimicrobial Agent Levofloxacin in Veterinary Medicine and its Pharmacokinetic-Pharmacodynamic Studies

Summary of the Doctoral Thesis for obtaining the scientific degree “Doctor of Science (*PhD*)”

Sector Group – Medical and Health Sciences
Sector – Basic Medicine
Sub-Sector – Pharmacology

Riga, 2024

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The Doctoral Thesis was developed at RSU Department of Pharmacology

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Abbreviations used in the Thesis

AUC	Area under the concentration vs. time curve
AUMC	Area under the first moment curve
BW	Body weight
CFU	Colony-forming units
C_{max}	Maximal plasma concentration
CI	Confidence interval
CLSI	Clinical and Laboratory Standards Institute
CV	Coefficient of variation
E	Antibacterial effect of levofloxacin
E_0	Log_{10} difference in the bacterial count from 0 to 24 hours of incubation in the control sample
EMA	European Medicines Agency
EU	European Union
F	Bioavailability
HM	Harmonic mean
HPLC	High Performance Liquid Chromatography
I_{max}	Difference between log_{10} difference in bacterial count between 0 and 24 hours in the control sample ($logE_0$) and the log_{10} difference in bacterial count in the sample incubated with levofloxacin for 24 hours when the limit of detection of 100 CFU/mL is reached
IC_{50}	AUC_{24}/MIC producing 50 % of the maximal antibacterial effect
IM	Intramuscular
IS	Internal standard
IV	Intravenous
LOD	Limit of detection
LOQ	Limit of quantification
MAT	Mean absorption time

MBC	Mean bactericidal concentration
MHB	Mueller Hinton Broth
MIC	Minimal inhibitory concentration
MRL	Maximum residue limits
MRT	Mean residence time
N/A	Not applicable
PK	Pharmacokinetics
PO	*per os*
SC	Subcutaneous
SD	Standard deviation
STT	Schirmer Tear Test
t	Time
$t_{1/2}$	Biological half-life
t_{max}	Time to reach maximum drug concentration
TSA	Trypticase soy agar
Vd	Volume of distribution
WHO	World Health Organization
λ_z	Slope of the elimination part of the curve
τ	Dosing interval

Introduction

Infectious diseases are a major problem in veterinary medicine and are associated with the need to administer antimicrobial agents to animals by their owners or people in charge. To make antimicrobial therapy more effective, an appropriate dosing regimen, based on pharmacokinetic and pharmacodynamic data is necessary for both infection treatment and limitation of proliferation of resistant bacterial strains (Toutain et al., 2002). This integrative approach is a proven tool for dose optimisation (Toutain & Lees, 2004). It utilises pharmacokinetic parameters such as area under the concentration vs time curve (AUC), maximal plasma concentration (C_{max}) and pharmacodynamic parameters – minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). The approach that is based on bacterial time-killing curves, actually, shows more rationality compared with the approach based only on MIC value, which is a static parameter (Ambrose et al., 2007). Fluoroquinolones are frequently used for the treatment of bacterial infections in both human and veterinary medicine. Pharmacokinetic-pharmacodynamic indices of fluoroquinolones indicate the effectiveness of this class of drugs. Levofloxacin, a potent third-generation antimicrobial fluoroquinolone drug, is used both in human clinical practice and to some extent in veterinary medicine (Sitovs et al., 2021). Its use in veterinary medicine is currently limited: it is completely banned for veterinary use in the EU and is only used off-label in companion animals in the USA. The pharmacokinetic parameters of levofloxacin have already been established in several domesticated mammalian species – pets, non-pets, and birds. Several research papers reporting on levofloxacin in non-human animals have been published in recent years (Kilburn et al., 2023; Madsen et al., 2019; Vercelli et al., 2020; Wang et al., 2021), indicating an increasing interest in levofloxacin as an off-label drug for use in animals. This interest is likely due to the fact that many of the currently licenced veterinary antimicrobials

do not meet the needs of veterinarians in the management of antibiotic resistant infections (Papich, 2021), and it implies that levofloxacin has promise in the treatment of infections in animals. At the time of the beginning of the work on this thesis, the published scientific studies on levofloxacin pharmacokinetics and activity in rabbits were scarce, and completely absent in geese.

Aim of the Thesis

To study the rationale for the use of levofloxacin as an antimicrobial agent in veterinary medicine.

Tasks of the Thesis

1. Summarise and review the existing scientific data from the veterinary field related to levofloxacin.
2. Assess and compare the pharmacokinetic profiles of levofloxacin in healthy domestic rabbits after intravenous, intramuscular and subcutaneous routes of administration.
3. Assess the pharmacokinetics of levofloxacin in geese after either intravenous and oral administration, and to evaluate the depletion profile in goose tissues.
4. Explore and evaluate levofloxacin antibacterial activity against common animal infection causative agents *P. multocida* and *E. coli* isolated from rabbits.

Hypotheses of the Thesis

Levofloxacin has the favourable properties to be used as an antimicrobial agent in veterinary medicine.

Novelty of the Thesis

This research identified, compiled and systemically arranged the scientific data on the studies of levofloxacin in the field of veterinary medicine. This information is now published at the international level for use by veterinary practitioners and scientists in making decisions regarding the levofloxacin use.

This is the first study to report pharmacokinetic parameters for levofloxacin in rabbits after the intramuscular and subcutaneous routes of administration, that could potentially be useful for off-label treatment of pet rabbits by their owners.

This is the first study to report pharmacokinetic profiles of levofloxacin in geese and its depletion profiles from the selected tissues.

This is the first study to evaluate the levofloxacin antimicrobial activity against *P. multocida* and *E. coli* isolated from rabbits and to propose daily doses for extravascular levofloxacin administration.

1 Materials and methods

1.1 Levofloxacin in veterinary medicine: a literature review

Levofloxacin possesses expanded activity against Gram-positive bacteria and atypical intracellular pathogens (North et al., 1998). Levofloxacin is being used in both human and veterinary medicine. The literature section of this thesis encompasses the key findings from published data related to levofloxacin in the veterinary field. The Scopus database (keywords: "levofloxacin" and "veterinary") and references of the research papers found were used as data sources. The review for the veterinary practitioners and scientists to make informed choices regarding appropriate levofloxacin use is published in the article "Levofloxacin in veterinary medicine: a literature review". The review of levofloxacin properties and use in veterinary medicine is published in the paper "Levofloxacin in veterinary medicine: a literature review" by Andrejs Sitovs, Irene Sartini, and Mario Giorgi. Research in Veterinary Science, 2021 Jul ; 137:111-126. doi: 10.1016/j.rvsc.2021.04.031. PMID: 33964616.

1.2 Pharmacokinetics of levofloxacin after intravenous, intramuscular and subcutaneous administration to rabbits

The study is described in the paper "Pharmacokinetic profiles of levofloxacin after intravenous, intramuscular and subcutaneous administration to rabbits (*Oryctolagus cuniculus*)" by Andrejs Sitovs, Laura Voiko, Dmitrijs Kustovs, Liga Kovalcuka, Dace Bandere, Santa Purvina and Mario Giorgi. Journal of Veterinary Science, 2020 Mar; 21(2):e32. doi: 10.4142/jvs.2020.21.e32.. PMID: 32233138; PMCID: PMC60 PMC7113567 24462. Paragraphs 1.2, 1.5, 2, 3.1 and 4.1 reference the aforementioned article.

1.2.1 Animals

Six cross-bred female rabbits (*Oryctolagus cuniculus*) (body mass 4.21 ± 0.74 kg), 6 months of age at the beginning of the study, were obtained from the animal facility of the Clinical Institute, Faculty of Veterinary Medicine, LBTU. Animals were determined to be healthy based on clinical examination, complete blood analysis, and complete ocular examination including biomicroscopy, indirect ophthalmoscopy, and tonometry. Animals received no drug treatment before the study and were allowed to acclimate in their cages for 7 days before the beginning of the study. Rabbits were housed individually in cages under 12-h light/12-h dark cycle with *ad libitum* access to drinking water and hay. Animals were fed standard pelleted food once daily (Purina Professional Rabbit Feed, Purina, USA). The room temperature was maintained at 20°C. Before the study, animals were randomly divided into 3 groups of 2 using research randomizer software.

1.2.2 Chemicals and reagents

Analytical standard (purity > 98 %) levofloxacin and enrofloxacin (used as the internal standard) and tetraethylammonium chloride were purchased from Sigma-Aldrich (USA). Acetonitrile, methanol, sodium dihydrogen phosphate, sodium hydrogen phosphate, chloroform, and isopropanol were of high-performance liquid chromatography grade. A levofloxacin solution (Levoflox 500 mg/100 mL; Claris, India) was used for administration to the animals.

1.2.3 Experimental design and sample collection

A 3-phase, 3-treatment cross-over study design was applied. The levofloxacin solution was administered as a single dose of 5 mg/kg body weight. In each phase, doses were administered as follows: IV route – as a 1 min bolus into the marginal ear vein; IM route – half of the dose was administered to each of the musculus biceps femoris consecutively; SC route – administered as an

injection in the back of the neck region. A fourteen-day washout period was applied, allowing animals to fully clear the drug and to recover from stress related to the experimental procedures. Animal groups for levofloxacin administration were rotated until all 3 phases of the study were completed. For each phase, a 24G catheter was placed in the central ear artery (for blood collection) and a second one into the marginal ear vein (for IV drug administration) prior to drug administration on the day of commencement of the experiment. The venous catheter was removed immediately after IV drug administration while the arterial one remained until blood collection at 10 hours post-administration. Catheters were flushed with heparin containing saline after blood collection. Blood samples (approximately 0.5 mL) were collected immediately before levofloxacin administration and at 0.083, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 10, 24, and 48 hours post-administration. Blood samples at 24 and 48 hours were collected by syringe from the jugular vein. Collected blood was immediately transferred to lithium-heparin containing test tubes, centrifuged at 1000 × g for 10 min, and the plasma harvested and stored at −20°C until analysis. At 0.5, 2, 4, 8 and 10 hours additional 0.5 mL of blood was collected in a test tube without anticoagulant, left at room temperature to coagulate and serum was harvested and stored at −80°C for the pharmacodynamic study.

1.2.4 Tear fluid collection and analysis

Tear fluid evaluations included tests of tear production and tear film osmolarity. All evaluations were conducted the day before levofloxacin administration to obtain baseline values, and then at 1, 4, 8, 10, 24, and 48 hours after each levofloxacin administration. Schirmer Tear Test (STT) values for tear production were obtained with standardized sterile Schirmer Tear Test I (Eickemeyer, Germany) tips that were inserted under the lower lateral eyelid margin for 1 min. The length of the wet section of the STT tip was immediately measured in millimetres (mm/min). Tear production was also evaluated by

applying I-TEAR TEST strips (I-MED Pharma Inc., Canada) into both eyes at the same period post levofloxacin solution administration as that for the STT-based evaluations. A strip was applied to the central lower lid tear meniscus without touching the cornea or conjunctiva in accordance with the manufacturer's instructions. The number of millimetres on the strip reached in 5 seconds was obtained (unit: mm/5 sec). Tear film osmolarity was assessed by applying the I-PEN VET device (I-MED Pharma Inc., Canada) immediately after the tear production tests were performed. The I-PEN VET sensor was applied to the palpebral conjunctiva until a sound signal, indicating the end of the measurement, was heard (unit: mOsms/L).

1.2.5 Plasma chromatographic analysis

Levofloxacin concentrations in plasma samples were assessed using a Waters Acquity H Class Ultra Performance Liquid Chromatography system equipped with a fluorescence detector (Waters Corporation, USA). The chromatographic analytical method and the sample extraction procedure were based on those previously described (Lee et al., 2017).

1.2.6 Chromatographic method validation

Drug-free rabbit plasma was used for both standard curve construction and quality control method validation in accordance with the Guideline on Bioanalytical Method Validation EMEA/CHMP/EWP/192217/2009 (EMA, 2018a). Drug-free pooled plasma was harvested from all 6 experimental rabbits (2 mL of blood collected) immediately before the beginning of the first phase of the experiment but after the catheters had been placed. The calibration curve was linear from 0.01 to 10 μg/mL (R^2 > 0.999). The levofloxacin recovery from plasma was 96 % ± 3.5 %. The lower limit of quantification was 0.01 μg/mL. Five level standards of levofloxacin quality controls of 0.01, 0.025, 0.05, 0.5, and 5 μg/mL.

1.2.7 Pharmacokinetic analysis

Individual pharmacokinetic parameters were estimated for every animal after treatment using all 3 administration routes. Estimation was performed using non-compartmental analysis and based on visual inspection of the obtained graph (ThothPro Version 1.6.66, Poland). Numerical differences of individual AUC_{0-last} values were lower than 20 % of AUC_{0-inf}, and the R^2 of the terminal phase regression line was > 0.85. Extraction ratio (E %) after IV administration was calculated using the clearance value after IV administration and the cardiac output value (i.e., E % = clearance/cardiac output ×100), where cardiac output = 180 × body weight$^{-0.19}$ (Toutain & Bousquet-Mélou, 2004b).

1.2.8 Pharmacokinetic-pharmacodynamic index

Because the levofloxacin concentrations were below the LOQ at 24 hours, in order to predict the AUC_{24} and to calculate the pharmacokinetic-pharmacodynamic surrogates, a dose 5 times that administered was modelled. The levofloxacin concentration values for all sampled times from 0.083 hours to 10 hours post-administration were multiplied by 5. Applying the superposition principle and assuming the same first-order kinetics (Gabrielsson & Weiner, 2001), approximate values of the concentration at 24 hours post-administration were calculated for each rabbit for all 3 routes of administration. The non-compartmental pharmacokinetic analysis was re-run to obtain an AUC_{24} value from this adjusted data, and the pharmacokinetic-pharmacodynamic surrogate AUC_{24}/MIC was calculated. Since fluoroquinolones produce a concentration-dependent antimicrobial effect over time (Brown, 1996), a target AUC_{24}/MIC ratio for fluoroquinolones of 72 was used (Madsen et al., 2019).

1.2.9 Drug accumulation prediction

A prediction based on a single administration was used to evaluate the possible accumulation ratio (R) at 12 h dosing intervals (τ). The following formula was used (Toutain & Bousquet-Mélou, 2004c):

$$R = \frac{1}{[1-(0.5)^{\frac{\tau}{t_{1/2}}}]} \qquad (1)$$

where τ is the dosing interval and $t_{½}$ is the half-life of elimination.

1.2.10 MIC breakpoints prediction

Based on the equation $AUC_{24}/MIC > 72$, the antimicrobial activity breakpoint for the theoretically computed dose of 25 mg/kg for rabbits, a $MIC < AUC_{24}/72$ was assumed to be effective (Madsen et al., 2019). The AUC was expressed in terms of the unbound drug; levofloxacin was previously reported to be 25 % bound to plasma proteins in rabbits (Destache et al., 2001).

1.2.11 Theoretical effective daily dose calculation

A theoretical optimal daily dosage was calculated for all 3 routes of administration based on the following formula (Toutain et al., 2002):

$$Dose\ per\ day = \frac{\frac{AUC_{24}}{MIC} \times \mathrm{MIC} \times \mathrm{Cl}}{f_u \times F} \times 24 \qquad (2)$$

where AUC_{24}/MIC is the ratio for optimal efficacy (= 72), Cl = clearance, f_u = free fraction of drug in plasma (= 0.75) and F = bioavailability (considered 1 if complete).

1.3 Levofloxacin pharmacokinetics and tissue residue concentrations after oral administration in geese

The study is described in the paper "Levofloxacin pharmacokinetics and tissue residue concentrations after oral administration in Bilgorajska geese" by Irene Sartini, Beata Łebkowska-Wieruszewska, Andrejs Sitovs, Andrzej Lisowski, Amnart Poapolathep and Mario Giorgi. British Poultry Science, 2021 Apr;62(2):193-198. doi: 10.1080/00071668.2020.1842855. Epub 2020 Nov 18. PMID: 33121260. Paragraphs 1.3, 1.5, 2, 3.2 and 4.2 reference the aforementioned article.

1.3.1 Animals

Geese were supplied by a local farm (Majątek Rutka, Puchaczów, Poland). Their health status was evaluated based on a complete physical examination by a veterinarian before the beginning of the study, and through daily observation of behaviour and appetite. Geese were acclimatised for one week in their new environment before the beginning of the trial, and a ring with an identity code was applied to the left leg for easy identification. Birds were housed in a 60 m^2 enclosed area with an indoor shelter of 8 m^2. Animals were allowed to graze freely during the day and were fed a balanced, drug-free pelleted diet (Purina Duck Feed pellets, Purina Animal Nutrition, Gray Summit, MO, USA) twice a day and water was supplied *ad libitum*. No pharmacological treatment was received by the birds before the experiment.

1.3.2 Chemicals and reagents

Levofloxacin and the internal standard (IS) enrofloxacin powder with a standard purity of 99.0 % were purchased from Sigma-Aldrich (Milan, Italy). High performance liquid chromatography (HPLC)-grade acetonitrile, methanol, trichloromethane and isopropanol were procured from Merck (Kenilworth, NJ, USA). Tetraethylamine was obtained from Sigma-Aldrich (St Louis, MI, US).

Orthophosphoric acid, sodium dihydrogen phosphate and potassium hydrogen phosphate were purchased from Carlo Erba Reagents (Milan, Italy). Deionised water was produced using a Milli-Q Millipore Water System (Millipore, Darmstadt, Germany).

1.3.3 Experimental design and sample collection

The study consisted of two parts – pharmacokinetic trial and a tissue depletion trial. The pharmacokinetic trial involved 16 healthy male Bilgorajska geese (body weight (BW), 3.4–4.9 kg; age, 3–4 years) which were randomly divided into two sub-groups (n = 8/group). Sub-group 1 received a single IV dose (2 mg/kg) of levofloxacin (levofloxacin TEVA 5 mg/mL; Teva Pharmaceutical, Hungary) into the left brachial vein using a sterile 26-gauge 1.75 cm needle. The geese in sub-group 2 were given a single oral dose (5 mg/kg) of levofloxacin. The oral doses were prepared by grinding, homogenising, and partitioning the marketed drug (Levofloxacin ACCORD 250 mg/tablet; Accord Healthcare Limited, UK) and dosed relative to the BW of each bird. The correct weight of the solid formulation was dissolved in water and administered via crop gavage using a rounded tip metal catheter 3 hours after being fed. Blood samples (1 mL) were collected in vacutainer lithium heparin tubes (BD, Vaud, Switzerland) from a 24-gauge catheter inserted immediately before the experiment in the right brachial vein at 0, 0.083, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 10, 24, 34, and 48 hours after IV and at 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 10, 12, 24, and 48 h after the last drug administration after per os (PO) treatment. After 12 hours, the catheter was removed, and blood was collected from the left brachial vein directly with a 24-gauge syringe. The catheter was cleaned by flushing with 1 mL of 0.9 % saline with the addition of 10 IU/mL heparin at each collection timepoint. For each blood collection, the first 0.2 mL of blood was discarded. Tubes were centrifuged at 1500 × g and the harvested plasma was stored at −20°C until analysis within 30 days of collection.

The tissue depletion trial involved 10 geese which were given an oral dose (5 mg/kg) of levofloxacin, as described for sub-group 2. Two animals were humanely killed by stunning and exsanguination at 6, 10, 24, 34 and 48 hours after treatment. Approximately 4 g of muscle, heart, liver, lung and kidney were collected and stored at −20°C until further analysis.

1.3.4 Plasma and tissue extraction procedure and chromatographic analysis

An aliquot (0.2 mL) of plasma was added to 0.1 mL of IS (0.1 μg/mL) solution in methanol and 0.8 mL of 0.1 M phosphate buffer at pH 7.1. After the addition of 6 mL of a mixture of trichloromethane and isopropanol (5:1 v/v %), the samples were shaken at 60 oscillations/minute for 10 minutes and centrifuged at 4000 × g for 5 minutes. Then 5 mL of the organic layer was transferred into a clean tube and dried at 40°C under a nitrogen stream. The residue was dissolved in 0.2 mL of mobile phase, vortexed and an aliquot (50 μL) was injected on to the HPLC system. Liver, kidney, lung, heart and muscle samples were thawed and immediately dissected into small pieces and an aliquot of 1 g per tissue was placed into 5 mL plastic tubes containing 3 mL of homogenisation solution (0.1 M phosphate buffer at pH 7.1). The suspension was homogenised for approximately 40 seconds and then 0.2 mL were processed, as described for the plasma samples.

The HPLC was an LC system (Jasco, Japan) consisting of a high-pressure mixer pump (model PU 980 Plus), spectrofluorometric detector (model 2020 Plus), auto sampler (model AS 950), and Peltier system (model CO-4062). The injection loop volume was set at 50 μL. Data was processed using the CromNav 2.0 software (Jasco, Inc., Japan). The chromatographic separation assay was modified from the method reported in the literature (Lee et al., 2017).

1.3.5 Chromatographic method validation

The quantitative HPLC method was fully validated for each tissue (liver, kidney, lung, heart and muscle) and plasma in terms of linearity, intra-day and inter-day precision, recovery, limits of detection (LOD) and quantification (LOQ), according to the EMA guidelines (EMA, 2018a). Linearity was determined by linear regression analysis, using calibration curves constructed using replicates (n=3) of samples from the control geese spiked with levofloxacin at concentrations of 0.005, 0.01, 0.1, 0.5, 1, 5 μg/mL. The intra- and inter-day precision was calculated after analysis of six plasma resamples spiked with levofloxacin at three different concentrations (0.005, 0.1 and 5 μg/mL) with the same instrument and the same operator on the same and on different days, respectively. Precision was calculated and expressed as the coefficient of variation (CV %). The extraction recovery experiment was carried out by analysing samples spiked with the same concentration (0.005, 0.1 and 5 μg/mL) by comparing the response (measured as area) of high, middle, low standards and the IS spiked into blank goose plasma (control), to the response of equivalent standards. Recovery was expressed as mean ± standard deviation (SD). The LOD was estimated as the plasma and tissue drug concentrations that produced a signal to noise ratio of 3 and LOQ was determined as the lowest plasma concentration that produced a signal to noise ratio of 10.

1.3.6 Pharmacokinetic analysis

Levofloxacin plasma concentration was modelled for each subject using a non-compartmental model using ThothPro 4.3.0 v software (www. thothpro.com, Gdansk, Poland).

A naïve pooled-data approach, using a non-compartmental analysis (Pouplin et al., 2016), was used to calculate the pharmacokinetic parameters for levofloxacin in all tissue samples. The penetration of levofloxacin into each

tissue was determined by comparing the AUC ratios between tissues and plasma ($AUC_{tissue}/AUC_{plasma}$) after PO administration (Sartini et al., 2020). Levofloxacin concentrations in the selected tissues were used to calculate preliminary withdrawal times using the software WT 1.4, developed by the European Medicines Agency (EMA, 2018b). The withdrawal time was established as being the time when the upper-one sided tolerance limit (99 %) with 95 % confidence interval (CI) was below the maximum residue limit of 0.1 µg/g levofloxacin, which reflected the MRL for fluoroquinolones in poultry liver (EMA, 1997, 1999, 2002).

1.4 *In vitro* and *ex vivo* antibacterial activity of levofloxacin against *Pasteurella multocida* and *Escherichia coli* isolated from rabbits

The study is described in the paper "*In vitro* and *ex vivo* antibacterial activity of levofloxacin against *Pasteurella multocida* and *Escherichia coli* isolated from rabbits (*Oryctolagus cuniculus*) – A preliminary study" by Andrejs Sitovs, Ingus Skadins, Santa Purvina and Dace Bandere. Journal of Veterinary Pharmacology and Therapeutics, 2023 Apr 15; Online ahead of print. doi: 10.1111/jvp.13383. PMID: 37060264. Paragraphs 1.4, 1.5, 3.3 and 4.3 reference the aforementioned article.

1.4.1 Bacterial isolates

This study included *P. multocida* clinical isolates (n = 10), *E. coli* isolates (n = 5) and commercially available *E. coli* ATCC 25922 (ATCC, ASV) as a reference strain. All *E. coli* isolates were collected from rectal swabs of clinically healthy rabbits that did not previously receive any treatment. Health status was verified by the veterinarian, based on the physical examination and complete blood analysis. Rabbits were housed on a farm near Riga, Latvia. Rectal swabs from were obtained using TRANSWAB® Gel Amies Plain (MWE, UK) with gel

media. Within the same day, the samples were transported to the laboratory of microbiology at Rīga Stradiņš University. Swabs were cultured on McConkey agar and identified with VITEK2 Compact system (bio- Mérieux, France). One *E. coli* isolate from one rabbit was selected. Isolates were considered part of commensal flora and not pathogenic. All *P. multocida* isolates were from rabbits with clinical rhinitis and/or pneumonia. Six *P. multocida* isolates were provided by the Faculty of Veterinary Medicine, Latvia University of Life Sciences and Technologies (Jelgava, Latvia), and 4 isolates were provided by the Institute of Food Safety Animal Health and Environment BIOR (Riga, Latvia). One *P. multocida* isolate from one rabbit was used in this study.

1.4.2 Determination of minimum inhibitory and minimum bactericidal concentrations in broth and serum

Minimum inhibitory concentration values were determined using the microdilution method according to the CLSI guidelines M100 (CLSI, 2018a, 2018b). Levofloxacin standard (> 99 %) was purchased from Sigma-Aldrich. Levofloxacin stock solution (5120 µg/ mL) was prepared in Milli-Q ultra-purified water (Millipore, USA) with the addition of 0.1 M NaOH and further diluted to working concentrations with cation-adjusted Mueller Hinton broth (MHB) or in commercially available drug-free sterile rabbit serum (Biowest, France), respectively. *Escherichia coli* MIC and MBC were detected in MHB and serum. *Pasteurella multocida* MIC and MBC were determined in MHB with the addition of 5 % defibrinated sheep blood (bioTRADING Benelux B.V., France) and in serum. After the overnight growth on agar plates, colonies were suspended in MHB to reach the same turbidity as the McFarland turbidity standard of 0.5. Each *E. coli* culture was diluted 1:100 in MHB to obtain a bacterial count of approximately 10^6 colony-forming units per millilitre (CFU/mL); each *P. multocida* culture was diluted 1:100 in MHB supplemented with 5 % defibrinated sheep blood. Levofloxacin 128 µg/mL working solutions

were prepared in MHB and in serum. Final incubation for 24 hours at 37°C was performed with levofloxacin serial dilutions from 64 to 0.004 μg/mL in both media in the presence of 5×10^5 CFU/mL of bacteria. After the incubation, *E. coli*-containing microdilution plates were read at 600 nm using Infinite F50 Plus reader (Tecan, Switzerland). MIC was reported as the lowest levofloxacin concentration, which showed no turbidity in the microdilution tray wells. For *P. multocida* in MHB with blood, MIC was reported as the lowest concentration where no colour change from red to brown was visually observed. To determine the MBC, 10 μL of the content of wells showing no bacterial growth was transferred to plates, containing Tryptic Soy Agar (TSA) for *E. coli* and TSA supplemented with 5 % defibrinated sheep blood for *P. multocida*. After incubation for 24 hours at 37°C, colonies were counted. The limit of detection was 100 CFU/mL. The lowest concentration showing no bacterial growth was reported as MBC. Reference culture *E. coli* ATCC 25922 MIC and MBC values were determined on MHB only. Experiments were performed in triplicate.

1.4.3 Levofloxacin serum samples for *ex vivo* bacterial killing curve evaluation

Serum samples containing levofloxacin at known concentrations were obtained from our rabbit levofloxacin pharmacokinetic profile study. There, after each drug administration, serum samples for *ex vivo* study were obtained after 0.5, 1.0, 2.0, 4.0, 8.0 and 10.0 hours. Pooled serum samples from experimental rabbits (3 mL) were used for the present study. Levofloxacin concentrations in pooled serum samples were determined prior to the time-killing study with a validated HPLC method (Sitovs et al., 2020).

1.4.4 *In vitro* bacterial killing curves for *Pasteurella multocida* and *Escherichia coli*

One isolate of *P. multocida* and one isolate of *E. coli* were chosen to be used in the bacterial time-killing curve study. The bacterial killing curve study protocol was based on the method described in the literature (Lee et al., 2017). Levofloxacin solutions in drug-free rabbit serum were prepared at concentrations relative to the MIC in the serum of the bacterial isolate. For *P. multocida*, concentrations were 0.00 μg/mL (control), 0.25, 0.5, 1, 2, 4, 8, 16, 32 and 64 times the MIC and for *E. coli* concentrations were 0.00 μg/mL (control), 0.5, 1, 2, 4, 8, 16 and 32 times the MIC. For each bacterial isolate, 8 colonies from overnight growth on agar plates were added to 9 mL of MHB and incubated for 20 hours at 37°C in presence of 5 % CO_2. Ten microlitres of broth culture were added to 1 mL of levofloxacin solutions in serum in order to reach the concentration of approximately 1.6×10^6 CFU/ mL for *P. multocida* isolate and 2×10^7 CFU/mL *E. coli*. Samples were incubated for 24 hours at 37°C in an orbital shaker; 20 μL from all samples were withdrawn at 3, 6 and 24 hours of incubation. Prior to withdrawal, samples were vortexed. Dilutions ranging from 10^{-1} to 10^{-8} in sterile 0.9 % saline were prepared to count the CFU. A 10 μL volume of each saline dilution was inoculated on a TSA plate and incubated for 16 hours. TSA plates for *P. multocida* samples were supplemented with 5 % defibrinated sheep blood. CFU were counted and the limit of detection was 100 CFU/mL. The count of bacteria in the initial inoculum was approved with the same dilution in the sterile saline method. All experiments were performed in triplicate.

1.4.5 *Ex vivo* bacterial killing curves for *Escherichia coli* and *Pasteurella multocida*

The same *P. multocida* and *E. coli* isolates, as for the *in vitro* bacterial killing study, were used in the *ex vivo* study. The study protocol was almost

identical, to the *in vitro* bacterial killing. The difference was that instead of levofloxacin dilutions in antibiotic-free rabbit serum, we used serum samples obtained from rabbits that received 5 mg/kg of levofloxacin parenterally. Pooled serum samples collected at 0, 0.5, 1, 2, 4, 8 and 10 hours after administration contained 0.00, 3.26, 2.64, 1.48, 0.58, 0.13 and 0.07 μg/mL for IM and 0.00, 2.59, 2.70, 1.91, 0.75, 0.14 and 0.08 μg/mL for SC routes of administration, respectively. All experiments were performed in triplicate.

1.4.6 Pharmacodynamic modelling and daily dose calculation

To determine AUC_{24}/MIC ratios, each in vitro levofloxacin concentration was multiplied by 24 (period of incubation) and then divided by the MIC value of each bacterial isolate tested, respectively. The relationship between *in vitro* AUC_{24}/MIC and $\log_{10}$ difference in bacterial count from the initial inoculum to the bacterial count after 24 hours of incubation for serum was evaluated by using the sigmoid inhibitory I_{max} model in Phoenix WinNonlin (Certara, USA). Akaike's Information Criterion was applied to determine the goodness of fit. The model is described with the following equation:

$$E = E_0 - \frac{I_{max} \times C^{\gamma}}{C^{\gamma} + IC_{50}^{\gamma}} \qquad (3)$$

E – antibacterial effect of levofloxacin; I_{max} – difference between $\log_{10}$ difference in bacterial count between 0 and 24 hours in the control sample ($\log E_0$) and the $\log_{10}$ difference in bacterial count in the sample incubated with levofloxacin for 24 hours when the limit of detection of 100 CFU/mL is reached; E_0 – $\log_{10}$ difference in the bacterial count from 0 to 24 hours of incubation in the control sample, antibiotic-free; IC_{50} is the AUC_{24}/MIC producing 50 % of the maximal antibacterial effect; C is the AUC_{24}/MIC in the effect compartment (serum); γ– the Hill coefficient which characterizes the slope of the AUC_{24}/MIC response curve.

The antibacterial activity of levofloxacin against both bacteria species in this study was assessed by calculation of AUC_{24}/MIC values required for bacteriostatic, bactericidal effects and bacterial elimination. AUC_{24}/MIC for bacteriostatic effect was calculated using E = 0, that is, no change in bacterial counts after the incubation for 24 h with levofloxacin. AUC_{24}/MIC for bactericidal effect was calculated using E = −3, that is, bacterial counts reduction by 99.9 % after the incubation for 24 hours with levofloxacin. AUC_{24}/MIC for bacterial elimination effect was calculated using the lowest E value when the maximal antibacterial effect was reached, that is, bacterial count reduction to the limit of quantification (100 CFU/mL) after the incubation for 24 hours with levofloxacin. Obtained from pharmacokinetic–pharmacodynamic integration, antibacterial effects AUC_{24}/MIC values were used to calculate optimal doses for three effect levels – bacteriostatic, bactericidal and bacterial elimination. The following formula (McKellar et al., 2004) was used:

$$Dose\ per\ day = \frac{\frac{AUC_{24}}{MIC} \times \mathrm{MIC} \times \mathrm{Cl}}{f_u \times F} \times 24 \qquad (4)$$

AUC_{24}/MIC are ratios for bacteriostatic, bactericidal and bacterial elimination effects, MIC is minimum inhibitory concentration in serum, Cl is clearance, F is bioavailability, and f_u is a free fraction of levofloxacin in plasma. The following values were used, Cl = 0.6 mL/g/h and F = 1 (Sitovs et al., 2020). Levofloxacin protein binding in rabbit plasma was 25 %, thus, f_u = 0.75 (Destache et al., 2001).

1.5 Ethics statement

For the levofloxacin pharmacokinetics and pharmacodynamics studies in rabbits, the experimental protocol was approved by the Animal Ethics Committee of the Republic of Latvia Food and Veterinary Service (Permission 025564). The study was performed according to the guideline for the care and

use of laboratory animals in accordance with the European law (2010/63/UE). For the levofloxacin pharmacokinetics and tissue depletion study in geese, the experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of Life Sciences (Lublin, Poland) and carried out in accordance with the European law (2010/63/UE).

2 Statistical Analysis

In the pharmacokinetics study after intravenous, intramuscular and subcutaneous administration of levofloxacin to rabbits, the statistical analysis of pharmacokinetic parameters was performed using SPSS (version 21.0; IBM Corporation, USA). Most statistical parameters are reported as mean ± standard deviation (SD) values. The exceptions are for plasma half-lives (harmonic means were calculated) and t_{max} (median values are reported). The normality of the data was assessed using the Shapiro-Wilk test. Paired t-tests were used to compare the statistical differences for pharmacokinetic parameters with normal data distributions in different administration groups. Where data did not have a normal distribution (e.g., V_{area}/F after IM or SC administration), the Wilcoxon test was applied. The p values lower than 0.05 were considered to indicate statistical significance. In the pharmacokinetics and tissue depletion study in geese, the pharmacokinetic parameters were checked for normal distribution by Shapiro–Wilk test and mean pharmacokinetic values were compared between the two routes of administration using unpaired t-tests using GraphPad Prism v 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). The p values lower than 0.05 were considered to indicate statistical significance.

3 Results

3.1 Pharmacokinetics of levofloxacin after intravenous, intramuscular and subcutaneous administration to rabbits

3.1.1 Animals

All 6 animals received levofloxacin via IV or IM routes; however, only 4 completed the SC administration. In the third phase of the cross-over study, 2 animals were excluded—one animal was excluded because of the inability to fix the catheter in either ear artery. The other animal suffered cramps post IV administration of levofloxacin and died within 48 hours post-administration. Post-mortem examination of this animal showed no respiratory tract, kidney, gastrointestinal tract, or liver abnormalities.

3.1.2 Pharmacokinetic parameters

For all 3 administration routes, the drug was quantifiable in plasma for up to 10 hours post-administration of 5 mg/kg. The semilogarithmic plots of mean levofloxacin plasma concentrations (± SD) after the 5 mg/kg single dose via all 3 routes of administration are presented in Figure 3.1. The mean values of pharmacokinetics parameters obtained (± SD) are reported in Table 3.1. The average AUC_{0-last} values were 9.03 (± 2.66), 9.07 (± 1.80) and 9.28 (± 1.56) μg×h/mL after IV, IM, and SC administration, respectively. Maximum plasma concentration reached 3.33 (± 0.39) and 2.91 (± 0.56) μg/mL after IM and SC administrations, respectively. The mean extraction rate after 5 mg/kg IV administration was 7.2 % ± 2.1 %.

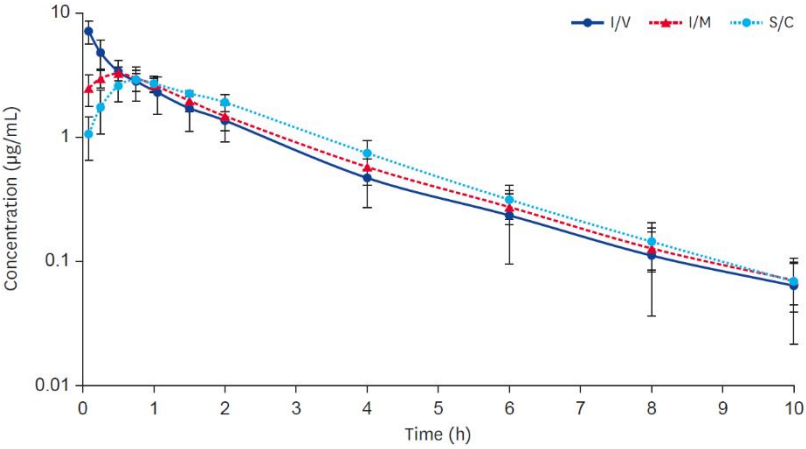

Figure 3.1 **Semilogarithmic plots of average levofloxacin plasma concentrations in rabbits (error bars represent standard deviations) after IV (n = 6), IM (n = 6), and SC (n = 4) levofloxacin administration of 5 mg/kg bodyweight**

IV, intravenous; IM, intramuscular; SC, subcutaneous.

Table 3.1

Mean (± SD) pharmacokinetic parameters of levofloxacin in plasma following IV, IM or SC administration to rabbits at a dose of 5 mg/kg bodyweight

PK parameter	Units	IV (n=6)	IM (n=6)	SC (n=4)
$AUC_{0\text{-last}}$	µg×h/mL	9.03±2.66	9.07±1.80	9.28±1.56
$AUC_{0\text{-inf}}$	µg×h/mL	9.08±2.64	9.07±1.80	9.31±1.50
$AUMC_{0\text{-last}}$	µg×h×h/mL	22.93±12.46	37.87±18.35•	36.62±17.35
$AUMC_{0\text{-inf}}$	µg×h×h/mL	23.64±12.17	37.89±18.34•	36.98±16.82
C_{max}	µg/mL	N/A	3.33±0.39	2.91±0.56
C_{first}	µg/mL	7.13±1.47	N/A	N/A
t_{max} MEDIAN	h	N/A	0.50 (0.08 – 0.75)	0.75
$t_{1/2}\lambda_z$ HM	h	2.06±0.18	2.01±0.24	1.80±0.14
λ_z	1/h	0.34±0.03	0.34±0.04	0.39±0.03
$MRT_{0\text{-last}}$ HM	h	2.19±0.83	3.75±1.16•	3.44±1.31

Table 3.1 continued

PK parameter	Units	IV (n=6)	IM (n=6)	SC (n=4)
$MRT_{0\text{-}inf}$ HM	h	2.27±0.80	3.75±1.16•	3.52±1.25
MAT HM	h	N/A	1.29±0.61	0.45±1.47
Cl	mL/g×h	0.60±0.18	N/A	N/A
Cl/F	mL/g×h	N/A	0.57±0.11	0.55±0.10
V_{ss}	mL/g	1.37±0.39	N/A	N/A
V_{area}/F	mL/g	N/A	1.66±0.34	1.42±0.18
F	%	N/A	105.69±27.50	118.93±40.51

PK, pharmacokinetic; AUC_{0-last}, area under the plasma-concentration time curve from zero to the last quantified sampling point time; $AUC_{0\text{-}inf}$, area under the plasma-concentration time curve from zero extrapolated to infinity; $AUMC_{0-last}$, area under the first moment curve from zero to the last quantified sampling point time; $AUMC_{0-inf}$, area under the first moment curve from zero extrapolated to infinity; C_{max}, maximum plasma drug concentration; C_{first}, concentration at first sample collection point; t_{max}, time of the maximum plasma concentration; $t_{1/2}\lambda_z$, half-life of the elimination part of the curve; λ_z, slope of the elimination part of the curve; MRT_{0-last}, mean residence time from zero to the last quantified sampling point time; MRT_{0-inf}, mean residence time from zero extrapolated to infinity; MAT, mean absorption time; Cl, total plasma clearance; Cl/F, plasma clearance corrected to the bioavailability; V_{ss}, volume of distribution at steady-state; V_{area}/F, volume of distribution corrected to the bioavailability; n, number of experimental animals receiving levofloxacin via the corresponding route of administration; IV, intravenous; IM, intramuscular; SC, subcutaneous; N/A, not applicable; HM, harmonic mean. •Significantly different from IV administration ($p < 0.05$); †Range reported.

3.1.3 Pharmacokinetic-pharmacodynamic index

The *in silico* obtained AUC_{24} values for the theoretical dose of 25 mg/kg were 44.98 ± 12.54 mg×h/L for IV administration, 43.11 ± 6.85 mg×h/L for IM administration, and 43.62 ± 13.65 mg×h/L for SC administration. The levofloxacin accumulation ratio when administered twice daily (τ =12 hours) was predicted to be 1.019 ± 0.006. To obtain the AUC_{24}/MIC of 72, considering that levofloxacin is 25 % bound to plasma proteins, it was calculated that 25 mg/kg of levofloxacin by IV administration would be effective against pathogens with a MIC < 0.47 μg/mL. In the case of IM and SC routes of administration, this dose would be effective against pathogens with a MIC < 0.45 μg/mL. Thus, an

effective daily dose against pathogens with a MIC of 0.5 μg/mL was calculated for the IV administration to be 29 ± 8 mg/kg body weight.

3.1.4 Effects on tear quality

Average tear production observed with STT was 6.4 ± 3.1 mm/min and 7.0 ± 3.1 mm/min, for left and right eyes, respectively (no significant difference, $p = 0.536$). Absolute values varied from 2 to 14 mm/min. No significant changes in tear production were observed among all routes of drug administration within 48 hours. Strip meniscometry values, obtained by following the manufacturer's instructions, of 5 mm and higher are considered to indicate normal tear production while smaller values suggest decreased tear production. The average SM measurement results were normal, 6.9 ± 1.3 mm/5 sec and 6.3 ± 1.9 mm/5 sec, for the left and right eyes, respectively (no significant difference, $p = 0.145$). No significant changes in tear production after levofloxacin IV, IM, and SC administration were observed. Tear osmolarity was 324 ± 21 mOsms/L and 331 ± 22 mOsms/L for both eyes (right and left) prior to drug administration, and the difference was not significant ($p = 0.255$). Mean tear osmolarity decreased in all 3 routes of administration within 48 h after treatment. Changes in tear osmolarity up to 48 hours after levofloxacin administration are summarized in Figure 3.2.

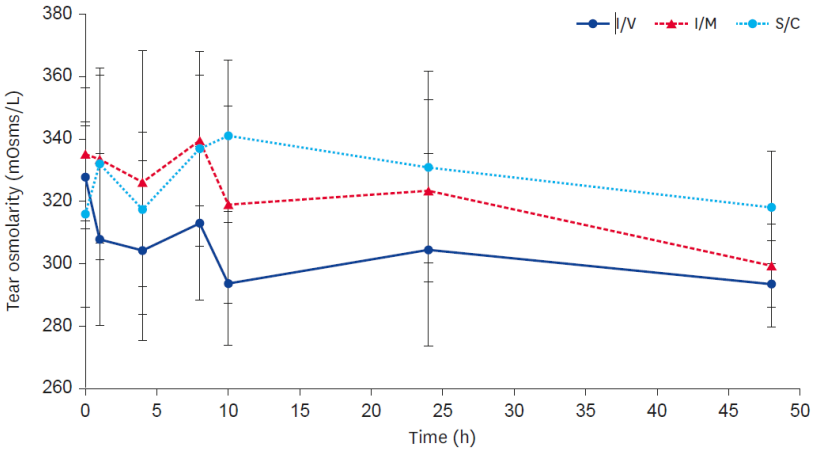

Figure 3.2 **Changes in tear osmolarity in rabbits after a single 5 mg/kg levofloxacin dose administered via IV (n = 6), IM (n = 6), or SC (n = 4) routes (mean values indicated; error bars represent standard deviation).**

IV, intravenous; IM, intramuscular; SC, subcutaneous

3.2 Levofloxacin pharmacokinetics and tissue residue concentrations after oral administration in geese

3.2.1 Animals

The geese did not show any adverse effects during or after drug treatments. All animals received levofloxacin via IV or PO routes and all of them completed the study.

3.2.2 Analytical method validation

The validated analytical method showed a good linearity in the range of 0.005 – 5 μg/mL for every matrix considered in this study. The main results from the analytical method validation in plasma and all tissues selected are reported in Table 3.2.

Table 3.2

Levofloxacin HPLC analytical method validation results in plasma and goose tissues

Parameter	Unit	Plasma	Muscle	Heart	Liver	Lung	Kidney
Inter-day CV	%	5.6	6.1	5.9	6.0	8.9	7.2
Intra-day CV	%	6.9	10.9	9.6	7.4	10.6	9.9
Recovery	%	96 ± 5	94 ± 10	95 ± 8	98 ± 3	93 ± 8	91 ± 9
LOD	µg/mL	0.001	0.001	0.001	0.001	0.001	0.001
LOQ	µg/mL	0.005	0.005	0.005	0.005	0.005	0.005

3.2.3 Pharmacokinetic results

The semilogarithmic plasma concentration vs time curves after IV and PO administration of a single dose of levofloxacin at 2 mg/kg and 5 mg/kg, respectively are shown in Figure 3.3. Plasma levofloxacin concentrations were quantifiable up to 24 hours in birds administered intravenously, and up to 48 hours after PO treatment. The slope of the elimination phase appears to be similar for both routes of administration (Table 3.3).

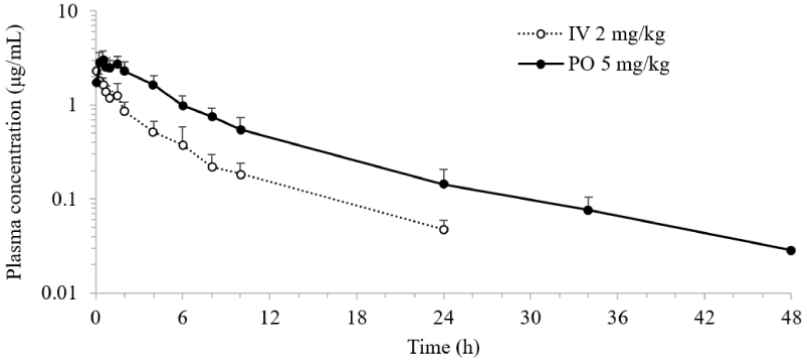

Figure 3.3 **Semilogarithmic plasma levofloxacin concentrations vs time curve following IV (–○–, n = 8) and PO (–●–, n = 8) administration to Bilgorajska geese at a dose of 2 mg/ kg BW and 5 mg/ kg BW, respectively**

Table 3.3 shows the main pharmacokinetic parameters for levofloxacin in geese. Levofloxacin was absorbed rapidly after PO administration displaying a high bioavailability. The drug showed a moderate volume of distribution and a fast clearance. The half-life was not statistically different between the two routes of administration. If normalised for the dose, C_{max} and AUC were not statistically different between the two different administration methods ($p > 0.05$).

Table 3.3

Mean pharmacokinetic parameters of levofloxacin in plasma following IV administration to geese at a dose of 5 mg/kg and PO administration to geese at a dose of 2 mg/kg

		IV (2 mg/kg)		PO (5 mg/kg)	
Parameter	**Unit**	**Mean**	**SD**	**Mean**	**SD**
$AUC_{0\text{-last}}$	mg×h/L	7.59	1.77	17.24	4.86
$AUC_{0\text{-inf}}$	mg×h/L	8.11	1.76	19.37	4.18
$MRT_{0\text{-last}}$	h	5.12	0.37	5.71	2.48
$MRT_{0\text{-inf}}$	h	7.08	0.97	7.65	2.17
λ_z	1/h	0.10	0.02	0.12	0.05
$t_{1/2}\lambda_z$	h	7.39	1.21	6.60	2.46
V_{ss}	mL/g	1.40	0.28	N/A	N/A
Cl	mL/g×h	0.28	0.06	N/A	N/A
V_{ss}/F	mL/g	N/A	N/A	1.63	0.49
Cl/F	ml/g×h	N/A	N/A	0.31	0.09
C_{max}	µg/ml	N/A	N/A	3.20	0.65
t_{max}†	h	N/A	N/A	0.38	(0.25 - 1.5)
F	%	N/A	N/A	95.57	20.61

$AUC_{0\text{-last}}$, area under the curve from 0 hours to last time collected samples; $AUC_{0\text{- inf}}$, area under the curve from 0 hours to infinity; $MRT_{0\text{-last}}$, mean residence time from 0 hours to last time collected samples; $MRT_{0\text{-inf}}$, mean residence time from 0 hours to infinity; λ_z, terminal phase rate constant; $t_{1/2}\lambda_z$, terminal half-life; V_{ss},volume of distribution; Cl, plasma clearance; V_{ss}/F, volume of distribution normalised for F; Cl/F, plasma clearance

normalised for F; C_{max} peak plasma concentration; t_{max}, time of peak concentration; F, bioavailability; † Median value and range; N/A, not applicable

3.2.4 Tissues residue analysis results

Results from tissue residue analysis are displayed in Figure 3.4 as semilogarithmic plots of tissue concentrations vs time curves. Drug residues were highest at 6 hours and decreased constantly, remaining over the LOQ up to 48 hours (last time-point of collection) in all selected tissues. Liver samples had the highest levofloxacin concentration, followed by kidney samples (Table 3.4).

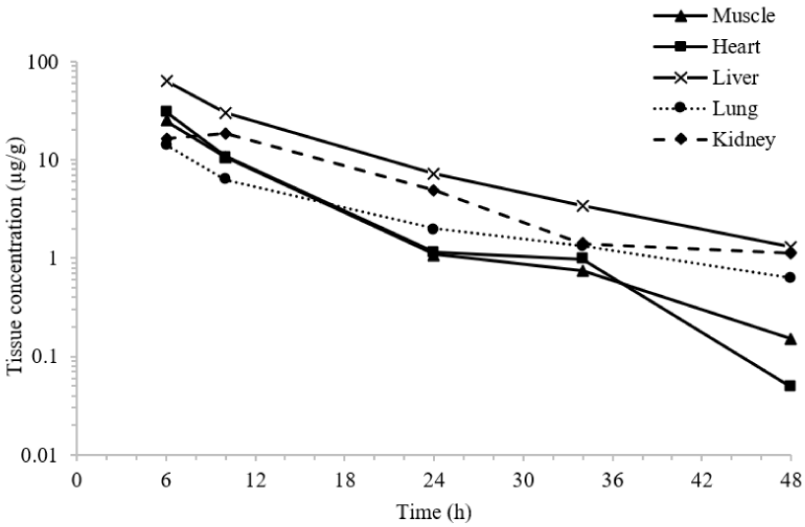

Figure 3.4 **Levofloxacin concentrations (logarithmic scale) in muscle, heart, liver, lung, and kidney following PO administration to Bilgorajska geese (n =2 /timepoint) at a dose of 5 mg/kg BW**

Table 3.4

Mean pharmacokinetic parameters, calculated by the naïve pooled-data approach for each tissue after PO administration to geese at a dose of 2 mg/kg

Parameter	Unit	Muscle	Heart	Liver	Lung	Kidney
$AUC_{0\text{-last}}$	μg×h/mL	218.72	249.8	687.94	165.26	329.51
$MRT_{0\text{-last}}$	h	10.41	9.94	12.56	14.31	13.58
$t_{1/2\lambda z}$	h	8.25	5.07	9.68	14.17	11.84
C_{max}	μg/mL	24.95	30.55	64.2	14.13	18.64
t_{max}	h	6	6	6	6	10
$AUC_{tissue}/AUC_{plasma}$		11.87	13.56	37.35	8.97	17.89

3.3 *In vitro* and *ex vivo* antibacterial activity of levofloxacin against *Pasteurella multocida* and *Escherichia coli* isolated from rabbits

3.3.1 Minimal inhibitory and minimal bactericidal concentration

All 10 isolates of *P. multocida* and all six isolates (including reference strain) of *E. coli* were susceptible to levofloxacin. None of the isolates were considered resistant. MIC and MBC values and MBC/MIC ratios in both media of all bacterial isolates are represented in Tables 3.5 and 3.6. Year of isolate collection is provided in Table 3.5, as well as diagnosis and origin of isolate.

Table 3.5

Minimal inhibitory concentration and minimal bactericidal concentration of *Pasteurella multocida* isolates from rabbits

	MIC_{broth} (µg/mL)	**MIC_{serum} (µg/mL)**	**MBC_{broth} (µg/mL)**	**MBC_{serum} (µg/mL)**	**MBC/MIC_{broth}**	**MBC/MIC_{serum}**	**Diagnosis and isolate origin**
P. multocida 297 (2021)	0.03	0.03	0.06	0.125	2	4	Nasal catarrh, pneumonia Nasal swab
P. multocida 320 (2021)	0.03	0.03	0.125	0.125	4	4	Rhinitis, Nasolacrimal flush fluid
P. multocida 306 (2021)	0.03	0.03	0.125	0.125	4	4	Rhinitis, Nasolacrimal flush fluid
P. multocida 122 (2021)	0.008	0.008	0.008	0.015	1	2	Rhinitis, Nasolacrimal flush fluid
P. multocida 2101 (2021)	0.008	0.008	0.015	0.015	2	2	Rhinitis, Nasal swab
P. multocida 298 (2021)	0.015	0.015	0.03	0.03	2	2	Rhinitis, Nasolacrimal flush fluid
P. multocida 7697[a] (2022)	0.015	0.015	0.03	0.03	2	2	Rhinitis, Nasal swab
P. multocida 3178 (2022)	0.008	0.008	0.125	0.125	16	16	Rhinitis, Nasolacrimal flush fluid
P. multocida 7042 (2022)	0.5	0.5	0.5	0.5	1	1	Rhinitis, Nasolacrimal flush fluid
P. multocida 0634 (2022)	0.5	0.5	0.5	0.5	1	1	Rhinitis, Nasolacrimal flush fluid

MIC - minimal inhibitory concentration, MBC minimal bactericidal concentration,
a - *P. multocida* isolate selected for *in vitro* and *ex vivo* bacterial time-killing study

Table 3.6

Minimal inhibitory concentration and minimal bactericidal concentration of *Escherichia coli* reference strain ATCC25922 and isolates from rabbits

	MIC_{broth} (µg/mL)	**MIC_{serum} (µg/mL)**	**MBC_{broth} (µg/mL)**	**MBC_{serum} (µg/mL)**	**MBC/MIC_{broth}**	**MBC/MIC_{serum}**
E.coli ATCC 25922	0.03	-	0.03	-	1	-
E. coli 1[a]	0.03	0.03	0.25	0.25	8	8
E. coli 2	0.008	0.008	0.03	0.03	4	4
E. coli 5	0.015	0.015	0.06	0.06	4	4
E. coli 11	0.015	0.015	0.03	0.06	2	4
E. coli 12	0.008	0.008	0.03	0.03	4	4

MBC, minimal bactericidal concentration; MIC, minimal inhibitory concentration.
[a]*E. coli* isolate selected for *in vitro* and *ex vivo* bacterial time-killing study

3.3.2 *In vitro* antibacterial activity of levofloxacin and time-killing curves

Figure 3.5 represents the time-dependent antibacterial activity of levofloxacin *in vitro* against a selected isolate of *P. multocida* (Isolate Nr. 7697, MIC = 0.015 µg/mL).

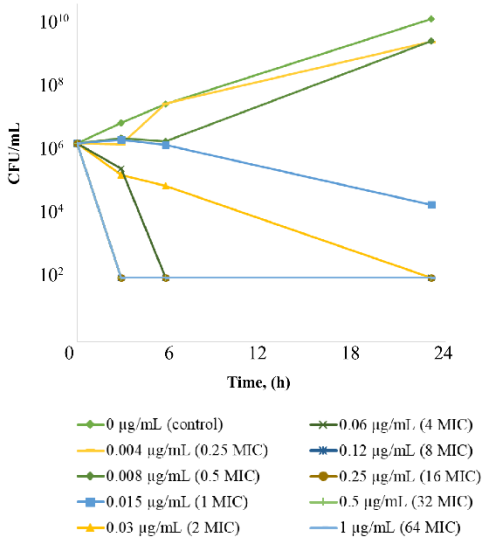

Figure 3.5 ***In vitro* time-killing curves representing the growth of *P. multocida* (Nr. 7697, MIC = 0.015 µg/mL) with different levofloxacin concentrations in rabbit serum**

In the absence of the drug, the 24-hour incubation resulted in bacterial growth of approximately 3 $\log_{10}$ CFU/mL. Levofloxacin concentrations equivalent to 0.25 and 0.5 MIC were not able to inhibit bacterial growth, and after 24 hours of incubation, bacterial counts exceeded the initial inoculum. One MIC concentration reduced the bacterial growth, but after 24 hours of incubation, the bacterial count was similar to the initial inoculum. Concentrations of levofloxacin equal to 2 and 4 MIC reduced the number of bacteria gradually at 3 and 6 hours of incubation and eradicated the bacteria at 24 hours of incubation.

Levofloxacin concentrations higher than 4 MIC decreased the number of bacteria to the limit of detection already at 3 hours of incubation.

Figure 3.6 shows the time-dependent antibacterial activity of levofloxacin *in vitro* against a selected isolate of *E. coli* (Isolate Nr. 1, MIC = 0.03 µg/mL).

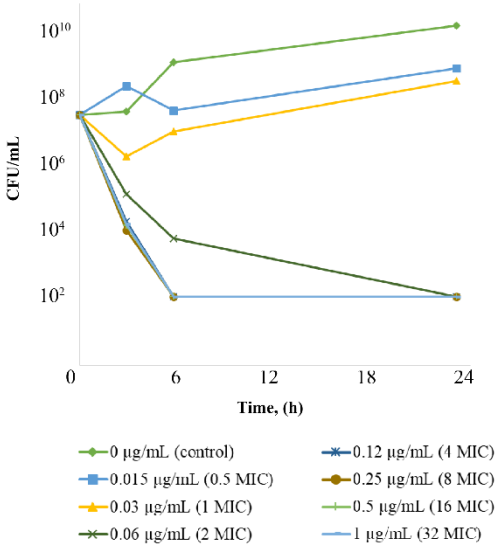

Figure 3.6 ***In vitro* time-killing curves representing the growth *E. coli* (Nr. 1, MIC = 0.03 µg/mL) with different levofloxacin concentrations in rabbit serum**

In the absence of the drug, the 24-hour incubation resulted in bacterial growth of approximately 3 $\log_{10}$ CFU/mL. Levofloxacin concentrations equivalent to 0.5 and 1 MIC were not able to inhibit bacterial growth, and after 24 hours of incubation, bacterial counts exceeded the initial inoculum. Concentrations of levofloxacin equal to 2 MIC reduced the number of bacteria gradually at 3 and 6 hours of incubation and eliminated the bacteria after 24 hours of incubation. Levofloxacin concentrations equal to and higher than 4 MIC decreased the number of bacteria to the limit of detection already at 3 hours of incubation.

3.3.3 *Ex vivo* antibacterial activity of levofloxacin after intramuscular and subcutaneous administration and time-killing curves

Figures 3.7 and 3.8 represent the bacterial time-killing curves for levofloxacin *ex vivo* against a selected isolate of *P. multocida* (isolate Nr. 7697, MIC = 0.015 µg/mL) after IM and SC dosage of 5 mg/kg body weight of levofloxacin solution to rabbits.

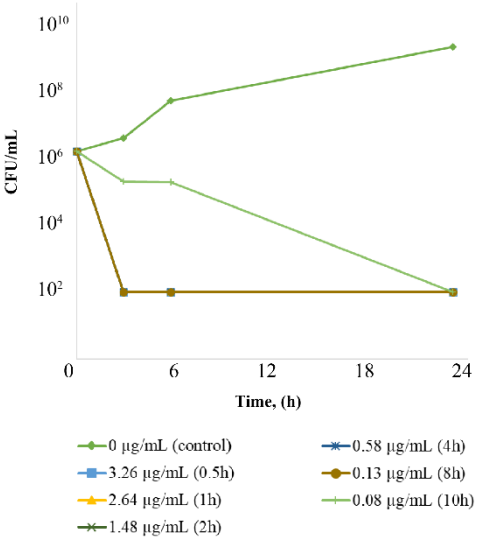

Figure 3.7 ***Ex vivo* time-killing curves representing the growth of *P. multocida* (Nr. 7697, MIC=0.015 µg/mL) with different levofloxacin concentrations in serum samples obtained after intramuscular administration of 5 mg/kg to healthy rabbits (n=6)**

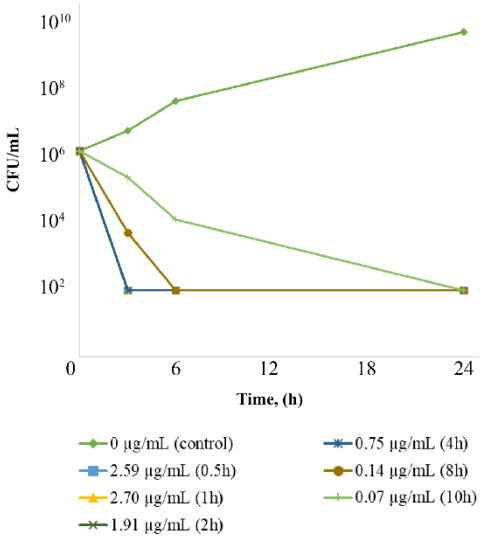

Figure 3.8 ***Ex vivo*** **time-killing curves representing the growth of *P. multocida* (Nr. 7697, MIC=0.015 µg/mL) with different levofloxacin concentrations in samples obtained after subcutaneous administration of 5 mg/kg to healthy rabbits (n=4)**

Concentrations of levofloxacin achieved in serum after 0.5, 1, 2 and 4 hours of both IM and SC administration reduced the bacterial count to the limit of detection already after 3 hours of incubation. Considering 25 % protein binding, free levofloxacin concentrations in these serum samples were 2.45 (163 MIC), 1.98 (132 MIC), 1.11(74 MIC) and 0.44 (29 MIC) µg/mL, and 1.94 (130 MIC), 2.03 (135 MIC), 1.43 (96 MIC) and 0.56 (38 MIC) µg/mL for IM and SC samples, respectively. After incubation for 24 hours, all serum samples containing levofloxacin were able to reduce the *P. multocida* bacterial count to the limit of quantification.

Figures 3.9 and 3.10 represent the bacterial time-killing curves for levofloxacin *ex vivo* against a selected isolate of *E. coli* (isolate No. 1, MIC = 0.03 µg/mL) after IM and SC dosage of 5 mg/kg body weight of levofloxacin solution to rabbits.

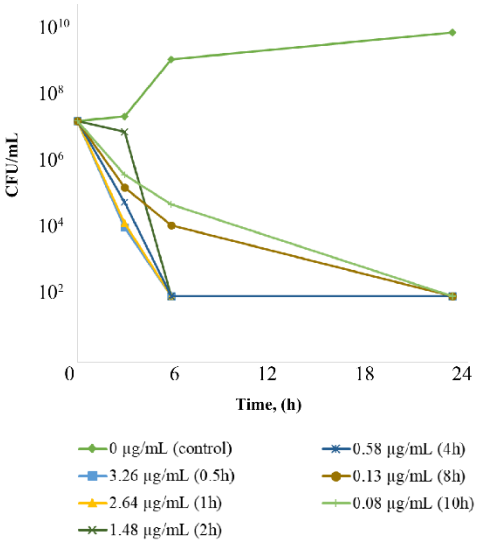

Figure 3.9 ***Ex vivo*** **time-killing curves representing the growth *E. coli* (No. 1, MIC=0.03 µg/mL) with different levofloxacin concentrations in samples obtained after intramuscular administration of 5 mg/kg to healthy rabbits (n=6)**

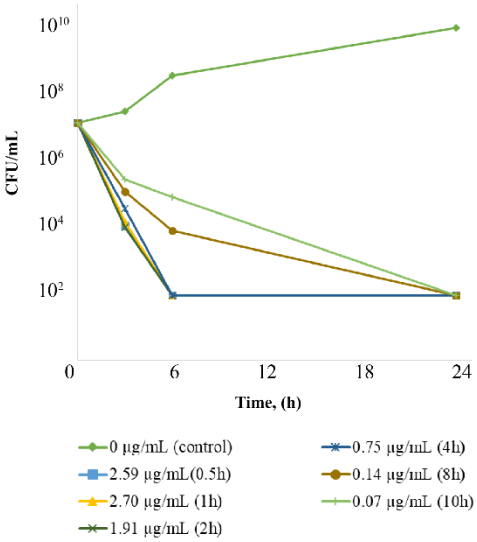

Figure 3.10 ***Ex vivo*** **time-killing curves representing the growth *E. coli* (No. 1, MIC=0.03 µg/mL) with different levofloxacin concentrations in samples obtained after subcutaneous administration of 5 mg/kg to healthy rabbits (n=4)**

Only serum samples collected at 0.5, 1 and 2 hours, representing the highest drug concentrations, were able to reduce the bacterial count to the limit of quantification after 3 hours of incubation. Considering 25 % protein binding, free levofloxacin concentrations in these serum samples were 2.45 (82 MIC), 1.98 (66 MIC) and 1.11 (37 MIC) μg/mL, and 1.94 (65 MIC), 2.03 (68 MIC) and 1.43 (48 MIC) μg/mL for IM and SC samples, respectively. After incubation for 24 hours, all serum samples containing levofloxacin were able to reduce the *E. coli* bacterial count to the limit of quantification.

3.3.4 Pharmacodynamic modelling and daily dose calculation

For the pharmacodynamic analysis, the plots of AUC_{24}/MIC ratios versus changes in bacterial counts after 24 hours of incubation for selected *P. multocida* and *E. coli* isolates are presented in Figures 3.11 and 3.12, respectively. Pharmacodynamic data obtained from the I_{max} model, namely, AUC_{24}/MIC required for bacteriostatic, bactericidal and bacterial elimination for selected *P. multocida* and *E. coli* isolates, are presented in Tables 3.7 and 3.8, respectively. Calculated daily doses of parenteral levofloxacin required to achieve antibacterial effects are reported in Table 3.9. Calculated daily doses for *P. multocida* isolates exhibiting highest MIC value (0.5 μg/mL) are 8.30, 11.55 and 30.18 mg/kg daily, for bacteriostatic, bactericidal and bacterial elimination effects, respectively.

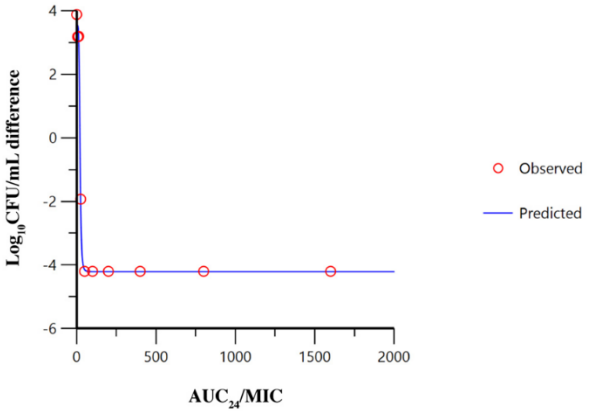

Figure 3.11 **Plot of *in vitro* AUC_{24}/MIC versus *P. multocida* (No. 7697, MIC = 0.015 µg/mL) bacterial count difference in levofloxacin containing rabbit serum**

Table 3.7

Pharmacokinetic-pharmacodynamic levofloxacin data integration of *P. multocida* (Nr. 7697, MIC=0.015 µg/mL) *in vitro* growth inhibition

Parameter	Units	Estimated value
I_{max}	Log_{10} CFU/mL	7.75
E_0	Log_{10} CFU/mL	3.54
E_0- I_{max}	Log_{10} CFU/mL	−4.21
IC_{50}	h	21.41
AUC_{24}/MIC Bacteriostatic	h	20.76
AUC_{24}/MIC Bactericidal	h	28.88
AUC_{24}/MIC Bacterial elimination	h	75.46
Slope (γ)	N/A	5.64

I_{max} – difference between log_{10} difference in bacterial count between 0 and 24 h in the control sample ($logE_0$) and the log_{10} difference in bacterial count in the sample incubated with levofloxacin for 24 hours when the limit of detection of 100 CFU/mL is reached; E_0 – log_{10} difference in the bacterial count from 0 to 24 hours of incubation in the control sample; E_0 - I_{max} – log_{10} difference in the bacterial count from 0 to 24 hours of incubation in samples incubated with levofloxacin when the detection limit of 100 CFU/mL is reached; IC_{50} – AUC_{24}/MIC producing 50 % of the maximal antibacterial effect; γ – the Hill coefficient, slope of the AUC_{24}/MIC response curve; N/A – not applicable

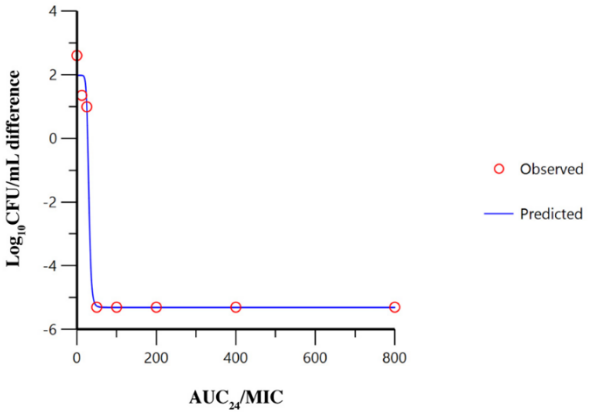

Figure 3.12 **Plot of *in vitro* AUC_{24}/MIC versus *E. coli* (Nr. 1, MIC = 0.03 µg/mL) bacterial count difference in levofloxacin containing rabbit serum**

Table 4.8

Pharmacokinetic-pharmacodynamic levofloxacin data integration of *E. coli* (Nr. 1, MIC = 0.03 µg/mL) *in vitro* growth inhibition

Parameter	Units	Estimated value
I_{max}	Log_{10} CFU/mL	7.28
E_0	Log_{10} CFU/mL	1.98
E_0- I_{max}	Log_{10} CFU/mL	−5.30
IC_{50}	h	30.08
AUC_{24}/MIC Bacteriostatic	h	27.25
AUC_{24}/MIC Bactericidal	h	32.49
AUC_{24}/MIC Bacterial elimination	h	59.62
Slope (γ)	N/A	9.98

I_{max} – difference between log10 difference in bacterial count between 0 and 24 hours in the control sample ($logE_0$) and the log10 difference in bacterial count in the sample incubated with levofloxacin for 24 hours when the limit of detection of 100 CFU/mL is reached E_0 – log_{10} difference in the bacterial count from 0 to 24 hours of incubation in the control sample E_0 - I_{max} – log_{10} difference in the bacterial count from 0 to 24 hours of incubation in samples incubated with levofloxacin when the detection limit of 100 CFU/mL is reached IC_{50} – AUC_{24}/MIC producing 50 % of the maximal antibacterial effect γ – the Hill coefficient, slope of the AUC_{24}/MIC response curve N/A – not applicable

Table 3.9

Calculated daily doses of levofloxacin for parenteral administration to rabbits against *P. multocida* (MIC=0.015 µg/mL) and *E. coli* (MIC=0.03 µg/mL)

Dose per day	*P. multocida* (MIC = 0.015 µg/mL)	*E. coli* (MIC = 0.03 µg/mL)
Bacteriostatic effect	0.25 mg/kg	0.65 mg/kg
Bactericidal effect	0.35 mg/kg	0.78 mg/kg
Bacterial elimination	0.91 mg/kg	1.43 mg/kg

4 Discussion

4.1 Pharmacokinetics of levofloxacin after intravenous, intramuscular and subcutaneous administration to rabbits

To the best of our knowledge, this is the first-time levofloxacin PK profiles after IM and SC administration in healthy rabbits were evaluated, although IV administration had been examined previously in rabbits infected by *S. pneumoniae*.

The 5 mg/kg dose used in this study was based on the dose used previously in a levofloxacin study involving broiler chicken (Lee et al., 2017). This dose is within the range of doses previously used in other mammalian and bird species (Aboubakr, 2012; Aboubakr & Soliman, 2014; Albarellos et al., 2005; Kumar et al., 2012; Urzúa et al., 2020; Varia et al., 2009); a dose associated with reduced risks of side effects. One rabbit died during the current experiment, and the death may be attributed to the stress of the sampling procedures. While necropsy showed no noticeable organ changes in the rabbit, a single IV dose of levofloxacin in humans has been reported to produce cardiovascular side effects – increased heart rate and QT interval prolongation (Basyigit et al., 2005). Thus, cardiovascular effects may also be involved in the lethal outcome in this individual.

All 3 routes of administration (IV, IM, and SC) used in this study produced very similar results for key pharmacokinetic parameters. This could be explained by the fast absorption and rapid distribution of the drug after the extravascular administration routes mimicking the pharmacokinetic profile of the IV administration. In this study, the AUC values for all 3 routes of administration were similar, and there was complete (calculated over 100 %) systemic bioavailability of levofloxacin reported following both IM and SC administration. Maximal plasma concentrations for both extravascular routes

were reached at around the same time (30–45 min post-administration) and were of similar value (around 3 μg/mL). Similar parallel results were observed for SC and IM mean residence times, clearances, and volumes of distribution compared to those for IV administration. These similarities in PKs suggest that the same drug efficacy should be expected for all 3 routes of administration when levofloxacin is given at a dose of 5 mg/kg. Moreover, previous studies of other fluoroquinolones in rabbits (Fernandez-Varon et al., 2005; Marín et al., 2008) and of levofloxacin in other animal species (Lee et al., 2017; Madsen et al., 2019; Patel et al., 2012a) showed very similar pharmacokinetic profiles after different routes of administration. The levofloxacin terminal plasma half-life appeared to be one of the shortest among the species tested (1.8–2.06 hours, depending on the route of parenteral administration).

The volume of drug distribution at a steady-state after IV administration of 1.37 mL/g suggests moderate penetration of the drug through the biological membranes of the body. This value is within the range reported in avian and mammalian species, 0.56 mL/g in sheep (Sartini et al., 2020a) and 2.88–3.25 mL/g in broiler chickens (Lee et al., 2017; Varia et al., 2009).

The results of the non-compartmental PK analysis showed that bioavailability values after IM and subcutaneous SC administration exceeded 100 %. Complete bioavailability of levofloxacin after extravascular administration has also been reported in other species (Vercelli et al., 2020, Goudah & Abo-El-Sooud, 2009; Lee et al., 2017; Madsen et al., 2019, Sartini et al., 2021). Interestingly, other fluoroquinolones studied in rabbits after IM and SC administration have also shown complete bioavailability, with actual values exceeding 100 % (Fernandez-Varon et al., 2007; Marín et al., 2008; Marín et al., 2018). This may be due to various factors that have already described in the literature (Brown, 1996; Martinez et al., 2006; Toutain & Bousquet-Mélou, 2004a), e.g., non-linear clearance. The IM administration of orbifloxacin,

norfloxacin, danofloxacin, and marbofloxacin have all been reported to exceed the 100 % bioavailability level in rabbits (Abo-El-Sooud & Goudah, 2010; Fernandez-Varon et al., 2005; Marín et al., 2008; Marín et al., 2018). Moreover, SC ofloxacin, orbifloxacin, and danofloxacin administration to rabbits also showed complete bioavailability (Fernandez-Varon et al., 2007; Marangos et al., 1997; Marín et al., 2008). These observations indicate that, in general, fluoroquinolones are well absorbed and widely distributed after IM or SC administration in rabbits. The application of compartmental PK analysis using PKanalix software (Lixoft, Simulations Plus, USA) to the same levofloxacin rabbit plasma concentrations supported the complete levofloxacin bioavailability in rabbits after the parenteral administration. IM administration data was best fitted to the two-compartmental with central and peripheral compartments and a linear elimination model with first order absorption. Akaike's Information Criterion was applied to determine the goodness of fit. The mean bioavailability was calculated to be 97 %. SC administration data was best fitted to the one compartment and a linear elimination model with first order absorption. The mean bioavailability was calculated to be 108 %.

Compared to the study in rabbits infected with *S. pneumoniae* (Destache et al., 2001), the AUC values of levofloxacin were much lower (at least twice corrected to the dose administered) in the present study. The plasma terminal half-lives of the drug were at least 3 times longer than that observed in our study. These differences might be due to differences in rabbit breed (New Zealand white vs. cross-bred in this study), size of the animals in the 2 studies (2–3 kg vs. 4.2 kg in the study performed in the scope of this Thesis) and the provision of other drugs (e.g. anaesthetic administration). Additionally, the presence of infection may have slowed the elimination of the drug from the body in a manner similar to that observed in a PK study of marbofloxacin in infected rabbits (Abo-El-Sooud & Goudah, 2010).

The AUC values reported for rabbits appear to be the lowest among the other species studied, considering the administered dose differences. This might be related to the rapid elimination of the drug from the rabbit body. The average plasma clearance of levofloxacin was 0.6 mL/g×h with some variability among the study animals. This is the highest clearance rate thus far reported in all previous mammalian and avian species studied, except sheep, which had similar reported clearance (0.55 mL/g×h (Patel et al., 2012a) vs. 0.6 mL/g×h in rabbits) and half-life of elimination (2.38 hours vs. 2.06 hours in rabbits) values. However, another study in sheep showed a lower clearance of 0.2 mL/g×h and a longer elimination half-life (3.3 hours), but that study was performed using sheep with a body mass almost twice as large, possibly, resulting in slower drug elimination (Goudah & Hasabelnaby, 2010). The longest levofloxacin elimination half-life after the extravascular administration is currently reported in Asian elephants (up to 12.11 hours) by Kilburn et al. (2022). The high rate of elimination in rabbits may be due to their high cardiac output and heart rate (Mitchell & Tully, 2008). Higher clearance in rabbits is observed after administration of other fluoroquinolones; orbifloxacin, norfloxacin, danofloxacin, and moxifloxacin are cleared even faster than levofloxacin with clearance values of 0.9, 0.8, 0.8, and 0.8 mL×g/h, respectively (Fernandez-Varon et al., 2005; Fernandez-Varon et al., 2007; Marín et al., 2008; Marín et al., 2018). These results indicate that parenteral fluoroquinolone administration in rabbits will require frequent dosing. Alternatively, the route of administration could be changed to consider practitioners' convenience and/or reduction of the handling stress of the infected animal.

A low extraction ratio (around 7 %) may indicate that levofloxacin is not fully metabolized and may be excreted unchanged by the kidney (Brown, 1996; Martinez et al., 2006). This suggests the use of orally administered dosage forms (Toutain & Bousquet-Mélou, 2004b). Although extraction ratio values were not

computed in other species in which levofloxacin pharmacokinetics were established, we calculated approximate extraction ratios for the above-mentioned studies. Low levofloxacin extraction ratios were predicted in cats, dogs, and rabbits (around 2 %) based on the clearance and mean animal body weights (Albarellos et al., 2005; Destache et al., 2001; Landoni & Albarellos, 2019; Madsen et al., 2019). In food-producing animals, the levofloxacin extraction rate is also low. Based on data provided in the literature for goats (Goudah & Abo-El-Sooud, 2009), sheep (Goudah & Hasabelnaby, 2010; Patel et al., 2012), and camels (Goudah, 2009) the values are 3.2 %, 3.9 %, and 9.5 %, respectively. The estimated extraction ratio values in all of the animal species investigated indicate similar drug elimination abilities among the species.

As the elimination half-life of levofloxacin for all 3 routes of administration was short, frequent administration, which is potentially stressful to the animal, would be required. The authors, therefore, do not suggest than any of these parenteral routes are suitable for regular clinical use of levofloxacin in the studied dosage form. While the therapeutic efficacy of fluoroquinolones may be inferred through pharmacokinetic-pharmacodynamic surrogate index assessment and the use of the AUC_{24}/MIC ratio, the low AUC value and the inability to quantify levofloxacin in rabbit plasma at 24 hours post drug administration resulted in the inability to perform these surrogate calculations based on the experimental data. Based on the results of this study, a dose of 5 mg/kg of levofloxacin is unlikely to produce a therapeutic effect in rabbits. The calculated effective daily dose for levofloxacin, based on an *Enterobacteriaceae* MIC value of 0.5 μg/mL reported in dogs (Madsen et al., 2019), was 29 ± 8 mg/kg. The estimate agrees with the oral dose of 25 mg/kg in dogs supposed to attain similar therapeutic targets. In rabbit management, the oral route for drug administration (in medicated feed or water) is the most common one used. Levofloxacin is reported to have complete oral bioavailability in 2 pet

mammalian species; dog (104 ± 30 %) (Albarellos et al., 2005; Madsen et al., 2019) and cat (86 ± 43 %) (Albarellos et al., 2005). If this trend in oral bioavailability is similar in rabbits, the effective daily dose of levofloxacin reported in our study could be added to pelleted rabbit food or drinking water. However, as infected animals may lose their appetite while maintaining water intake, we suggest the daily dose could be prepared in 50–100 mL of drinking water (i.e., the average daily water intake of rabbits) (Harcourt-Brown, 2002).

This study is the first to investigate the effect of systemic administration of levofloxacin on some ocular parameters. The high variability in the qualitative parameters of tears between individual animals before and after treatment with levofloxacin made identification of trends difficult. The authors suggest that the dose may have been too small or a single administration insufficient to produce any discernible effects on tear production. The basal level of the tear production assessed with STT method (7 ± 3 mm/min) was slightly higher than those reported for English angora rabbits and Dutch rabbits (5.4 and 4.6 mm/min, respectively) (Rajaei et al., 2016). Regardless, tear osmolarity appeared to decrease slightly but significantly ($p = 0.002$) at 48 hours after drug administration. Therefore, we suggest that levofloxacin administration at 5 mg/kg is unlikely to cause major changes in the qualitative and quantitative properties of tears. However, studies with multiple-dose administration and a larger number of animals are warranted to make solid conclusions.

According to obtained study results, a levofloxacin dose of 5 mg/kg is unlikely to be effective in rabbits. Moreover, a single administration of that dose is unlikely to have any effect on tear parameters. Based on the calculations, a daily dose of 29 mg/kg may be effective for IV administration of levofloxacin.

4.2 Levofloxacin pharmacokinetics and tissue residue concentrations after oral administration in geese

The geese did not show any adverse effects during or after drug treatments. The dose was chosen on the basis of a previous study on chickens (Lee et al., 2017). This is the first study which dealt with the pharmacokinetics of levofloxacin in geese. The drug showed a moderate half-life (7.39 hours) comparable with results from chickens (6.93 hours, (Lee et al., 2017), but was longer than in ducks (2.76 hours), with a slower clearance (geese, 0.28 mL/g×h; ducks, 0.41 mL/g×h) (Aboubakr & Soliman, 2014). The V_{ss} in geese (1.40 mL/g) was in line with the value found in ducks (1.37 mL/g). Levofloxacin showed higher AUC (7.59 µg×h/mL), if normalised for dose, than values reported in ducks (4.89 µg×h/mL) and chicken (5.09 µg×h/mL) (Aboubakr & Soliman, 2014; Lee et al., 2017). Species specific differences, such as variations in metabolic pathways, plasma protein binding or differences in absorption processes, may have caused these variances. After oral administration, levofloxacin showed faster (t_{max}) and higher (C_{max}) absorption in geese than ducks, turkeys and chickens (Aboubakr & Soliman, 2014; Aboubakr et al., 2014; Lee et al., 2017; Patel et al., 2012b; Varia et al., 2009). The different formulations administered, variability in experimental design, climatic conditions or feed management might have contributed to such differences. Levofloxacin's oral bioavailability is high in avian species in general (ducks, 73.6 %; chickens, 59.5 %; leghorn hens, 71.6 %; turkeys, 79.9 %), but is highest in geese (95.6 %), suggesting that the oral route is an appropriate route of administration in birds, and especially geese (Aboubakr & Soliman, 2014; Aboubakr et al., 2014; Patel et al., 2012; Varia et al., 2009).

The MIC of levofloxacin has not yet been determined for bacteria isolated from geese. Regarding the AUC_{24} value obtained in the present study after oral administration (5 mg/kg), levofloxacin in geese appeared be effective against

bacteria at an MIC <0.24 μg/mL. For the MIC against *E. coli* isolated in broilers (0.125 μg/m, Lee et al., 2017), an AUC_{24}/MIC ratio of 136 was obtained, which suggests that the dose regimen in the present study might be effective in geese. Levofloxacin's plasma protein binding has not been evaluated in geese, but has resulted in a low percentage (25 %) in broilers (Lee et al., 2017) and may be considered negligible for the pharmacokinetic-pharmacodynamic surrogate calculation. However, further studies are required to establish if the plasma protein binding of levofloxacin in geese is in line with that found in other avian species.

Levofloxacin was detected in all tissues selected, and the concentration was highest at 6 hours and gradually decreased over 48 hours. Considering that in humans approximately 90 % of levofloxacin is rapidly absorbed from the intestinal tract into the hepatic portal vein and, similarly to other fluoroquinolones, is primarily excreted unchanged from the kidney in the urine (Fish & Chow, 1997). Hence, it was reasonable to expect a higher drug residue in liver and kidney in geese. Probable tropisms related to levofloxacin have not yet been evaluated. The tissue depletion profile found in the present study was in line with that found in chickens (Kyuchukova et al., 2013; Lee et al., 2017). In this study, muscle levofloxacin concentrations, normalised for dose, were higher than concentrations found in chickens (Kyuchukova et al., 2013; Lee et al., 2017). These differences could be due to species specific difference, or the diverse analytical techniques used.

The MRL for fluoroquinolones in poultry liver is about 0.1 μg/g (EMA, 1997, 1999, 2002). On the basis of this value, a preliminary withdrawal time has been computed with the CI of 95 % for liver, resulting in a time of 89.7 hours. Despite the fact that this matched well with the data reported in chickens – 4 days (Ravikumar et al., 2015), caution should be taken because of the small population sample size. Further studies are required to confirm this finding. Drug

penetration in tissue can be described using the $AUC_{tissue}/AUC_{plasma}$ ratio. A ratio value over 1 indicates relatively higher drug concentrations in the tissue than in blood, with potential for tissue accumulation (Bellmann et al., 2004). The $AUC_{tissue}/AUC_{plasma}$ ratios in our study were high in all tissues, and especially in liver. Further studies could clarify this point (e.g. whether levofloxacin may be stored specifically in hepatocytes).

4.3 *In vitro* and *ex vivo* antibacterial activity of levofloxacin against *Pasteurella multocida* and *Escherichia coli* isolated from rabbits

To the best of our knowledge, this study describes for the first-time levofloxacin time-killing curves for *P. multocida* and *E. coli* isolates from rabbits. None of the bacterial isolates included in this study showed resistance to levofloxacin. However, reports are indicating cases of *P. multocida* and *E. coli* resistance to this drug (Saha et al., 2021; Sitovs et al., 2021). MIC values for both *P. multocida* and *E. coli* were low, compared to other pathogens' MIC reported in the literature. Two *P. multocida* isolates (Nr. 7042 and 0634) showed relatively high MIC (0.5 μg/mL). As no clinical breakpoints for levofloxacin for *P. multocida* isolates from rabbits currently exist, applying CLSI M100 (CLSI, 2018b) levofloxacin breakpoints, these isolates could be considered susceptible. Applying fluoroquinolone clinical breakpoints for respiratory *P. multocida* (pradofloxacin, enrofloxacin and danofloxacin) according to the CLSI VET08 (CLSI, 2018a), these isolates would not be considered susceptible, anymore (susceptible defined as MIC ≤ 0.25 μg/mL), but rather intermediate. All other *P. multocida* isolates showed MIC values (0.008–0.03 μg/mL) in line with MIC_{90} values reported for veterinary fluoroquinolones and their active metabolites – difloxacin, enrofloxacin, ciprofloxacin, marbofloxacin, orbifloxacin and pradofloxacin (0.008–0.05 μg/mL) against *P. multocida* (Riviere & Papich, 2018). MIC_{90} values for the same veterinary fluoroquinolones against *E. coli*

(0.03–0.39 μg/mL) were slightly higher compared to *E. coli* MIC values obtained in the present study (0.008–0.03 μg/ mL). Only 15 bacterial isolates were used in our study; thus, it is not yet obvious that levofloxacin is significantly superior to other veterinary fluoroquinolones.

Minimal bactericidal concentration/MIC ratios of levofloxacin were not high in the present study. The median ratios for *P. multocida* and *E. coli* isolates were 2 and 4, respectively. That is similar to ratios obtained from isolates from humans in which, levofloxacin was reported to achieve a reduction in CFU/ mL of ≥ 99.9 % of most aetiology of bacteraemia faster compared to other fluoroquinolones (Akinjogunla et al., 2022). MBC/MIC ratios > 8 were reported to be associated with antibiotic tolerance (Gonzalez et al., 2013). Our pharmacodynamic study results do not suggest levofloxacin tolerance in rabbits.

AUC_{24}/MIC is described as the most important factor to determine efficacy of concentration-dependent antibacterial drugs, including fluoroquinolones (Aliabadi & Lees, 2001). In the present study, the use of *ex vivo* AUC_{24}/MIC was not suitable for pharmacokinetic–pharmacodynamic modelling. The reason for that was bacterial count reduction to the detection limit after 24 hours of incubation with all experimentally obtained levofloxacin concentrations in rabbit serum. All samples from time points collected after IM and SC dose of 5 mg/kg had levofloxacin concentrations higher than 1 MIC for both bacterial isolates used in the time-killing study. *In vitro* AUC_{24}/MIC data were used for modelling instead. AUC_{24}/MIC values obtained for lower levofloxacin concentrations (0.25, 0.5 and 1 MIC, which did not reduce the bacterial counts to the detection limit) provided more data for creating the model. When time-killing curves for *in vitro* and *ex vivo* experiments were visually compared, their similarity provided almost identical bacterial killing patterns. That justifies the use of *in vitro* AUC_{24}/MIC data for modelling.

Slightly slower killing rate was observed in the *in vitro* study compared to the *ex vivo* study. That could be attributable to chemical differences between experimental rabbit serum and commercially available rabbit serum used for the *in vitro* study. Hill coefficient values in both models in this study were high, 5.64 for *P. multocida* and 9.98 for *E. coli*, respectively. These values illustrate the rapid increase in levofloxacin activity with the small increase in the concentration. A slightly less steep slope of 5.21 for levofloxacin against *E. coli* isolated from broiler chickens is reported (Lee et al., 2017). Levofloxacin in this study showed similar AUC_{24}/MIC ratios required for bacteriostatic, bactericidal and bacterial elimination effects for *P. multocida* (20.76, 28.88 and 75.46 hours), compared to marbofloxacin, (20.9, 45.2 and 71.7 hours) for *P. multocida* isolates from pigs (Dorey et al., 2017) and slightly lower than marbofloxacin for isolates from calves (48.6, 64.9 and 74.8 hours, respectively) (Potter et al., 2013). AUC_{24}/MIC ratios for bacteriostatic, bactericidal and bacterial elimination effects in this study for *E. coli* (27.25, 32.49 and 59.62 hours) were higher compared to values reported in chickens – 18.77, 24.02 and 36.27 hours, respectively (Lee et al., 2017). AUC_{24}/MIC ratios obtained by for danofloxacin against *E. coli* isolated from turkeys were significantly lower (0.42, 1.90 and 6.73 hours) (Haritova et al., 2006) and for enrofloxacin against *E. coli* isolated from chickens were much higher (257.40 and 2794.40 hours for bacteriostatic effect and bacterial elimination, respectively) (Haritova & Russenova, 2010). Despite the previous conclusion from our levofloxacin pharmacokinetic study in rabbits, that a dose of 5 mg/kg levofloxacin is unlikely to be effective in rabbits, the *ex vivo* time-killing curves showed a reduction of the bacterial counts to the limit of quantification at 24 hours. Calculated daily doses appear to be even lower. In our pharmacokinetic-pharmacodynamic study, proposed doses per day required for bacteriostatic, bactericidal and bacterial elimination effects (0.25–1.43 mg/kg daily) were lower compared to the levofloxacin doses

calculated for broilers (1.1–4.3 mg/kg daily) (Lee et al., 2017) and for rabbits – 29 mg/kg daily (Sitovs et al., 2020). Previously reported dose was up to 100-fold higher that doses obtained in this study. Compared to the dose reported by previously, this study utilizes experimental pharmacodynamic data from susceptible bacterial time-killing curves, while previous pharmacodynamic data were from the published literature. Difference in doses between two studies originates from the higher AUC_{24}/MIC used in calculations – 72 hours, as reported the literature (Madsen et al., 2019) and with lower MIC values used in calculations. In the current study, doses were calculated based on the experimentally obtained MIC values, while previously we used MIC = 0.5 μg/mL (Sitovs et al., 2020). Doses calculated using highest *P. multocida* MIC (0.5 μg/mL) are less different from the dose reported in the rabbit levofloxacin pharmacokinetics study, 8.30, 11.55 and 30.18 vs. 29 mg/kg daily. Real, rather than theoretical MIC values were used in dose calculations here. As we determined that levofloxacin bioavailability in rabbits after IM and SC routes of administration is around 100 % it is considered complete. From the point of view of bioavailability, there is no difference between IM and SC administration for suggested daily doses. However, compared to SC, the IM administration is generally more painful and considering relatively small muscle mass in rabbits, rarely used (Shellim, 2011). Additional factors that can contribute to the calculation of daily doses are associated with changes in fluoroquinolone pharmacokinetics in rabbits in the diseased state. For example, *P. multocida* infection results in the change in the primary pharmacokinetic parameter clearance for marbofloxacin (Abo-El-Sooud & Goudah, 2010). If the same could apply to levofloxacin, that may impact the calculation of the dose. To prove this, an additional pharmacokinetic study of levofloxacin in infected animals would be required. There are also some known limitations in our study. First, a small number of animals in the pharmacokinetic study do not cover all possible inter-

animal difference in clearance, necessary for dose calculation. Impact of infection was not considered in this study, as serum samples from healthy rabbits were used. Small number of bacterial isolates used in this study does not represent all MIC variability within one isolate and among population of wild-type pathogenic bacteria in rabbits. The *ex vivo* study does not consider the immune response of the animal organism, which could contribute to the elimination of bacteria and possibly allow lower doses of the antimicrobial agent to be used. The effect of inoculum concentration was not assessed in terms of antimicrobial activity of levofloxacin. Finally, this study did not predict further resistance development against levofloxacin for the tested microbial isolates, and no mutant prevention concentrations values were obtained in this study. However, fluoroquinolone resistance is an important issue in global health (Brown, 1996; WHO, 2019). Lastly, consideration of antimicrobial stewardship principles (Lloyd & Page, 2018) in the selection and possible use of levofloxacin in rabbits has to be considered.

Conclusions

Levofloxacin shows favourable pharmacokinetic profiles and is generally well tolerated in rabbits and geese. Levofloxacin dose of 5 mg/kg is likely to be effective in studied animal species and even lower doses are active for highly susceptible bacteria. Our studies provide preliminary examination of key elements of the dose regimen in rabbits and geese. Highest concentrations of levofloxacin were observed in the liver and kidneys, suggesting possible drug accumulation.

The results of this study do not encourage the use of levofloxacin instead of conventional veterinary antibiotics, but provide and up-to-date information on levofloxacin, that will help veterinary practitioners and scientists to make informed choices regarding appropriate levofloxacin use.

Proposals

Safe and effective use of an antibiotic requires require additional issues to be addressed.

1. Despite susceptibility of microbial isolates have been reported in multiple studies, it does not exclude further resistance development. The resistance development mechanisms and resistance possibility against levofloxacin for the microbial isolates of interest is advised to be evaluated using the mutant-prevention concentration determination.
2. The dose optimization for levofloxacin in veterinary medicine is advised to be performed. This could be achieved by using population pharmacokinetics methods and utilizing extensive MIC data from microorganisms of interest.
3. The impact of the infected state is advised to be evaluated in order to account for the pharmacokinetic differences in real clinical cases where levofloxacin could be used.
4. Levofloxacin MRL values for food producing are advised to be defined in countries where levofloxacin is used in food-producing animals.

List of publications, reports and patents on the topic of the Thesis

Publications

1. Sitovs, A., Voiko, L., Kustovs, D., Kovalcuka, L., Bandere, D., Purvina, S., & Giorgi, M. (2020). Pharmacokinetic profiles of levofloxacin after intravenous, intramuscular and subcutaneous administration to rabbits (*Oryctolagus cuniculus*). Journal of veterinary science, 21(2), e32. https://doi.org/10.4142/jvs.2020.21.e32

2. Sartini, I., Łebkowska-Wieruszewska, B., Sitovs, A., Lisowski, A., Poapolathep, A., & Giorgi, M. (2021). Levofloxacin pharmacokinetics and tissue residue concentrations after oral administration in Bilgorajska geese. British poultry science, 62(2), 193–198. https://doi.org/10.1080/00071668.2020.1842855

3. Sitovs, A., Sartini, I., & Giorgi, M. (2021). Levofloxacin in veterinary medicine: a literature review. Research in veterinary science, 137, 111–126. https://doi.org/10.1016/j.rvsc.2021.04.031

4. Sitovs, A., Skadins, I., Purvina, S., & Bandere, D. (2023). *In vitro* and *ex vivo* antibacterial activity of levofloxacin against *Pasteurella multocida* and *Escherichia coli* isolated from rabbits (*Oryctolagus cuniculus*) - A preliminary study. Journal of veterinary pharmacology and therapeutics, 10.1111/jvp.13383. Advance online publication. https://doi.org/10.1111/jvp.13383

Reports and theses at international congresses and conferences

1. Sitovs A., Kustovs D., Giorgi M., Kovalcuka L., Voiko L., Purviņa S., Bandere D. (2019). Levofloxacin Assay in Rabbit Plasma: UPLC Method Optimisation and Validation – Rīga Stradiņš University International Conference on Medical and Health Care Sciences "Knowledge for Use in Practice": Abstracts April 1–3, 2019, 396

2. Sitovs A., Kustovs D., Giorgi M., Kovalcuka L., Voiko L., Purviņa S., Bandere D. (2019). Pharmacokinetics of levofloxacin after three different routes of single parenteral administration to domestic rabbits – 4th conference "Current approach to health and diseases in animals and humans" Lublin, Poland: Abstracts, September 19–20, 2019, Oral presentation II.O.7

3. Sitovs, A., Voiko L., Purviņa S., Bandere D., Giorgi M. (2020). Levofloxacin pharmacokinetics and efficacy prediction in domestic rabbits (*Oryctolagus cuniculus*). ICECVM 2nd International Conference of the European College of Veterinary Microbiology, Online: Abstracts, October 25–27, 2020, Oral presentation

4. Sitovs A., Skadins I., Dovbenko A., Purvina S., Bandere D. (2021). Levofloxacin efficacy against *E. coli* isolated from pet rabbits: a pilot study – Rīga Stradiņš University International Conference on Medical and Health Care Sciences "Knowledge for Use in Practice": Abstracts March 24–26, 2021, 393

5. Sartini I., Łebkowska-Wieruszewska B., Sitovs A., Lisowski A., Poapolathep A., Giorgi M. (2021). Levofloxacin pharmacokinetics and tissue residues after oral administration in geese - Rīga Stradiņš University International Conference on Medical and Health Care Sciences "Knowledge for Use in Practice": Abstracts March 24–26, 2021, 394

6. Sitovs A., Sartini I., Giorgi M. (2021). The role of levofloxacin in veterinary medicine currently - Rīga Stradiņš University International Conference on Medical and Health Care Sciences "Knowledge for Use in Practice": Abstracts March 24–26, 2021, 398

7. Sitovs A., Skadins I., Bandere D. (2023). Levofloxacin Antibacterial Activity against *Pasteurella multocida* Isolated from Domestic Rabbits - Rīga Stradiņš University International Conference on Medical and Health Care Sciences "Knowledge for Use in Practice": Abstracts March 30 – 31, 2023

8. Sitovs A., Skadins I., Purvina S., Bandere D. (2023). *In vitro* and *Ex vivo* Levofloxacin Antibacterial Activity against *Escherichia coli* Isolated from Domestic Rabbits - Rīga Stradiņš University International Conference on Medical and Health Care Sciences "Knowledge for Use in Practice": Abstracts March 30–31, 2023

9. Sitovs A., Skadins I., Purvina S., Bandere D. (2023). *In vitro* and *ex vivo* antibacterial effects of levofloxacin against *Pasteurella multocida* and *Escherichia coli* isolated from rabbits- EAVPT 15th International congress of the European Association for Veterinary Pharmacology and toxicology, Bruges, Belgium: Abstracts July 2–5, 2023

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