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Pharmacokinetic studies of different drugs in geese

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Abstract

This PhD research is composed by different pharmacokinetic studies and tissue residue analysis performed in geese, an underestimated species that in some countries have been domesticated since long time for the production of meat, eggs, and feathers.

One of the main objectives of veterinary pharmacology is preserve food producing animals' health, preventing potential diseases, increasing the animals' performances and, on the other hand, to guarantee an adequate amount of food with the lowest economic losses. It is well known that for a good livestock production the use of drugs is necessary. Pharmaceutical companies invest big budgets and efforts to elucidate the pharmacokinetics, pharmacodynamics and safety profile of drugs in major food producing species. Unfortunately, the economic return of these studies is not positive when minor species are accounted. Often even minor species require therapeutic treatments, and since no information is available, doses and potential therapeutic effects are extrapolated by other animals. This dangerous practice can lead to drug ineffectiveness or toxicity and since no MRL and withdrawal time are available residues may be present in the animal's products intended for human consumption.

The goose belongs to the minor species food producing animal class and very little information is present about drugs in this bird. A deep knowledge of the pharmacokinetic characteristics and tissue residue profile of a drug in geese is the starting point of the process that is essential for the rational drug usage, ensuring an efficacious treatment in the goose as well as the consumers' safety.

In order to achieve this target, different drugs belonging to the most important classes of drugs used in veterinary medicine have been studied. Five antimicrobial drugs (marbofloxacin, danofloxacin, levofloxacin, doxycycline, amoxicillin), two painkiller drugs (meloxicam, acetaminophen) and one antiparasitic drug (ivermectin). For each of these drugs an appropriate analytical method has been validated for their quantification according to the European Medicines Agency guideline. The selected drugs, and in some cases their metabolites, have been quantified in different matrices (e.g. plasma, different animal tissues) using high-performance liquid chromatography with ultraviolet or fluorescence detector as well as liquid chromatography - tandem mass spectrometry. The pharmacokinetic parameters have been carried out using a pharmacokinetic software and drug plasma concentration profiles have been provided after different routes of administration. The residues analysis has been performed to describe the accumulation of the drug in the edible tissues and for the calculation of an appropriate withdrawal time, a crucial factor for ensuring food safety. In some cases, an *in silico* pharmacokinetic simulation has been performed to reproduce the in-field breeding condition. The PK/PD surrogates (when available) have been used to predict if the achieved plasma concentrations might be therapeutic.

In addition, under the same attitude to "bring new knowledge in the veterinary pharmacology field" novel drugs have been tested in different animal species and reviews published to facilitate the studies of other colleagues.

1. GEESE: A BRIEF OVERVIEW

Increasing abundance of geese in North America and Europe constitutes a major conservation success of the last century. Almost 60 different goose breeds exist, with many located in Eastern Europe (Buckland and Guy 2002). Geese belong to the family Anatidae which includes two genera, *Anser* and *Branta* which differ for a number of physical characters. For instance, *Anser* genera tend to have uniform plumage coloring, in greys, brown and white, while their bills and legs are pink, orange or yellow. The *Branta* geese have more boldly patterned black or dark brown and white plumage, and their bills and legs are black. *Anser* geese have quite prominent tooth-like serrations along the cutting edge of their upper mandible which are very small or absent in the *Branta* genera. Geese, included in the genus *Anser,* were one of the first animals to be domesticated. There are two main types of domestic geese. The first are thought to have their origins in Europe, descendants of the wild Greylag goose (*Anser anser*) and the second are thought to have their origins in Asia, descendants of the wild Swan goose (*Anser cygnoide*s). Bilgorajska geese, the subject of this thesis, belong to the *Anser* genera have the physical characteristics typical of this genus. The Bilgorajska goose (*Anser anser domesticus*) is a primitive breed from north-eastern Poland (Bilgoraj region) and is actively preserved because of its genetic significance (Książkiewicz 2006). These birds are entirely white, with feathers that closely adhere to the body. The beak and legs are orange-red colored. They are characterized by a carcass low in fat and solid muscles (Pudyszak 2006).

Figure 1. Bilgorajska geese

Geese are defined as an underestimated species from FAO (Food and Agriculture Organization), since have remained neglected despite the relevant advantages in their breeding (FAO, 2019). From a practical point of view, geese adapt easily to captivity, to different climates and are easily managed. Moreover, it is one of the fastest-growing avian species, characterized by strong adaptability. Geese have a large size, and this make it less vulnerable to predator attacks and seem to be more resistant to different diseases compared to common other avian species. Geese supply nutritious meat, eggs, and fat for cooking, as well as soft down and feathers for bedding and clothing, which may represent a supplementary income for the farmers (Kozák 2021).

Consumption of goose products is of relatively minor importance but has increased greatly during the last decades. The goose meat is consumed for its tenderness and its delectable taste (Kozák 2021). It has low-fat content high in monounsaturated oleic, linolic and arachidonic acid. In particular, the meat of geese raised on grassland is high in polyunsaturated fatty acids. Thus, goose meat can be considered as a healthy food (Okruszek et al 2013). In the world's overall meat production, goose meat is of minor importance compared to other poultry species such as

chickens (Pingel 2004). According to FAO data the total production of goose meat reached 2.7 million tons in 2019: 95.9% of goose meat was produced in Asia (China, with a 2.6 million tons) and 2.4% in Europe (i.e., Poland, Hungary) (FAO, 2019).

Moreover, a large part of the population considered the fatty liver (foie gras) as the most delicious part of the goose. Nowadays, in Europe, the countries with a great tradition of goose liver production are France and Hungary (Kozák 2021). Fatty liver is produced via cramming following the Commission Regulation/EC/No 543/2008, and only goose liver weighing above 400 g can be marketed as foie gras. However, fatty liver production has been banned in some countries such as Norway and Germany due to the animal welfare criticism. Even if in some cases goose eggs are not eaten due to consumer habits, in some countries are also used for human consumption. In fact, nutrient composition of the goose egg is comparable to that of other poultry species, and result important for their rich nutriment, essential amino and fatty acid, vitamin and mineral contents. Finally, feather and down remain important base material in several products of the textile industry (Kozák, 2021).

The health and productive performance of commercial geese is supported via modern pharmaceutical management and facilities, nutritional practices and genetic improvement. Infections, caused by pathogens such as *Mycoplasma spp*. or *Pseudomonas spp*., are common in geese, chickens, turkeys, ducks and ostriches. These pathogens can infect eggs, destroy embryos and, consequently, lead to a significant economic loss (De Vos et al. 2009; Stipkovits and Szathmary 2012). Thus, poultry health is an important factor that constantly requires new protocols in

pathogen prevention, control and treatment. In large-scale farms, administering pharmacological treatments is challenging and generally drugs are provided using medicated feed or drinking water. However, geese are often considered domestic animals or included in production settings backyard geese and small flock free-ranging operations where specific pharmacological treatments may be required.

Nevertheless, when goose breeding is intended for human consumption the assessment of the amounts of residues in goose tissues is required to ensure the safety of the consumers.

2. CHEMICAL AND PHARMACOLOGICAL PROPERTIES OF THE SELECTED DRUGS

2.1 Antimicrobial drugs

Antimicrobial drugs are one of the most important pharmaceutical classes used in domestic and food-producing animals. The most commonly used antimicrobial drugs in animals belong to six major classes: β-lactams, tetracyclines, aminoglycosides, macrolides, sulphonamides and fluoroquinolones. The development of resistance to these different classes has been reported and become a recent and urgent issue among microbiologists, physicians and veterinarians (Nakamura 1997, European Food Safety Authority, 2012). The spread of bacterial resistance in animals and humans is mainly due to over- and/or improper- use of antimicrobial drugs. To prevent the occurrence of resistant strains, it is necessary a deep knowledge on the pharmacokinetic and pharmacodynamic profile of a drug in the species of interest. Significant inaccuracies may result when doses and effects of drugs for the species of interest are predicted based on extrapolation from other

species which might have marked differences in their metabolism of drugs and physiological processes (Toutain et al. 2010). Thus, some of the drugs more commonly used in poultry have been investigated in the selected species in the present work. These drugs and their classes are listed and briefly detailed below.

2.1.1 Fluoroquinolones

The fluoroquinolones represent an expanding class of broad-spectrum antibacterial which cover a multitude of Gram-negative and anaerobic species. They belong to the last generations classes of quinolones and have a larger spectrum of activity due to some modification on the chemical structure. A fluorine molecule at the 6-position of the basic quinolone structure and a piperazine substitution at the 7-position was found to enhance quinolone antibacterial activity and to increase the extent of oral drug absorption and tissue distribution (Figure 2) (Riviere and Papich 2010).

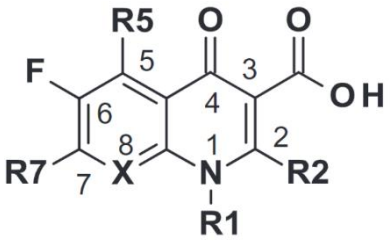


Figure 2. General fluoroquinolone's chemical structure

Fluoroquinolones can inhibit two enzymes involved in DNA synthesis, both of which are DNA topoisomerases (DNA gyrase and topoisomerase IV), that are missing in human cells and that are essential for bacterial DNA bacterial replication, resulting in a specific and bactericidal effect (Figure 3). DNA topoisomerases are responsible for the separation of the strands of duplex bacterial DNA, inserting

another strand of DNA through the break, and then resealing the originally separated strands (Blondeau 2004). DNA gyrase introduces negative super helical twists in the bacterial DNA double-helix ahead of the replication fork, catalyzing the separation of daughter chromosomes. Its action is essential for initiation of DNA replication and allows for binding of initiation proteins. Topoisomerase IV is responsible for decatenation, allowing segregation into two daughter cells at the end of a round of replication (Blondeau 2004).

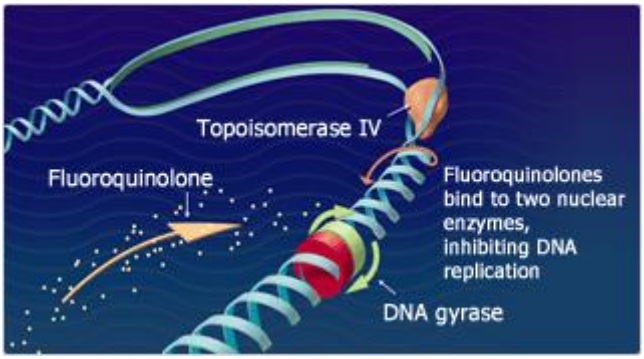


Figure 3. Fluoroquinolone's mechanism of action

The activity of the early compounds of this class was limited to the treatment of urinary tract infections due to their limitations in absorption and distribution, while fluoroquinolones showed significant potency not limited to Gram-negative, but also against Gram-positive bacteria such as *P. aeruginosa*.

In Figure 4 the observed structure-activity relationships (SARs) of fluoroquinolone core substitutions are depicted.

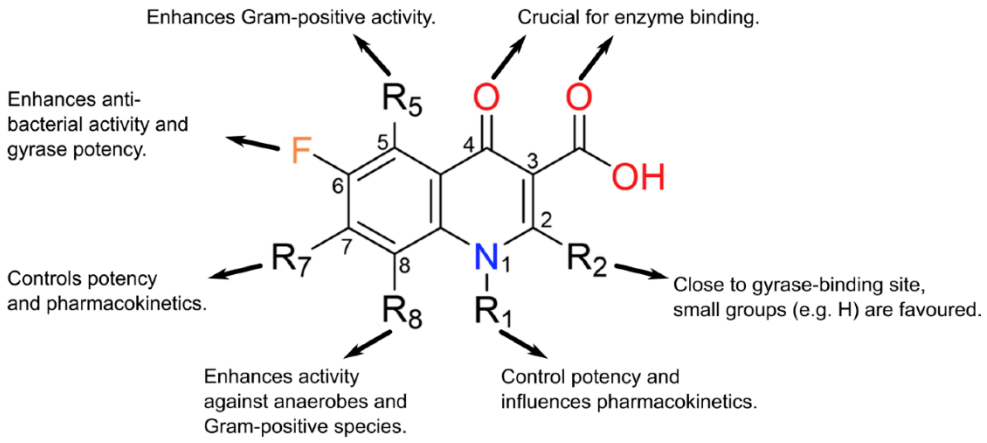


Figure 4. structure-activity relationships of fluoroquinolones.

The carboxyl group in position 3 and the ketone at position 4 are essential for antibacterial activity and no better modifications are possible. Fluoride in position 6 gives a wider spectrum action, a greater potency than any other halogen or substituent. The N substitution in position 1 with an ethyl, cyclopropyl, fluorophenyl, leads to a wider spectrum of action and a substitution at position 7, with a piperazine group, increases antibacterial activity against *Pseudomonas.* Position 8 is associated to side effects at the central nervous system level (CNS). Thus, it is preferable to have carbon instead of nitrogen in this position. Although, the substitutions with a methoxy or cyan group, in this position, can increase the activity against anaerobic bacteria (Pham et al. 2019).

2.1.1.1 Marbofloxacin

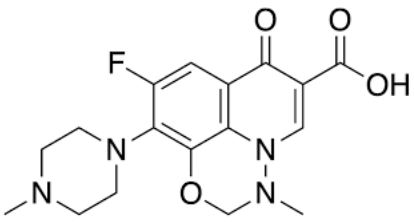


Figure 5. Chemical structure of marbofloxacin

Marbofloxacin (Figure 5) is a veterinary fluoroquinolone antimicrobial with a bactericidal broad spectrum of activity. Susceptible bacteria include *Staphylococcus spp*., *Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*, and *Pasteurella spp. Pseudomonas aeruginosa* is moderately susceptible but requires higher concentrations. Marbofloxacin has poor activity against streptococci and anaerobic bacteria. It has been developed exclusively for veterinary use. Its use is approved in cats, dogs, horses and in different food-producing animals (i.e., swine, cattle). Infections treated with marbofloxacin include skin and soft tissue, bone, urinary tract infections, pneumonia, and infections caused by intracellular organisms (Papich 2015). Within birds, pharmacokinetic studies have shown several differences in area under the concentration-time curve (AUC) and maximum concentration (C_{max}) in plasma after administration of the same dose of marbofloxacin (Carpenter et al. 2006; Yuan et al. 2011; Lashev et al. 2015; Urzúa et al. 2016), suggesting variable effective doses between different avian species.

2.1.1.2 Danofloxacin

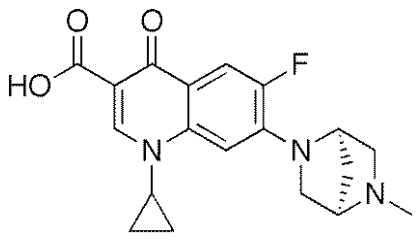


Figure 6. Chemical structure of danofloxacin

Danofloxacin (which molecular structure is reported in Figure 6), like other fluoroquinolones, has activity against a broad spectrum of bacteria, including Gram-negative bacilli, especially *Enterobacteriaceae* (*Escherichia coli, Klebsiella*,

and *Salmonella spp*.) and some Gram-positive cocci, such as *Staphylococcus spp*. It has good activity against pathogens in cattle, such as *Pasteurella multocida*, *Mannheimia haemolytica*, and *Histophilus somni*. It is used in domestic animals (cats and dogs), but also in cattle to treat bovine respiratory disease, in swine and horses (Papich 2015). Danofloxacin is available as injectable solution or oral formulation and several previous studies have described its therapeutic efficacy against different bacterial and *Mycoplasma*-related infectious diseases in poultry (Jordan et al. 1993; Migaki et al. 1993; Charleston et al. 1998; Zhang et al. 2017). Further, several pharmacokinetic studies have been performed in avian species, including chickens (Lynch et al. 1994; Knoll et al. 1999; Zeng et al. 2010), turkeys (Haritova et al. 2006), chukar partridges (Corum et al. 2019), pheasants, guinea fowls, quails (Haritova et al. 2013; Dimitrova et al. 2014), pelicans (Schmitt et al. 2019) and ducks (Goudah and Mouneir 2009). Several differences in pharmacokinetic profiles and parameters have been reported among these birds suggesting variable effective doses.

2.1.1.3 Levofloxacin

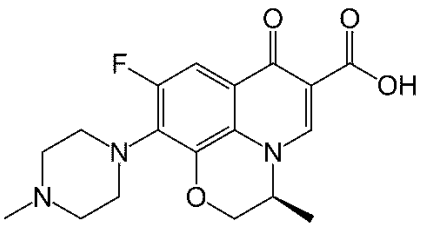


Figure 7. Chemical structure of levofloxacin

Levofloxacin (which molecular structure is reported in Figure 7) is the L-isomer (S-enantiomer) of ofloxacin and is more active *in vitro* against Gram-positive bacteria and anaerobes than some other fluoroquinolones. Levofloxacin is a human-labeled antibiotic in most of the world areas, but it is also approved as veterinary drug to treat various bacterial infections in some countries. Its spectrum of activity includes Gram-negative bacilli of the *Enterobacteriaceae* and fluoroquinolone-susceptible *Pseudomonas aeruginosa*. It also has activity against some Gram-positive cocci, including *Staphylococcus spp*. It also has activity against some anaerobic bacteria and other organisms (e.g., *Chlamydia, Mycoplasma, and Mycobacterium spp*.) (Riviere and Papich 2010). Levofloxacin is a human drug without an approved label indication for veterinary species. Although not approved for animals in Europe and the US, in some country, such as Argentina, Russia and China it can be prescribed by veterinarians to treat a variety of infections, including skin infections, pneumonia, and soft tissue infections (Landoni and Albarellos 2018; Sitovs at al. 2021). For this reason, detailed research was needed to clarify the use of levofloxacin in veterinary species worldwide. Thus, in the last year we have published a review to clarify this issue (Sitovs at al. 2021). Levofloxacin pharmacokinetics have been investigated in ducks (Aboubakr and Soliman 2014), chickens (Varia et al. 2009; Patel et al. 2012; Kyuchukova et al. 2013; Lee et al. 2017), quails (Aboubakr 2012) and turkeys (Aboubakr et al. 2014). Several differences in pharmacokinetic profiles and parameters have been reported among these birds suggesting variable effective doses.

2.1.2 Tetracycline

The tetracyclines are a group of four-ringed amphoteric compounds that differ by specific chemical substitutions at different points on the rings (Figure 8).

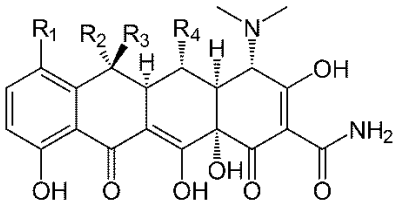


Figure 8. Basic structure of a tetracycline.

Tetracyclines have activity against both Gram-positive and Gram-negative bacteria. They also have activity against atypical pathogens such as *Mycoplasma*, blood-borne pathogens, and organisms such as *Rickettsia* transmitted by ticks and other parasites. Tetracyclines acts by binding to the 30S ribosomal subunit of the bacteria. Consequently, these compounds interfere with the binding of aminoacyl-tRNA to the messenger RNA molecule/ribosome complex, thereby affecting the bacterial protein synthesis (Figure 9). The action of tetracyclines is usually bacteriostatic (Riviere and Papich 2010).

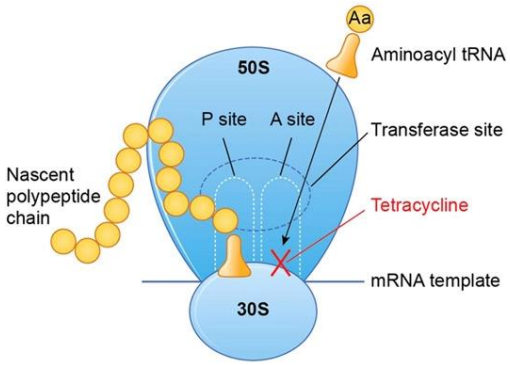


Figure 9. Mechanism of action of tetracycline.

2.1.2.1 Doxycycline

Doxycycline is one of the most popular tetracycline used in small animals and birds (Figure 10).

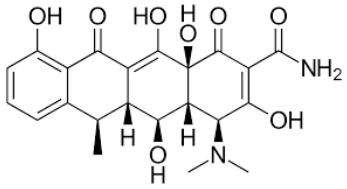


Figure 10. Doxycycline chemical structure.

Its broad spectrum of activity includes many bacteria, some protozoa, *Rickettsia spp*., and *Ehrlichia spp*. Doxycycline is usually used in the treatment of vector-borne diseases in animals, such as those transmitted by ticks and fleas. It is used for treating infections caused by bacteria, some protozoa, *Rickettsia spp*., and *Ehrlichia spp*. Doxycycline has been used in cats, dogs and to treat infections in other species, including respiratory tract disease and systemic colibacillosis in poultry and anaplasmosis in splenectomies calves (Papich 2015). An important use of doxycycline is in birds: it has become a treatment of choice for psittacosis caused by *Chlamydophila psittaci* due to its good oral absorption, tolerance, and efficacy. Doxycycline is indicated for the prevention and treatment of respiratory and gastrointestinal infections in poultry caused by different bacterial pathogens (EMA, 2010). Doxycycline is used in birds at doses of 10–20 mg/kg for 3–5 days as water-soluble doxycycline hyclate powders or oral solutions for administration via drinking water (EMA, 2010). The pharmacokinetics of doxycycline have been established in various avian species including chickens (Anadón et al. 1994; El-Gendi et al. 2010; Laczay et al. 2001; Hantash et al. 2008; Soliman et al. 2015;

Yang et al. 2016, 2018), ostriches (Abu-Basha et al. 2006), ducks (Bratoev et al. 2016; Yang et al. 2015a) and turkeys (Santos et al. 1996).

2.1.3 β-lactams

β-lactam antibiotics have a 4-membered β-lactam ring in their structure which is the active chemical component (Figure 11).

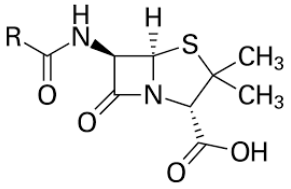


Figure 11. Penicillin chemical structure.

β-lactams includes 6-membered ring–structured penicillins, monobactams, and carbapenems; and 7-membered ring–structured cephalosporins and cephamycins. These classes differ in their chemical structure and in the β-lactams susceptibility to β-lactamase action (Riviere and Papich 2010).

β-lactam antibiotics are considered to be bactericidal in a time-dependent manner. Their mechanism of action is based on the inference on the bacterial cell wall synthesis and on the disruption of the bacterial cell wall integrity. Thus, these drugs kill bacteria by inhibiting the synthesis or damaging the cell wall. In brief, β-lactams inhibit the transpeptidation reaction responsible for the cross-linking formation between the N-acetylglucosamine and N-acetylmuramic acid units which formed the cell bacterial wall. Consequently, the cell wall becomes weak resulting in the rupture of the bacteria. Inhibition of this transpeptidation reaction by acetylating the enzyme is one of the sites of action for β-lactams. The binding sites for β-

lactams are called penicillin-binding proteins (PBPs), which are the enzymes that form the cell wall (Riviere and Papich 2010).

The β-lactam antibiotics are time dependent in their activity. These compounds are slowly bactericidal because of the slow rate of acetylation of the PBP, and the time of drug concentration above the minimum inhibitory concentration (MIC) (T> MIC) is an index which can predict their clinical success (Toutain et al. 2002).

Penicillins contains a fused ring system, the β-lactam thiazolidine and are divided in categories based on their synthesis and spectrum of action. These include the natural penicillins (e.g., penicillin G), aminopenicillins (e.g., ampicillin, amoxicillin), antistaphylococcal penicillins (e.g., oxacillin), and the extended-spectrum penicillins (e.g., piperacillin).

Aminopenicillins are active against many *Streptococcus spp*., non-penicillinase-producing *Staphylococcus spp*. and some Gram-positive such as penicillin G. Moreover, they can penetrate through the outer membrane of Gram-negative bacilli better than penicillin, and this increases their spectrum of activity. In fact, it comprises some *Enterobacteriaceae*, including strains of *E. coli*, *Proteus mirabilis*, and *Salmonella*. However, different bacteria are resistant (Papich 2015).

2.1.3.1 Amoxicillin

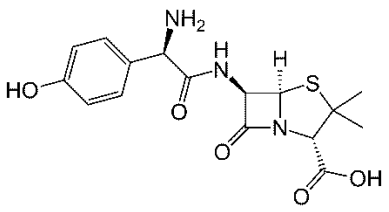


Figure 12. Amoxicillin chemical structure.

Amoxicillin (Figure 12) belongs to the subclass of aminopenicillins, and it has been used in the treatment of a variety of diseases in domestic animals. It has a broader spectrum of activity compared to penicillin G, and it can be administered orally. It is relatively inexpensive and safe. Aminopenicillins have a wide spectrum of activity since they can penetrate the outer layer of Gram-negative bacteria better than penicillin G. In fact, amoxicillin spectrum of action also includes some of the Gram-negative bacteria (e.g., susceptible *Enterobacteriaceae*). To overcome resistance the β-lactamase inhibitors clavulanic acid is used in association with amoxicillin to increase the spectrum (Riviere and Papich 2010).

Amoxicillin is used for a variety of infections in all species, including lower urinary tract infections, soft tissue infections, and pneumonia. Because of a short half-life, frequent administration is needed for treating Gram-negative infections. It is used to treat certain bacterial infections in poultry, included respiratory tract infections (Papich 2015). It is often used in cases of injury, to help prevent pasteurellosis from bites or scratches (Elviss et al. 2009). The pharmacokinetics have been previously investigated in chickens (Anadón et al. 1996; Abo El-Sooud et al. 2004; Krasucka and Kowalski 2010; Kandeel 2015; Ledesma et al. 2018).

2.2 Painkiller drugs

Treatment of pain conditions in veterinary medicine is challenging, especially nowadays, considering the growing interest in animal welfare. The concepts of "Replacement, Reduction and Refinement", called the '3Rs', is applied in laboratory animals as well in farm animals to minimize pain incidence (Russell and Burch 1959). A study should be design and performed to minimize unnecessary

pain in experimental animals based on a new approach the '3S' "Suppress, Substitute and Soothe pain". It is based on the possibility to "suppress" the procedures or environments that are a source of pain, to "substitute" such procedures by others causing less pain and to "soothe" pain when it cannot be avoided (Guatteo et al. 2012). This approach is used to minimized pain in farm animals.

The owners demand of a higher level of care lead to the need of more effective and innovative veterinary therapies (Giorgi and Kim 2013). That can be apply not only for domestic, but also for food producing animals since, as a basis, analgesia should be administered to animal species when dealing with conditions known to be painful in humans. It can happen in case of injury, external trauma or in case of painful pathology such as arthritis. Furthermore, in some painful procedure such as piglets' tail-docking or ruminant dehorning the use of painkiller drugs is recommended. Nowadays, different species such as geese or swine are considered domestic animals rather than food producing animals and owners require for safe and effective analgesic or anti-inflammatory treatments.

However, the challenge in the pain management of bird (geese), is to recognize and evaluate the pain status. Pain evaluation is complicated in this species because of several factors such as differences between acute and chronic pain, behavioral differences between domestic and wild animals and inter subject variability to pain sensitivity. However, if an injury or a procedure (e.g., plucked practice) involves pain, tissue damage, changes in posture, temperament or behavior can be observed. Recently, pain has been shown to affect animal welfare and production, and the interest in the field of analgesia has been drastically increasing (De Vito 2015). As

a basis, analgesia should be administered to birds when dealing with conditions known to be painful in humans. Joint and bone lesions received extensive attention in the poultry industry, and accounts for significant morbidity and economic losses in young broilers and turkeys. Moreover, waterfowl are usually affected by degenerative joint disease and osteoarthritis. Persistent pain can have a negative effect on homeostasis and healing. Untreated, this could eventually manifest as debilitating chronic pain. In some cases, geese are considered domestic animals, and a treatment for pain may be useful in urbanizing setting such as production settings backyard geese and small flock free-ranging operations.

One of the most used classes to manage pain, fever and inflammation in veterinary species are the non-steroidal anti-inflammatory drugs. The reasons for their large use are diverse: they have a good efficacy, are not restricted substances, are generally well oral absorbed, and their safety profiles have been defined in most of the animals. From the other side it is even known that some drugs of this class can produce severe (life-threatening) side effects in some animals' species. Thus, before recommending the use of these drugs in a new animal species specific PK/PD studies need to be undertaken in the target animals.

2.2.1 Non steroidal-anti inflammatory drugs (NSAIDs)

NSAIDs are commonly used in veterinary medicine to treat pain, fever and inflammation in various conditions.

NSAIDs act inhibiting the Cyclo-Oxygenase (COX) enzyme in different organs (Riviere and Papich 2010). COX is the enzyme that converts arachidonic acid to form prostanoids, including prostaglandins (PG) and thromboxanes. These

compounds are essential biological mediators during the inflammation process. Two different COX isoforms have been discovered. COX-1 is a constitutive enzyme found in many organs under normal conditions and it is involved in body's homeostasis with functions including maintenance of mucosal epithelium integrity and control of renal hemodynamics and glomerular filtration rate. COX-2 is the isoform which is mainly up-regulated during inflammatory processes since it is responsible of the production of prostanoids such as PGE_2 and PGI_2 that are just involved in inflammatory and pathological processes (Giorgi and Kim 2013; Graham et al. 2013). A third COX isoform (COX-3) was discovered but his role is not fully understood (Graham et al. 2013).

2.2.1.1 Meloxicam

Meloxicam (Figure 13) is an NSAID of the oxicam class, which shows a relatively COX-1 sparing compared with older NSAIDs, and a preferential inhibition of COX-2. For this reason, meloxicam may be associated with a decreased incidence of adverse effects (Gates et al. 2005).

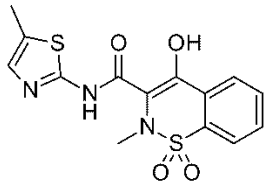


Figure 13. Meloxicam chemical structure.

Meloxicam is used to decrease pain, inflammation, and fever. It has been used for the acute and chronic treatment of pain and inflammation in dogs and cats. One of the most common uses is osteoarthritis, but it has also been used for pain associated with surgery. Meloxicam is also successfully used extra label in many exotic and

zoo animals, including reptiles and birds, for treatment of pain and inflammation. It has shown high variability in pharmacokinetics among bird species such as domestic chickens (Baert and De Backer 2003; Souza et al. 2018), rock pigeons, common ostriches, mallard ducks, domestic turkeys (Baert and De Backer 2003), various vulture species such as *Gyps coprotheres* (Naidoo et al. 2008), parrots (Molter et al. 2013; Montesinos et al. 2017), red-tailed hawks, great horned owls (Laçasse ate al. 2013), American kestrels (Summa et al. 2017), and some flamingo species such as *Phoeconipterus ruber* (Lindemann et al. 2016)

2.2.1.2 Acetaminophen

Acetaminophen (Figure 14), also known as paracetamol (N-acetyl-para-aminophenol) is extensively used as analgesic and antipyretic agent in human and veterinary medicine.

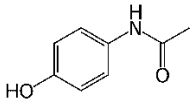


Figure 14. APAP chemical structure.

It is generally not considered an NSAID because of its little anti-inflammatory activity. Some studies report that acetaminophen inhibits centrally mediated pain transmission via inhibition of COX-3 in the central nervous system. Other mechanism of action that has been proposed is the acetaminophen inhibition of prostaglandins in some cells and tissue in which low concentrations of arachidonic acid are present. It has been hypothesized that the site of acetaminophen action may be the peroxidase enzyme component of PGH_2 synthase. Therefore, COX enzyme

inhibition may occur at site specific tissues, sparing the gastrointestinal mucosa, platelets, and kidneys but acting centrally (Riviere and Papich 2010).

Acetaminophen can stimulate the inhibitory pain pathway mediated by serotonin suggesting that it may directly activate serotonin receptors. An additional mechanism of action may be the activation transient receptor potential vanilloid 1 (TRPV1) receptor (Mallet et al. 2010). This action may play a role in some pain syndromes, but the relevance is undetermined for clinical use.

Acetaminophen is used for the treatment of painful disease states associated with fever in swine and, occasionally, in the control of postoperative pain in dogs in association with codeine. In some countries, it is administered to cattle for fermentation disorders and acetonemia and in poultry for the treatment of painful diseases and pyrexia. Few and scattered reports only on acetaminophen pharmacokinetics in poultry are present in the literature. Its pharmacokinetics were reported following a single oral 10 mg/kg dose in chickens and turkeys and after an intraperitoneal injection of 100 mg/kg in chicks (Neirinckx et al. 2010; Mohammad et al. 2012). No residue depletion studies for poultry have been described. Acetaminophen demonstrated good anti-nociceptive properties in pigeons (Brune et al. 1974). Additionally, it seems to be safer than diclofenac or other NSAIDs in poultry since no nephrotoxic signs were observed after a 10 mg/kg intramuscular injection daily for seven days (Jayakumar et al. 2010).

2.3 Antiparasitic drug

The economic importance of parasites infections in livestock is one of the primary causes of the development of antiparasitic drug specific for animal therapy. Despite

significant treatment advances, parasites remain a major threat to livestock farming causing large deficits for the agricultural economy. Effective parasite control is thus essential for profitability in intensive livestock production. However, the investment in control measures does not always result in the expected therapeutic success. Management strategies are often underrated and are not well integrated with chemotherapy procedure. Moreover, the incorrect use of anthelmintic drugs due to insufficient knowledge of their pharmacological features may lead to an ineffective therapy and /or to development of resistance.

2.3.1 Avermectin

Avermectin structures are complex 16-membered macrocyclic lactones which are produced as a mixture of different components from fermentation of *Streptomyces avermitilis*. These natural products are indicated with A (those containing a methoxy group at the 5-position) and B (with a hydroxy group at the 5-position). B1 homologs is correlated to the highest anthelmintic potency (Õmura 2008; Riviere and Papich 2010).

This class, along with milbemycin, is considered as endo-ectoparasiticidal drugs due to their wide spectrum of action which include insects, acarines, and nematodes. However, they do not possess efficacy against cestode and trematode parasites (Õmura 2008).

These drugs act on the nervous system of the parasite, inducing reduction in motor activity and paralysis in both arthropods and nematodes. The paralytic effects are mediated through GABA and/or glutamate-gated chloride channels (GluCl), collectively known as ligand-gated chloride channels. The drug-receptor binding

causes a slow and irreversible increase in chloride membrane conductance, hyperpolarization, and flaccid paralysis of the invertebrate somatic muscles.

Avermectin exert their paralytic effects on the pharyngeal pump also, affecting nutrients ingestion, and on the parasite somatic musculature limiting parasite mobility. The selective toxicity is due to the fact that the same GluCl channels are members of the ligand-gated ion channel superfamily uniquely found in invertebrates (Õmura 2008).

The avermectin family includes a series of natural and semisynthetic molecules, such as abamectin, ivermectin, doramectin, eprinomectin, and selamectin.

2.3.1.1 Ivermectin

Ivermectin consists in a mixture at least 80% of 22-23 dihy- droavermectin B_{1a} and less than 20% 22-23 dihydroavermectin B_{1b} (Figure 15).

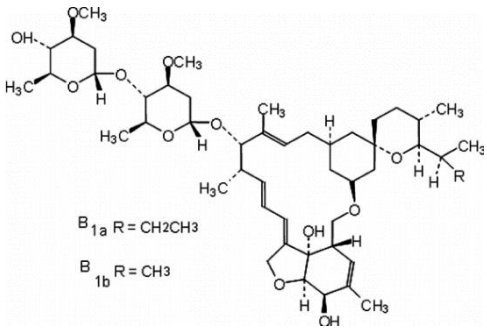


Figure 15. Ivermectin chemical structure.

Ivermectin is highly lipophilic compound, and it is characterized by a long persistence in the body with large volume of distribution. Its protracted half-life which has been found in all the animals studied, allows for infrequent administration to achieve clinical effects (Riviere and Papich 2010).

It has been the most commonly used antiparasitic agent in cattle, horses, sheep, and swine in many countries. Its spectrum of activity covers a wide variety of nematodes, microfilaria, and external parasites of domestic species. Dosage regimens vary, depending on the species and parasite treated and huge number of pharmaceutical formulations such as oral, topic, or injectable are available (Papich 2015).

Ivermectin is use in an extra-label manner in broiler poultry, due to its effectiveness against *Ascaris galli*, *Heterakis gallinarum*, and *Capillaria* after oral (in food or water) or topically (in the cloaca) administrations (Moreno et al. 2015). The studies available regard mainly tissue/egg residues in broiler chickens and laying hens (Moreno et al. 2015; Mestorino et al. 2017; Cirak et al. 2018), but no information is reported in waterfowl such as geese.

3. CONCENTRATIONS IN PLASMA AND SELECTED TISSUES OF MARBOFLOXACIN AFTER ORAL AND INTRAVENOUS ADMINISTRATION IN BILGORAJSKA GEESE (*Anser anser domesticus*).

3.1 Aim of the study

This study was aimed to:

1. determine the pharmacokinetic profiles of 2 mg/kg marbofloxacin in geese after IV and PO administration;
2. assess the tissue depletion of marbofloxacin in geese after oral administration;
3. calculate the daily dose from the experimental data and to compare it with that calculated by allometric scaling.

3.2 Material and methods

3.2.1 Chemicals, reagents, and solutions

Marbofloxacin and the internal standard (IS) enrofloxacin powder with a standard purity of 99.0% were purchased from Sigma-Aldrich (Milan, Italy). HPLC-grade acetonitrile and dichloromethane were procured from Merck (Kenilworth, NJ, USA). Triethylamine was obtained from Sigma-Aldrich (St Louis, MI, US). Orthophosphoric acid, potassium dihydrogen phosphate and sodium hydrogen phosphate were purchased from Carlo Erba Reagents (Milan, Italy). Deionized water was produced using a Milli-Q Millipore Water System (Millipore, Darmstadt, Germany).

3.2.2 Animal experiment

Ten clinically normal female Bilgorajska geese, with a mean bodyweight (BW) of 4.7 kg (min 4.1, max 5.2), supplied by a local farm (Majątek Rutka, Puchaczów, Poland), were used for the study. The animal experiment was approved by the Institutional Animal Care and Use Committee of the University of Lublin (Poland) and carried out in accordance with European law (2010/63/UE). Geese were judged to be in good health based on physical examination at the time of acquisition and at the start of the study, and through daily observation of behavior and appetite. Geese were randomly divided into two groups; two were untreated (controls) and eight were treated. The treated group was used in a three-phase study design, with the three phases separated by 3-week wash-out periods.

In the first phase, the treated birds received an IV bolus of 2 mg/kg of marbofloxacin in the left brachial vein using a sterile 26-gauge 1.75 cm needle. In the second and third phases, they received 2 mg/kg marbofloxacin as a solution administered via crop gavage using a rounded tip metal catheter, 3 hours after being fed. Prior to use, all volumes for injection or oral dosing of marbofloxacin (Marbocyl 10%; Vetoquinol, Towcester, UK) were made up to a volume of 2 ml with sterile saline. This was to reduce dosing errors due to the bias in administering injections of different volumes. The two control birds received oral administration of a volume of saline equal to that given to the treated group (2 ml) at the beginning of any phase. Immediately before the start of the first and second phases, a 24-gauge catheter was inserted in the right brachial vein for the collection of blood samples. Blood samples (1 ml) were collected at 0, 5, 15, 30, 45 minutes and 1, 1.5, 2, 4, 10, 24, 34, and 48 hours after IV administration of marbofloxacin during the first phase.

During the second phase, the same blood collection schedule was followed except no samples were collected at 15 minutes. Samples were collected via the catheter up to 24 hours and then using a syringe and 25-gauge needle. To ensure patency, the catheter was flushed with 1 ml of 0.9% saline with the addition of 10 IU/ml heparin. For each blood collection, the first 0.2 ml of blood was discarded. Blood was immediately transferred to heparinized blood tubes (Sarstedt; Nümbrecht, Germany). Tubes were centrifuged at 1,500x g and the harvested plasma stored at −20°C until analysis within 30 days of collection.

During the third phase, two birds were sacrificed and exsanguinated at 6, 24, 34, 48 hours after oral administration of marbofloxacin, and the following tissues were collected: entire liver, kidneys, heart, and ∼40 g lungs and breast muscle. The two control birds were sacrificed 24 h after saline administration and the same tissues collected. Tissues were placed in cryobags and immediately stored at −20°C for 2 weeks until analysis

3.2.3 Analytical method

3.2.3.1 Instrumentation and analytical conditions

The analytical method was performed at the University of Pisa, Department of Veterinary Sciences, Italy. The HPLC system was a LC system (JASCO International Co. Ltd., Tokyo, 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 analysis was performed using a Luna analytical column (150 × 4.6 mm inner diameter, 3 µm particle size; Phenomenex, Torrance, CA, USA) maintained at 25°C. The mobile phase consisted

of acetonitrile:aqueous solution (20:80% v/v) at a flow rate of 1 ml/min. The aqueous solution consisted of potassium dihydrogen phosphate (0.02 M), phosphoric acid (0.006 M) and triethylamine (0.012 M) in water (pH 4.6). Excitation and emission wavelengths were set at 295 and 500 nm, respectively. Data were processed using CromNav 2.0 software (Jasco Inc.). The analytical method was validated based on a method previously described by Vercelli et al. (2016) with slight modifications.

3.2.3.2 Sample preparation

An aliquot (0.2 ml) of plasma were added to 0.1 ml of internal standard (500 ng/ml enrofloxacin, IS) and diluted with 0.8 ml of 0.1M phosphate buffer at pH 7.4. After adding 6 ml of a mixture of dichloromethane, the samples were shaken and centrifuged at 4,000x*g* for 5 minutes. Then 5 ml of the organic layer was transferred into a clean tube and dried at 40°C under nitrogen stream. The residue was dissolved in 0.2 ml mobile phase, vortexed and an aliquot was injected onto the HPLC system.

Liver, kidneys, lungs, heart and muscle were thawed and immediately dissected into small pieces. Samples of 1 g per tissue were placed into 5 ml plastic tubes with 3 ml of homogenization reagent (0.1 M phosphate buffer at pH 7.4). The suspension was homogenized for approximately 40 seconds. Aliquots of 0.2 ml were processed as described for plasma samples

3.2.3.3 Sample quantification

The quantitative HPLC method was validated for each of the tissues (liver, kidney, lung, heart and muscle) and plasma. Linearity was determined by linear regression analysis, using calibration curves constructed using replicates (n=3) of samples

from control geese spiked with marbofloxacin at concentrations of 10, 50, 100 and 500 ng/ml. The intra-day variation was calculated from six replicates of three samples from control geese, spiked with 10, 100, and 1,000 ng/ml marbofloxacin. The inter-day variation was determined from results for six replicate samples from control geese containing 1, 100 and 1,000 ng/ml marbofloxacin over three consecutive days (total of 18 runs).

The extraction efficiency (recovery) was determined by comparing the peak areas of control samples spiked with 1, 100 and 1,000 ng/ml marbofloxacin, with the peak areas of the same standards prepared in mobile phase. The limit of quantification was determined as 10:1 signal-to-noise ratio, while limit of detection was determined at a ratio of 3:1. Concentrations of marbofloxacin in plasma, liver, kidney, lung, heart and muscle were determined using calibration curves constructed using samples from control geese spiked with standard stock solutions of marbofloxacin to obtain concentrations of 1, 10, 100, 500 ng/ml and, 1, 5 μg/ml.

3.2.4 Plasma protein binding

Protein binding was determined by ultracentrifugation (Optima™ Max-XP; Beckman Coulter, Inc, USA). An aliquot of fresh plasma sample (0.9 ml) from control geese were added to 0.1 ml of marbofloxacin in saline to obtain final concentrations of marbofloxacin of 0.1, 1, 10 μg/ml, with three replicates for each concentration. Samples were centrifuged at 120,000x*g* for 2.30 h. Plasma samples and their corresponding ultrafiltrate were assayed by HPLC as mentioned above. The percentage of plasma protein binding fraction was calculated according to equation 1.

Protein binding (%) = (total – ultrafiltrate concentration) / total concentration*100 (1)

3.2.5 Pharmacokinetic analysis and statistical analysis

The concentrations of marbofloxacin in samples with respect to time were analysed using a non-compartment model using ThothPro software (Gdansk, Poland). The maximum plasma concentration (C_{max}) and time to reach it (T_{max}) were determined directly from the concentration-time curves. The elimination half-life ($t_{1/2kel}$) was calculated using least squares regression analysis of the concentration-time curve, and the area under the curve (AUC) was calculated by linear log trapezoidal and the linear-up log-down rule to the final concentration-time point for IV and PO administration, respectively. From these values, the apparent volume of distribution (V_{ss}), mean residence time (MRT), mean absorption time (MAT) and systemic clearance (Cl) were calculated. A naïve pooled-data approach using a non-compartmental analysis (Pouplin et al. 2016) was used to calculate the pharmacokinetic parameters for marbofloxacin in the tissues.

The extraction ratio for marbofloxacin after IV administration was calculated for each bird according to the formula (Toutain and Buosquet-Melou 2004a):

$$E=CL/Q° \quad (2)$$

where CL is the value of clearance reported for each animal after IV administration, while Q° (ml/min) is the cardiac output calculated according to the allometric equation:

$$Q°=290.7\ W^{0.69} \quad (3)$$

where W stands for BW (kg) of each animal (Grubb 1983).

As minimum inhibitory concentration (MIC) data for geese is not available, an MIC of 0.125 μg/ml, previously described for *E. coli* isolates from turkeys (Haritova et al. 2006), was used to estimate the optimal daily dose of marbofloxacin in geese, using the equation:

$$\text{Dose (per day)} = \frac{(AUC_{(0\text{-}24)}/MIC)_{\text{break point}} \times Cl \times MIC}{fu \times F \times 24h} \quad (4)$$

where AUC/MIC is the PK/PD breakpoint (predictive index for fluoroquinolone efficacy, 125), clearance is either observed or predicted, and fu is the free fraction of the drug, unbound to plasma proteins (Toutain et al. 2002; Toutain 2003).

For the allometric calculation of Cl (Cl pred) the following formula was used:

$$Cl\ pred = 0.23\ W^{0.73} \quad (5)$$

where W stands for the animal BW (kg) (Lashev and Haritova, 2012).

The concentrations of marbofloxacin in different tissues at 6, 24, 34, and 48 h after oral administration were used to calculate preliminary withdrawal times (WT) using the software WT 1.4, developed by the European Medicines Agency (Anonymous 2018). The withdrawal time was established as being the time when the upper one-sided tolerance limit (99%) with 95% CI was below the maximum residue limit of 150 μg/kg marbofloxacin for bovine and porcine tissues (Anonymous 1999).

The pharmacokinetic parameters were normally distributed (tested by Shapiro–Wilk test) and mean values were compared between the two routes of administration using paired t-tests using GraphPad Prism v 5.0 (GraphPad Software, Inc., La Jolla, CA, USA).

3.3 Results

3.3.1 Analytical method validation

The method had good linearity (R^2> 0.987) and satisfactory recoveries (83–94%),

Parameter	Unit	Plasma	Liver	Kidney	Lung	Heart	Muscle
Range	ng/ml	1-5000	1-5000	1-5000	1-5000	1-5000	1-5000
R^2		0.998	0.993	0.997	0.994	0.987	0.992
Intra-assay							
CV	%	<8.3	<10.2	<5.8	<6.8	<9.4	<8.7
Inter-assay							
CV	%	<7.9	<8.4	<7.5	<11.1	<9.7	<9.9
LOQ	ng/ml	1	10	10	10	1	1
LOD	ng/ml	0.3	3	3	3	0.3	0.3
Recovery	%	92±5	85±7	89±8	83±9	90±6	94±7

with intra- and inter-day CV < 12% (Table 1).

Table 1. Results of the validation of the HPLC assay used for measurement of concentrations of marbofloxacin in plasma and different tissues of geese.

3.3.2 Pharmacokinetic analysis

Concentrations were always higher following IV administration compared with PO administration and were detectable in plasma up to 24 h after administration by either route but was below the LOQ by 34 h after administration (Figure 16).

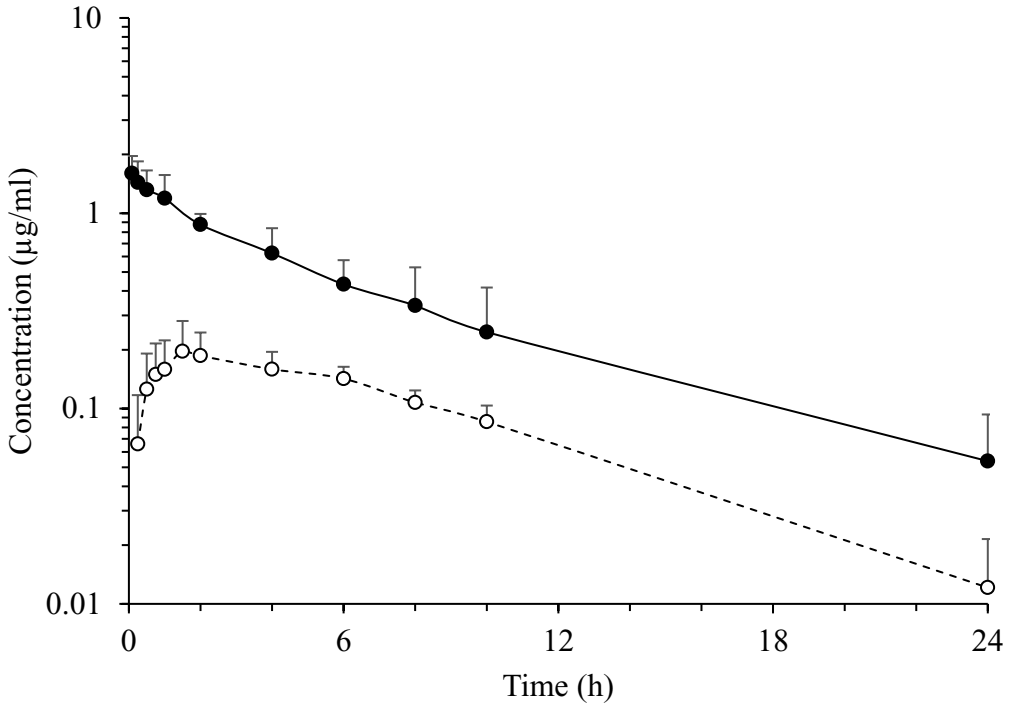


Figure 16. Semi logarithm observed mean plasma concentrations of marbofloxacin after 2 mg/kg IV (●, *n*=8) and PO (○, *n*=8) administration.

Mean bioavailability following oral administration was 26.5±7.7% (Table 2), the mean extraction ratio was 5.7±2.7% and mean binding of marbofloxacin to serum proteins was 30±5% (min 25%, max 38%).

Parameter	Unit	IV	PO
$t_{1/2kel}$	h	6.31±0.87	6.22±1.77
T_{max} §	h	N/A	1.75 (1.5-6)
C_{max}	µg/ml	N/A	0.21±0.07
AUC_{last}	h µg/ml	8.47±3.83	1.92±0.32*
AUC_{inf}	h µg/ml	9.03±4.26	2.11±0.34*
V_{ss}	l/kg	1.52±0.33	N/A
Cl	l/h kg	0.26±0.08	N/A
MRT_{last}	h	5.92±0.76	7.62±1.35
MRT_{inf}	h	7.47±1.09	10.01±2.31
MAT		N/A	1.77±1.74
F	%	N/A	26.46±7.68

$t_{1/2kel}$ = elimination half-life; AUCinf = area under the curve from zero to infinity; AUC_{last} = area under the curve from zero to 24 h; C_{max} = maximum concentration; T_{max} = time at maximum concentration; MRT_{inf} = mean residence time from zero to infinity; MRT_{last} = mean residence time from zero to 24 h; N/A = not applicable; §Median (max, min). *Values differ within rows ($p < 0.05$).

Table 2. Mean (±SD) pharmacokinetic parameters of marbofloxacin in plasma following IV or PO administration to geese (*n*=8) at a dose of 2 mg/kg BW.

3.3.3 Tissue residue analysis

Concentrations of marbofloxacin in all tissues tested were highest at 6 h and then decreased consistently up to 34 h after oral administration (Figure 17).

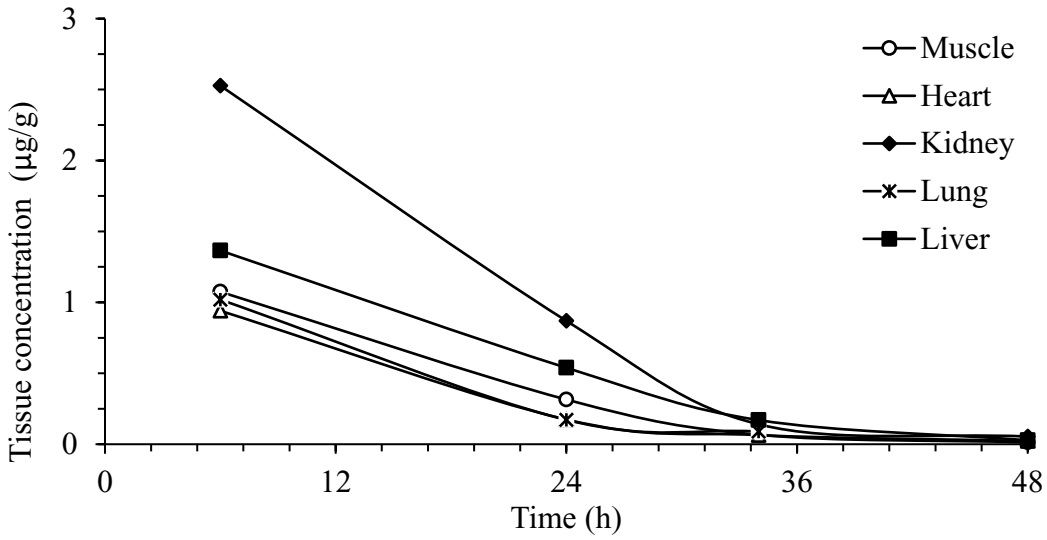


Figure 17. Concentrations in muscle, heart, kidney, lung, and liver of marbofloxacin following PO administration (2 mg/kg) to geese (*n*=2/time point).

Marbofloxacin was detected in all tissues up to 48 h, except in lung where residues were below the LOQ after 34 hours. Concentrations in the kidney were higher compared to the other tissues from 6–24 h. The pharmacokinetic parameters for tissue depletion are presented in Table 3.

Parameter	Unit	Kidney	Liver	Lung	Muscle	Heart
$t_{1/2kel}$	h	7.26	7.08	7.83	9.86	7.26
C_{max}	µg/ml	0.94	2.53	1.02	1.37	1.07
T_{max}	h	6	6	6	6	6
AUC_{last}	h µg/ml	14.52	43.42	15.04	25.54	17.79
MRT_{last}	h	10.94	12.22	9.73	13.61	11.68
$AUC_{tissue/plasma}$		8.2	22.5	7.09	18.5	7.73

$t_{1/2kel}$ = elimination half-life; AUC_{last} = area under the curve from zero to 24 hours; $AUC_{tissue/plasma}$ = area under the curve ratio of tissue:plasma; C_{max} = maximum concentration; MRT_{last} = mean residence time zero to 24 hours; T_{max} = time at maximum concentration.

Table 3. Pharmacokinetic parameter estimates calculated by the naïve pooled-data approach for marbofloxacin in different tissues following PO (2 mg/kg) administration to geese.

3.3.4 Estimated daily dose

The optimal mean daily dose calculated using the observed clearance and based on an MIC of 0.125 µg/ml, for IV and PO administration of marbofloxacin was 6.06±1.53 and 10.36±2.18 mg/kg, respectively. If the observed clearance was replaced by predicted clearance (based on BW), the calculated mean daily dose was 3.39±0.08 and 5.54±0.14 mg/kg, respectively.

3.3.5 Estimated withdrawal time (WT)

The preliminary WT for a MRL of 0.15 mg/kg calculated for muscle was 38.4 h, heart 33.6 h, kidney 48.3 h, lung 47.7 h and liver 49.3 h.

3.4 Discussion and conclusions

Following oral administration, the bioavailability of marbofloxacin was only 26.5%. This is lower than the previously reported 56% in Japanese quails (Coturnix japonica; Haritova et al. 2013) and 98% in healthy chickens (Urzúa et al. 2016). This difference might be due the kind of feed administered, the drug formulation, or species-specific differences. A limitation of the present study was the use of the IV formulation for PO administration. This should have limited likely errors from drug compounding but could have affected the oral bioavailability of marbofloxacin. No pilot study has been performed to investigate the bioequivalence between the PO and IV formulation when administered by PO route.

The V_{ss} for marbofloxacin was moderate (1.5 l/kg) and Cl was fast (0.26 h l/kg) following IV administration, resulting in a short $t_{1/2kel}$ (6.3 h), which reflected the fast elimination of the drug from plasma and tissues. The mean $t_{1/2kel}$ was within the range reported for other birds, from 1.96 h in ostriches (Struthio camelus; de Lucas et al. 2005) to 12.51 h in vultures (Gyps fulvus; Garcia-Montijano et al. 2011). To carry out a physiological interpretation of plasma Cl, a minimal physiological model for total body clearance was applied in order to calculate the E, which can be regarded as the percentage of drug cleared by the entire body during a single passage through the different organs contributing to the body clearance. E found in this study of 5.7%, can be considered to be low, based on the recommendations of Toutain and Buosquet-Melou (2004a) that it should be considered high if >35%, medium if around 15%, and low if around 5%. The value obtained in this study was similar to the 8.1% calculated for chickens (Urzúa et al. 2016) and 6.0% for parrots

(Carpenter et al. 2006), and higher than the 2.4% in Muscovy ducks (Yuan et al. 2011). However, all these values are considered as low values.

The effectiveness of fluoroquinolones is known to be concentration-dependent (Forrest et al. 1993), and the ratios of AUC/MIC and C_{max}/MIC are considered as PK/PD indexes to predict their antimicrobial effect (Turnidge 1999). The AUC/MIC index has been proposed to be at least 100–125 for fluoroquinolones against Gram-negative bacilli and from 25 to 30 for fluoroquinolones against *Streptococcus pneumoniae*, to achieve maximum clinical effect, and a C_{max}/MIC > 10 has been proposed for bacterial eradication (Craig 1998; Levison and Levison 2009). MIC for marbofloxacin have not been reported for geese, therefore based on an MIC of 0.125 μg/ml reported for *E. coli* isolated in turkeys (Haritova et al. 2006), the calculated AUC/MIC and C_{max}/MIC for the oral administration were 17.24 and 1.56, respectively. Neither of these indexes reach the proposed values, suggesting that 2 mg/kg marbofloxacin given orally might not be effective against *E. coli* in geese. This dose of marbofloxacin should be effective for Gram-negative bacteria with MIC <0.015 μg/ml and for *S. pneumoniae* with MIC of 0.06 μg/ml. Further studies in infected geese are needed to confirm these estimations.

The optimal daily dose, calculated based on an MIC of 0.125 μg/ml for *E. coli* isolated from turkeys, was 6.06 and 10.36 mg/kg, after IV and PO administration, respectively, using the observed Cl, and 3.39 and 5.54 mg/kg, respectively, using predicted clearance. Therefore, the maintenance doses calculated using predicted clearance are about half of the doses calculated using observed clearance. This suggests that the prediction of the clearance by allometry might not be appropriate in geese administered marbofloxacin.

Concentrations of marbofloxacin in selected tissues were detected from 6 to 34 h after PO administration. An earlier study also orally administered 2 mg/kg marbofloxacin to healthy chickens and reported that residues were detected in muscle for up to 48 h, with a C_{max} of 0.351 μg/mg (Urzúa et al. 2016), compared to the C_{max} of 1.07 μg/g found in the current study. Those authors also reported a C_{max} in plasma of 0.8 μg/ml after PO administration. The difference between studies might be due to the different drug formulation administered, to species-specific differences, or other aspects related to the experiment, i.e. temperature, stressogenic insults, feed and season. The WT of 49 h for liver and 48 h for kidney calculated in the present study should be regarded as preliminary because they were calculated using only two birds per time-point, however they are in agreement with the 2 days reported for the same tissues in broiler chickens (Yang et al. 2014).

In conclusion, it seems that marbofloxacin orally administered at a daily dose of 2.0 mg⁄kg might not be effective for treatment of *E. coli* in geese. The calculated dose based on observed clearance was approximately twice that based on clearance predicted from allometric scaling. Further PK/PD studies in geese are recommended to determine the marbofloxacin dose regimen and its clinical efficacy in geese.

Reference: Sartini I, Łebkowska-Wieruszewska B, Lisowski A, Poapolathep A, Owen H, Giorgi M, 2019. Concentrations in plasma and selected tissues of marbofloxacin after oral and intravenous administration in Bilgorajska geese (*Anser anser domesticus*). New Zealand Veterinary Journal, 22:1- 16.

4. DANOFLOXACIN PHARMACOKINETICS AND TISSUE RESIDUES IN BILGORAJSKA GEESE

4.1 Aim of the study

The aim of the study was three-fold:

1. to assess the pharmacokinetic profile of danofloxacin after single IV and PO administrations;
2. to evaluate the tissue residues in muscle, heart, liver, kidney and lung;
3. to simulate the concentration-time curves after multiple-dose administration in the practical context of the large-scale breeding establishment and to observe the potential differences with a classical multiple-dose simulation model.

4.2 Material and methods

4.2.1 Chemicals, reagents, and solutions

Danofloxacin and marbofloxacin (internal standard, IS) both with a standard purity of 99.0%, were purchased from Sigma-Aldrich (Milan, Italy). HPLC-grade acetonitrile and trichloromethane were procured from Merck. Triethylamine was obtained from Sigma-Aldrich (St Louis, MI, US). Orthophosphoric acid, sodium dihydrogen phosphate, and potassium dihydrogen phosphate were purchased from Carlo Erba Reagents (Milan, Italy). Deionized water was produced using a Milli-Q Millipore Water System (Millipore, Darmstadt, Germany).

4.2.2 Animal experiment

Twenty-four adult (2 years old) healthy male Bilgorajska geese, weighing 4.6 – 6.4 kg (average, 5.4 kg) were used in this study. Geese were supplied by a local farm (Majątek Rutka, Puchaczów, Poland) and a ring with an identity code was applied to the left leg for easy identification. The animal experiment was approved by the Institutional Animal Care and Use Committee of the University of Lublin (Poland) and carried out in accordance with European law (2010/63/UE). All animals were judged to be in good health based on physical examination, serum chemistry and haematological analyses performed before the beginning of the study from the supervising veterinarian. The geese were monitored daily through observation of behaviour and appetite and were acclimatised for 1 week into the new environment before beginning the study. They 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. Geese were fed with a drug-free pelleted diet twice a day and water was supplied *ad libitum*.

Geese were randomly divided into three groups, each composed of eight animals. Groups 1 and 2 were used to provide the pharmacokinetic profiles of the drug and group 3 was used for the evaluation of danofloxacin depletion in tissues.

Group 1 was treated with a single IV injection (5 mg/kg, Advocin®, Pfizer) in the left brachial vein using a sterile 26-gauge 1.75 cm needle. Group 2 and 3 received a single PO dose (5 mg/kg, Advocin®, Pfizer) by crop gavage. A 24-gauge catheter was inserted in the right brachial vein immediately before the experiment commenced to facilitate blood collection for the first 10 h. Blood samples (1 ml)

were collected in lithium heparin tubes (BD, Vaud, Switzerland) at 0, 5, 15, 30, 45 minutes and 1, 1.5, 2, 4, 6, 8, 10 and 24 h after IV administration (group 1) and at 15, 30, 45 min and 1, 1.5, 2, 4, 6, 8, 10, 24, and 48 h after PO administration (group 2). After 10 h, the catheter was removed, and blood was collected directly with a 24-gauge hypodermic needle syringe from the left brachial vein. To ensure patency, the catheter was flushed 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 1500x*g* and the harvested plasma was stored at −20°C until analysis (within 30 days of collection).

Two animals from group 3 were humanely killed by stunning and exsanguinated at selected time-points: 6, 10, 24, 48 h after PO treatment. Approximately 20 g of muscle, heart, liver, lung, and kidney were collected and stored at -20°C until further analysis.

4.2.3 Analytical method

4.2.3.1 Instrumentation and analytical conditions

The HPLC system used was an LC system (Jasco, Japan) consisting of a high-pressure mixer pump (model PU 980 Plus), a spectrofluorometric detector (model 2020 Plus), an auto sampler (model AS 950), and a 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.). The chromatographic separation assay, modified from Garcia et al. (2000), was performed using a C18 Gemini analytical column (250 × 4.6 mm inner diameter, 5 µm particle size, Phenomenex, Torrance, California, USA) at 25°C. The mobile phase consisted of acetonitrile:aqueous

solution (15:85 v/v %) at a flow rate of 1 ml/min. The aqueous solution consisted of potassium dihydrogen phosphate (0.02 M), phosphoric acid (0.006 M), and tetraethyl amine (0.012 M) in water (pH 4.1). Excitation and emission wavelengths were set at 338 and 425 nm, respectively, for both danofloxacin and IS.

4.2.3.2 Sample preparation

The analytical procedure was validated for plasma and for all tissues collected from the geese according to the method previously described by Garcia et al. (2000), with a few modifications. An aliquot (0.2 ml) of plasma was added to 0.1 ml of IS (50 µg/ml) solution in water and 0.8 ml of 0.1 M phosphate buffer at pH 7.1. Four ml of trichloromethane was added and samples were shaken for 10 minutes and centrifuged at 4500x*g* for 10 minutes. Three ml of the organic layer was transferred into a clean tube and dried at 45°C under nitrogen stream. The residue was reconstituted with 0.2 ml of mobile phase, vortexed and 50 µl of this latter solution was injected onto the HPLC system.

Muscle, heart, liver, kidney, and lung samples were thawed and immediately dissected into small pieces (Garcia et al. 2000; Sartini et al. 2020b). An aliquot of 1 g per tissue was placed into 5 ml plastic tube with 3 ml of homogenisation solution (0.1 M phosphate buffer at pH 7.1). This suspension was homogenised for approximately 30 seconds and then, 0.2 ml was processed as previously described for plasma samples.

4.2.3.3 Sample quantification

The quantitative HPLC method was fully validated for each of the goose tissues (liver, kidney, lung, heart and muscle) and plasma in terms of linearity, intra-day

and inter-day precision, recovery, LOQ and LOD according to the European Medicines Agency (EMA) guidelines (Anonymous, 2012).

Linearity was determined by linear regression analysis, using calibration curves constructed using replicates (n=3) of control samples from control goose matrix spiked with danofloxacin at concentrations of 0.005, 0.01, 0.05, 0.1, 0.5 μg/ml. When danofloxacin concentrations exceeded 0.5 μg/ml, the samples were diluted with a control goose matrix. The intra-day and inter-day precision were calculated after analysis of six plasma samples spiked with danofloxacin at three different concentrations (0.005, 0.05 and 0.5 μg/ml), and is expressed as the percentage coefficients of variation (CV %). The extraction recovery experiment was carried out by comparing the response (in area) of high, middle, low standards (0.005, 0.01 and 0.5 μg/ml) and the IS, spiked into blank goose matrix (control), to the response of equivalent standards. The LOD was estimated as the plasma drug concentration that produced a signal to noise ratio of three and LOQ was determined as the lowest plasma concentration that produced a signal to noise ratio of ten.

4.2.4 Pharmacokinetic analysis and statistical analysis

The pharmacokinetic analyses were performed using ThothPro software (Gdansk, Poland). For the plasma samples, standard approaches to both non-compartmental and compartmental analysis were evaluated on each individual animal data set. The optimal model was determined based on the visual inspection of individual fits and the Akaike's information criterion. The plasma IV and PO concentration-time profiles were best described by a two- and one-compartmental model, respectively.

The Non-Linear Optimization method was used for the one-compartment analysis using the Levenberg-Marquardt algorithms (Kargar et al. 2018).

The absolute oral bioavailability was calculated as:

$$F(\%) = \frac{AUC_{PO}}{AUC_{IV}} \times 100 \quad (6)$$

The pharmacokinetic parameters are reported as geometric mean and ranges (Julious and Debarnot 2000).

The Wilcoxon's rank sum test was used for the statistical comparison of pharmacokinetic data between the two routes of administration (Powers 1990).

For the prediction of danofloxacin antimicrobial effect, the PK/PD index $AUC_{(0-24)}$/MIC was used (Turnidge 1999). As MIC data for geese is not available, a MIC of 0.25 μg/ml, previously described for *Mycoplasma gallisepticum* isolates from poultry (Cooper et al. 1993) was used to estimate the optimal daily dose of danofloxacin in geese, using equation (4).

4.2.5 Tissue residue analysis

A naïve pooled-data approach using a non-compartmental analysis (Pouplin et al. 2016) was used to calculate the pharmacokinetic parameters for danofloxacin in all the selected tissues.

The drug tissue penetration was determined considering the ratios between the AUC value found in each tissue and in plasma ($AUC_{tissue/plasma}$) after PO administration (Sartini et al. 2020a).

Explorative WT were computed for danofloxacin in goose liver and kidney using the software WT 1.4, developed by the EMA (Anonymous 2018). The WT was established as being the time when the upper-one sided tolerance limit (99%) with 95% confidence interval was below the MRL. In the present study, the MRL (0.4 μg/g) reported for danofloxacin in poultry tissues by the EMA was used (EU Regulation 37/2010).

4.3.6 Multiple-dose simulations

The modelling of a daily oral dose regimen of 5 mg/kg/day administered for 5 days was computed applying the superposition principle and assuming by visual inspection the same first-order kinetics (Gabrielsson and Weiner 2016). In the first simulation (A), the drug was assumed to be administered to the animals in a single daily administration. In the second simulation (B), the dose of 5 mg/kg was split in 14 equal doses (0.357 mg/kg) every hour for the first 14 h, with the drug not being administered for the remaining 10 h (replicating a rough light[feeding]-dark[fasting] cycle).

The potential accumulation ratio (R) at 24 h dosing intervals (τ) following both simulations was determined using the following formula:

$$R = \frac{1}{[1-(0.5)^{\tau/t_{1/2}}]} \quad (7)$$

where τ is the dosing interval and $t_{1/2}$ is the half-life of elimination (Toutain and Bousquet-Mèlou 2004b).

Fluctuations of drug plasma concentration at the steady state peak and trough concentrations were calculated with the following equation:

$$\frac{P}{T} = \frac{C_{max_{ss}}}{C_{min_{ss}}} = 2^{\tau}/t_{1/2} \quad (8)$$

where P/T is the peak/trough concentration ratio at steady-state, τ is the dosing interval, $t_{1/2}$ is the half-life of elimination and C_{maxss} and C_{minss} are the steady state peak and trough concentrations, respectively (Toutain and Bousquet-Mèlou 2004b).

4.3 Results

4.3.1 Analytical method validation

The analytical method showed an optimal recovery, low LOQs and a good linearity in the range of 0.005 – 0.5 µg/ml for every matrix considered (Table 4).

Parameter	Unit	Plasma	Muscle	Heart	Liver	Lung	Kidney
Equation		y = 8.7496x - 0.0111	y = 8.6971x + 0.0014	y = 8.7195x + 0.0030	y = 8.7191x + 0.0051	y = 8.6901x + 0.0043	y = 8.7292 x + 0.0026
R^2		1	0.999	0.999	0.999	0.999	0.998
Inter-day CV	%	11	10.7	11.1	4.9	12.4	16.9
Intra-day CV	%	2.9	3.8	9.3	5.4	7.9	6.4
Recovery	%	101	97	98	99	98	99
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

Table 4. Results of the HPLC method validation for danofloxacin quantification in plasma and different tissues in geese.

4.3.2 Pharmacokinetic analysis

Danofloxacin plasma concentrations were quantifiable up to 24 h (*n*=8) after IV treatment. After PO administration the drug was quantifiable up to 24h (*n*=6) or 48 h (*n*=2) (Figure 18).

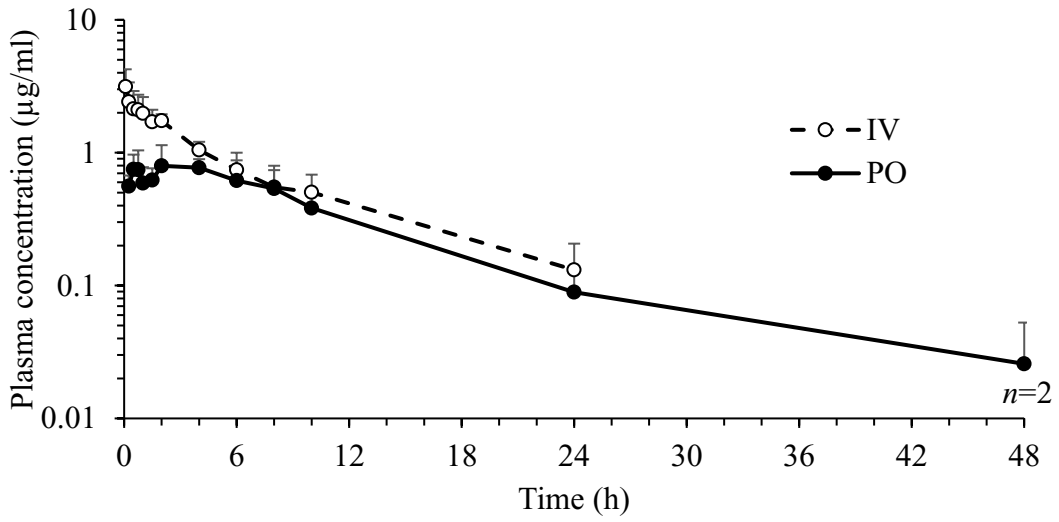


Figure 18. Semi-logarithmic mean plasma concentrations of danofloxacin (±SD) *vs* time curve after a single IV (···○···) and PO (—●—) administration at a dosage of 5 mg/kg (*n*=8) in geese.

A moderate half-life, a slow clearance and a large volume of distribution was observed. The oral bioavailability was moderate (57.95%) (Table 5).

		IV			PO		
Parameter	Unit	Geometric mean	Min	Max	Geometric mean	Min	Max
AUC_{last}	µg h/ml	14.27	10.44	19.80	8.30*	5.66	10.27
AUC_{inf}	µg h/ml	15.35	11.10	23.39	9.14*	7.02	11.03
k_{el}	1/h	0.10	0.08	0.12	0.17	0.09	0.33
k_a	1/h	N/A	N/A	N/A	0.32	0.20	0.47
k_{10}	1/h	0.25	0.14	0.53	N/A	N/A	N/A
k_{21}	1/h	1.14	0.33	3.15	N/A	N/A	N/A
k_{12}	1/h	1.34	0.79	2.08	N/A	N/A	N/A
$t_{1/2kel}$	h	6.61	5.64	8.99	4.12	2.12	7.80
C_{max}	µg/ml	N/A	N/A	N/A	0.96	0.75	1.57
T_{max}	h	N/A	N/A	N/A	1.70	0.50	4.00
Cl	ml/g h	0.35	0.25	0.48	N/A	N/A	N/A
V_1	ml/g	1.46	0.80	2.05	N/A	N/A	N/A
V_2	ml/g	1.71	1.11	2.74	N/A	N/A	N/A
F	%	N/A	N/A	N/A	57.95	44.47	69.87

AUC_{last}, area under the curve from zero to the last detectable timepoint; AUC_{inf}, area under the curve from zero to infinity; k_{el}, elimination rate constant; k_a, absorption rate constant; k_{10}, rate constant from compartment one to compartment 0; k_{21}, rate constant from compartment 2 to compartment 1; k_{12}, rate constant from compartment 1 to compartment 2; $t_{1/2kel}$, terminal half-life; C_{max}, maximum concentration; T_{max}, time at maximum plasma concentration; Cl, plasma clearance; V_1, volume of distribution in the first compartment; V_2, volume of distribution in the second compartment; F, bioavailability. N/A = Not applicable. *Significantly different between the groups (p<0.05).

Table 5. Geometric mean (range) of danofloxacin pharmacokinetic parameters in plasma following IV (n=8) and PO administration (n=8) to geese at a dose of 5 mg/kg.

4.3.3 Tissue residues analysis

Danofloxacin was detected in all tissues at every time point, with concentrations being highest at 6 h and gradually decreasing up to 48 h (Figure 19).

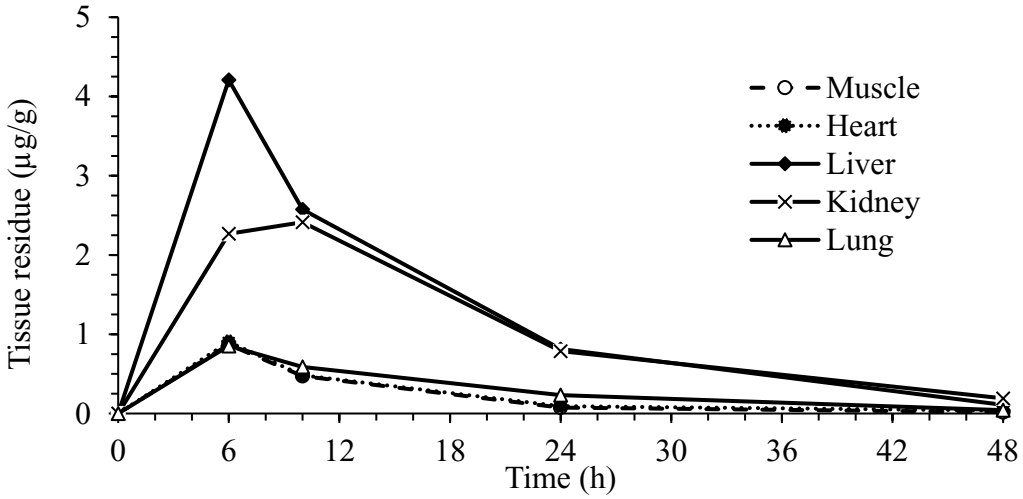


Figure 19. Mean concentrations in muscle, heart, kidney, lung and liver of danofloxacin following PO administration (5 mg/kg) to geese (*n*=2/time point).

The drug residue was higher in kidney and liver compared to the other tissues evaluated, with an $AUC_{tissue/plasma}$ of 5.39 and 6.32, respectively (Table 6). The explorative WT was 2.6 and 3.8 days for liver and kidney, respectively.

Parameter	unit	Heart	Kidney	Liver	Lung	Muscle
AUC_{last}	mg h/ml	11.07	50.31	60.97	14.48	10.23
$MRTl_{ast}$	h	12.14	14.99	12.98	14.43	11.01
k_{el}	1/h	0.06	0.07	0.08	0.07	0.08
$t_{1/2kel}$	h	10.81	10.51	8.31	10.02	8.19
C_{max}	µg/ml	0.90	2.41	4.21	0.85	0.87
T_{max}	h	6.00	10.00	6.00	6.00	6.00
$AUC_{tissue/plasma}$		1.17	5.39	6.32	1.53	1.06

AUC_{last}, area under the curve from zero to 48 h; MRT_{last}, mean residence time zero to 48 h; $t_{1/2kel}$, terminal half-life; C_{max}, maximum concentration; T_{max}, time at maximum concentration; $AUC_{tissue/plasma}$ = area under the curve ratio of tissue:plasma.

Table 6. Pharmacokinetic parameters for danofloxacin in different tissues following PO administration to geese at a dose of 5 mg/kg.

4.3.4 Multiple-dose simulations

Figure 20 displays the two multiple-dose simulations computed at a dosage of 5 mg every 24 h for 5 days (simulation A) and 0.357 mg/kg every hour for 14 h (5 mg/kg/day) for 5 days (simulation B). The predicted plasma concentrations reached steady state after the third day, leading to an accumulation index of 1.3 and 1.6, and a P/T ratio 4.1 and 1.8, for the A and B simulations, respectively.

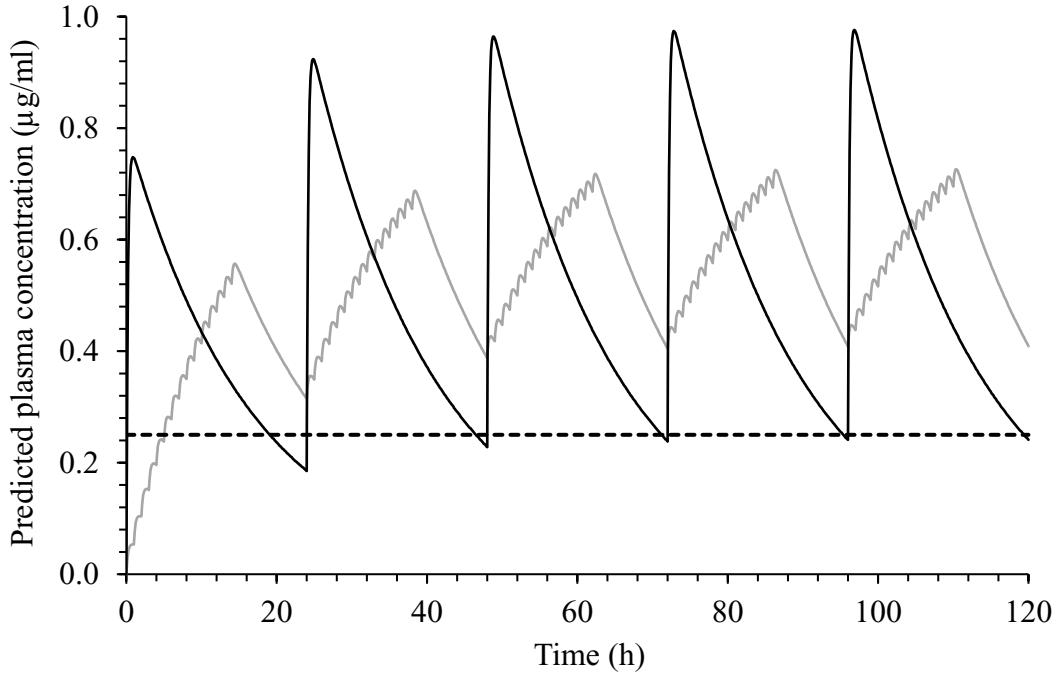


Figure 20. *In silico* plasma concentration of danofloxacin *vs* time curve following a simulated PO multiple dose rate of 5 mg/kg every 24 h for 5 days (black line - simulation A) and at 0.357 mg/kg every h for 14 h (5 mg/kg/day) for 5 days (grey

line - simulation B) in geese. The horizontal dashed line represents the MIC value for *M. gallisepticum* (0.25 µg/ml).

4.4 Discussion and conclusions

Danofloxacin at a single dose of 5 mg/kg administered IV or PO produced no observable adverse effects in geese. The dose was chosen based on the recommended therapeutic regimen for the treatment of respiratory diseases in poultry (Charleston et al. 1998; Zhang et al. 2017). At this dose, danofloxacin showed activity against *Mycoplasma spp*., such as *M. gallisepticum*, a common pathogen which can cause a chronic respiratory disease in poultry, affecting growth rate and egg production (Cooper et al. 1993; Zhang et al. 2017).

In the current study, danofloxacin in geese showed a higher C_{max} and a lower T_{max} than those reported in chickens and ducks (Knoll et al. 1999; Goudah and Mouneir 2009; Zeng et al. 2010). AUC was the only parameter with statistically different results between the two animal groups, resulting in an absolute oral F% of 58%. Pharmacokinetic profiles of enrofloxacin (Shi et al. 2014) and marbofloxacin (Abo-EL-Sooud et al. 2020; Sartini et al. 2020a) were previously investigated in geese. Danofloxacin oral F resulted in an intermediate value compared to that found for enrofloxacin (74.16%, Shi et al. 2014) and marbofloxacin (26.5%, Sartini et al. 2020a) in geese. Moreover, a higher danofloxacin oral F% has previously been reported for ducks (89.2%) and chickens (99.2%) (Knoll et al. 1999; Goudah and Mouneir 2009). The reason of this variance may be related to: species specific differences in the absorption or metabolism of the drug; differences in feed consumption; or discrepancies in carrying out the experiment (Toutain and Bousquet-Mélou 2004c). Several studies in the literature highlight how different

drug formulations might influence PK parameters (Tikhomirov et al. 2018; 2020). In the current study the IV danofloxacin formulation was used for PO administration, while Knoll et al. (1999) administered chickens with medicated drinking water. This aspect may has contributed to the above-mentioned variations. The $t_{1/2kel}$ values found in the present study were higher to those found in ducks (3.91 h, Goudah and Mouneir 2009), but lower than those found in chickens (13.05 h, Zeng et al. 2010).

It is suggested that the critical break point determining the efficacy of fluoroquinolones is $AUC_{(0-24)}$/MIC with a value of 100-125 and 25-30 against Gram-negative bacteria and *S. pneumoniae,* respectively (Walker 2000; Toutain et al. 2002). The danofloxacin protein plasma binding in geese was not calculated in the present study, however, danofloxacin has a low capacity to bind to plasma protein in other bird species. It was 17% in ducks (Goudah and Mouneir 2009) and 27% in turkeys (Haritova et al. 2006). The MIC of danofloxacin for bacteria isolated from geese has not been determined yet. Assuming the percentage of plasma protein binding reported for ducks, the dose regimen used in the present study might be effective in goose treatment for Gram-negative bacteria and *S. pneumoniae* with a MIC<0.076 µg/ml and MIC<0.29 µg/ml, respectively.

Although no MIC value for *M. gallisepticum* from geese has been reported yet, if the same MIC of *M. gallisepticum* isolated from poultry (Hannan et al. 1997) is assumed, the therapeutic daily dose would be 9.10 mg/kg. It is higher than the dose used in this study, so further safety and PD studies would be required to evaluate therapeutic efficacy.

Oral multiple-dose simulations from a single-dose study are generally developed with the assumption of a single bolus administered at defined time intervals (e.g., 5 mg/kg every 24 h for 5 days – simulation A, Figure 20). However, in large-scale field conditions, drugs are often administered to poultry as medicated feed or dissolved in drinking water, which is then consumed *ad libitum* during waking hours. Thus, to reproduce a practical dosing regimen in a large-scale breeding system, a dosage of 0.357 mg/kg every hour for 14 h with the remaining 10 h with no drug administration was simulated, with the dose being repeated for 5 days (simulation B, Figure 20). The two multiple-dose simulations were compared to highlight potential differences and to understand if these disparities can influence the interpretation of a predicted multiple dose regimen. No substantial differences in AUC_{ss} values were found, and hence there was negligible plasma accumulation (R= 1.3 [A] *vs* R=1.6 [B]).

The peak and trough ratio at steady state in simulation B (1.8) was 44% of the value calculated from simulation A (4.1). This aspect might be useful for likely considerations regarding danofloxacin efficacy if the predicted plasma concentrations remain above the MIC value. Simulation B provides predicted plasma concentrations that remain above the MIC of 0.25 μg/ml, while simulation A provides concentrations that for a short period of time are below the MIC (Figure 20). For drugs with a concentration-dependent profile (such as fluoroquinolones) this difference may be more pronounced and relevant.

Moreover, differences in the fluctuations of drug plasma concentration at the steady state may be relevant when a drug with a narrow therapeutic window is evaluated

since lower or higher plasma concentrations may cause serious therapeutic failures or adverse drug reactions (Toutain and Bosquet-Melou 2004b), respectively.

As seen with other fluoroquinolones, such as marbofloxacin (Sartini et al. 2020a), the kidney and liver were found to have higher danofloxacin residues compared to muscle, heart, and lung in geese. Kidney and liver are the organs which have a higher exposure to fluoroquinolones since they are rapidly absorbed from the intestinal tract into the portal system and primarily excreted unchanged in the urine (Brown 1996). In-line with the present findings, these two organs were also found to have the highest danofloxacin concentration in chickens (Lynch et al. 1994; Zeng et al. 2010) and in ducks (Goudah and Mouneir 2009). In the present study the $AUC_{tissue/plasma}$ ratio was highest for liver and kidney suggesting a wide penetration in these two organs. However, the $AUC_{tissue/plasma}$ ratio found in this study was lower compared to the ratio found for liver (20.62) and kidney (15.43) in chickens by Zeng et al. (2010).

An explorative WT was computed assuming an MRL of 0.4 µg/g. The WT was 2.6 and 3.8 days for liver and kidney, respectively. This is similar to the reported WT in chickens (Yang et al. 2015b), but lower than that reported in ducks (Goudah and Mouneir 2009). However, caution should be taken in the use of the WTs reported in the current study, because they were computed using a limited number of geese.

In conclusion, if the $AUC_{(0-24)}$ value found in the present study is included in the PK/PD index for the prediction of fluoroquinolones' efficacy, danofloxacin seems to be effective in geese against Gram-negative bacteria with a MIC <0.076 µg/ml and against *S. pneumoniae* with a MIC<0.29 µg/mL after a single PO dose of 5

mg/kg. The predicted daily therapeutic dose for *M. gallisepticum* was 9.10 mg/kg. Kidney and liver showed the highest drug tissue penetration value, with an explorative WT of 2.6 and 3.8 days, respectively. The in-field multiple-dose regimen simulation did not suggest any plasma drug accumulation and it may be more predictive for use of drugs with narrow therapeutic windows in large-scale poultry breeding system.

Reference: Sartini, I, Łebkowska-Wieruszewska, B, Lisowski, A, Poapolathep, A, Giorgi, M, 2021. Danofloxacin pharmacokinetics and tissue residues in Bilgorajska geese. Research in Veterinary Science, 136: 11-17.

5. LEVOFLOXACIN PHARMACOKINETICS AND TISSUE RESIDUE CONCENTRATIONS AFTER ORAL ADMINISTRATION IN BILGORAJSKA GEESE

5.1 Aim of the study

The aims of this study were to:

1. assess the pharmacokinetics of levofloxacin in geese after a single IV (2 mg/kg) or PO (5 mg/kg) administration
2. evaluate the drug residue concentrations in selected tissues (muscle, heart, liver, kidney, lung) after a single PO 5 mg/kg levofloxacin administration.

5.2 Material and methods

5.2.1 Chemicals, reagents, and solutions

Levofloxacin and the internal standard (IS), enrofloxacin, powders with a standard purity of 99.0% were purchased from Sigma-Aldrich (Milan, Italy). HPLC-grade acetonitrile, methanol, trichloromethane and isopropanol were procured from Merck (Kenilworth, NJ, USA). Triethylamine 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). Deionized water was produced using a Milli-Q Millipore Water System (Millipore, Darmstadt, Germany).

5.2.2 Animal experiment

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 behavior and appetite. Geese were acclimatized 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 twice a day and water was supplied ad libitum. No pharmacological treatment was received by the birds before the experiment. The animal experiment was approved by the Institutional Animal Care and Use Committee of the University of Lublin (Poland) and carried out in accordance with European law (2010/63/UE).

There were two parts of the study, pharmacokinetic and a tissue depletion. The pharmacokinetic trial involved 16 healthy male Bilgorajska geese (BW, 3.4–4.9 kg; age, 3–4 years) which were randomly divided into two groups (n=8/group). 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 group 2 were given a single oral dose (5 mg/kg) of levofloxacin. The oral doses were prepared by grinding, homogenizing, 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 h after being fed. Blood samples (1 ml) were collected in vacutainer lithium heparin tubes from a 24-gauge catheter inserted immediately before the experiment in the right brachial vein at 0, 5, 15, 30, 45 min and 1, 1.5, 2, 4, 10, 24, 34, and 48 h after IV and at 15, 30, 45 min and 1, 2, 4, 6, 8,

10, 12, 24, and 48 h after the last drug administration after PO treatment. After 12 h, 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 1500x*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 group 2. Two animals were humanely killed by stunning and exsanguination at 6, 10, 24, 34 and 48 h after treatment. Approximately 4 g of muscle, heart, liver, lung and kidney were collected and stored at −20 °C until further analysis.

5.2.3 Analytical method

5.2.3.1 Instrumentation and analytical conditions

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.). The chromatographic separation assay, modified from Lee et al. (2017), was performed using a Gemini analytical column (250 × 4.6 mm inner diameter, 5 µm particle size, Phenomenex, Torrance, California, USA) at 15°C. The mobile phase consisted of acetonitrile: aqueous solution (20:80 v/v%) at a flow rate of 1 ml/min. The aqueous solution consisted of potassium dihydrogen phosphate

(0.02 M), phosphoric acid (0.006 M) and tetraethyl amine (0.012 M) in water (pH 4.0). Excitation and emission wavelengths were set at 295 and 490 nm, respectively.

5.2.3.2 Sample preparation

The procedure was validated for plasma and all tissues collected from the geese, according to Lee et al. (2017), with slight modifications. 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/min for 10 min and centrifuged at 4000 x g for 5 min. 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 (Sartini et al. 2020a). An aliquot of 1 g per tissue was placed into 5 ml plastic tubes containing 3 ml of homogenization solution (0.1 M phosphate buffer at pH 7.1). The suspension was homogenized for approximately 40 s and then 0.2 ml were processed, as described for the plasma samples.

5.2.3.3 Sample quantification

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, LOD and LOQ, according to the EMA guidelines (Anonymous 2012). 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 and tissue samples 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 CV%. The extraction recovery experiment was carried out by analyzing 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 and tissues (control), to the response of equivalent standards. Recovery was expressed as mean±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.

5.2.4 Pharmacokinetic analysis and statistical analysis

Levofloxacin plasma concentration was modelled for each subject using a non-compartmental model using ThothPro software (Gdansk, Poland). C_{max} and T_{max} were determined directly from the concentration *vs* time curves. $t_{1/2kel}$ was calculated using least squares regression analysis of the concentration-time curve, and the AUC was calculated by linear log trapezoidal and the linear-up log-down rule was applied to the final concentration-time points for both IV and PO administration, respectively. From these values, the V_{ss}, MRT and Cl were calculated. Pharmacokinetic estimates were calculated only if the individual value of $AUC_{rest\%}$ was lower than 20% of AUC_{inf} and the square of coefficient of determination (R^2) of the terminal phase regression line was >0.85. Absolute oral F was calculated using the following formula:

$$F\ (\%) = \frac{AUC_{PO\ individual} \times dose_{IV}}{AUC_{IV\ avarage} \times dose_{PO}} \times 100 \quad (9)$$

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 using the $AUC_{tissue/plasma}$ ratio after PO administration (Sartini et al. 2020a). Levofloxacin concentration in the selected tissues were used to calculate preliminary WTs using the software WT 1.4, developed by the European Medicines Agency (Anonymous 2018). The WT was established as being the time when the upper-one sided tolerance limit (99%) with 95% CI was below the MRL of 0.1 μg/g levofloxacin, which reflected the MRL for many fluoroquinolones in poultry liver (Anonymous 1997, 1999, 2002). The pharmacokinetic parameters were normally distributed (tested by Shapiro–Wilk test) and mean 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).

5.3 Results

5.3.1 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 the present study (Table 7).

Parameter	Unit	Plasma	Muscle	Heart	Liver	Lung	Kidney
Equation		y=2.5446x - 0.0611	y=0.1113x - 0.0029	y=0.1356x - 0.0035	y=0.1894x - 0.0079	y=0.1722x - 0.0099	y=0.1454x - 0.0063
R^2		0.999	0.998	0.997	1	0.998	0.996
Inter-day CV	%	5.6	6.1	5.9	6	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

Table 7. Results of the validation of the HPLC assay used for levofloxacin quantification in plasma and different tissues of geese.

5.3.2 Pharmacokinetic analysis

Plasma levofloxacin concentrations were quantifiable up to 24 h in birds administered IV, and up to 48 h after PO treatment. The slope of the elimination phase appears to be similar for both routes of administration (Figure 21).

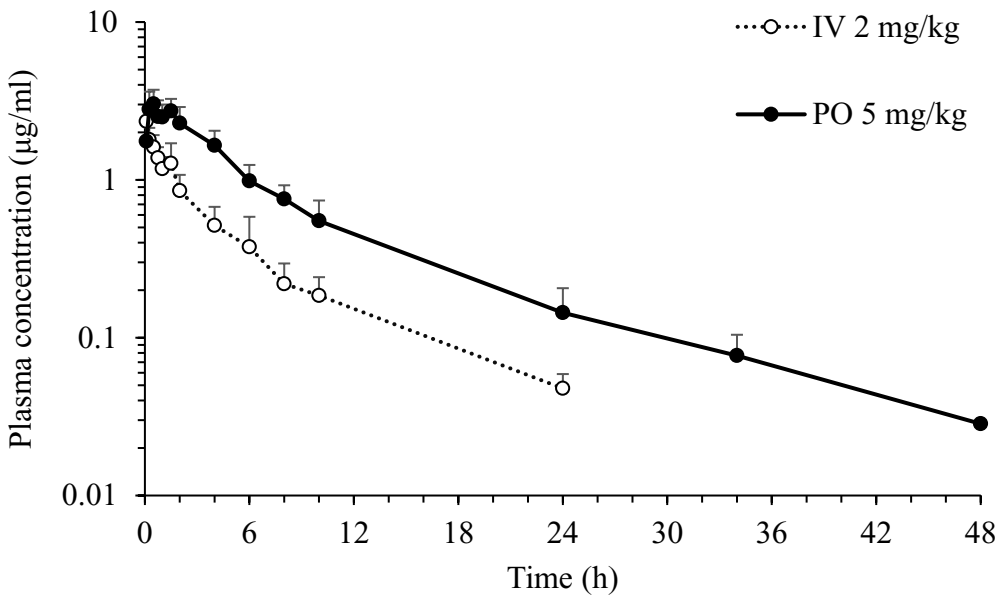


Figure 21. Semilogarithmic plasma levofloxacin concentrations *vs* time curve following IV (··○··, *n*=8) and PO (–●–, *n*=8) administration to geese at a dose of 2 and 5 mg/ kg, respectively.

Levofloxacin was absorbed rapidly after PO administration displaying a high F. The drug showed a moderate V_{ss} and a fast Cl. The $t_{1/2kel}$ was not statistically different between the two routes of administration. If normalized for the dose, C_{max} and AUC were not statistically different between the two different administrations ($p > 0.05$) (Table 8).

		IV (2 mg/kg)		PO (5 mg/kg)	
Parameter	Unit	Mean	SD	Mean	SD
AUC_{last}	mg h/ml	7.59	1.77	17.24	4.86
AUC_{inf}	mg h/ml	8.11	1.76	19.37	4.18
MRT_{last}	h	5.12	0.37	5.71	2.48
MRT_{inf}	h	7.08	0.97	7.65	2.17
k_{el}	1/h	0.10	0.02	0.12	0.05
$t_{1/2kel}$	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	0.085
C_{max}	µg/ml	N/A	N/A	3.20	0.65
$T_{max}^{\dagger}$	h	N/A	N/A	0.38 (0.25 - 1.5)	
F	%	N/A	N/A	95.57	20.61

AUC_{last}=area under the curve from 0 h to last time collected samples, AUC_{inf}=area under the curve from 0 h to infinity, MRT_{last}=mean residence time from 0 h to last time collected samples, MRT_{inf}=mean residence time from 0 h to infinity, k_{el}=terminal phase rate constant, $t_{1/2kel}$=terminal half-life, V_{ss}= volume of distribution, Cl=plasma clearance, V_{ss}/F=volume of distribution normalized for F, Cl/F=plasma clearance normalized for F, C_{max}=peak plasma concentration, T_{max}=time of peak concentration, F= bioavailability. †Median value and range.

Table 8. Mean (±SD) pharmacokinetic parameters of levofloxacin in plasma following IV (2 mg/kg, *n*=8) or PO (5 mg/kg, *n*=8) administration to geese.

5.3.3 Tissue residue analysis

Drug residues were highest at 6 hours and decreased constantly remaining over the LOQ up to 48 h (last time-point of collection) in all selected tissues (Figure 22).

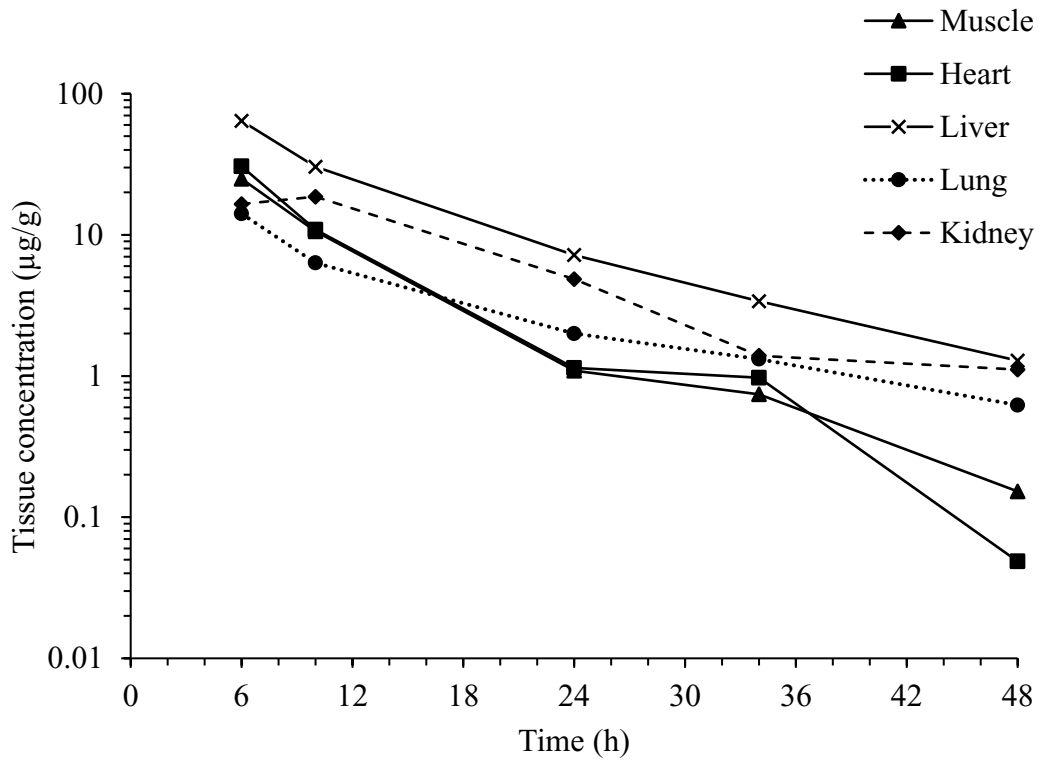


Figure 22. Levofloxacin concentrations (logarithmic scale) in muscle, heart, liver, lung, and kidney following PO administration to geese (*n*=8) at a dose of 5 mg/kg.

Liver was found as the organ with a highest levofloxacin concentration, followed by kidney (Table 9).

Parameter	Unit	Muscle	Heart	Liver	Lung	Kidney
AUC_{last}	mg h/ml	218.72	249.8	687.94	165.26	329.51
MRT_{inf}	h	10.41	9.94	12.56	14.31	13.58
$t_{1/2kel}$	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
$AUC_{tissue/plasma}$		11.87	13.56	37.35	8.97	17.89

AUC_{last}=area under the curve from zero to 48 h; MRT_{last} =mean residence time zero to 48 h; $t_{1/2kel}$=terminal half-life; C_{max}=maximum concentration; T_{max}=time at maximum concentration $AUC_{tissue/plasma}$=area under the curve ratio of tissue:plasma.

Table 9. Pharmacokinetic parameter calculated by the naive pooled-data approach for levofloxacin in different tissues following oral administration to geese at a dose of 5 mg/kg.

5.4 Discussion and conclusions

The geese did not show any adverse effects during or after drug treatments. The dose was chosen based on a previous study on chickens (Lee et al. 2017). The drug showed a moderate half-life (7.39 h) comparable with results from chickens (6.93 h, Lee et al. 2017), but was longer than in ducks (2.76 h), with a slower Cl (geese, 0.28 ml/g h; ducks, 0.41 ml/g h). 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 normalized for dose, than values reported in ducks (4.89 μg h/ml) and chicken (5.09 μg h/ml) (Aboubakr and 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 (Varia et al. 2009; Patel et al. 2012; Aboubakr et al. 2014; Aboubakr and Soliman 2014; Lee et al. 2017). 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 (Varia et al. 2009; Patel et al. 2012; Aboubakr and Soliman 2014; Aboubakr et al. 2014). Fluoroquinolones are drugs that act in a concentration-time dependent manner (Forrest et al. 1993), and the ratio of AUC/MIC is considered the PK/PD index to predict their antimicrobial effects (Turnidge 1999). It has been proposed that a value of 72 for fluoroquinolones can indicate maximum clinical effect in dogs (Madsen et al. 2019). The MIC of levofloxacin has not yet been determined for bacteria isolated from geese. Regarding the $AUC_{(0–24)}$ value obtained in the present study after oral administration (5 mg/kg), levofloxacin in geese appeared be effective against bacteria at a MIC <0.24 µg/ml. For the MIC against *E. coli* isolated in broilers (0.125 µg/ml, Lee et al. 2017), an AUC/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 resulted in a low percentage (25%) in broilers (Lee et al. 2017) and may be considered negligible for the PK/PD 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 h and

gradually decreased over 48 h. In humans approximately 90% of levofloxacin is rapidly absorbed from the intestinal tract into the hepatic portal vein and, as with other fluoroquinolones, is primarily excreted unchanged from the kidney in the urine (Fish and Chow 1997). Hence, it was reasonable to expect a higher drug residue in liver and kidney (Figure 21, Table 9). 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, normalized for dose, were higher than concentrations found by Lee et al. (2017) and Kyuchukova et al. (2013) in chickens. These differences could be due to species specific difference, or the diverse analytical techniques used. The MRL for many fluoroquinolones in poultry liver is about 0.1 μg/g (Anonymous 1997, 1999, 2002). Based on this value, a preliminary WT has been computed with the CI of 95% for liver, resulting in a time of 89.7 h. Even though this matched well with the data reported by Ravikumar et al. (2015) in chickens (4 d), 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 $AUC_{tissue/plasma}$ 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 $AUC_{tissue/plasma}$ ratios in the current study were high in all tissues, and especially in liver (Table 9). Further studies are needed to clarify this point (e.g. whether levofloxacin may be stored specifically in hepatocytes).

In conclusion, a single oral dose (5 mg/kg) of levofloxacin might be effective against bacteria with a MIC<0.24 μg/ml in geese. However, further PD studies are

needed to assess the efficacy of levofloxacin in healthy, as well as diseased, geese. Liver had the highest concentrations of levofloxacin compared to other organs tested, suggesting that drug accumulation might be an issue. The authors would like to emphasize that the results of this study were purely experimental and the use of levofloxacin in avian species is not encouraged.

Reference: Sartini, I, Łebkowska-Wieruszewska, B, Sitovs, A, Lisowsk,i A, Poapolathep, A, Giorgi, M, 2020. Levofloxacin pharmacokinetics and tissue residue concentrations after oral administration in Bilgorajska geese. British Poultry Science, 62: 193-198.

6. DOXYCYCLINE PHARMACOKINETICS IN GEESE

6.1 Aim of the study

This study was aimed to:

- determine the pharmacokinetics of doxycycline following a single IV and PO 20 mg/kg dose in the goose
- perform two simulations of multiple dose treatments at 10 and 20 mg/kg administered daily for 5 days to determine the predicted plasma concentrations.

6.2 Material and methods

6.2.1 Chemicals, reagents, and solutions

Doxycycline and oxytetracycline (IS) powders with a standard purity of 99.0% were purchased from Sigma-Aldrich (Milan, Italy). HPLC-grade acetonitrile was purchased from Merck (Kenilworth, NJ, USA). Trifluoracetic acid (TFA) was obtained from VWR International Bvba (Leuven, Belgium). Deionized water was produced using a Milli-Q Millipore Water System (Millipore, Darmstadt, Germany).

6.2.2 Animal experiment

Ten male Bilgorajska geese underwent a two-phase cross-over study design with a washout period of two weeks. The animals were approximately 2 years of age and their median BW was 3.21 kg (2.88 - 4.28 kg). The animal experiment was approved by the Institutional Animal Care and Use Committee of the University of Lublin (Poland) and carried out in accordance with European law (2010/63/UE).

All animals were judged to be in good health based on physical examination, serum chemistry and haematological analyses performed before the study commencement. The geese were monitored daily through observation of behaviour and appetite. They were acclimatised for 1 week in a 60 m^2 enclosed area with an indoor shelter of 8 m^2 before beginning the study. Animals could graze freely during the day as a ring with an identity code was applied to the left leg for easy identification. Geese were fed with a drug-free pelleted diet twice a day and water was supplied *ad libitum*.

Geese were randomly divided in two groups. In the first phase, group 1 (n=5) was treated with 20 mg/kg doxycycline (Doxycyclinum TZF (0.02 g/mL), Polfa SA Tarchomin, Warszawa, Poland) IV using a sterile 20-gauge 3.75 cm needle in the left ulnar vein, while group 2 (n=5) received a single oral dose of doxycycline (20 mg/kg) (Doxycyclinum 200 Biofaktor, 0.2 g/g, Biofaktor, Skierniewice, Poland) by crop-gavage. The powder was dissolved in sterile water at a concentration of 40 g/l for an easily administration. In the second phase the groups were inverted, with group 2 receiving doxycycline IV and group 1 receiving doxycycline PO at the same dosages.

Blood samples (approximately 1 ml) were collected from a pre-implanted 22-gauge catheter in the right ulnar vein. After each sample collection the catheter was flushed with 1 ml of 0.9% saline containing 10 IU/ml heparin. Prior to each blood collection, the first 0.2 ml of blood was discarded. Blood was collected at 0 (before drug treatment), 0.085, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 10, 24, and 48 h after IV administration. After PO administration, blood was collected at 0 (before drug treatment), 0.025, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 10, 24 and 48 h. Blood was collected

in heparinized tubes and centrifuged at 1500×*g*. The harvested plasma was stored at −20°C and analyzed within 30 days of collection.

6.2.3 Analytical method

6.2.3.1 Instrumentation and analytical conditions

The HPLC system was a LC Jasco (Como, Italy) consisting of a ternary gradient system (PU 980), in line degasser (DG-2080-53), autosampler (AS-2055) and an UV multiple wavelength detector (MD-1510). The chromatographic separation assay was performed with a Luna C18 analytical column (250 × 4.6 mm inner diameter, 5 µm particle size, Phenomenex) maintained at 30 °C using a Peltier system (CO-4062) (Jasco, Como, Italy). The mobile phase consisted of acetonitrile:0.1% TFA (21:79% v:v) in water with a flow rate of 1 ml/min. The optimal wavelength for the quantification was set at 350 nm.

6.2.3.2 Sample preparation

Sample purification was performed using protein precipitation. Two hundred µl of plasma were spiked with 20 µl of IS (10 µg/ml) solution in water. After the addition of 1 ml of acetonitrile and 20 µl of TFA, each sample was vortexed, shaken for 10 min and centrifuged at 4000×*g* for 10 min. One ml of the upper layer was transferred into a clean tube and dried at 45 °C under a gentle nitrogen stream. The residue was dissolved in 200 µl of mobile phase, vortexed and an aliquot of 50 µl was injected onto the HPLC system.

6.2.3.3 Sample quantification

The quantitative HPLC method was fully validated for goose plasma in terms of linearity, intra-day and inter-day precision, recovery, LOD and lower limit of

quantification (LLOQ), according to the EMA guidelines (Anonymous 2012). Doxycycline (1 mg/ml) and IS (1 mg/ml) stock solutions and all related dilutions were produced in water. Linearity was assessed using goose plasma spiked with low (0.1, 0.25, 0.5, 1, 5, 10 µg/ml) or high (10, 25, 50, 100, 250, 500 µg/ml) concentrations. Three replicates of each concentration were analyzed, with two calibration curves constructed using standard doxycycline concentrations *vs* ratio of doxycycline/IS peak areas. Intra-day and inter-day precision were calculated after analysis of six plasma samples spiked with doxycycline at three different concentrations (QC; 0.25, 10 and 250 µg/ml), and expressed as CV (%). Sample recovery was evaluated by comparing the response (in area) of high (250 µg/ml), middle (10 µg/ml), low (0.25 µg/ml) concentration spiked samples, and the IS to the response of equivalent standards. Recovery is expressed as mean±SD. The LOD was estimated as the plasma drug concentration that produced a signal-to-noise ratio of three and LLOQ was determined as the lowest plasma concentration that produced a signal-to-noise ratio of five. The mean concentration was within 15% and 20% of the nominal values for the QCs and LLOQ samples, respectively.

6.2.4 Pharmacokinetic analysis and statistical analysis

The data were pharmacokinetically analysed using a non-compartmental approach (ThothPro software, Gdansk, Poland). C_{max} and T_{max} were determined directly from the concentration *vs* time curves; $t_{1/2kel}$ was calculated using least squares regression analysis of the concentration-time curve. The AUC was calculated by linear log trapezoidal (IV administration) and the linear-up log-down rule (PO administration). From these values, the V_{ss} and Cl were calculated. The individual

value of $AUC_{rest\%}$ was lower than 20% of AUC_{inf} and the R^2 of the terminal phase regression line was >0.85.

The absolute oral F was calculated using the earlier-mentioned formula (6).

The E for doxycycline after IV administration was calculated using equation (2).

Wilcoxon's rank sum test was used to statistically compare the pharmacokinetic data between the two routes of administration (Powers, 1990).

6.2.5 Multiple-dose simulations

The modelling of a daily oral dose regimen of 10 and 20 mg/kg/day administered for 5 days was computed applying the superposition principle and assuming first-order kinetics (Gabrielsson and Weiner 2016) using ThothPro software (Gdansk, Poland).

The potential accumulation ratio following both simulations was determined using equation (7), while the fluctuations of drug plasma concentration at the steady state peak and trough concentrations were calculated using equation (8).

The pharmacokinetic parameters are reported as geometric mean and ranges, except for T_{max} (categorical variable) which is expressed as the median value and range (Julious and Debarnot 2000).

6.3 Results

6.3.1 Analytical method validation

The analytical method demonstrated linearity in the low and high concentration ranges, with R^2 of 0.997 (y = 0.7868x - 0.1987) and 0.999 (y = 0.9913x - 0.5293), respectively. The LOD and LLOQ was 0.03 and 0.1 µg/ml, respectively, and the

mean extraction recovery was 96 ± 17%. The inter- and intra-day precision showed a CV% of 15.3 and 9.1, respectively. For the LLOQ, the CV% was lower than 20%.

6.3.2 Pharmacokinetic analysis

No adverse effects were observed during or after drug administration in any of the geese. Plasma doxycycline concentrations were always higher than the LLOQ of the analytical method (Figure 23).

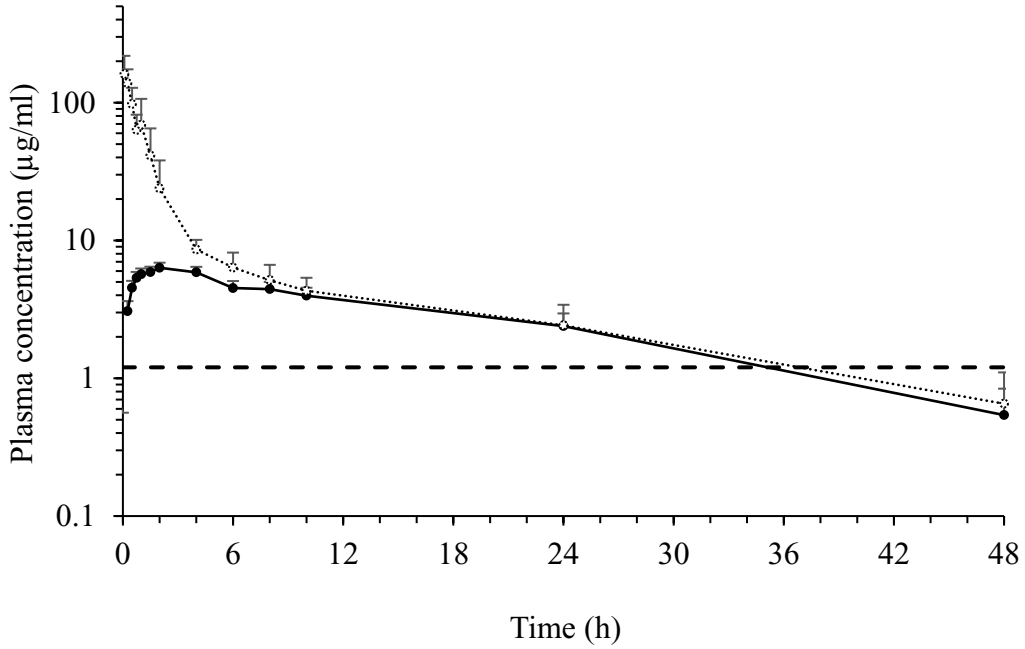


Figure 23. Semi logarithmic doxycycline plasma concentration-time curves after a single 20 mg/kg IV (*n*=7, ···○···) or PO (*n*=10,−●−) dose in geese. The horizontal dashed line represents the MIC value for *M. gallisepticum* (1.20 µg/ml).

The elimination slope of the IV and PO plasma concentration-time curve was similar, with a long elimination $t_{1/2}$ (13.95 h, IV and 13.35 h, PO). The extraction ratio was low (2%). Three animals showed an $AUC_{rest\%}$ higher than 20% in the IV treatment, and so were excluded from the IV pharmacokinetic assessment. Cl was slow, and the V_{ss} was moderate. The oral F was moderate, with a significant difference between AUC_{IV} and AUC_{PO} (Table 10).

		IV			PO		
		Geometric mean	Min	Max	Geometric mean	Min	Max
AUC_{last}	µg h/ml	273.99	202.70	411.05	120.1*	73.26	173.07
AUC_{inf}	µg h/ml	287.62	214.20	427.24	131.2*	82.98	187.48
k_{el}	1/h	0.05	0.04	0.06	0.05	0.05	0.06
$t_{1/2kel}$	h	13.95	11.73	17.84	13.35	11.98	15.13
C_{max}	µg/ml	N/A	N/A	N/A	6.67	4.45	8.99
T_{max}	h	N/A	N/A	N/A	2.00	1.00	4.00
Cl	ml/g h	0.07	0.05	0.10	N/A	N/A	N/A
V_{ss}	ml/g	0.58	0.31	1.03	N/A	N/A	N/A
F	%	N/A	N/A	N/A	42.79	31.62	54.14

AUC_{last}, area under the curve from zero to the last detectable timepoint; AUC_{inf}, area under the curve from zero to infinity; k_{el}, elimination rate constant; $t_{1/2kel}$, terminal half-life; C_{max}, maximum concentration; T_{max}, time at maximum plasma concentration; V_{ss}, volume of distribution; Cl, plasma clearance; F, bioavailability. N/A = Not applicable. *Significantly different between the groups (p<0.05).

Table 10. Pharmacokinetic parameters of doxycycline after a single 20 mg/kg IV (n=7) or PO (n=10) dose in geese.

6.3.3 Multiple-dose simulations

After the third dose of the multiple dose simulated treatment (Figure 24) the steady state was reached with a P/T value of 3.43. The accumulation index was 1.4.

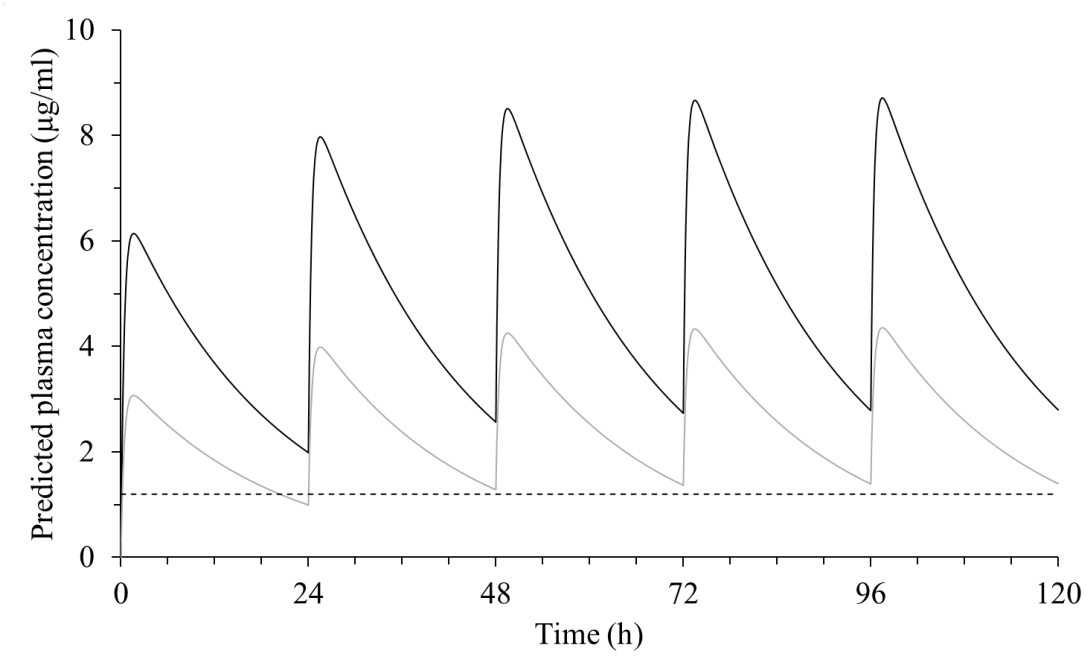


Figure 24. Multiple PO dose simulations of doxycycline in geese at 10 mg/kg (grey line) and 20 mg/kg (black line) daily for 5 days. The horizontal dashed line represents the MIC value for *M. gallisepticum* (1.20 µg/ml).

6.4 Discussion and conclusions

The pharmacokinetic profile of doxycycline in geese was similar to that reported in ducks by Yang et al. (2015a). The Cl was similar to ducks; however, the half-life was longer (ducks, 21 h; geese, 13 h). The V_{ss} in geese was comparable to most other avian species such as ducks (Yang et al. 2015a) and laying hens (Yang et al. 2016), however higher than reported in chickens by Anadón et al. (1994). It has been demonstrated that waterfowl have physiological differences in renal morphology compared to galliform birds, which may result in species differences in renal elimination and/or reabsorption of drugs (Warui 1989). Differences in the activity of liver enzymes between waterfowl and galliform birds may also be a potential cause in the difference in the V_{ss} (Warui 1989).

The absorption profile does not differ appreciably farom that observed in chickens given the same dose (Soliman et al. 2015; Hsiao et al. 2016; Hantash et al. 2008). In chickens the peak plasma concentrations in three studies occurred at 1.30 h, 2.07 h and 3.60 h, respectively (Soliman et al. 2015; Hsiao et al. 2016; Hantash et al. 2008), whereas in geese the C_{max} was reached in 2 h. Substantial differences were observed with the results of Anadón et al. 1994, where the C_{max} in broiler chickens (also administered 20 mg/kg) was much higher than that reported in geese and achieved much faster. Even ducks, another waterfowl species, had a different C_{max} when treated at the same dosage (Yang et al. 2015a). Discrepancies in the experimental methodology, including the different drug formulation used (different excipients) may have contributed to these differences (Toutain and Bousquet-Mélou 2004c). For example, the present study administered an oral powder formulation for poultry to geese, whereas a commercial doxycycline hyclate formulation for injection was used orally in ducks (Yang et al. 2015a). Additionally, differences in the characteristics of the animals such as the age (geese, 2 years; ducks, 6 months) or BW (geese, 3.31 kg; ducks, 1.52 kg), or differences in the status of the animals (e.g., feeding conditions, diet) may have also influenced the drug absorption (Yang et al. 2015a).

Oral F of doxycycline in different avian species has consistently been reported as moderate, both in the current study and in other avian species (Yang et al. 2015a; Yang et al. 2016; Anadón et al. 1994).

The PK/PD index T>MIC (the duration of plasma concentrations exceeding the MIC) has been proposed to predict the success of doxycycline therapy as it is a time-dependent antibiotic (Toutain et al. 2002). MIC values reported in the

literature for doxycycline against different *Mycoplasma spp*. isolated from geese and ducks vary significantly between strains (Grózner et al. 2016; Gyuranecz et al. 2020). *M. gallisepticum* and *M. synoviae* are considered the most relevant pathogens in the poultry industry, with reported MIC values in avian species of 1.20 µg/mL (Zhang et al. 2017) and 0.625-1 µg/ml (Kreizinger et al. 2017; Catania et al. 2019), respectively. The optimal %T>MIC value has been reported as 54.36% during a 48h treatment period with doxycycline (Zhang et al. 2016). In the present study, doxycycline plasma concentration remained above the MIC value of 1.2 µg/ml almost 34 h after PO administration, exceeding the PK/PD index (71%).

Since a multiple dose schedule in the range of 10 – 20 mg/kg is used in practical clinical conditions, two simulations were carried out to predict the plasma concentrations reached after 5 day's treatment with these doses (10 - 20 mg/kg/day). The multiple dose simulation showed that steady state was reached after the third dose. An accumulation index of 1.42 was found, suggesting a slight plasma accumulation. The predicted plasma concentration after both simulations exceeded the MIC (1.2 µg/ml) value (Zhang et al. 2017), suggesting that doxycycline could be a promising therapeutic treatment in geese for *Mycoplasma spp*.. A dosage of 10 mg/kg/day for 5 days seems to be adequate to reach the appropriate plasma levels for clinical efficacy without the need for higher doses and unnecessarily high plasma concentrations (20 mg/kg/day simulation).

Further consideration should be made of the following: (1) since in practice doxycycline would typically be given in drinking water, the drug/water intake could differ between animals. It is therefore reasonable to hypothesize that in a practical context the predicted plasma concentration may be lower than that found in the

present simulation, with a lower P/T ratio (Sartini et al. 2020a, 2021a). Further, the duration of medicated water availability could affect T>MIC and clinical efficacy; (2) the protein plasma binding of doxycycline in geese is not available in the literature and was not evaluated in the present study. In many species it is reported to be high, so may be an important factor in PK/PD analysis; (3) the presence of resistant strains and cross-resistance phenomena may result in ineffective drug treatment in cases where a higher MIC is required. Some studies reported *Mycoplasma* strains requiring an MIC value >10 µg/ml for doxycycline (Grózner et al. 2016; Kreizinger et al. 2017). Thus, it is fundamental to highlight the importance of susceptibility testing before therapy commencement, and antimicrobial stewardship.

In conclusion, doxycycline showed a long half-life with a moderate bioavailability after oral administration. The PK/PD index in the 48 h after a single PO treatment of 20 mg/kg doxycycline (%T>MIC 71%) suggests this dose would be effective against some *Mycoplasma spp*. in the goose. However, further studies are needed to clarify the free fraction of the drug. The multiple dose simulations aimed to reflect clinical use in poultry, and these showed a low accumulation index. A dosage of 10 mg/kg/day for 5 days seems to be adequate for good therapeutic efficacy without achieving unnecessarily high plasma concentrations. Due to the potential variability in drug intake associated with drinking water dosing in clinical practice, and the possible presence of resistant pathogen species, further studies are warranted to confirm these findings.

Reference: Sartini, I, Łebkowska-Wieruszewska, B, Lisowski, A, Poapolathep, A, Sitovs, A, Giorgi, M, 2021. Doxycycline pharmacokinetics in geese. Journal of Veterinary Pharmacology and Therapeutics, 44: 975– 981.

7. SINGLE AND MULTIPLE ORAL AMOXICILLIN TREATMENT IN GEESE: A PHARMACOKINETIC EVALUATION.

7.1 Aim of the study

The study aimed to:

- evaluate the pharmacokinetics of amoxicillin after a single oral dose (20 mg/kg) in geese;
- perform a multiple dose study to evaluate the drug plasma concentration reached after 20 mg/kg was given daily for 4 days in geese
- carry out an *in-silico* simulation to evaluate if a computer driving modeling could predict amoxicillin plasma concentrations at the steady state.

7.2 Material and methods

7.2.1 Chemicals, reagents, and solutions

Pure amoxicillin and acetaminophen (internal standard, IS) powders with a standard purity of 99.0% were purchased from Sigma-Aldrich (Milan, Italy). HPLC-grade acetonitrile and methanol were procured from VWR International S.A.S. (Fontenay-sous-Bois, France). Triethylamine was obtained from Sigma-Aldrich (St Louis, MI, US). Orthophosphoric acid, sodium dihydrogen phosphate, and ammonium acetate were purchased from VWR International Bvba (Leuven, Belgium). Deionized water was produced using a Milli-Q Millipore Water System (Millipore, Darmstadt, Germany).

7.2.2 Animal experiment

A total of 20 geese randomly selected from a larger group were enrolled in this study. The animal experiment was approved by the Institutional Animal Care and Use Committee of the University of Lublin (Poland) and carried out in accordance with European law (2010/63/UE). In the first period, 10 geese (group I) were randomly selected using software (Research Randomizer) and treated with a single dose of amoxicillin (Biomox 10g/100g, Vetoquinol Biowet Sp. o.o., Gorzów Wielkop., Poland) by crop-gavage at 20 mg/kg. In the second period of the study (after three months), the remaining 10 geese (group II) underwent a multiple dose administration. A four-day period was chosen for the achievement of the steady state based on the $t_{1/2kel}$ value of amoxicillin reported in poultry (Abo El-Sooud et al. 2004; Krasucka and Kowalski 2010; Kandeel 2015). Amoxicillin (Biomox 10g/100g, Vetoquinol Biowet Sp. o.o., Gorzów Wielkop., Poland) was given by crop-gavage at 20 mg/kg every day for 4 consecutive days. For ease of administration, the drug was dissolved in sterile water at a concentration of 40 g/l and approximately a volume of 2 ml was given to each animal.

The animals were approximately 2 years of age, and the average BW in groups I and II was 3.32 kg (2.60–3.73 kg) and 4.02 (3.60–4.65 kg), respectively. All animals were judged to be in good health based on physical examination, serum chemistry and hematological analyses performed before the study commencement. The geese were monitored daily through observation of behavior and appetite. They were acclimatized for 1 week in a 60 m^2 enclosed area with an indoor shelter of 8 m^2 before beginning the study. Animals could graze freely during the day as a ring

with an identity code was applied to the left leg for easy identification. Geese were fed with a drug-free pelleted diet twice a day and water was supplied *ad libitum*.

Blood samples (approximately 1 ml) were collected from the right ulnar and jugular vein by direct venipuncture. Concerning the single dose treatment (group I), the blood was collected at 0 (before drug treatment), 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 10, 24 and 48 h after the treatments. After the multiple dose treatment (group II), blood was collected before and at 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8 and 10 h after the first dose administered. A single blood collection was withdrawn immediately after the second (24 h) and third (48 h) dose. Finally, blood was collected at 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 10, 24 and 48 h after the fourth and last administration.

Blood was collected in heparinized tubes and centrifuged at 1500x*g*. The harvested plasma was stored at −20°C and analyzed within 30 days of collection.

7.2.3 Analytical method

7.2.3.1 Instrumentation and analytical conditions

The HPLC system was a LC Jasco consisting of a ternary gradient system (PU 2089), in line degasser (DG-2080-53), autosampler (AS-2055) and an UV detector (UV-975). The chromatographic separation assay was performed with a BDS Hypersil TM C18 analytical column (150 × 4.6 mm inner diameter, 5 μm particle size, Thermo Scientific). The mobile phase consisted of methanol: aqueous solution (9:91 v/v %) at a flow rate of 1 ml/min. The aqueous solution consisted of potassium dihydrogen phosphate (0.01 M), phosphoric acid (0.006 M), and tetraethyl amine (0.012 M) in water (pH = 5.2). The optimal wavelength for amoxicillin quantification was set at 228 nm.

7.2.3.2 Sample preparation

An aliquot of plasma (0.2 ml) was spiked with the IS (10 µg/ml) and 0.5 ml of an acetate buffer 0.1 M at pH 5 was added. One ml of acetonitrile was added for the extraction and each sample was vortexed, shaken and centrifuged at 4000x*g* for 10 min. The supernatant (1.5 ml) was collected in a clean tube and dried at 40°C under a gentle nitrogen stream. The residue obtained was solubilized in 0.2 ml of mobile phase, sonicated for 10 min and centrifuged at 4000x*g* for 5 min. An aliquot of this latter solution (50 µl) was injected onto the HPLC system.

7.2.3.3 Sample quantification

The quantitative HPLC method was fully validated for goose plasma in terms of linearity, intra-day and inter-day precision, recovery, LOD and LOQ, according to the EMA guidelines (Anonymous 2012). Linearity was determined by linear regression analysis, using calibration curves constructed using replicates (n=3) of control samples from control geese matrix spiked with amoxicillin at concentrations of 0.1, 0.5, 1, 5, 10, 50 µg/ml. The intra-day and inter-day precision and accuracy were calculated after analysis of six plasma samples spiked with amoxicillin at three different concentrations (0.25, 5 and 50 µg/ml). The intra-day and inter-day precision is expressed as the percentage coefficients of variation (CV %). The extraction recovery experiment was carried out by comparing the response (in area) of high, middle, low standards (0.25, 5 and 50 µg/ml), and the IS, spiked into blank goose matrix (control), to the response of equivalent standards. The LOD was estimated as the plasma drug concentration that produced a signal to noise ratio of

3, while LOQ was determined as the lowest plasma concentration that produced a signal to noise ratio of 10.

7.2.4 Pharmacokinetic analysis and statistical analysis

The pharmacokinetic analyses were performed using ThothPro software (Gdansk, Poland). Amoxicillin plasma concentration was modeled for each subject using a non-compartmental approach. C_{max} of amoxicillin and T_{max} were obtained directly from the data. The $t_{1/2kel}$ was calculated using linear least squares regression analysis of the concentration-time curve. The $AUC_{0\text{-}inf}$ was calculated using the linear-up log-down rule. Pharmacokinetic estimates were calculated only if the individual value of $AUC_{rest\%}$ was lower than 20% of $AUC_{0\text{-}inf}$ and R^2 of the terminal phase regression line was > 0.85.

The pharmacokinetic parameters are reported as geometric mean and ranges, except for T_{max} (categorical variable) which is expressed as the median value and range (Julious and Debarnot 2000).

Statistical analysis was carried out using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Wilcoxon's rank sum test was used to statistically compare the pharmacokinetic data between the different drug of administrations.

7.2.5 Multiple-dose simulations

The modelling of a daily oral dose regimen of 20 mg/kg/day administered for 4 days was computed applying the superposition principle and assuming first-order kinetics (Gabrielsson and Weiner 2016) using ThothPro software (Gdansk, Poland).

7.3 Results

7.3.1 Analytical method validation

The analytical method demonstrated an optimal specificity and linearity in the concentration range, with R^2 of 0.999 (y = 0.0558x - 0.0042). The LOD and LOQ were 0.05 and 0.25 µg/ml, respectively, and the mean extraction recovery was 79±13%. The inter- and intra-day precision showed a maximum CV% of 10.9 and 8.8, respectively. The intra-day and inter-day accuracy were 83 and 80%, respectively.

7.3.2 Pharmacokinetic analysis

No adverse effects were observed during or after drug administration in the selected geese. After the single oral administration, amoxicillin showed a rapid absorption (Table 11) and plasma concentrations remained detectable up to 8 hours (Figure 25).

		Geometric mean	Min	Max
AUC_{last}	mg h/l	22.51	14.88	51.80
k_{el}	1/h	0.52	0.31	1.25
$t_{1/2kel}$	h	1.33	0.56	2.23
C_{max}	µg/ml	6.79	4.08	11.44
T_{max} §	h	0.50	0.25	1.00

Note: AUC_{last}, area under the curve from last detectable time point; k_{el}, elimination rate constant; $t_{1/2kel}$, terminal half-life; C_{max}, maximum concentration; T_{max}, time at maximum plasma concentration. § Median value.

Table 11. Main pharmacokinetics parameters of amoxicillin in geese after a single 20 mg/kg oral dose (*n*= 10).

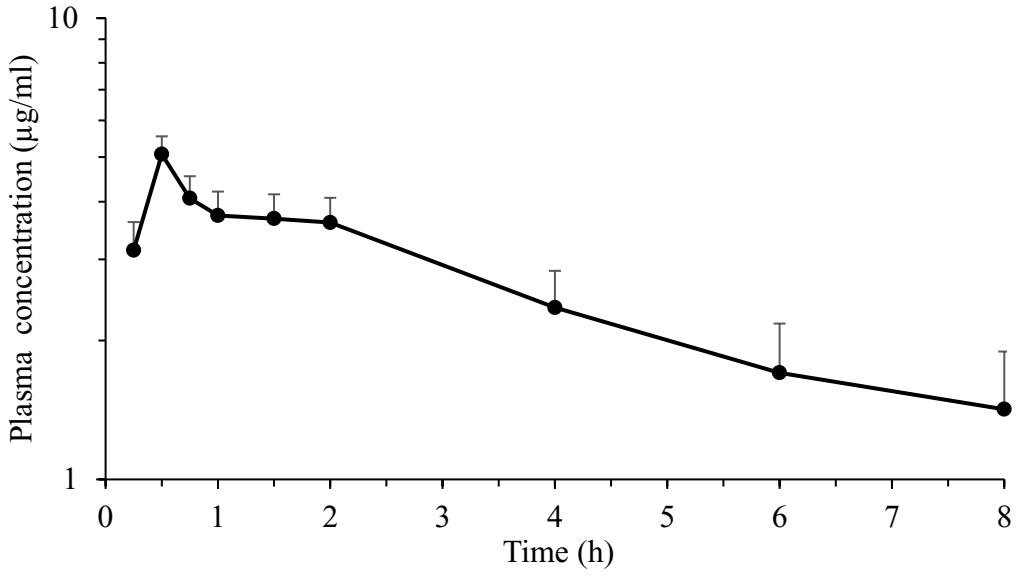


Figure 25. Semi logarithmic plasma concentration vs time curve after a single oral dose (20 mg/kg) of amoxicillin in geese (*n*= 10).

7.3.3 Multiple-dose simulations

Amoxicillin plasma concentrations were quantifiable in all the samples collected up to 48 h after the last administration of the multiple dose treatment (Figure 26).

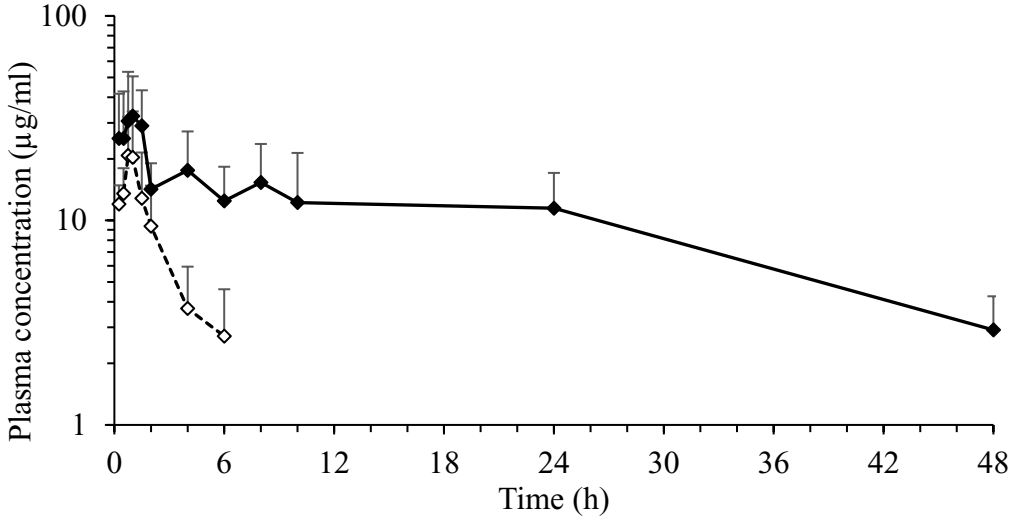


Figure 26. Semi logarithmic plasma concentration vs time curve after the first (···◊···) and the last dose (—♦—) of amoxicillin (20 mg/kg/daily for 4 days) in geese (*n*=10).

The pharmacokinetic profile after the first administration was dramatically different from that found after the last administration. Consequently, the AUC and $t_{1/2kel}$ values were found to be significantly different. Concerning the pharmacokinetic parameters calculated in group I and those calculated after the first dose in group II, C_{max}, T_{max} and AUC values differed statistically (Table 12).

		First dose			Last dose		
		Geometric mean	Min	Max	Geometric mean	Min	Max
AUC_{last}	mg h/l	44.13[a,c]	26.16	74.35	465.46	234.38	853.43
k_{el}	1/h	0.80	0.25	4.36	0.04	0.03	0.06
$t_{1/2kel}$	h	0.86[c]	0.16	2.80	15.59	11.16	20.50
C_{max}	µg/ml	23.99 [a]	11.13	44.02	41.43	20.42	81.21
T_{max}[§]	h	0.88[a]	0.50	1.50	0.75	0.25	1.50

AUC_{last}, area under the curve from last detectable time point; k_{el}, elimination rate constant; $t_{1/2kel}$, terminal half-life; C_{max}, maximum concentration; T_{max}, time at maximum plasma concentration. [§] Median value. [a] Significant difference (p>.05) between the parameters found after the first administration of the multiple dose treatment and those found after the single treatment. [c] Significant difference (p>.05) between the parameters found after the first and the last administration of the multiple dose treatment.

Table 12. Main pharmacokinetics parameters of amoxicillin in geese after the first and last dose administered in the oral multiple-dose study at 20 mg/kg/day for 4 days (*n*= 10).

7.4 Discussion and conclusions

Since these earlier findings showed that a 10 mg/kg dose cannot produce optimal plasma concentrations for an appropriate treatment efficacy in chickens (Abo El-Sooud et al. 2004; Krasucka and Kowalski 2010; Kandeel 2015; Ledesma et al. 2018), a dose of 20 mg/kg was selected in the current study.

This is the first study performed on waterfowls evaluating amoxicillin pharmacokinetics. Although it has been extensively studied in chickens, it is unsafe to extrapolate these findings to geese. Indeed, physiological differences in activity of liver enzymes, as well as in the renal function, of drugs between waterfowl and galliform birds have been demonstrated (Warui 1989).

Amoxicillin absorption in geese after a single dose seems to be faster than in chickens, while the elimination half-life seemed in the same range (1.13-2.47 h). AUC and C_{max} values, if normalized for the dose, were similar to those found in chickens by Abo El-Sooud et al. (2004), and Kandeel (2015). However, the variability of these parameters was considerable in geese. Variances in the BW between the animals might be an additional explanation for the observed differences in the elimination rate (Poźniak et al. 2020). Large differences were observed compared to the study of Anadón et al. (1996), where the drug exposure was dramatically higher in terms of AUC (1534 µg/ml h, chickens vs 22.51 µg/ml h, geese). Moreover, the $t_{1/2kel}$ found in geese (1.33 h) was 4-fold lower compared to that found by Anadón et al. (1996) (9.16 h). These variations are more likely due to differences in experimental conditions and to species specific differences rather than due to the analytical method. Indeed, in all the studies in chickens, the HPLC-

UV system was used for the analysis, but with different clean-up procedures. The sensitivity of our method was similar in terms of LOQ compared to the other studies. This allowed the quantification of amoxicillin up to 8-10 hours after the administration of the drug, as reported in early studies (Abo El-Sooud et al. 2004; Krasucka and Kowalski 2010; Kandeel 2015).

In the present study, a multiple administration treatment was performed since administration of this drug is expected to be repeated daily (Anonymous 2020). Significant differences were found in the drug exposure in terms of AUC (and C_{max}) between the single dose administration and the first dose of the repeated treatment. Although the geese were selected randomly from the same larger group containing animals with similar features (e.g., age, breed, BW), a three-month period was observed between the two experimental phases, thus some environmental conditions might have changed and affected the results.

After the fourth administration of amoxicillin, the plasma concentration decreased rapidly up to 2 h and then decreased slowly (Figure 3). This phase of slow decrease was not found after the first dose (group I and II). A possible explanation for this difference may be a variation in the Cl values, which may have led to a longer $t_{1/2kel}$ (1.33 h, group I *vs* 0.86 h, first dose group II *vs* 15.59 h, fourth dose group II). The difference in the $t_{1/2kel}$ could be due to variation either in the elimination or distribution processes. It is unlikely that the absorption can have played a critical role in this difference. Indeed, even if in the multiple administration group the drug absorption was increased drastically the $t_{1/2kel}$ was not expected to change significantly. Unfortunately, it was not possible to administer the drug IV, due to poor solubility. Thus, the absolute Cl and Vd values were not calculated.

Another possible explanation might be the formation of metabolites or other compounds which were coeluted with amoxicillin during the analytic separation. They might have not had time to be formed after the first administration but only after multiple treatments. In line with this, it is reported that the metabolic pathways of amoxicillin in human liver microsomes includes the formation of seven different metabolites (Szultka et al. 2014). In addition, a study on the metabolome modifications in various chicken tissues after amoxicillin administration, Hermo et al. (2014) showed that the main metabolite clearly evidenced in kidney tissue was diketopiperazine-2',5'-dione derivative. Unfortunately, its concentrations could not be calculated since the commercial standard was not available. Thus, the last shallow part of the pharmacokinetic curve found in this study might not be representative of pure amoxicillin. In fact, the evaluation of the plasma accumulation using the ratio AUC^{1st}/AUC^{4th} gave an exaggerated value which cannot be considered reliable. Further analysis with LC/MS-MS (not available in the present study) are warranted to clarify this issue (Toutain and Bousquet-Melou 2004b).

Assuming that the elimination phase described after the first administration of amoxicillin in the multiple dose study can be totally attributed to amoxicillin, an *in-silico* simulation was performed at the same dosage schedule. The *in-silico* simulation produced a different pharmacokinetic profile after the last dose (Figure 27).

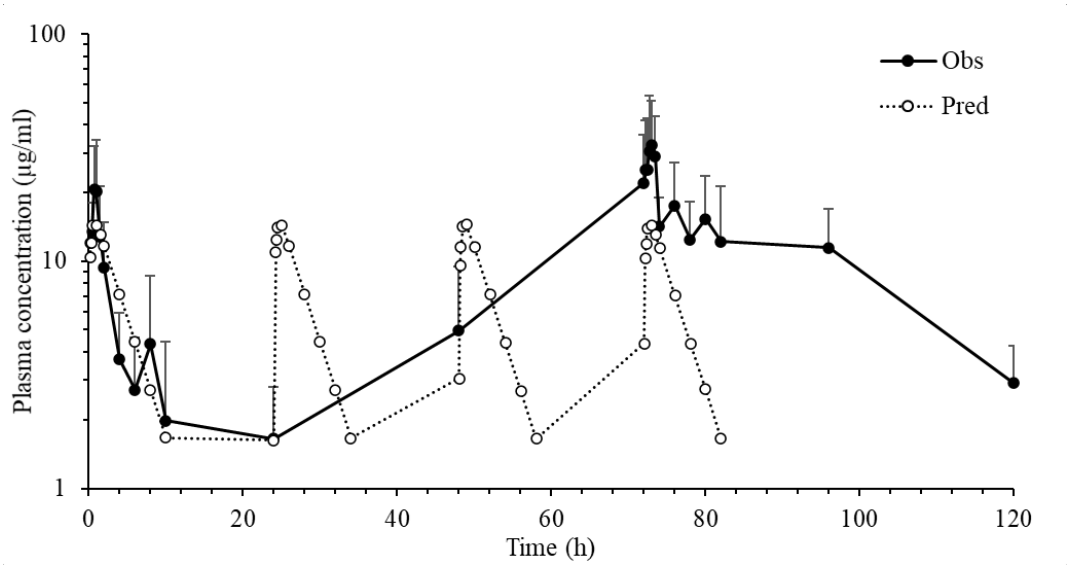


Figure 27. Semi logarithmic observed plasma concentration vs time curve (—●—) after a dose of amoxicillin (20 mg/kg/daily for 4 days) in geese (*n*=10) and the predicted plasma concentration vs time curve of the in-silico simulation (---○---) at the same repeated dosage.

Indeed, due to the short $t_{1/2kel}$, no accumulation in plasma was predicted and no statistical differences were found in terms of AUC after the first dose and the last simulated dose (data not shown). This difference may be due to the assumption for the superposition model, namely that subsequent dosing events will not be affected by drug that is already circulating in the blood (Gabrielsson and Weiner 2016). This assumption might not be reflected in amoxicillin multiple doses. Another potential cause might be that liver or kidney have been affected by multiple drug administration consequently reducing the drug elimination rate. Further studies are warranted to clarify these findings.

Further speculations on the clinical relevance of amoxicillin in geese were avoided due to some limitations of the study such as the high variability of the

pharmacokinetic parameters and the fact that the oral administration was not performed following the “in field condition” (e.g., via drinking water, Sartini et al. 2021b).

In conclusion, amoxicillin showed a rapid absorption after a single dose treatment, with an elimination half-life of approximately 1 h. A high variability in the pharmacokinetic profiles and a potential drug accumulation was observed during the multiple dose treatment. Moreover, in this study, results of the *in-silico* simulation do not predict those from the experimental conditions.

Un-publish data: Sartini I, Łebkowska-Wieruszewska B, Fadel C, Lisowski A, Poapolathep A, Giorgi M. “Single and multiple oral amoxicillin treatment in geese: a pharmacokinetic evaluation” has been accepted to British Poultry Science (19th December 2021).

8. PHARMACOKINETIC PROFILES OF MELOXICAM AFTER SINGLE IV AND PO ADMINISTRATION IN BILGORAJSKA GEESE.

8.1 Aim of the study

The aim of this study was two-fold:

- to provide a pharmacokinetic profile of meloxicam in geese after IV and PO administration;
- to assess the tissue residues of meloxicam in muscle, heart, kidney, lung, and liver after PO administration.

8.2 Material and methods

8.2.1 Chemicals, reagents, and solutions

The pure powder of meloxicam (purity > 99.8%) and of piroxicam (internal standard, IS, purity ≥98%) were provided by Sigma-Aldrich. Acetonitrile, sodium chloride (NaCl), potassium dihydrogen phosphate, methanol, and phosphoric acid were purchased from VWR chemicals (Oud-Heverlee, Belgio). Deionized water was produced by a Milli-Q Millipore Water System (Millipore).

8.2.2 Animal experiment

Ten male Bilgorajska geese were used in this study. The BW ranged from 4.1 to 6.6 kg. Geese were examined to be clinically healthy based on blood analysis (complete blood count) and through daily observation of behavior and appetite. These observations were prepared by licensed veterinary personnel. Before the beginning of the study, geese were randomly divided into two groups: group 1 was composed of treated animals (n=8) and group 2 of control animals (n=2). Animals

were acclimatized for 1 week to the new environment: each group was housed in a 60 m^2 enclosed area with an indoor shelter of 8 m^2. Geese were fed twice a day with a pelleted diet and let free to graze during the day. A ring with the assigned ID code was applied to the right leg for ease of identification. The animal experiment was approved by the Institutional Animal Care and Use Committee of the University of Lublin (Poland) and carried out in accordance with European law (2010/63/UE).

Group 1 underwent a 3-phase parallel study design (phase I, II, III) with a washout period of 1 week between the phases. In phase I, group 1 was intravenously administered (0.5 mg/kg) using a sterile 20-gauge 3.75 cm needle in the left ulnar vein. In phase II (blood collection) and III (tissue collection), group 1 was treated orally with the same dosage (0.5 mg/kg) with a solution administered via crop gavage by a rounded tip metal catheter, 3 h after feeding. Group 2 (control for tissue collection) was administered PO with a volume of saline equal to that given to the treated group. Blood samples (1 ml) were collected from a pre-implanted 22-gauge catheter in the right ulnar vein, at 0, 0.085, 0.25, 0.5, 1, 2, 4, 6, 8, 10, 24, 34, and 48 h in phase I (IV) for group 1. The catheter was flushed with 1 ml of 0.9% saline with 10 IU/ml heparin added. Prior to each blood collection, the first 0.2 ml of blood was discarded. In phase II (PO), blood was collected at 0, 0.085, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 10, 24, 34, and 48 h for group 1. Blood was immediately transferred to tubes containing lithium heparin. Tubes were centrifuged at 1,500x*g* and the harvested plasma stored at −20°C and analyzed within 30 days of collection. During phase III, two animals were sacrificed at each time point (10, 24, 34, and 48 h) in order to collect the following organs: liver, kidneys, lungs, heart, and breast muscle. The animals in group 2 were sacrificed after 24 h following saline administration,

and the organs were collected as described for phase III. Organs were placed in cryobags and immediately stored at −20°C until the analysis.

8.2.3 Analytical method

8.2.3.1 Instrumentation and analytical conditions

Meloxicam in goose plasma was determined using a modified published HPLC method (De Vito et al. 2018; Kimble et al. 2013). The chromatographic separation assay was performed with an Omnishep C18 analytical column (250 × 4.6 mm inner diameter, 5 μm particle size, Agilent) maintained at 25°C. The mobile phase consisted of acetonitrile:0.05 M phosphate buffer pH 3.2 with a flow rate of 1 ml/min. The wavelength was set at 365 nm. The analytical method was re-validated for geese plasma and each tissue sample according to the EMA guidelines on bioanalytical method validation (Anonymous 2012) by examining the within-run precision calculated from similar responses for six repeats of three control samples (0.1, 0.5, and 1 μg/ml) in one run. The between-run precision was determined by comparing the calculated response of the low (0.1 μg/ml), middle (0.50 μg/ml), and high (1 μg/ ml) concentration control samples over three consecutive daily runs (total of 6 runs). The assay accuracy for within-run and between-runs was established by determining the ratio of calculated response to expected response for low (0.1 μg/ml), middle (0.5 μg/ml), and high (1 μg/ml) concentration control samples over 6 runs. LOQ was determined as signal-to-noise ratio of 10 and the LOD as the signal-to-noise ratio of 3.

8.2.3.2 Sample preparation

In a 15 ml polypropylene snap cap tube containing 500 µl of plasma, a volume of 100 µl of IS solution (2 µg/ml) was added. After vortexing, 2.2 ml of acetonitrile was added to the samples and vortexed again. Hundred mg of NaCl was added for the optimal separation of the organic and aqueous phases and vortexed. Then, samples were shaken and centrifuged for 10 min at 4,500x*g*. The organic layer (2 ml) was transferred into a clean 15 ml polypropylene snap cap conical tube and evaporated under a gentle stream of nitrogen at 30°C. Finally, the residue collected was reconstituted with 200 µl of the mobile phase and 50 µl of this latter solution was injected into the HPLC system. Liver, kidney, lung, heart, and muscle samples were thawed and immediately dissected into small pieces. A total of 1 g per sample was placed into 5 ml plastic tubes and was added to 3 ml of homogenization reagent consisting of 0.1 M phosphate buffer at pH 7.4. The suspension was homogenized using a tissue homogenizer for around 40 s. Aliquots of 500 µl were processed as described for plasma samples.

8.2.3.3 Sample quantification

Meloxicam and IS singular stock solutions in MeOH were prepared at the concentration of 1 mg/ml, diluted to reach a final concentration of 100 µg/ml and stored at −20°C. Meloxicam solution was diluted in glass tubes (10 ml) to reach final concentrations of 5, 2.5, and 1 µg/ml, these were stored at +4°C. This last concentration was then diluted at the following concentrations: 0.5, 0.1, 0.05, 0.025, and 0.015 µg/ ml to prepare calibration curves of meloxicam in plasma and tissue matrices. Standard curves were constructed with standard meloxicam

concentrations versus ratio of drug/internal standard peak areas. The analyte was stable for at least 20 weeks if stored at +4°C. Linearity of the regression curve for plasma and tissues were assessed based on the residual plot, the fit test and the back calculation. The efficiency of extraction method was evaluated by comparing the response (in area) of high, middle, low concentration standards, and the IS, spiked into blank plasma or organs, to the response of equivalent standards.

8.2.4 Pharmacokinetic analysis and statistical analysis

Meloxicam plasma concentration *vs* time curves were modelled for each subject using non-compartmental analysis. The pharmacokinetic calculations were carried out using ThothPro software (Gdansk, Poland). C_{max} was peak plasma concentration, T_{max} was time at peak plasma concentration. The $t_{1/2kel}$ was calculated by linear regression on the log-transformed concentration data in the terminal phase, and the AUC was calculated by the log trapezoidal method for the IV group and by the linear-log method for the PO group, to the final concentration-time point. Area under the first moment curve (AUMC) was calculated as $\int_0^\infty c\ t\delta t$. From these values, the Vdss, MRT and Cl were determined. The PO F% was calculated using equation (6).

Individual values between AUC_{inf} and AUC_{last} were lower than 20% of AUC_{inf} and R^2 of the terminal phase regression line was > 0.85.

The MAT was obtained using the following equation:

$$MAT_{PO} = MRT_{PO} - MRT_{IV} \quad (10)$$

Paired Student's *t*-test was used to verify statistically significant differences in pharmacokinetic parameters between groups performed by GraphPad InStat

(GraphPad Software, La Jolla, CA, USA). The normal data distribution was tested by the Shapiro-Wilk test. The pharmacokinetic parameters are presented as means±SD and T_{max} (categorical variable) was expressed as median and range. In all experiments, differences were considered significant if $p < 0.05$.

8.3 Results

8.3.1 Analytical method validation

Parameter	Unit	Plasma	Liver	Kidney	Lung	Heart
Equation		y = 1.3498x + 0.0129	y = 0.9556x - 0.0392	y = 0.8992x - 0.0273	y = 0.9849x - 0.0299	y = 0.952x + 0.0324
Correlation coefficient	R	0.997	0.999	0.996	0.999	0.998
Intre-day precision	%	<8.0	<6.1	<7.8	<11.0	<8.9
Interday-precision	%	<6.9	<8.9	<7.5	<8.3	<10.6
LOQ	µg/ml	0.015	0.015	0.015	0.015	0.015
LOD	µg/ml	0.005	0.005	0.005	0.005	0.005
Recovery	%	96±8	91±4	84±9	93±8	86±9

The method showed an optimal linearity in the range of 0.015-2.5 µg/ml (Table 13).

Table 13. Summary of the main parameters for the analytical method validation of meloxicam in geese plasma and different tissues.

8.3.2 Pharmacokinetic analysis

No adverse effects at the point of injection and no behavioral or health alterations were observed in experimental animals during or after the study. Meloxicam plasma concentrations were quantifiable up to 24 hr. The elimination phase from plasma was similar in both the administration groups (Figure 28).

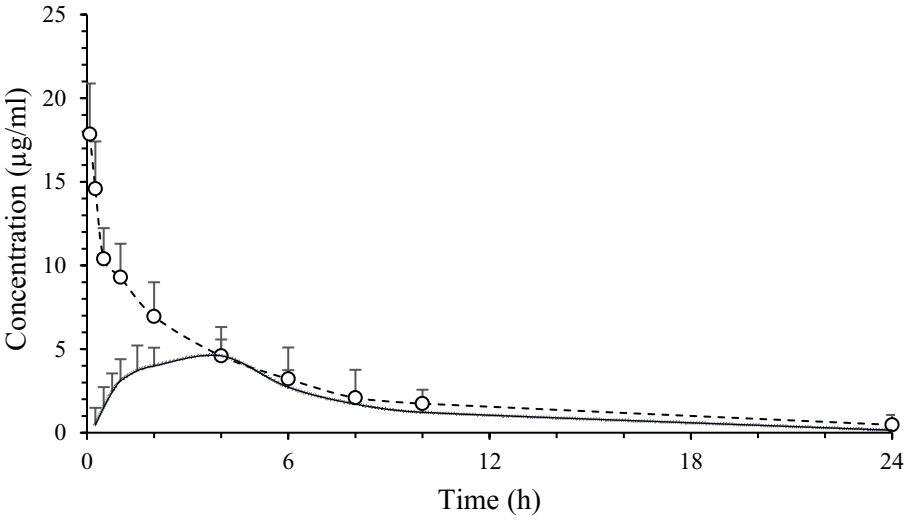


Figure 28. Plasma meloxicam concentration following single IV (—o—, *n*= 8) and PO (—●—, *n*= 8) administration in Bilgorajska geese (0.5 mg/kg).

One goose showed higher plasma concentrations at each time point compared with other geese. Since its individual dataset was an outlier after both routes of administration, it was not excluded from the study, although this increased the

variability of the data. The average absolute PO bioavailability was 64.2 ± 24.0% (Table 14).

Parameter	Units	IV	PO
AUC_{inf}	mg h/l	66.13±47.13	38.08$^{\$}$±19.90
AUC_{last}	mg h/l	64.81±44.57	37.84$^{\$}$±19.49
$AUMC_{last}$	mg h h/l	497.86±700.88	334.16$^{\$}$±329.75
$AUMC_{inf}$	mg h h/l	571.64±870.78	345.75$^{\$}$±352.50
C_{max}	µg/ml	N/A	5.19±1.25
$T_{max}^{\S}$	h	N/A	2 (1-4)
k_{el}	1/h	0.15±0.04	0.22±0.06
$t_{1/2kel}$	h	5.06±2.32	3.65±1.74
Cl	ml/h g	0.01±0.003	N/A
V_{ss}	ml/g	0.05±0.01	N/A
F	%	N/A	64.2±23.9
MRT	h	6.41±3.70	7.83±4.20
MAT	h	N/A	2.37±3.11

AUC_{inf}, area under the curve from 0 h to infinity; AUC_{last}, area under the curve from 0 to last time collected samples; $AUMC_{last}$, area under the first moment of the curve from 0 to last time collected samples; $AUMC_{inf}$, area under the first moment from 0 h to infinity; C_{max}, peak plasma concentration; T_{max}, time of peak concentration; k_{el}, terminal phase rate constant; $t_{1/2kel}$, terminal half-life; Cl, plasma clearance; Vss, volume of distribution at the steady state; F, bioavailability; MRT, mean resident time; MAT, mean absorption time. §Median and range. $^{\$}$ $p < 0.05$

Table 14. Pharmacokinetic parameters of meloxicam after single IV and PO administration (0.5 mg/kg) in Bilgorajska geese (*n*=8).

8.3.3 Tissue residue analysis

Meloxicam residues were lower than the LOD in any tested tissue and at any collection time.

8.4 Discussion and conclusions

The AUC value after IV administration was in line with that reported in chickens, but higher than those found in pigeons, ducks, ostriches and turkeys treated with the same dose (Baert and De Backer 2003). The small V_{ss} reported in this study was in line with those reported in other domestic birds (Baert and De Backer 2003). The Cl value is in the range reported for other domestic birds of similar size, but lower than those displayed in red-tailed hawks, great horned owls (Laçasse et al. 2013) and ostriches (Baert and De Backer 2003). This difference might be due to different food habits, metabolic processes, or plasma protein binding. Generally, NSAIDs are characterised by a small volume of distribution, and this can be due to binding to serum albumin, which can exceed 99% for some compounds in some species. Differences in protein binding across species would be expected to affect clearance, volume of distribution and the fraction of the dose that is able to interact with receptors (Riviere et al. 1997). Unfortunately, in this study plasma protein binding was not calculated and no data is available for geese in the literature. The $t_{1/2kel}$ is in line with those reported for pigeons and chickens, higher than those found in ducks, turkeys, ostriches (Baert and De Backer 2003), red-tailed hawks, great horned owls (Laçasse et al. 2013) and lower than that in African grey parrots (Montesinos et al. 2017). The variabilities of meloxicam half-life values in birds is in line with that earlier reported among mammalian species (Lees and Aliabadi

1991). Following PO administration AUC, T_{max} and C_{max} values found in the present study, if normalized for the dose, are in line with those reported in domestic chickens (Souza et al. 2018). Few studies report meloxicam PO bioavailability in avian species. The PO F% found in the present study was higher than that reported in African grey parrots (Montesinos et al. 2017). This difference could be due to the different feed given to the animals, to species specific differences or to other unknown factors.

One of the subjects in the present study showed 2-fold higher plasma concentrations compared to the other subjects. As the phenomena was observed in both phase I and II, it could be related to peculiar characteristics of this individual rather than random errors incurred in the experimental method.

The therapeutic plasma concentration of meloxicam in geese is yet to be determined. In the literature minimum effective concentrations (MECs) are reported in different species: 833 ng/ml in the dog; 735 ng/ml in the horse and 347 ng/ml in the cat (Toutain and Cester 2004; Toutain and Lassourd 2002). In Hispaniolan Amazon parrots, it has been suggested that a mean plasma concentration of 3.5 µg/ml should adequately provide analgesia (Molter et al. 2013). In the present study, the plasma concentration after PO administration achieved this value for 5 out of 24 h. To provide the average plasma concentration of 3.5 µg/ml/day, the AUC_{0-24} value should be 84 mg h/l. If meloxicam is anticipated to be a concentration dependent drug in geese, a dosage of 1.26±0.39 mg/kg should be used. However, species specific differences need to be considered; PK/PD assessments for the avian species of interest are required in order to evaluate meloxicam efficacy.

The analytical method described in the present study was not able to quantify any residues in any organs as reported in poultry by Bisht et al. (2019). However, the method used in the present study had a LOQ of 0.1 µg/kg that is higher than the MRLs values of meloxicam reported by EMA in muscle, liver and in kidney (20 µg/kg, 65 µg/kg and 65 µg/kg, respectively; Anonymous 2000) for cattle and pigs. The possibility that quantifiable meloxicam residues may be present in the first 10 h following PO administration cannot be excluded (not tested in the present research). A more sensitive technique, such as HPLC-MS/MS, might be necessary to determine likely residue concentrations which remain under the LOD of the present study.

In conclusion, the dosage used in this study achieved the plasma concentration which provides analgesia in *Hispaniolan Amazon* parrots for 5 out of 24 h after PO administration. Meloxicam plasma concentrations were quantifiable up to 24 h and residues were lower the LOQ in all the tissues from 10 to 48 h. Further studies are warranted to clarify the appropriate dose of meloxicam in order to produce effective analgesia in geese.

Reference: Sartini I, Łebkowska-Wieruszewska B, Lisowski A, Poapolathep A, Owen H, Giorgi M, 2020. Pharmacokinetic profiles of meloxicam after single IV and PO administration in Bilgorajska geese. Journal of Veterinary Pharmacology and Therapeutics, 43: 26– 32.

9. ACETAMINOPHEN PHARMACOKINETICS IN GEESE

9.1 Aim of the study

The aims of the study were two:

- evaluate the pharmacokinetics of acetaminophen (APAP) after an IV and PO single dose (10 mg/kg) in geese and to quantify APAP and its main metabolites, paracetamol glucuronide (PG) and glucuronide sulphate (PS) in goose tissues;
- a histopathologic evaluation of goose stomach, duodenum, liver, and kidney tissues was carried out after a single PO administration of APAP (10 mg/kg) to observe potential signs of toxicity.

9.2 Material and methods

9.2.1 Chemicals, reagents, and solutions

APAP, PS potassium, PG sodium salt, and 4-Acetamidophenyl β-D-glucuronide-d3 sodium salt (internal standard, IS) were obtained from Sigma-Aldric (St. Louis, MO, USA). Acetonitrile, methanol, and SiOH 500 mg SPE cartridges were purchase from Avantor Performance Materials (Poland, Gliwice, Poland). Formic acid (99%) was acquired from VWR Chemicals (Randor, USA) and Ultrapure water was obtained using a purification system (Milli-Q purification system, Millipore, France).

9.2.2 Animal experiment

Twenty-four adult male geese (*Anser anser domesticus*) were randomly selected from a larger flock and randomly divided in three groups each composed of eight

animals using a software. BW and age of the geese ranged from 4.6-5.5 kg (average of 5.1 kg) and 3-4 years (average of 3.6 years), respectively. The animal experiment was approved by the Institutional Animal Care and Use Committee of the University of Lublin (Poland) and carried out in accordance with European law (2010/63/UE). They were judged to be in good health based on physical examination before the beginning of the study and were monitored during the experimental trial through daily observation of behaviour and appetite. Birds were acclimatised for two weeks into a 60 m^2 enclosed area with an indoor shelter of 8 m^2 before the beginning of the study. Geese were fed with a drug-free pelleted diet twice a day and water ad libitum. Animals could graze freely during the day and were identified using an identity code ring applied on the right leg.

Group I was administered with APAP 10 mg/ml (Paracetamol, 10 mg/ml, solution for infusion, B. Braun, Germany) at 10 mg/kg in the left brachial wing vein using a sterile 26-gauge 1.75 cm needle.

Group II was PO treated with APAP 500 mg (Paracetamol 500 mg, tablets, Accord, GB) at the same dosage by crop gavage 3 h after being fed. The marketed drug was grounded, homogenized, partitioned, and dosed according to the body weight of each bird. The correct weight of the powder was dissolved in 2 ml water for easily administration using a rounded tip metal catheter. Approximately 1 ml of blood was collected in lithium heparin tubes at 0, 0.083, 0.25, 0.5, 1, 1.5, 2, 4, 6, 8, 10, 24 h and 0, 0.25, 0.5, 1, 1.5, 2, 4, 6, 8, 10, 24 h after IV and PO administration, respectively, using a 24-gauge catheter inserted after the manual removal of the few feathers in the right brachial wing vein and fixed with some tape. The catheter was flushed with 1 ml of 0.9% saline with the addition of 10 IU/ml heparin at each

collection time-point and the first 0.2 ml of blood was discarded before each blood withdrawal. Tubes were centrifuged at 1500x*g* and the plasma was harvested and stored at −20 °C until further analysis.

Group III was PO administered with 10 mg/kg APAP as previously described for group II. Two animals were humanely sacrificed by stunning and exsanguinated at each selected time–points (1, 4, 10, 24 h) for tissue collection. A portion of stomach, duodenum, liver, and kidney was removed and prepared for the histopathological evaluation. A portion of muscle, heart, lung, liver, and kidney was collected at the same time-points for the quantification of APAP and its metabolites.

9.2.3 Analytical method

9.2.3.1 Instrumentation and analytical conditions

APAP and its metabolites were quantified using an UHPLC-MS/MS method previously developed and validated in tissues by Pietruk et al. (2021).

The LC-MS/MS analysis was performed using UHPLC system connected to a triple quadrupole mass spectrometer (SCIEX 4500 triple quadrupole mass spectrometer, Sciex, MA, USA). Separation of the analytes was performed using a column (50 x 2.1mm, 1.8µm, Agilent Zorbax RRHD, Agilent, St Clara, CA, USA) maintained at 45 °C coupled with a guard column. The mobile phase composition was 0.1 % formic acid (A) and 0.1% formic acid in acetonitrile (B). Gradient elution was performed with the following program: 0-5 min 95% A, 5-6.3 min 15% A and finally from 6.31 to 8 min back to 95% A at a flow rate of 0.6 ml/min. Detection was conducted in positive and negative electrospray ionization mode. Two

transitions were monitored for paracetamol and metabolites and one transition for IS using Analyst 1.6.2 software (SCIEX, Framingham, MA, USA).

9.2.3.2 Sample preparation

Briefly, 2 g of heart, muscle, liver, lung, or kidney sample was spiked with 10 µl of IS working solution (5 µg/ml). Then, 4 ml of acetonitrile and 4 ml of 0.1% formic acid in methanol was added and vortexed. The sample was centrifuged for 10 min at 20 °C and 6 ml of the supernatant was passed through a C18 SPE cartridge, pre-conditioned with 2 ml of methanol. The filtrated extract was dried using nitrogen stream at 45°C. The residue was dissolved in 600 µl of 0.1% formic acid and filtered into vials. Ten µl of this latter solution was injected into the chromatography system.

Two hundred µl of plasma was placed into the micro-centrifuge tube, fortified with 40 µl of IS at a concentration of 1 µg/ml. Then, 460 µl of methanol was added, mixed for 30 s and centrifuged for 5 min. Six hundred µl of supernatant was transferred into a glass tube and evaporated to dryness under a stream of nitrogen at 45°C. The dry residue was then reconstituted with 300 µl 0.1% formic acid and transferred into LC-vials before injection into the chromatography system.

9.2.3.3 Sample quantification

The method for the determination of APAP and its metabolites in goose plasma was validated based on linearity, selectivity, precision, recovery, limits of detection and quantification. Two different calibration curves (10-500 ng/ml and 1000-10000 ng/ml) were prepared using drug-free goose plasma samples spiked at 5 concentration levels. The specificity was evaluated by analysing different blank

goose plasma samples (*n*=6) and checked for potential interferences with endogenous substances. Precision, repeatability and within laboratory reproducibility were calculated by the repeated analysis of drug-free plasma samples (*n*=6) fortified with APAP and its metabolites at 3 concentration levels (50, 100 and 250 ng/ml). The CV values were calculated. For repeatability, samples were analysed on the same day by the same operator and with the same instrument. For within laboratory reproducibility, another two sets of fortified samples at the same concentration levels as for the repeatability were analysed on two different days with different operators and the same instrument. The average recovery was investigated by comparing the mean measured concentration with the fortified concentration of the samples. The limits of detection and quantifications were calculated as a signal to noise ratio of three and ten, respectively, of each analyte in fortified (lowest detectable concentration level) samples. Validation results for tissues have been reported previously (Pietruk et al. 2021).

9.2.4 Histopathological evaluation

Immediately after the sacrifice of the animals, a portion of proventriculus (glandular stomach), gizzard (muscular stomach), duodenum, liver and kidney was collected. The material was rinsed with saline, fixed in buffered 10% formalin, and embedded in paraffin blocks using a routine histological technique. Ten µm thick sections of the selected organs were obtained using a microtome and placed on the SuperFrost Plus slides (Fisher Scientific, Bishop Meadow Road, Loughborough, Leicestershire, LE11 5RG). Then, all sections were stained with the Mayer's haematoxylin and eosin (H + E). The stained slices were observed, analysed, and

photographed using the Olympus BX51 light microscope with the digital Olympus Color View III camera (Olympus, Tokyo, Japan).

9.2.5 Pharmacokinetic analysis and statistical analysis

The pharmacokinetic analysis was performed using a pharmacokinetic software (ThotPro™ software, Gdansk, Poland). A non-compartment approach was used for data evaluation obtained after IV and PO administration. C_{max} and T_{max} of APAP, PG and PS were determined from the raw data. The elimination half-life was calculated by linear regression on the log-transformed concentration data in the terminal phase. The AUC of APAP was calculated by linear log trapezoidal and the linear-up log-down rule to the final concentration–time point for the IV and PO group, respectively. From these values, APAP V_{ss}, MRT and systemic Cl were determined. The absolute oral bioavailability was calculated using equation (6).

Individual values between AUC_{inf} and AUC_{last} were lower than 20% of AUC_{inf} ($AUC_{rest\%}$<20%), and R^2 was >0.85.

The E for APAP after IV administration was calculated using formula (2).

A naïve pooled-data approach using a non-compartmental analysis was used to calculate the pharmacokinetic parameters for APAP and its metabolites in all the selected tissues (Pouplin et al. 2016).

The drug tissue accumulation was determined considering the ratios $AUC_{tissue/plasma}$ after PO administration.

All pharmacokinetic values are presented as geometric mean and range, while T_{max} as a categorical variable, was expressed as median and range. Statistical analysis

was performed using a statistical software (GraphPad Prism v 5.0, GraphPad Software, Inc., La Jolla, CA, USA) (Julious and Debarnot 2000). Mean values were compared between the two routes of administration using Wilcoxon rank-sum test.

9.3 Results

9.3.1 Analytical method validation

The developed method was successfully validated in goose plasma, the matrix-matched curves exhibited good linearity for all the analytes through the coefficient of correlation r^2>0.996 (Table 15). The method specificity showed that no potential interfering compounds were detected at the retention time of the APAP and metabolites.

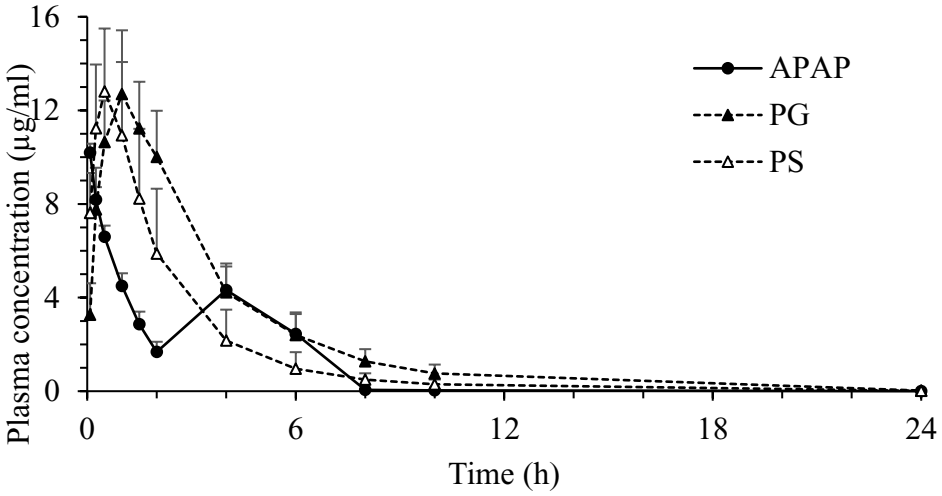
Analyte	Repeatability †	Within-lab Reproducibility†	LOD	LOQ	Recovery†
	CV%	CV%	ng/ml	ng/ml	%
APAP	7.7 ± 2.6	11.3 ± 1.8	5.0	10.0	87.4 ± 4.6
PS	9.2 ± 2.3	13.2 ± 2.8	5.0	10.0	111.2 ± 3.7
PG	11.7 ± 2.7	14.8 ± 3.1	5.0	10.0	92.8 ± 5.1

†average±SD (*n*=3) for each validation level

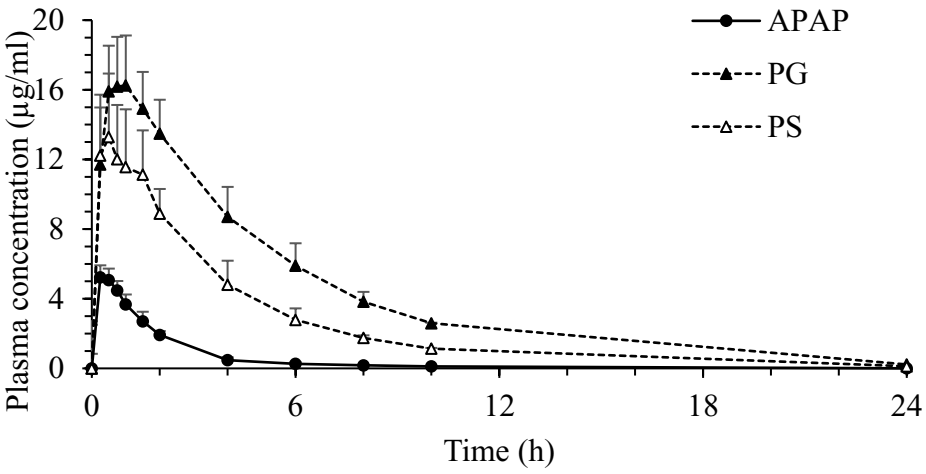
Table 15. Validation parameters of the analytical method used for the quantification of APAP, PG and PS in goose plasma.

9.3.2 Pharmacokinetic analysis

APAP plasma concentrations were lower compared to those of the metabolites at almost all the selected time points after both treatments. It is noteworthy to report that a double peak phenomenon was observed in plasma APAP concentration 4 h after IV administration in all the animals (Figure 29).



(a)



(b)

Figure 29. Mean APAP, PG and PS plasma concentration *vs* time curve after a single 10 mg/kg IV (a) and PO (b) administration of APAP in geese (n=8).

After IV treatment, the AUC_{APAP} value was statistically higher ($p < 0.001$) than that found after PO administration, resulting in an oral bioavailability of 46%. In contrast, PO AUC_{PS} and AUC_{PG} were statistically higher (double) than those found after IV administration ($p < 0.001$) (Table 16).

		APAP					
		IV			PO		
Parameter	Unit	Geom mean	Min	Max	Geom mean	Min	Max
AUC_{last}	µg h/ml	24.91	20.61	33.92	10.52***	8.37	12.89
AUC_{inf}	µg h/ml	24.95	20.65	33.95	11.55***	9.23	13.36
MRT_{last}	h	3.08	2.65	3.49	2.31***	1.71	2.78
MRT_{inf}	h	3.11	2.70	3.51	4.04	2.43	15.86
k_{el}	1/h	0.15	0.13	0.19	0.14	0.02	0.35
$t_{1/2kel}$	h	4.66	3.70	5.33	5.01	1.97	29.60
C_{max}	µg/ml	N/A	N/A	N/A	5.31	4.37	6.50
T_{max}†	h	N/A	N/A	N/A	0.25	0.25	0.50
Cl	ml/g h	0.40	0.30	0.49	N/A	N/A	N/A
V_{ss}	ml/g	1.23	1.03	1.36	N/A	N/A	N/A
F	%	N/A	N/A	N/A	46.29	34.49	59.56

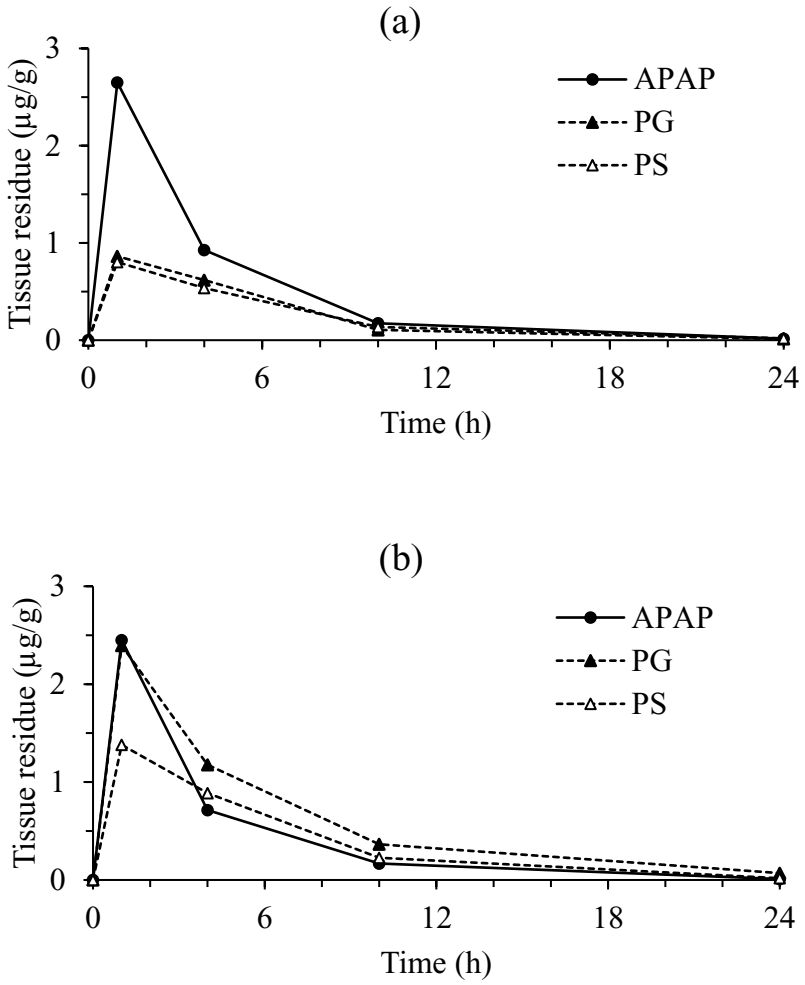
		PG					
		IV			PO		
Parameter	Unit	Geom mean	Min	Max	Geom mean	Min	Max
AUC_{last}	µg h/ml	48.09	37.17	64.45	89.04***	61.73	108.50
AUC_{inf}	µg h/ml	48.14	37.17	64.67	93.06***	71.14	108.92
MRT_{last}	h	3.51	2.97	4.27	4.68**	3.43	5.50
MRT_{inf}	h	3.54	2.97	4.32	5.30***	4.95	5.69
k_{el}	1/h	0.30	0.23	0.36	0.20***	0.18	0.24
$t_{1/2kel}$	h	2.34	1.94	3.06	3.41***	2.87	3.90
C_{max}	µg/ml	12.96	10.90	18.50	16.55	13.40	21.40
T_{max}†	h	1.00	0.50	1.50	0.75	0.75	1.50
		PS					
		IV			PO		
Parameter	Unit	Geom mean	Min	Max	Geom mean	Min	Max
AUC_{last}	µg h/ml	31.46	22.22	60.72	53.02**	38.42	79.18
AUC_{inf}	µg h/ml	31.56	22.30	60.74	57.44***	48.31	80.11
MRT_{last}	h	2.56	2.09	3.19	3.96***	2.66	4.85
MRT_{inf}	h	2.64	2.18	3.65	5.32***	4.25	11.65
k_{el}	1/h	0.23	0.14	0.33	0.17	0.05	0.27
$t_{1/2kel}$	h	3.02	2.10	4.94	4.13	2.59	14.07
C_{max}	µg/ml	13.66	10.80	18.30	13.57*	11.13	20.10
T_{max}†	h	0.38	0.25	1.00	0.50	0.25	1.50

AUC_{inf}, area under the curve from 0 h to infinity; AUC_{last}, area under the curve from 0 to last time collected samples; MRT_{inf}, mean resident time from 0 h to infinity; MRT_{last}, mean resident time from 0 to last time collected samples C_{max}, peak plasma concentration; T_{max}, time of peak concentration; k_{el}, terminal phase rate constant; $t_{1/2kel}$, terminal half-life; Cl, plasma clearance; Vss, volume of distribution at the steady state; F, bioavailability. N/A = Not applicable. † Median value. Significantly difference between the treatments, *p<0.05; **p<0.01; ***p<0.001.

Table 16. Main pharmacokinetic estimates (geometric mean) of APAP, PG, and PS in geese after a single IV and PO APAP administration (*n*=8, 10 mg/kg).

9.3.3 Tissue residue analysis

APAP residues were higher in liver and lung compared to the other tissues with higher AUC and C_{max} values. In liver and lung, the tissue accumulation ratio was the highest (Figure 30, Table 17).



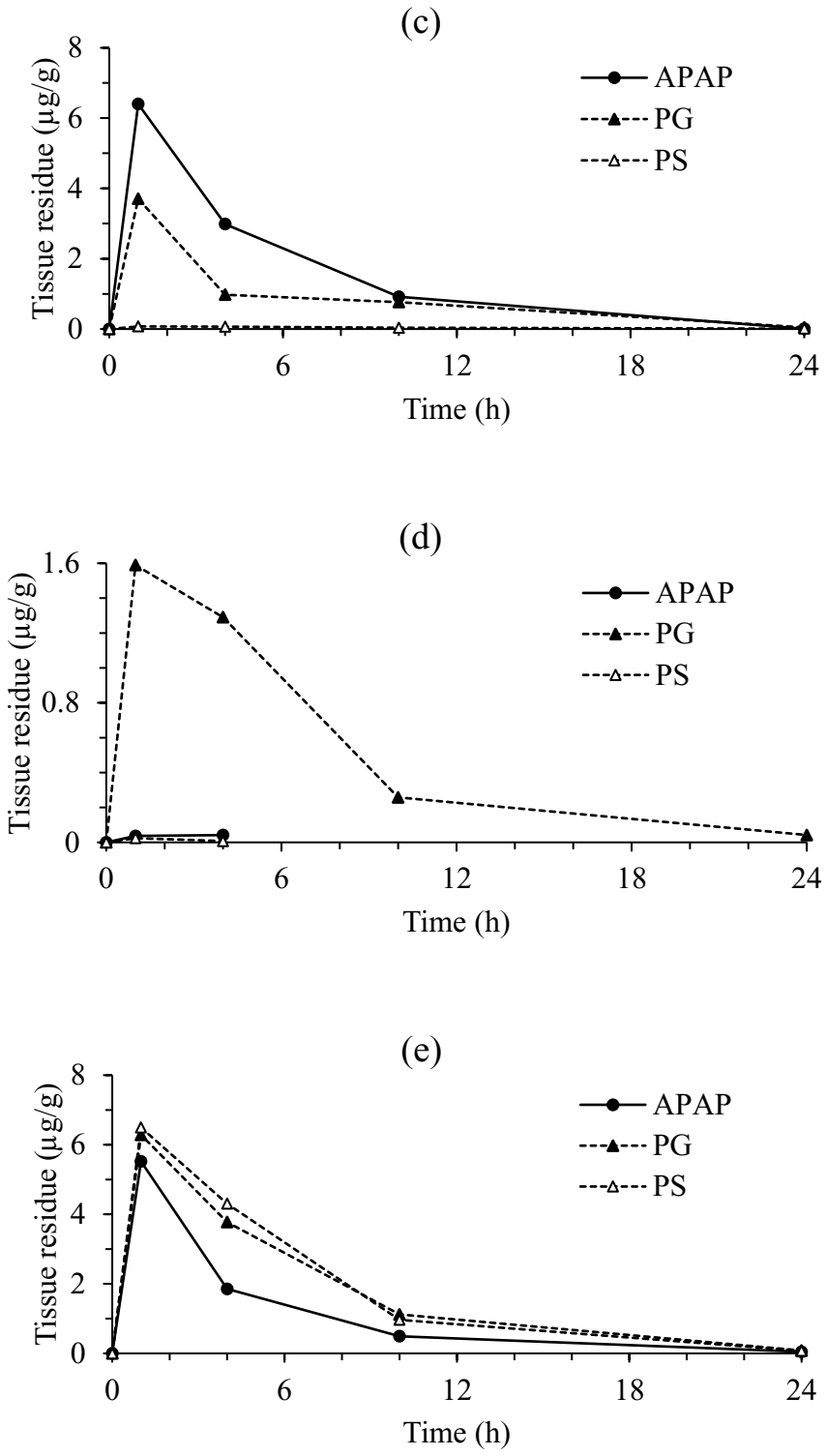


Figure 30. Mean APAP, PG and PS tissue concentration *vs* time curve after a single PO administration (10 mg/kg) of APAP in goose muscle (a), heart (b), liver (c), kidney (d) and lung (e) (*n*=2 per time-point).

	Parameter Unit	AUC_{last} µg h/ml	C_{max} µg/ml	T_{max}† h	Accumulation index
Muscle	APAP	11.30	2.63	1.00	1.06
	PG	5.65	0.86	1.00	0.06
	PS	10.30	2.63	1.00	0.19
Heart	APAP	9.21	2.44	1.00	0.86
	PG	14.19	2.39	1.00	0.16
	PS	9.10	1.35	1.00	0.17
Liver	APAP	32.38	6.40	1.00	3.04
	PG	19.76	3.70	1.00	0.22
	PS	0.67	0.09	2.50	0.01
Kidney	APAP	/	/	/	/
	PG	11.84	1.73	2.50	0.13
	PS	/	/	/	/
Lung	APAP	24.64	5.49	1.00	2.31
	PG	41.33	6.28	1.00	0.46
	PS	42.50	6.44	1.00	0.80

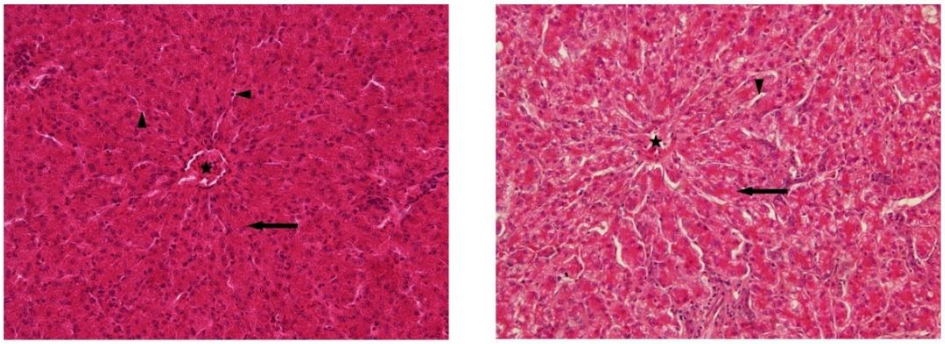
Table 17. Pharmacokinetic parameters (geometric mean) for APAP, PG and PS in different tissues following PO administration of APAP to geese (*n*=2/timepoint) at a dose of 10 mg/kg.

In the liver, PS concentrations were very close to the limit of quantification, while it was the highest in the lung (Figure 29e). The AUC_{PG} and AUC_{PS} values were

similar in the lung, while in the liver, AUC_{PG} was about 30-fold higher than AUC_{PS} (Table 17). The kidney showed low drug and metabolites levels, but the PG residue was much higher compared to APAP and PS levels. The APAP and PS levels were quantifiable only for the first two collection time points (1 and 4 h) and their PK parameters could not be calculated. APAP concentrations in heart and muscle were similar.

9.3.4 Histopathological results

No histological alterations in the morphology of stomachs, duodenal wall and kidneys were observed in any of the examined birds (data not show). The microscopic analysis showed no differences in the structure of the goose liver from the experimental groups compared to the control animals in the first hours after administration. A normal and typical structure of the liver was found in all the geese at 1, 4 and 10 h after drug administration. However, the goose liver samples collected at 24 h after drug administration showed dilated capillaries and lipid accumulation in hepatocytes (Figure 31a,b).



Central vein (*); sinusoids (◀); hepatocytes (◀—). The magnification of objective x 40.

Figure 31. Fragment of the hepatic lobule from the control group (a) and treated group 24 h after administration of APAP (10 mg/kg) (b).

9.4 Discussion and conclusions

9.4.1 Pharmacokinetic analysis

APAP pharmacokinetics have been previously investigated in avian species such as chickens and turkeys. PG and PS plasma levels were evaluated in turkeys only and were always higher than APAP concentrations. A similar situation was observed in the present research. APAP was eliminated at a slower rate than in chickens and turkeys (Neirinckx et al. 2010). V_{ss} in geese was in line with those found in other avian and in some mammalian species (Engelking et al. 1987; Ali et al. 1996; Janus et al. 2003; Neirinckx et al. 2010). APAP is a small lipophilic molecule with a low molecular weight, and it is found in a unionized form at all physiological pH values (Ali et al. 1996; Bertolini et al. 2006). These features, including the low plasma protein binding (15%, swine; 21%, humans) (Gazzard et al. 1973), may contribute to its wide distribution. Geese Cl was slower compared to those reported in chickens and turkeys and the E value was found to be low (0.05) as extrapolated from other studies in poultry (chickens, E = 0.1; turkeys, E = 0.08) (Neirinckx et al. 2010).

APAP C_{max} and T_{max} resulted in an intermediate value between those reported in chickens and turkeys administered the same dose (Neirinckx et al. 2010). The difference in gastric emptying and feed status between the studies may have influenced these parameters (Clements et al. 1978). Moreover, the different formulation administered, different excipients and carrier, may be another reason of this variation.

AUC of APAP was statistically different between the PO and IV treatments. Even if AUC was higher in geese compared with those found in chickens and turkeys,

the oral bioavailability was moderate (46%) and in line with those from other avian species (chickens, 42%; turkeys; 39%) (Neirinckx et al. 2010). This suggests that although species specific differences may occur in drug exposure, the bioavailability of the drug is similar. Species specific differences in the absorption process and the different formulation used may have contributed to the discrepancy in AUC values between the above-mentioned avian species. For instance, in the study of Neirinckx et al. (2010) the IV formulation was also used for the oral administration.

Some differences have been reported in AUC and/or F% value of APAP between avian and mammalian species (turkeys, chickens, geese *vs* pigs, horse) (Neirinckx et al. 2010). These may be related to species specific differences in the extent of the first-pass hepatic extraction or in the absorption process (Clements et al. 1984; Neirinckx et al. 2010). Indeed, in most animal species, humans included, APAP is rapidly and predominantly absorbed from the gastrointestinal tract, but it is not completely available to the systemic circulation due to the first-pass metabolism (Forrest et al. 1982; Clements et al. 1984; Gramatté et al. 1994; Neirinckx et al. 2010). Other metabolic processes have been documented to occur in the intestine during absorption (Josting et al. 1976; Prot et al. 2014).

APAP exhibited a secondary peak in plasma profile 4 h after IV administration in all the geese. Surprisingly, this peak did not occur after PO treatment nor in metabolite concentration profiles. It might be attributed to the phenomenon of enterohepatic recycling (Clements et al. 1978; Siegers et al. 1983; Davies et al. 2010). Although this is the first time that enterohepatic circulation is speculated to be relevant in APAP metabolism in geese, other NSAIDs like rofecoxib, carprofen

and diclofenac undergo enterohepatic recirculation in veterinary species. In addition, a secondary peak concentration after IV administration of rofecoxib and diclofenac has been reported in rats (Peris-Ribera et al. 1991; Eeckhoudt et al. 1997; Priymenko et al. 1998; Baillie et al. 2001).

After PO administration, AUC_{PG}/AUC_{APAP} (1.93) and AUC_{PS}/AUC_{APAP} (8.06) ratios were higher compared to those found after IV administration (1.27 and 4.97, respectively). This may be related to some enzymes being present in the intestine which may have a different influence on the oral formulation administered (Josting et al. 1976; Prot et al. 2104). These IV ratios (metabolite/APAP) are not in line with the results reported in goats and camels (Ali et al. 1996). It should be noted that this comparison is being made on significantly different species (avian *vs* mammals) and on data obtained with different analytical methods (Toutain and Bousquet-Mélou 2004b).

9.4.2 Tissue residue analysis

Higher APAP concentrations were found in the liver and lung, compared to muscle, heart, and kidney. Tissue accumulation occurred in liver and lung, with an accumulation index of 3.04 and 2.31, respectively. Concerning liver, this might be related to the fact that APAP is metabolized predominantly in the liver and/or to the possible covalent binding of APAP to hepatocytes (Graham et al. 2013).

As far as lung is concerned, APAP seemed to penetrate quite well in goose lungs. This behavior might be related to the physiological and anatomical species characteristics and to the physicochemical properties of the drugs: it may lead to a high affinity to the alveolar epithelial lining fluid and/or bronchoalveolar lavage

cells as it happened for other drugs such as tulathromycin (Villarino et al. 2013). Although few studies are available in the literature on the effects of APAP on human lungs, it has been speculated that APAP may be present at this site (McBride et al. 2011; Kennedy et al. 2019; Bjerg 2020). However, no information is available on lung residues in avian species and the explanation for its high accumulation index is uncertain and needs further investigations.

Higher PS concentrations were found in the kidney compared to APAP. This is in line with early studies in animal species and humans where APAP is renally eliminated (approximately 90% in humans) mostly as metabolites, while only 2 to 5 % of the unchanged drug is excreted in the urine (Forrest et al. 1982; Clements et al. 1984; Ali et al. 1996; Neirinckx et al. 2010).

9.4.3 Histopathological results

No adverse effects were observed during or after the experimental trial in any of the animals. No signs of toxicity were found in the kidney which is the main site of toxicity for NSAIDs (Swan et al. 2006; Akhter and Sarker 2015; Palocz et al. 2016). This is in line with the results of Jayakumar et al. (2010) where no histopathological changes were found in chicken kidneys after 7-day administration at 10 mg/kg. Severe hepatotoxicity signs were observed in chicks after 650 mg/animal of APAP given for 7 days with an increase in liver AST, ALT and ALP, while a single dose of 2 g/kg was found to be lethal (Joulideh Pour 2016; Marmat et al. 2015). In the present study, minor liver alterations were observed in both geese liver samples collected at 24 h after administration, suggesting fatty degeneration of hepatocytes. Oxidative stress caused by the toxic metabolite of APAP – N-acetyl-p-

benzoquinone (NAPQI) – might be responsible for these effects. Unfortunately, in the present study it was not possible to obtain data related to the toxic metabolite NAPQI, limiting the reliability of this latter speculation. However, consistent with the present results, signs of liver congestion were previously observed in poultry after a single dose of APAP (10 mg/kg), while severe alterations such as progressive granular degenerative changes and diffuse or focal necrosis were reported in poultry treated with higher dosages (20 and 40 mg/kg) (Hedau and Bhandarkar 2010). The toxicity evaluations performed in the present research cannot lead to a rigorous conclusion since multiple dose and efficacy studies are required to better assess the severity of hepatotoxicity at the therapeutic dose (unknown yet) in geese. Thus, it can also be concluded that doses higher than 10 mg/kg, should be avoided since mild or severe hepatotoxic effects may occur.

Unpublish data: Sartini I, Łebkowska-Wieruszewska B, Gbylik-Sikorska M, Pietruk K, Krawczyk A, Gajda A, Lisowski A, Poapolathep A, Giorgi M. “Acetaminophen pharmacokinetics in geese” has been accepted to Journal of the American Veterinary Medical Association (12th April 2021).

10. ORAL PHARMACOKINETICS OF IVERMECTIN IN GEESE

10.1 Aim of the study

The study was aimed to:

- describe the pharmacokinetic profiles of ivermectin in geese after PO and IV administration (0.2 mg/kg)

10.2 Material and methods

10.2.1 Chemicals, reagents, and solutions

Ivermectin and the internal standard (IS, moxidectin) both with a standard purity of 99.0% were purchased from Sigma-Aldrich (Milan, Italy). HPLC-grade acetonitrile was purchased from VWR International Bvba (Leuven, Belgium), while deionized water was produced using a Milli-Q Millipore Water System (Millipore, Darmstadt, Germany).

10.2.2 Animal experiment

Ten male Bilgorajska geese were selected randomly from a wider group and enrolled in this study. They were judged to be in good health based on serum chemistry, physical examination and hematological analyses and acclimatized for 1 week in a 60 m^2 with an indoor shelter of 8 m^2 before the commencement of the study. Geese were fed with a drug-free pelleted diet twice a day and water was supplied *ad libitum*. Geese were daily monitored from a certified veterinarian through observation of behavior and appetite. The animal experiment was approved by the Institutional Animal Care and Use Committee of the University of Lublin (Poland) and carried out in accordance with European law (2010/63/UE).

A two-phase parallel design with a washout period of three months was observed. In the first phase (June 2021) the ten geese were administered orally with 0.2 mg/kg ivermectin (Vetamectin®, 10 mg/ml) via crop gavage by a rounded tip metal catheter. In this period the goose BWs were between 4.35 and 5.7 kg (with an average of 5.0 kg). After a wash out period of three months (September 2021) the animals were IV treated (Vetamectin®, 10 mg/ml) at the same dosage using a sterile 20-gauge 3.75 cm needle in the left-wing vein. In this second phase the BW of the animals ranged between 3.1 and 6.1 kg (with an average of 4.85 kg). Blood (approximately 5 ml) was collected from the right-wing vein by direct venipuncture at 1, 3, 6, 12, 24, 48, 96, 120, 144, 192, 240, 360, 480 h after both treatments. Blood was collected in heparinized tubes and centrifuged at 1500x*g*. The harvested plasma was stored at −20°C and analyzed within 30 days of collection.

10.2.3 Analytical method

10.2.3.1 Instrumentation and analytical conditions

The HPLC system was a LC Jasco (Como, Italy) consisting of a ternary gradient system (PU 980), in line degasser (DG-2080-53), autosampler (AS-2055) and an UV multiple wavelength detector (MD-1510). The chromatographic separation assay was performed with a Gemini C18 analytical column (250 × 4.6 mm inner diameter, 5 μm particle size, Phenomenex) maintained at 30 °C using a Peltier system (CO-4062) (Jasco, Como, Italy). The mobile phase consisted of acetonitrile:water (90:10 % v:v) at a flow rate of 1 ml/min and the optimal wavelength for the quantification was set at 242 nm.

10.2.3.2 Sample preparation

Samples were de-frozen and processed according to the method described by Zhao et al. (2005) with some modifications. Sample purification was performed using protein precipitation. Briefly, 0.5 ml of plasma was spiked with 100 µl of IS (10 µg/ml) solution in methanol. After the addition of 1 ml of acetonitrile each sample was vortexed, shaken and centrifuged at 4000x*g* for 10 min. The supernatant was transferred into a clean tube and dried at 40 °C under a gentle nitrogen stream. The residue was dissolved in 100 µl of mobile phase, vortexed, sonicated and centrifuged at 4000x*g* for 10 min. An aliquot of 50 µl was injected onto the HPLC system. CromNav 2.0 (Jasco) software was used to extract and analyze chromatograms.

10.2.3.3 Sample quantification

The quantitative HPLC method was fully validated for goose plasma in terms of linearity, intra-day and inter-day precision, recovery, LOD and LOQ according to the EMA guidelines (Anonymous 2012). Ivermectin (1 mg/ml) and IS (1 mg/ml) stock solutions and dilutions were prepared in methanol. Linearity was assessed using goose plasma spiked with different concentrations (0.0025, 0.05, 0.1, 0.5, 1, 10 µg/ml). Intra-day and inter-day precision were calculated after analysis of six plasma samples spiked with ivermectin at three different concentrations (0.05, 1 and 10 µg/mL), and expressed as CV%. Sample recovery was evaluated by comparing the response (in area) of high (10 µg/mL), middle (1 µg/mL), low (0.05 µg/mL) concentration spiked samples, and the IS to the response of equivalent standards. Recovery is expressed as mean ± SD. The LOD was estimated as the plasma drug concentration that produced a signal-to-noise ratio of three and LOQ

was determined as the lowest plasma concentration that produced a signal-to-noise ratio of ten. The mean concentration was within 20% of the nominal values.

10.2.4 Pharmacokinetic analysis and statistical analysis

The data obtained after IV and PO administrations were analyzed using a non-compartmental approach (ThothPro software, Gdansk, Poland). The pharmacokinetic parameters are reported as geometric mean and ranges (Julious and Debarnot 2000). The Wilcoxon's rank sum test was used for the statistical comparison of pharmacokinetic data between the two routes of administration (Powers 1990).

10.3 Results

10.3.1 Analytical method validation

The analytical method showed an optimal linearity (R^2=1; y=0.372x-0.0064) in the range of 0.0025 – 10 µg/ml. The recovery was found to be 94±3.2%. The inter- and intra-day precision were (CV%) <4.02, while the LOD and LOQ were 0.001 and 0.0025 µg/ml, respectively.

10.3.2 Pharmacokinetic analysis

The animals did not show any adverse effect during or after the treatments. Ivermectin showed a long $t_{1/2kel}$ value which significantly differed between the two treatments: after the IV administration the $t_{1/2kel}$ was two-fold higher compared to the value found after PO administration (Table 18).

		IV			PO		
Parameter	Unit	Geometric mean	Min	Max	Geometric mean	Min	Max
AUC_{last}	mg h/l	18.20	14.07	21.49	2.72*	1.61	4.62
k_{el}	1/h	0.004	0.001	0.01	0.01*	0.008	0.02
$t_{1/2kel}$	h	165.26	67.44	677.74	52.99*	33.92	88.87
Cl	ml/g h	0.01	0.009	0.014	N/A	N/A	N/A
V_{ss}	ml/g	0.97	0.76	1.27	N/A	N/A	N/A
C_{max}	µg/ml	N/A	N/A	N/A	0.08	0.06	0.12
T_{max} §	h	N/A	N/A	N/A	3.00	3.00	3.00
F	%	N/A	N/A	N/A	19.63	12.66	28.75

AUC_{last}, area under the curve from zero to the last detectable timepoint; k_{el}, elimination rate constant; $t_{1/2kel}$, terminal half-life; C_{max}, maximum concentration; T_{max}, time at maximum plasma concentration; Cl, plasma clearance; V_{ss}, volume of distribution in the first compartment; F, bioavailability. N/A = Not applicable. § Median. *Significantly different between the groups (p<0.05).

Table 18. Geometric mean (range) of the pharmacokinetic parameters found after IV and PO ivermectin administration (0.2 mg/kg, n=10).

The AUC value was also statistically different between the two treatments. After the IV dose drug exposure was 6-fold higher compared to the value found after the PO administration. After the IV administration ivermectin was quantifiable up to 240 h while after PO treatment up to144 h in 8/10 geese (Figure 32).

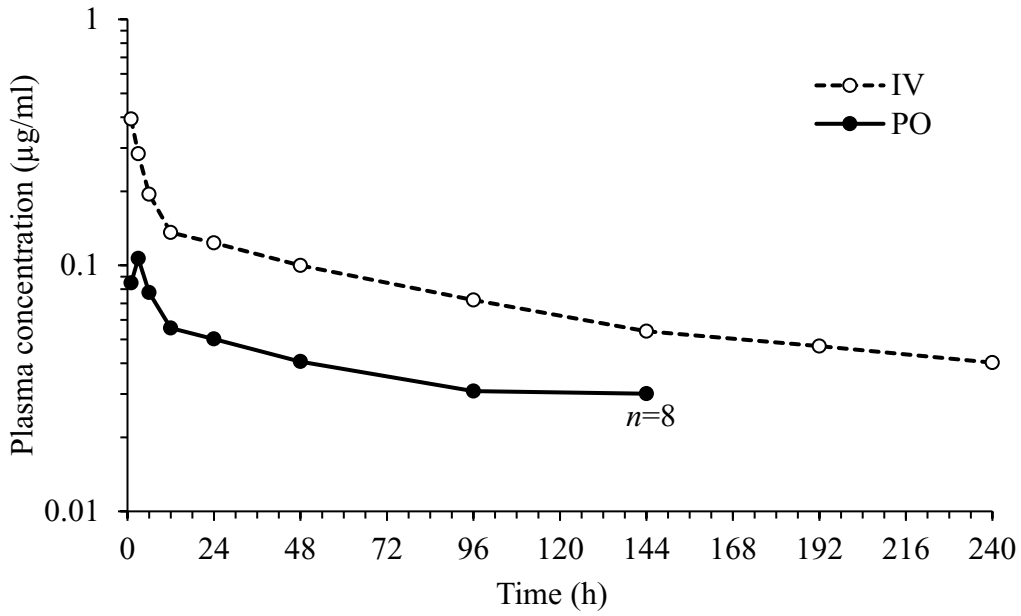


Figure 32. Semi-logarithmic mean plasma concentrations of ivermectin (±SD) *vs* time curve after a single IV (---○---) and PO (—●—) administration at a dosage of 0.2 mg/kg in geese (*n*=10, except for the 144h sample in the oral group).

10.4 Discussion and conclusions

This is the first work which describe the pharmacokinetic of ivermectin in geese. Ivermectin was characterized by a long persistence in the goose plasma, as it occurs in laying hens (Cirak et al. 2018, Moreno et al. 2020). In fact, the plasma drug concentrations were detectable up to 240 h (10 days) and 144 h (6 days, *n*=8/10) after IV and PO administration, respectively.

A significant difference between the AUC_{PO} and AUC_{IV} was found in geese. It occurred also in laying hens with a similar AUC_{IV}/AUC_{PO} ratio. Consequently, in both studies a very low oral bioavailability was found (19.6%, geese; 11.9%, laying hens) (Cirak et al. 2018). It should be noted that geese were fasted before the

treatment to avoid variation in the absorption, and this may have led to a decrease in drug absorption. Due to the high lipophilicity of ivermectin, the oral bioavailability might be higher in fed status. Furthermore, it is well known that the gastrointestinal tract is not only a site for drug absorption but can acts also as a metabolic and immunological organ (Adedokun Olojede 2019). Thus, it can be speculated that some metabolic interactions in the gastrointestinal tract might occurred in poultry compromising the drug absorption process. In addition, as suggested by Svihus and Itani (2019) the retention time in the digestive tract of poultry is remarkably short (5 – 6 h) compared to other mammals or ruminants, and this can be linked to the low PO bioavailability of ivermectin. A further explanation may be absorption or binding to the particulate phase of the digesta which has been shown to influence the pharmacokinetics of some endectocides (McKellar and Gokbulut 2012).

Although the same trend, AUC_{PO} and AUC_{IV} values were approximately 20-fold higher compared to that found in laying hens (Cirak et al. 2018). The large variation between the AUC (and in the C_{max}) values found in geese *vs* laying hens might be explained by the different pharmaceutical formulations used. Different excipients may lead to different absorption: in the present study the formulation Vetamectin 10 mg/ml was used for both the routes of administration, while in laying hens the drug was diluted in propylene glycol for the IV administration and the formulation Baymec 1% was used for the PO treatment.

After the PO treatment a faster elimination seemed to occur: the $t_{1/2kel}$ after PO treatment was significantly lower compared to the IV administration. It can be observed a similar trend in laying hens (Cirak et al. 2018). The reason is not clear;

however, a possible explanation may be related to variation of the clearance in the animals between the two treatments. Ivermectin and/or its metabolites are primarily eliminated in the feces in all species (González Canga et al. 2007). The primary excretory way is bile, since the P-glycoprotein was also found in biliary canaliculus, and it may contribute to the drug's high fecal excretion (Laffont et al. 2002). Biliary and small intestinal clearances of ivermectin were found to account for 5.5 and 27% of ivermectin total (plasma) clearance, respectively (Laffont et al. 2002). According to the study's findings of Moreno et al. (2020), hens can presumably metabolize nearly 15% of ivermectin. No information is available in geese; some metabolic process may have affected the liver function and consequently the clearance in the oral treated group, leading to a faster drug elimination.

However, the $t_{1/2kel}$ values were lower in laying hens compared to those found in geese. It may reflect the differences in clearance. Indeed, clearance was slower in chickens (Cirak et al. 2018, Moreno et al. 2020) compared to geese and this may be due to the different size of the animals (chickens, 1.7 – 2.2 kg BW; geese, 4.35 – 5.7 kg BW).

From these findings it seems that the PO administration in geese is not the optimal one due to the low bioavailability. Due to this, ivermectin may be active against GI parasites, although it should be investigated. In addition, further research on the tissue residues should be performed to evaluate the withdrawal time to ensure consumer safety.

11. SUMMARY OF ADDITIONAL PROJECTS CARRIED OUT

My research activity led to additional studies in food producing and companion animals reported here below.

11.1 Sheep

11.1.1 Pharmacokinetics of levosulpiride after single dose administration by different routes in sheep (Ovis aries Linnaeus)

Reference: Łebkowska-Wieruszewska B, Sartini I, Barsotti G, Camillo F, Rota A, Panzani D, Poapolathep A, Giorgi M, 2019a. Pharmacokinetics of levosulpiride after single dose administration by different routes in sheep (*Ovis aries Linnaeus*). Small Ruminant Research, 179: 39-42.

The pharmacokinetics of levosulpiride after IV, intramuscular (IM) and PO administration at a dosage of 50 mg was assessed, since in small ruminants this drug was previously proposed to be a valid alternative to the current drugs used for the synchronization (Rubianes et al. 2003; Abecia et al. 2012). The results showed that the PO administration of levosulpiride in sheep is not recommended due to the low plasma concentration and oral bioavailability. On the other hand, the IV and IM administration showed comparable PK profiles (Łebkowska-Wieruszewska et al. 2019a).

11.1.2 Pharmacokinetics and tissue analysis of levofloxacin in sheep (Ovis aries Linnaeus) after multiple-dose administration

Reference: Sartini I, Łebkowska-Wieruszewska B, Kim TW, Lisowski A, Poapolathep A, Giorgi M, 2020c. Pharmacokinetics and tissue analysis of levofloxacin in sheep (*Ovis aries Linnaeus*) after multiple-dose administration. Research in Veterinary Science, 128: 124-128.

The pharmacokinetics of levofloxacin was described in sheep together with its residue depletion profile in different tissues after IV and PO administration of 2 mg/kg once a day for 5 days. Fluoroquinolones are used in veterinary medicine after strong evidence for their efficacy and when there are no alternative treatment options. The interest in the use of fluoroquinolones in veterinary medicine has led to the initiation of several studies on the pharmacokinetics of levofloxacin in various animal species, but not data were available in sheep. In the present study, the IV and PO administration of levofloxacin showed no significant differences in the PK parameters. The results suggest that the treatment may be considered effective for bacteria with an MIC of 0.049–0.061 μg/ml that is lower than the MIC reported for ruminant pathogens. Tissue residues were highest in the kidney and liver, but the exposure of drug tissue to plasma ratio indicated that levofloxacin is not likely to accumulate in the plasma or organs (Sartini et al. 2020c).

11.2 Goats

11.2.1 Impact of lactation on pharmacokinetics of meloxicam in goats

Reference: Kim TW, Sartini I, Łebkowska-Wieruszewska B, Lisowski A, Poapolathep A, Giorgi M, 2020. Impact of lactation on pharmacokinetics of meloxicam in goats. Journal of Veterinary Pharmacology and Therapeutics, 43: 13–18.

The impact of lactation on pharmacokinetics of meloxicam in goats has been investigated after IV and IM administration. A number of studies have been conducted in goats to find a rational dose for therapeutic off label use of meloxicam. Despite various pharmacokinetic studies being performed, there were no reports on the implication of lactation on meloxicam pharmacokinetics in goats. In addition, due to likely harmful consequences due to the milk drug residue, it was important

to understand whether different routes of administration play a role in the depletion of meloxicam in milk. From this study was concluded that the pharmacokinetics of meloxicam in goats were not affected by the lactating condition of the animals. Thus, a different dosage regime for lactating animals is probably not needed. The approximate milk WT was found to be over 70 h (Kim et al. 2020).

11.2.2 *Single intravenous and oral pharmacokinetics of danofloxacin in the goat*

Reference: Sartini I, Łebkowska-Wieruszewska B, Lisowski A, Poapolathep A, Llewelyn V, Giorgi M, 2021c. Single intravenous and oral pharmacokinetics of danofloxacin in the goat. Small Ruminant Research, 200: 106393.

Since different studies have demonstrated the benefits of danofloxacin in the treatment of a range of infectious pathologies in goats, including contagious caprine pleuropneumonia and mammary infections. The pharmacokinetics of danofloxacin have been investigated in healthy goats after a single IV or PO administration of danofloxacin at 6 and 12 mg/kg, respectively. Results from the present study suggest that a single PO dose of 12 mg/kg may be effective in maintaining a therapeutic PK/PD index against *M. haematolytica* in goats for up to 22 h. However, the oral route of administration may be inappropriate in goats due to a long and variable absorption phase (Sartini et al. 2021c).

11.3 Dogs

11.3.1 Pharmacokinetics of acetaminophen after intravenous and oral administration in fasted and fed Labrador Retriever dogs

Reference: Sartini I, Łebkowska-Wieruszewska B, Lisowski A, Poapolathep A, Cuniberti B, Giorgi M, 2021d. Pharmacokinetics of acetaminophen after intravenous and oral administration in fasted and fed Labrador Retriever dogs. Journal of Veterinary Pharmacology and Therapeutics, 44: 28-35.

In some countries acetaminophen is clinically used in a safe range (10–15 mg/kg two or three times daily) for management of acute pain in dogs, but not pharmacokinetic data was available in Labrador Retrivers a breed more representative of the whole canine population than Beagle dogs. Thus, this study was aimed to assess the pharmacokinetic profile of acetaminophen in this specific dogs' breed after a single IV and PO dose (20 mg/kg). The drug was administered in fasted and fed conditions to evaluate the feeding influence on the drug absorption. Pharmacokinetic simulations were performed to predict the plasma concentrations reached after a multiple dose treatment trying to reproduce the clinical situation. The present results showed that feeding did not significantly affect acetaminophen pharmacokinetics; thus, it may be easily administered by the owner regardless of food consumption. Moreover, the multiple oral simulated doses of 20 mg/kg every 6 h provide an average plasma concentration close to the effective analgesic concentration found in humans, suggesting that this dosage regime might be effective in dogs (Sartini et al. 2021d)

11.3.2 Propacetamol in dogs: first description of its pharmacokinetics after intravenous and oral administration

Reference: Sartini I, Łebkowska-Wieruszewska B, Gajda A, Pietruk K, Gbylik-Sikorska M, Lisowski A, Kim TW, Poapolathep A, Giorgi M, 2021. Propacetamol in dogs: first description of its pharmacokinetics after intravenous and oral administration. Research in Veterinary Science, submitted R1

Since drug armamentarium in veterinary medicine to manage pain conditions is little and sometimes obsolete compared to that in humans, the prodrug of acetaminophen (propacetamol), used in humans postoperative care, was

investigated in dogs for the first time (data not publish yet). In this study its IV and PO pharmacokinetics in terms of acetaminophen (APAP) was evaluated in dogs as well as the formation of acetaminophen inactive (paracetamol sulfate [PS], paracetamol glucuronide [PG]) and toxic (N-acetyl-p-benzoquinone imine [NAPQI]) metabolites. Briefly, PS and PG exposures were higher than that of acetaminophen, while NAPQI concentrations were constantly below the detection limit of the analytical method. IV propacetamol administration produced 30% more APAP than oral administration. However, propacetamol released a significantly lower amount of active moiety in dogs than in humans. The propacetamol dose administered in this study did not produce plasma APAP concentrations above the threshold sufficient to provide analgesia in adult humans (4 μg/ml). Can be only concluded that a direct IV injection of APAP instead of propacetamol might be a better clinical option for pain relief in dogs.

11.3.3 Pharmacokinetics of thalidomide in dogs: can feeding affect it? A preliminary study

Reference: Pierini A, Sartini I, Giorgi M, Łebkowska-Wieruszewska B, Lisowski A, Poapolathep A, Marchetti V, 2020. Pharmacokinetics of thalidomide in dogs: can feeding affect it? A preliminary study. Journal of Veterinary Science, 21:e60.

Thalidomide is used as a has been used in canine chemotherapy for the treatment of hemangiosarcoma, pulmonary and mammary carcinoma due to its anti-angiogenic effects. However, the dose of thalidomide proposed for the treatment of tumors in canine patients has been empirically selected, with studies using a wide range of doses. In the present study the oral pharmacokinetics of thalidomide was investigated in dogs at a dosage of 400 mg/dog. The main relevant result reported

from this study is that the feeding significantly affects thalidomide pharmacokinetics, and this should be considered by veterinarians when using this drug in a clinical setting (Pierini et al. 2020).

11.3.4 Effect of feeding on the pharmacokinetics of vilazodone in dogs

Reference: Sartini, I, Gbylik-Sikorska, M, Łebkowska-Wieruszewska, B, Gajda, A, Lisowski, A, Kowalski, CJ, Posyniak, A, Poapolathep, A, Giorgi, M, 2019b. Effect of feeding on the pharmacokinetics of vilazodone in dogs. Research in Veterinary Science, 125:309-314.

Psychiatric disorders such as depression, anxiety, mania, obsession, sleep deprivation and hyperactivity have been recognized to affect not only human beings but also pets, especially in dogs. Consequently, drugs labelled for humans have started to be clinically used off-label in pets. The main issue is related to the fact that their administrations were often based on the dose regimen for men without any specific pharmacokinetic or pharmacodynamic rationale. Thus, two antidepressant drugs vilazodone and agomelatine were considered. This latter differ from the classical antidepressants from the selective serotonin reuptake inhibitors (SSRIs), noradrenaline reuptake inhibitors (NARIs) and serotonin/noradrenaline reuptake inhibitors (SNRIs).

Vilazodone is a phenylpiperazine chemical derivative approved for the treatment of major depressive disorder in humans. Its structure is related to trazodone which has gained popularity as adjunctive off label medication for long term treatment of anxiety disorders in dogs. Its antidepressant efficacy seems to be correlated to its peculiar mechanism of action. The present study evaluated the pharmacokinetics of a single oral 40 mg dose of vilazodone in Labrador dogs in fasted and fed conditions. Vilazodone concentrations were quantified using LC-MS/MS in dogs'

plasma in two different windows of time: 30 min to 10 h for the fasted group and 4 h to 35 h for the fed group, suggesting that the unfed condition decreases the relative oral bioavailability to about 30% (Sartini et al. 2019b).

11.3.5 Agomelatine: a novel melatonergic antidepressant. Method validation and first exploratory pharmacokinetic study in fasted and fed dogs

Reference: Łebkowska-Wieruszewska, B, Ziółkowski, H, Sartini, I, Lisowski, A, Kowalski, C, Poapolathep, A, Giorgi, M, 2021a. Agomelatine: a novel melatonergic antidepressant. Method validation and first exploratory pharmacokinetic study in fasted and fed dogs. Research in Veterinary Science, 139: 140-144.

Agomelatine, on the other hand, is a structural analogue of melatonin (MT), representing the only available MT1/MT2 receptor agonist. It has also little affinity for other receptors, including adrenoceptors, dopamine, GABA, muscarinic, histamine, benzodiazepine and sigma receptors, as well as ion channels and a minimal affinity for the serotonin 5-HT2C, 5-HT1A and 5-HT2B receptors. Since agomelatine represents an appealing and innovative approach towards improved treatment of depression focuses acting on the regulation of circadian rhythms, its plasma concentration *vs* time profile in Labrador dogs in fasted and fed conditions were evaluated. Unfortunately, the findings were too variable to evaluate the effect of feed on the F% of agomelatine in dogs because of the unanticipated broad variability and the consequent reduction of power of the study. As the plasma concentrations are broadly variable from dog to dog, agomelatine cannot be recommended for off-label therapies in canine species so far. Further studies are requested to evaluate the factors that produce the inter-subject variability (Łebkowska-Wieruszewska et al. 2021a).

11.4 Analytical method validation studies

11.4.1 Analytical method validation of vilazodone with spectrofluorimetric detection in rabbit plasma.

Reference: Sartini I, Salvadori M, Łebkowska-Wieruszewska B, Poapolathep A, Giorgi M, 2019c. Analytical method validation of vilazodone with spectrofluorimetric detection in rabbit plasma. American Journal of Animal and Veterinary Sciences, 14: 50-56

This paper describes an analytical method developed and validated vilazodone in rabbit plasma. Few analytical methods to detect this drug in plasma have been reported in the literature, but they were mainly based on mass spectrometry detection. A sensible, selective, and accurate analysis of vilazodone using the HPLC with spectrofluorimetric detection, without the need for expensive clean up steps, solvent consuming flows, or expensive devices was developed (Sartini et al. 2019c).

11.4.2 Development of a multimatrix UHPLC-MS/MS method for the determination of paracetamol and its metabolites in animal tissues.

Reference: Pietruk, K, Gbylik-Sikorska, M, Łebkowska-Wieruszewska, B, Gajda, A, Giorgi, M, Sartini, I, Jedziniak, P, 2020. Development of a multimatrix UHPLC-MS/MS method for the determination of paracetamol and its metabolites in animal tissues. Molecules, 26: 2046.

The quantification of acetaminophen and its metabolites (including NAPQI) in animals' tissues was optimized using a UHPLC-MS/MS method. Since no information on the presence and concentration of its main metabolites in animal tissue are available in literature, the method developed may be a useful tool to investigate the metabolism of acetaminophen in different veterinary species and to assess tissues' residues profile (Pietruk et al. 2020).

11.4.3 Cebranopadol a novel first-in-class drug candidate. Method validation and first exploratory pharmacokinetic study in rabbits

Reference: Łebkowska-Wieruszewska, B, Gbylik-Sikorska, M, Gajda, A, Sartini, I, Lisowski, A, Poapolathep, Giorgi, M, 2021b. Cebranopadol a novel first-in-class drug candidate. Method validation and first exploratory pharmacokinetic study in rabbits. Journal of Veterinary Pharmacology and Therapeutics, 44: 516-521.

In order to contributed to the drug armamentarium in veterinary medicine, a novel UHPLC-MS/MS method was developed and validated to quantify cebranopadol in rabbit plasma. Cebranopadol is a novel, centrally acting, potent, first-in-class analgesic drug candidate with a unique mode of action that combines nociceptin/orphanin FQ peptide receptor and opioid peptide receptor agonism. In this view, it could have several advantages including multiple level targeting of the pain pathway, a small molecular size and as such a high CNS permeability. The method resulted selective, repeatable, accurate, precise, and robust with a lower limit of quantification. In addition, it was applied to perform a first exploratory cebranopadol pharmacokinetic study in rabbits after subcutaneous administration (Łebkowska-Wieruszewska et al. 2021b).

11.5 Review

11.5.1 Levofloxacin in veterinary medicine: a literature review

Reference: Sitovs A, Sartini I, Giorgi M, 2021. Levofloxacin in veterinary medicine: a literature review. Research in Veterinary Science, 137: 111-126.

The use of levofloxacin remains controversial in veterinary medicine, since it is used in some extra-EU countries (e.g., Argentina, India, China, and Russia), while it is completely banned for veterinary use in the EU and used extralabel in only

companion animals in the USA (as mentioned in Chapter 2.1.1.3). In order to clarify this issue, I contributed to write a literature review highlighting the most clinically relevant and scientifically important levofloxacin studies linked to the field of veterinary science (Sitovs et al. 2021).

11.5.2 Paracetamol: A Focus on Dogs

Reference: Fadel, C, Sartini, I, Giorgi, M, 2021. Paracetamol: A Focus on Dogs. American Journal of Animal and Veterinary Sciences, 16: 247-262.

This review aimed to clarify the use of paracetamol (acetaminophen) in canine clinical practice. This study is a documentation of all the available data on the therapeutic, toxic, and lethal doses of acetaminophen found in literature, as well as the therapeutic effects, clinical application, mostly for the control of post-operative pain and its toxic effects in dogs (Fadel et al. 2021).

11.5.3 Grapiprant: A snapshot of the current knowledge

Reference: Sartini I, Giorgi, M, 2021. Grapiprant: A snapshot of the current knowledge. Journal of Veterinary Pharmacology and Therapeutics, 44: 679– 688.

Grapiprant is the pioneer member of the novel piprant class, a potent and specific antagonist of the prostaglandin E2 receptor 4. It has been approved in veterinary medicine for the control of pain and inflammation associated with osteoarthritis in dogs at the dose regimen of 2 mg/kg once a day by the FDA and EMA (for pain only) in 2016 and 2018, respectively. The aim of this narrative review was to report the analytical methods, pharmacokinetics, pharmacodynamics and safety of grapiprant in several animal species using the best available published scientific evidence. In conclusion, most of the analytical methods proposed for grapiprant

detection are simple, reliable, sensitive and validated. Some discrepancies reported in the pharmacokinetics between animal species. The therapeutic efficacy seems more suited to chronic rather than acute pain (Sartini and Giorgi, 2021).

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