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**Prudent use of fluoroquinolones in avian species:  
pharmacokinetics of flumequine and enrofloxacin  
for PK/PD modelling in turkey.**

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# **CHAPTER 1**

## **Foreword**





# 1. Foreword

## 1.1 Antimicrobial use in veterinary medicine

The introduction of antimicrobial agents in human and veterinary medicine has been one of the most significant medical achievements of the 20th century. The first antimicrobial agents were introduced in the 1930's, and a large number of new compounds were discovered in the following decades. However, shortly after the introduction, resistance began to emerge. Formerly the same Alexander Fleming, discoverer of penicillin, had seen the future of antibiotic resistance risk. "The time may come when penicillin can be bought by anyone in the shops", he said in his Nobel lecture; "then there is the danger that ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant" (Fleming, 1945). In all known cases emergence of antimicrobial resistance has eventually followed the introduction of a new antimicrobial compound (EMA, 2006).

In veterinary medicine antibiotics are used in animals for therapeutic and prophylactic purposes in order to cure and prevent bacterial diseases. In these cases, antimicrobial are administered under veterinary control. Another use of antimicrobial agents in animals, although banned since 2006 in the European Union but still allowed in other non-European countries(USA, Japan, Australia, among others), is as growth promoters. In this case, drugs are added in sub-therapeutic doses and for long periods of time in the feed of healthy animals, conditions that could further contribute to the selection of resistant bacteria (McEwen and Fedorka-Cray, 2002).

It is estimated that the volumes of antimicrobials used in animals exceeds the use in human medicine worldwide, and nearly all the classes of antimicrobials that are used for humans are also being used in food animals, including the newest classes of drugs such as third- and fourth-generation cephalosporins, fluoroquinolones, glycopeptides, and streptogramins (Aarestrup et al., 2008).

In animal production systems with high density of animals or poor biosecurity, the spread of infectious diseases is favoured, which leads more frequently to antimicrobial treatment and increases the need for prevention of those diseases (EMA, 2006).

Regarding the knowledge about veterinary antimicrobials use in Europe, huge improvement has been made through the activities of the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC). The third ESVAC report presented the results on antimicrobial consumption in animals in 25 European Countries. In this report, the magnitude of the animal population is quantified by means of the population correction unit (PCU). This PCU is a technical unit of measurement based on the estimated weight at treatment of livestock and slaughtered animals; one PCU is the representation of 1 kg of different

categories of livestock and slaughtered animals. As reported by the ESVAC report in 2011, a total of 8,481 tonnes of active ingredient of antimicrobials have been sold for veterinary use in the 25 EU countries. The sales of tetracyclines, penicillins and sulfonamides, in mg/PCU, accounted for more than 71% of the total sales (range 53%–88%). Among the so-called CIA (Critically Important Antibiotics), designated by the WHO as the molecules critical to human health, the sales percentages of 3<sup>rd</sup> and 4<sup>th</sup> generation cephalosporins, fluoroquinolones and macrolides were, respectively, 0.2%, to 1.6% and 8% of total sales in the 25 countries in 2011. Another important finding was that the total sales, both in tonnes and in mg/PCU, of veterinary antimicrobial agents in the 25 EU/EEA countries were mainly accounted for pharmaceutical forms applicable for mass treatment (premixes, oral powder and oral solution) and this type of treatment is considered even more risky for the development of antimicrobial resistance. In Italy, a decline in antimicrobials sales (in mg/PCU) of 13% from 2010 to 2011 has been observed and the decline has been reported for almost all antibiotic classes (ESVAC, 2013).

## **1.2 Overview of poultry production**

During the last 50 years, avian production increased enormously; the industry developed to be highly integrated, with fewer companies controlling most sources of birds, feed mills, farms, slaughter and processing facilities. Integration led to standardized management practices, including drug treatment policies and procedures and to many successes in the prevention and control of infectious diseases (McEwen and Fedorka-Cray, 2002).

The European Union (EU) is one of the world's top producers in poultry meat and a net exporter of poultry products. Over the years the market organisation for poultry sector was improved to ensure the development of the sector, the quality of the products and consumers protection while harmonizing the entire market. In 2012, the total poultry meat production in the EU was 12.9 million tonnes, with an increase of 14% compared to 2007. Broilers meat is the main poultry meat with a total production of 9.9 million tonnes in 2012. In Europe, the leading producers of broilers meat, with a production above 0.7 million tonnes each, are the UK, Poland, Germany, France, Spain, Italy and the Netherlands. All these countries together are responsible for 76% of the EU's poultry meat production. Besides broilers, turkeys are also an important subsector. In 2012, total turkey meat production in the EU was 1.9 million tonnes. The main producing countries of turkey meat are France, Germany, Poland, Italy and the UK, with a common share of 81% of the EU total (AVEC annual report, 2014).

### 1.3 Antimicrobial drug use in poultry industries

In highly organized and intensive avian production, every disease outbreak has a major effect on health and welfare, leading to a decreased technical performance and profitability. Shorter production cycles, through improved genetics and diet, have led to the fact that there is little recovery time for animals after a disease outbreak. In case of endemic diseases, their outbreaks will predominantly result in economic losses for individual farmers, whereas in case of epidemic diseases, the entire poultry production sector may be involved through mandatory preventive measures such as quarantine or destruction of poultry (Gelaude et al., 2014).

Since commercial poultry are food animals the choice of antimicrobials is affected by the issue of drugs residues in edible tissues. In Europe, only those antimicrobials for which a maximum residue level (MRL value) is set can be used in poultry flocks. The withdrawal period indicates the time which pass between the last dose given to the animals and the time when animals can enter in the food chain. The relatively short lifetime of the meat producing poultry species and the importance to guarantee the withdrawal time can significantly limit the availability of therapeutic options (Lohren et al., 2008).

Another important aspect in determining the drug to use is the economical factor: in avian species the low economic value of individual birds makes single therapy cost-prohibitive. Therefore, due to the enormous number of animals bred, the oral mass therapy becomes a forced choice (Hofacre et al., 2013).

The most important antimicrobial classes used in avian medicine are listed in the table below (Lohren et al., 2008):

Antimicrobial class	Drug name	Type of activity
Aminoglycosides	apramycin gentamicin neomycin spectinomycin streptomycin	Bactericidal
Sulfonamides	sulfadiazine sulfadimethoxine	Bacteriostatic
Potentiated sulfonamides	trimethoprim and sulfonamides	Bactericidal
$\beta$ -Lactames	benzylpenicillin potassium penicillin G ampicillin amoxicillin ceftiofur	Bactericidal
Fluoroquinolones	enrofloxacin difloxacin flumequine	Bactericidal
Lincosamides	lincomycin	Bacteriostatic
Macrolides	erythromycin	Bacteriostatic

	spiramycin tylosin tilmicosin	
Pleuromutilines	tiamulin	Bacteriostatic
Polypeptides	colistin sulfate	Bactericidal
Tetracyclines	tetracycline chlortetracycline oxytetracycline doxycycline	Bacteriostatic

For poultry, the preferred method of treatment is oral medication, via drinking water or feed medication. This is because large numbers of birds can be medicated at the same time. However, in the case of acute disease with serious losses of animals, parenteral therapy offers a valuable alternative; the method of medication is often complicated by other factors such as animal welfare, avoidance of tissue damage and stress (Vermeulen et al., 2002). The major consideration in determining the method of administration to birds is ease of management. The formulation of a drug intended for use in avian species is frequently dictated by the need for a practical and economical method of administration. When a feed-based antimicrobial is prescribed, the time required for the manufacturing, transporting and delivering through the feeding system at the farm must be taken into account; whereas the administration of the antibiotic in drinking water allows faster treatment (Hofacre et al., 2013). Drinking water therapy offers several advantages and few disadvantages. Important advantages are the low cost of management, the low work load, the ease of administration and a quick change of drug and/or dose is possible. Besides, diseased birds usually tend to stop eating, but they will continue to drink. The main disadvantages are: drug uptake can vary dramatically as a function of the animal, unprofessional use and a wrong preparation of the medical solution by the farmer and possible solubility and stability problems (Vermeulen et al., 2002). At first sight, this technique seems simple but many more variables have to be taken into account, these include: the water quality, the individual water uptake, the influence of drinking water system and the lighting periods. Basically, the water should be clean, cool and neutral in taste. The water should be replaced daily and soiling of the drinking water should be avoided because of possible drug inactivation. Several water quality parameters which may influence the use of an oral medication are hardness, the pH values, dissolved solids and a high mineral content (Esmail, 1996).

Medication use is mainly indicated as mg per kg body weight and this means that information on daily water intake as function of poultry species, flock size, weight and age on the moment of treatment must be available. The drinking water intake varies greatly depending upon different factors such as

environmental temperature, density and composition of the diet (Vermeulen et al., 2002).

Finally, the treatments via drinking water can be conducted following two types of schemes: continuous administration during the entire light period or pulsed administration for a limited period between 4 and 10–12 h (Charleston et al., 1998).

#### **1.4 Antimicrobial resistance**

Overuse and misuse of antimicrobial drugs have favoured the growth of resistant organisms and resistance can spread to other microbial populations, jeopardizing human and animal health (Toutain et al., 2002).

Antimicrobial resistance, defined as the ability of a microorganism to withstand the effect of a normally active concentration of an antimicrobial agent, is a global phenomenon and a well-recognised threat to public as well as animal health. The causal factors in the development of resistance are many and complex, but originate generally from exposure of microorganisms to antibiotics (Marshall and Levy, 2011).

The development of resistance is a natural process which increases every time an antibiotic is used. The initial emergence of resistance is thought to be a random occurrence that arose during replication of bacteria. However, once resistance has occurred, the use of an antibiotic kills or inhibits competing bacteria present in the animal or human, thereby favouring the spread of those bacteria that have become resistant. This defensive mechanism whereby resistance genes are selected in the face of exposure to a particular antibiotic is known as selection pressure. The more often exposure occurs, the greater the risk that resistance will develop. As bacteria reproduce very rapidly, organisms with the resistant gene can rapidly become dominant in a bacterial population with an individual animal or human (Coates, 2012).

In human medicine it is generally agreed that the improper use of antimicrobial agents is the most important factor in the selection of resistance in bacteria and that, in general, a close association exists between the rate of resistance development and the quantities of antimicrobial agents used (Aarestrup, 1999). Human infections caused by resistant bacteria are more frequently associated with higher morbidity and mortality than those caused by susceptible pathogens. In areas of concentrated use, such as hospitals, this has led to lengthened hospital stays, increased health care costs and, in extreme cases, to untreatable infections (Boerlin and White 2006).

Antimicrobial resistance mechanisms have been reported for all known antibiotics currently available for clinical use in human and veterinary medicine. Therefore, successful sustainable management of current antimicrobials and the continued development of new ones and of alternatives to antimicrobial drugs

are vital to protecting animal and human health against infectious microbial pathogens (Martinez and Silley, 2010).

#### *1.4.1 Resistance mechanism*

Antimicrobial resistance mechanisms can be classified into four major categories: 1) the antimicrobial agent can be prevented from reaching its target by reducing its penetration into the bacterial cell; 2) the antimicrobial agent can be expelled out of the cell by general or specific efflux pumps; 3) the antimicrobial agent can be inactivated by modification or degradation, either before or after penetrating the cell; and 4) the antimicrobial target can be modified or protected by another molecule preventing access of the antibiotic to its target, so that the antimicrobial cannot act on it anymore. Alternatively, the antimicrobial agent target can be rendered dispensable by the acquisition or activation of an alternate pathway by the microorganism (Boerlin and White, 2013).

#### *1.4.2 Types of antimicrobial resistance*

In the context of antimicrobial resistance, bacteria display three fundamental phenotypes (Boerlin and White2006):

- **susceptibility** that implies that isolates are inhibited by the usually achievable concentrations of antimicrobial agent when the recommended dosage (dosage regimen) is used for that site of infection (CLSI, 2007)
- **intrinsic resistance**, that is the innate ability of a bacterial species to resist the activity of a particular antimicrobial agent through its inherent structural or functional characteristics, which allow tolerance of a particular drug or antimicrobial class (Boerlin and White2006).
- **acquired resistance**, it occurs when a particular microorganism obtains the ability to resist the activity of a particular antimicrobial agent to which it was previously susceptible. This can result from the mutation of genes involved in normal physiological processes and cellular structures, from the acquisition of foreign resistance genes or from a combination of these two mechanisms. Acquired resistance can be manifested as resistance to a single agent, to some but not all agents within a class of antimicrobial agents, to an entire class of antimicrobial agents, or even to agents of several different classes. (Boerlin and White2006).

In the great majority of cases, a single resistance determinant encodes resistance to one or several antimicrobial agents of a single class of antimicrobials or of a group of related classes of antimicrobials (Mayer, 2009).

### 1.4.3 Transfer of antimicrobial resistance from animals to human

The use of antibiotics in food producing animals contributes to the selection of resistant bacteria that can be transferred from animals to humans. The food chain is considered as a transmission vehicle of resistant bacteria to humans. The risk to transfer antimicrobial resistance arises via the consumption of product (milk, eggs, honey, meat) from treated animals, but also by the contact with treated animals, being they pets or food-producing animals, or their environment (Aarestrup, 1999).

Nowadays, there is considerable evidence that antimicrobial use in animals selects for resistance in zoonotic enteropathogens and in commensals bacteria (Garcia-Migura et al., 2014). Antimicrobial resistance in zoonotic enteropathogens (e.g., *Salmonella*, *Campylobacter*, *Yersinia*, and some strains of pathogenic *E. coli*) and commensals (e.g., enterococci, mostly *E. coli*) is of special concern to human health and various European countries have implemented national monitoring programs [e.g. Denmark (DANMAP), France (FARM), Netherlands (MARAN), Norway (NORM-VET), Sweden (SVARM) and Spain (VAV)] to assess susceptibility to antibiotics among these bacteria isolated from healthy food animals (DANMAP report, 2013, FARM report, 2013, MARAN report, 2013, NORM-VET report, 2013, SVARM report, 2013, VAV report, 2005).

If commensal bacteria, which are naturally occurring in the host, are exposed to antimicrobial agents, they may become resistant and be able to transfer resistance genes to pathogenic bacteria. Antimicrobial resistance in the commensal bacteria of humans and animals results largely from the selective pressure of antimicrobial agents use and reflects the genetic elements that may transfer to pathogens. The above cited zoonotic bacteria are considered responsible of important infections in food animal species; fluoroquinolones and cephalosporines are antimicrobials frequently used against these pathogens. An increase in resistant strains of *E. Coli*, *Campylobacter* and *Salmonella* has been reported in recent years. In particular high level of resistance was reported in bacteria strains isolated from poultry and turkeys (EFSA, 2010).

In the United States in 2005, two fluoroquinolones that were approved for the control of colibacillosis in poultry were banned. The primary reason for this ban was exactly to allay concerns regarding rising fluoroquinolones resistance rates in human cases of campylobacteriosis. (FDA, 2005).

In Countries where fluoroquinolones are not used in poultry, such as Australia, the resistance phenomenon in *Campylobacter*, *Salmonella* and *E. coli* are rare and consequently resistance in human is also much lower than in most countries (Cheng et al., 2012).



## 1.5 Judicious use of antimicrobial drugs

In order to minimize the possible impact of animal antimicrobial usage on public and animal health, various international organizations such as WHO, OIE, FAO and EU Commission have, in recent years, emphasized the importance of prudent and rational use in veterinary medicine . This has been recognized by professional associations as well as by national and international authorities. All these institutions have underlined to a lesser or greater degree that prudent antimicrobial use is important, not only to safeguard the efficacy of antimicrobial drugs in veterinary medicine but, even more so, to prevent the emergence and spread of undesirable resistance phenotypes in zoonotic pathogens as well as in commensal bacteria that can be transmitted between animals and humans (Teale and Moulin, 2012).

There are no finite definitions of “prudent” and “rational” in relation to antimicrobial use. Both terms are frequently used to suggest a responsible attitude to antimicrobial use, aimed at minimizing the development and spread of antimicrobial resistance while maximizing therapeutic efficacy. This attitude, and its objectives, apply both to human and veterinary medicine. Sometimes the terms prudent and rational are used more or less synonymously. However, they refer to slightly different aspects. **prudent use** has the overall goal of reducing antibiotic usage, with particular emphasis on the relative use of broad spectrum and critically important drugs. **Rational use** refers to rational administration of antimicrobials to the individual with the purpose of optimizing clinical efficacy while minimizing development of resistance. Judicious use of antibiotics, both in humans and in animals, is a key factor to minimize the risk of selecting resistant bacteria and help keep antibiotics effective for future generations (Guardabassi and Kruse, 2008).

The best way to reduce the need for and use of antimicrobials and thereby aiding the containment of resistance phenomenon, is by preventing disease. Prevention is better than cure, not only in relation to antimicrobial resistance, but also from an animal welfare prospective and from economic viewpoint. In this regard several management techniques including good biosecurity rules and specific vaccination programs are routinely used in intensive farming in order to limit as much as possible the need for antibiotics (Laanen et al., 2014).

In veterinary medicine, prudent use of antibiotics is an integral part of good veterinary practices. Prudent use principles should not be interpreted so restrictively as to replace professional judgment of practitioners or to compromise animal health or welfare. In all cases, animals should receive prompt and effective treatment as deemed necessary by the prescribing or supervising veterinarian (FVE, 1999).

General prudent use guidelines have been developed in recent years by most national and international veterinary organizations, mostly providing statements

of principles of judicious antimicrobial use. For example the Food and Drug Administration, Center for Veterinary Medicine, in cooperation with the American Veterinary Medical Association has drafted a list of points to consider in order to optimize the use of antibiotics in poultry farms (FDA, CVM):

- **Preventive strategies, such as appropriate husbandry and hygiene, routine health monitoring, and immunization, should be emphasized.**

The foundation of the success in the poultry industry is through disease prevention management. Farms utilizing all-in-all-out production minimize the presence of multiple ages of flocks on farms to help in disease prevention. Biosecurity programs in place on poultry farms prevent the introduction of diseases.

- **Other therapeutic options should be considered prior to antimicrobial therapy.**

The poultry industry approaches the treatment of diseases with antimicrobial agents very seriously. Because of the cost of disease treatment with antimicrobials, therapeutic antimicrobial intervention is used only as a tool to treat active disease.

- **Judicious use of antimicrobials, when under the direction of a veterinarian, should meet all requirements of a valid veterinarian-client-patient relationship.**

Poultry veterinarians, in integrated companies, closely monitor antimicrobial use in their poultry flocks. They maintain close contact with service technicians and managers related to the use of antimicrobials. Veterinarians should be involved in the training of all individuals that will ultimately be following veterinary directions for antimicrobial use. Antimicrobials should be used always under the direction and knowledge of the company veterinarian or veterinary consultant.

- **Regimens for therapeutic antimicrobial use should be optimized using current pharmacological information and principles.**

For the purpose of correct use a precise diagnosis and antimicrobial susceptibility testing are essential. The choice of an appropriate drug product and the administration route should also be considered when you have to treat diseased animals. Likewise an appropriate dosage regimen (dose level, dose interval and treatment duration) is of fundamental importance to minimize therapy failures, exploit the efficacy potential of the drug and comply with the regulated withdrawal times. Low doses, increased intervals and reduced treatment periods can lead to redevelopment of the infection and may increase the risk of selecting resistant organisms.

- **Therapeutic exposure to antimicrobials should be minimized by treating only for as long as needed for the desired clinical response and therapy should be limited to ill or at risk animals.** In population medicine involving flocks, it is recognized that in a disease outbreak, all birds are not infected at the same time with the disease to which antimicrobial therapy is warranted. However, birds in the same house are “at risk” to the same primary disease that often results in secondary bacterial infections. Only birds within the same house ill or at risk are treated.
- **Minimize environmental contamination with antimicrobials whenever possible.**

Even more importance and attention is now given to the prudent use of medically important antimicrobial drugs, a term that refers to those drugs for human therapeutic use. In recent years the European Medicines Agency (EMA), acting at the request of the European Commission, has reviewed the indications and conditions under which a number of the more modern antibiotic classes (fluoroquinolones, macrolides and cephalosporins) are used in veterinary medicine. These reviews have resulted in the elaboration of more precise recommendations for use and new warnings which must be taken into account by vets when prescribing these antibiotics (EMA, 2011).

In some marketing authorizations in the EU, special precautions for use have been added to the Summary of Product Characteristic (SPCs) of fluoroquinolone products. For the fluoroquinolones, at EU level, the risk evaluation is still ongoing and it has been decided that risk management measures has to be implemented to maintain their efficacy for veterinary use. These include the following: fluoroquinolones should be reserved for the treatment of clinical conditions which have responded poorly, or are expected to respond poorly, to other classes of antimicrobials; their dosage regimens should be carefully determined on the basis of and pharmacodynamic properties to ensure optimal efficacy and reduce selection of resistance (EMA, 2006).

## **1.6 Pharmacokinetic/pharmacodynamics approach to a rational dosage regimens**

There are several possibilities to limit the development of antimicrobial resistance phenomenon. They include research into the mechanisms and diffusion of resistance; the search of new drugs and alternative to antibiotic use; proper attention to preventive measures and commitment to prudent use (Wise, 2003).

For a rational and judicious antibiotic therapy, dosage regimens have to be optimized, both to guarantee clinical efficacy and to minimize the selection of

resistant pathogens. In veterinary practice, one of the most important risk factor for emergence of resistance is repeated exposure of bacteria to sub optimal concentrations of antibiotics (Toutain and Lees, 2006). This event is even more frequently when considering the mass treatment with feed or water medication often adopted in animal husbandry (Wise, 2003).

In humans many studies have been carried out to define precise dosages, to improve drug activity and reduce selection of resistance in antimicrobial therapy (Rybak, 2006). Many of these studies use “so called” models (PK/PD) that correlate the antimicrobial pharmacokinetics (PK) with the action on pathogens by pharmacodynamics (PD). PK, is the study of time course of absorption, distribution, metabolism and elimination of drugs, while PD is the study of the efficacy of the drug on causative bacteria determining the minimum inhibitory concentration (MIC) (Toutain et al., 2002).

PK/PD modelling provides surrogate markers for clinical and bacteriological efficacy based on the relationship between serum/tissue concentrations of antimicrobial agents and MIC and has great potential for optimal dosage regimen determination (Toutain and Lees, 2006).

The use of PK/PD principles for evaluation of antimicrobial compounds has become common also in the veterinary literature. Most papers published in the last few years in veterinary journals discuss the features of PK in light of the PD of the drug and how this relates to rational dosage regimens; the results confirm the high potential of PK/PD modeling to define more rational dosages and the significant variable to consider is the plasma concentration time profile (Papich, 2014).

The differences in pharmacokinetics between different species exist and are well documented. Studies to define the dosage are often made in the species considered major, but to optimize the effectiveness and avoid underdosing precise studies should be carried out in the target species of the drug (Toutain et al., 2010).

One mechanism to reduce the risk of emergence of resistant bacteria is to use the agents currently available to veterinarians more effectively. This means administration of sufficient dosages and appropriate regimens that meet the PK/PD targets for each antimicrobial drug class. In veterinary therapy the efficacy confirmation of dosage schemes is necessary, correlating the kinetic profile of both authorized doses and higher doses, often used in practice, with the *in vitro* efficacy evaluation of field isolated strains, known as more resistant (Mc Kellar et al., 2004).

To determine bacteriostatic or bactericidal effects, main PK/PD indices result from combination of blood concentration parameters, as maximum or minimum concentrations, ( $C_{\max}$  and  $C_{\min}$ , respectively), half-life, area under the curve (AUC) with PD characteristics, as sensitivity, MIC, minimum bactericidal

concentration (MBC). The indices that best correlate kinetics and efficacy are the ratio between AUC calculated from 0 to 24 h ( $AUC_{0-24}$ ) and MIC ( $AUC_{0-24}/MIC$ ), between  $C_{max}$  and MIC ( $C_{max}/MIC$ ) and the time during which blood concentrations exceed the MIC ( $T > MIC$ ). All these indices (breakpoints) are particularly useful to optimize efficacy and minimize resistance of antimicrobials used in therapy (Toutain and Lees, 2006).

The PK/PD indices are also now used to derive breakpoints by the Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) subcommittee on Veterinary Antimicrobial Susceptibility Testing (VAST) (Papich, 2014).

## 1.7 Fluoroquinolones

### 1.7.1 History of fluoroquinolones

The fluoroquinolones, also known as quinolones, 4-quinolones and quinolone carboxylic acids, are a large and expanding group of synthetic antimicrobial agents. The history of quinolone agents began with the discovery of nalidixic acid in 1962 as an accidental byproduct during the synthesis of antimalarial compound, chloroquine (Leshner and Froelich, 1962). The nalidixic acid was the first 4-quinolone marketed for clinical use. However, the effectiveness of nalidixic acid was limited due to poor absorption and distribution, narrow spectrum of activity including the tendency to select for resistant organisms during the course of therapy, and its toxic effect on the host. Over the next two decades hundreds of analogues of nalidixic acid were synthesized but only a few (such as piperimic acid, oxolinic acid and flumequine) were used clinically. Despite chemical modifications to the basic 4-quinolone molecule, these products had many of the same limitations that affected the clinical use of nalidixic acid, restricting their use principally to the treatment of urinary infections. The success for this class of antibacterial agents came when a fluorine atom and a piperazine ring were attached to the 6- and 7- positions, respectively, of the basic quinolone nucleus. These substitutions increased absorption, increased antibacterial activity and reduced toxicities (Ball, 2000). The first of these newer agents used clinically was norfloxacin that exhibited activity against many common bacterial pathogens but was less active against more troublesome organisms such as *Pseudomonas aeruginosa* (Koga et al., 1980). Structurally, norfloxacin differed from nalidixic acid and the substitutions performed increased the activity against Gram-positive and Gram-negative bacteria (Schentag and Scully, 1999). Compared to original quinolones, these compounds possess superior pharmacokinetic characteristics, such as good bioavailability when given orally, greater tissue penetration, and a longer half-life. Since then, a large number of new molecules, many of them polifluorate derivatives, have been synthesized (Escribano et al., 1997).

The fluoroquinolones are classified into different groups or generations based on their chemical structure or their biological activities. The biological classification places fluoroquinolones in three groups of generations; the first-generation molecules are those with antibacterial activity restricted to the *Enterobacteriaceae*. Second-generation quinolones have an extended spectrum of activity whereas the third-generation exhibited considerable improving of activity also against streptococci and obligate anaerobes (Martinez et al., 2006).

Just at the beginning of the 1980's, the first older generation quinolones (e.g. oxolinic acid and flumequine) were licensed for use in food animals and during the late 1980's and early 1990's, also the first fluoroquinolones. To date there have been eight fluoroquinolones approved for use in veterinary medicine: danofloxacin, difloxacin, enrofloxacin, ibafloxacin (Europe only at this time), marbofloxacin, orbifloxacin, pradofloxacin and sarafloxacin (Giguère and Dowling, 2013).

### *1.7.2 Mechanism of action*

Fluoroquinolones act by inhibiting two enzymes involved in bacterial DNA synthesis, both of which are DNA topoisomerases that human cells lack and that are essential for bacterial DNA replication, thereby enabling these agents to be both specific and bactericidal. DNA topoisomerases are responsible for separating the strands of duplex bacterial DNA, inserting another strand of DNA through the break, and then resealing the originally separated strands. Specifically, fluoroquinolones inhibit DNA gyrase (topoisomerase II) and topoisomerase IV. DNA gyrase introduces negative superhelical twists in the bacterial DNA doublehelix ahead of the replication fork, thereby catalyzing the separation of daughter chromosomes (Blondeau, 2004). This activity is essential for initiation of DNA replication and allows for binding of initiation proteins. The topoisomerase IV enzyme is a secondary fluoroquinolone target and it is responsible for decatenation that is, removing the interlinking of daughter chromosomes thereby allowing segregation into two daughter cells at the end of a round of replication. Fluoroquinolones interact with the enzyme-bound DNA complex (i.e., DNA gyrase with bacterial DNA or topoisomerase IV with bacterial DNA) to create conformational changes that result in the inhibition of normal enzyme activity (Martinez et al., 2006). As a result, the new drug–enzyme–DNA complex blocks progression of the replication fork, thereby inhibiting normal bacterial DNA synthesis and ultimately resulting in rapid bacterial cell death (Blondeau, 2004).

A peculiarity of these antimicrobials is their biphasic concentration-response curve (paradoxical effect). Survival curves show that when the fluoroquinolone concentration is near the minimal inhibitory concentration (MIC) of bacterium, the drug has a static effect on bacterial growth (bacteriostatic). As the

concentration increases relative to the MIC, bacterial killing increases up to a certain drug concentration termed the optimum bactericidal concentration. As concentration exceed the optimum bactericidal concentration, further increases in drug concentration can lead to a decrease in bacterial killing. Initially these concentration-related differences in drug effect may be associated with the difference between concentrations needed to inhibit DNA supercoiling versus those needed to inhibit bacterial growth. In general, it appears that the supercoiling reaction of gyrase is less sensitive to the drugs than is bacterial growth by one or two orders of magnitude (Martinez et al., 2006).

### 1.7.3 Spectrum of activity

The fluoroquinolones have excellent activity *in vitro* against a wide range of aerobic Gram-negative bacteria, including the Enterobacteriaceae, *Actinobacilluspleuropneumoniae*, *Histophilussomni*, *Mannheimiahaemolytica*, and *Pasteurella* spp. including *P. multocida*. They are also active against *Bordetellabronchiseptica*, *Brucella* spp., and *Chlamydia/Chlamydophila* spp. (Giguère and Dowling, 2013). Fluoroquinolones also have significant activity against veterinary mycoplasmas (Govendir et al., 2011). Activity against *Pseudomonasaeruginosa* is dependent on the compound, with ciprofloxacin being the most potent agent against this bacterium (Van Bambeke et al., 2005). Generally, the first- and second-generation fluoroquinolones are less active against Gram-positive bacteria, especially enterococci, and have poor activity against anaerobic bacteria. The third-generation of fluoroquinolones target this deficiency. For example, trovafloxacin, moxifloxacin, and gatifloxacin are newer fluoroquinolones with good *in vitro* activity against obligate anaerobes (Stein and Goldstein, 2006). Most fluoroquinolones approved for use in veterinary medicine should be considered to be ineffective against obligate anaerobes. The only exception is pradofloxacin, which is active against anaerobic bacteria from dogs and cats including *Clostridium* spp., *Bacteroides* spp., *Fusobacterium* spp., and *Prevotella* spp. (Silley et al., 2007).

### 1.7.4 Resistance mechanisms

Resistance to the fluoroquinolones occurs by target modification, decreased permeability, efflux and/or target protection. Each of these fluoroquinolones resistance mechanisms can occur simultaneously within the same cell, thereby leading to very high resistance levels (Giguère and Dowling, 2013).

The major mechanism of quinolone resistance is alteration of the target enzymes of fluoroquinolones, DNA gyrase and topoisomerase IV. Both enzymes are composed of 2 pairs of subunits: *GyrA* and *GyrB* in DNA gyrase, *ParC* and *ParE* in DNA topoisomerase IV. Resistance to fluoroquinolones occurs as a result of changes in amino acid composition, particularly in the Quinolone-Resistance-

Determining Regions (QRDRs) within *GyrA* and *ParC*, making the target enzymes less susceptible to fluoroquinolones (Fàbrega et al., 2008). Among Gram-negative organisms, quinolone resistance typically develops in a stepwise manner. A single QRDR mutation, usually at serine 83 (Ser83), confers resistance to nalidixic acid and decreases susceptibility to fluoroquinolones; secondary mutations in the *GyrA* QRDR lead to overt fluoroquinolone resistance. However, this does not apply to all Gram-negative bacteria. In *Campylobacter*, which lacks topoisomerase IV, a single mutation in *GyrA* is sufficient to impart high-level of MICs (Wang et al., 1993). This feature helps explain the higher prevalence of resistance in *Campylobacter*, compared with *E. coli*, from food animals exposed to fluoroquinolones (Van Boven et al., 2003). While mutations in the quinolone target genes are required to achieve a clinical level of resistance, several other mechanisms may also contribute to resistance, including decreased intake of the drug due to the loss of a membrane-bound porin; drug extrusion via efflux pumps, some of which may have a broad substrate specificity; or one of the more recently described plasmid-mediated quinolone resistance (PMQR) mechanisms (Karczmarczyk et al., 2011).

#### 1.7.5 Pharmacokinetic properties

Generally these compounds are characterized by a high volume of distribution, long biological half-life, low serum protein binding, elimination by renal and extrarenal mechanisms with high total and renal clearances, limited biotransformation and moderate to excellent bioavailability after oral administration (Giguère and Dowling, 2013).

The fluoroquinolones used in veterinary medicine are primarily administered orally although parental formulations are available for administration to dogs, cats, horses and food animals. Although there is considerable individual variation among the different compounds and in the different animal species, the fluoroquinolones are rapidly absorbed following oral administration in monogastric species; bioavailability is rather low in ruminants although the reason for has not been determined (Brown, 1996).

When fluoroquinolones are co-ingested with food the time to reach peak concentration ( $T_{max}$ ) may be delayed but the maximum concentration ( $C_{max}$ ) is unaffected. However, concomitant administration with products that contain metal cations (such as Fe, Ca, Mg), will adversely affect the  $C_{max}$ . Parenteral bioavailability of most quinolones is approximately complete in pre-ruminants and ruminants cattle (Thomas et al., 1994a).

Following absorption, fluoroquinolones exhibit rapid and extensive tissue distribution because of their physicochemical properties; moreover they have the capacity to penetrate nearly all organs and cells. Their apparent volumes of distribution exceed total body water ( $> 1$  L/kg). As with other antibiotics which



exhibit intracellular accumulation (e.g. tetracyclines, macrolides), the concentrations of fluoroquinolones in different tissues are often higher than the concurrent serum levels. Fluoroquinolones are able to concentrate within phagocytic and non-phagocytic cells and remain active against different facultative, obligate, intracellular pathogens (Brown, 1996). In most species, this distribution volume over 3 times greater than that of most  $\beta$ -lactam antibiotics and aminoglycoside, and probably represents intracellular sequestration of the drug in various tissues. The very good distribution of these drugs is also due to their low plasma protein binding (<50%) (Giguère and Dowling, 2013).

Fluoroquinolones undergo a partial hepatic metabolism and give rise to active metabolites that are excreted in bile and urine. Fluoroquinolone metabolic pathways include glucuronidation, N-oxidation and desmethylation. Generally, metabolism involves the CYP 450 system (Martinez et al., 2006).

For example, the major metabolite of enrofloxacin is ciprofloxacin; the amount of ciprofloxacin produced varies with different species, with some producing ciprofloxacin concentrations that exceed the MIC of some pathogens (Kung et al., 1993).

Elimination may be via liver or kidneys or both depending on the compound. The fluoroquinolones are predominantly excreted as unchanged drug in the urine by glomerular filtration and active tubular secretion. The exception is difloxacin, where 80% is excreted in the feces. The elimination half-life of the fluoroquinolones is dependent on the drug and the animal species, and may also be dose dependent. The long elimination half-lives make these compounds ideal for every 24 or 48 hour dosing regimens (Giguère and Dowling, 2013).

#### *1.7.6 Pharmacodynamic properties and PK/PD correlation*

With ideal pharmacokinetic characteristics but a potential to select for resistant bacteria, optimal therapeutic dosage regimens for fluoroquinolones requires, as previously described, the PK/PD integration (Giguère and Dowling, 2013).

The universally recognized variable, which provides a quantitative index of drug efficacy and potency, is MIC and it is defined as the lowest concentration which completely inhibits bacterial growth. MBC is an alternative, but less frequently used, measure of potency. It is the drug concentration that produces a 99.9% reduction in bacterial count. Another PD variable, used specifically in relation to antimicrobial resistance acquired by mutation (e.g. quinolone resistance), is mutant prevention concentration (MPC), which is defined as the concentration that does not allow any mutant to be recovered from a population of more than  $10^{10}$  microorganisms (Lees et al., 2008).

The best parameters associated with fluoroquinolones efficacy are  $AUC_{0-24}/MIC$  or  $C_{max}/MIC$  ratios (Toutain and Lees, 2006). For fluoroquinolones, several authors reported that resistance selection may be reduced by achieving an

$AUC_{0-24}/MIC$  ratio  $> 100$  h or a  $C_{max}/MIC$  ratio  $> 8$  for concentration-dependent antimicrobial drugs. Other authors recommended, ratios  $> 125$  h for  $AUC_{0-24}/MIC$  or a  $C_{max}/MIC > 10$  to achieve high efficacy (McKellar et al., 2004). Furthermore, differences result if efficacy is determined against Gram-negative or Gram-positive strains. For the former microorganisms the ratio to ensure bacterial cure and prevent the selection of resistant bacteria should be approximately 125, while with the latter the ratio can be as low as 30-50 (Martinez et al., 2006). The exact  $AUC/MIC$  ratio that would predict outcome of infection in domestic animals would likely vary according to animal species, infectious agent, site of infection, immune status of the host, and specific fluoroquinolones selected (Giguère and Dowling, 2013).

### *1.7.7 Adverse effects*

Fluoroquinolones are relatively safe antimicrobial drugs. When administered at therapeutic doses, toxic effects are mild and generally limited to gastrointestinal disturbances such as nausea, vomiting, and diarrhea. Chronic, high-dose fluoroquinolone therapy causes articular cartilage lesions in juvenile dogs, particularly in weight bearing joints (Burkhardt et al., 1992).

Retinal degeneration has been reported in cats treated with high doses (20 mg/kg every 24 hours) of enrofloxacin (Wiebe and Hamilton, 2002). Neurotoxic effects causing central nervous system disturbances (seizures, dizziness, ataxia, insomnia, restlessness, somnolence, tremors) are common adverse effects of fluoroquinolones in humans and have been reported in horses, dogs and cats treated with enrofloxacin (Papich and Riviere, 2009). Photosensitivity and Achilles tendon rupture has been associated with the use of fluoroquinolones in humans but has not been reported in animals (Brown, 1996).

### *1.7.8 Clinical applications*

Quinolones are indicated for the treatment of local and systemic infections caused by susceptible microorganisms, particularly against deep-seated infections and intracellular pathogens. Therapeutic success has been obtained in respiratory, intestinal, urinary, and skin infections, as well as in bacterial prostatitis, meningoencephalitis, osteomyelitis, and arthritis. In horses they are useful for the treatment of a variety of Gram-negative infections caused by susceptible bacteria resistant to alternative, first-choice drugs. Several fluoroquinolone products are approved for use in swine to treat respiratory disease and Metritis-Mastitis-Agalactia syndrome (Papich and Riviere, 2009). In companion animals these drugs can be used to treat a large number of diseases such as prostatitis and mastitis caused by susceptible bacteria; urinary tract

infections; respiratory infections including rhinitis and pneumonia and osteomyelitis and soft tissue infection (Giguère and Dowling, 2013).

In avian species respiratory and systemic infections caused by avian pathogenic *Escherichiacoli* (APEC) constitute the most prevalent and economically most important primary or secondary bacterial diseases (Lutful Kabir, 2010). Fluoroquinolones are considered potentially useful drugs in the treatment of colibacillosis and other infections caused by *E. coli* in chickens and turkeys, in particular when first and second choice drugs had failed.

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# **CHAPTER 2**

## **Objectives**



## 2. Objectives

Due to a wide understanding of the effects of antibiotics and the increase of the phenomena of microbial resistance to these drugs, a particular attention has been devoted, during the last years, to antibacterial use in humans and animals and in particular to the methods oriented to a correct evaluation of efficacious dosages for a more prudent and targeted use of antimicrobials in veterinary species.

The PK/PD approach gives tools to improve efficacy in field through the relationship between the efficacy values of the antimicrobial drug studied *in vitro* (PD) with the main kinetic parameters obtained by studying the fate of a drug in a target specie (PK). Therefore the PK/PD relationship can serve to obtain estimates of doses that may be needed to achieve a desired clinical response or to modify a dosing regimen based upon susceptibility information on the considered pathogen.

The present study was focused on a particular avian species, turkeys that is considered a “minor species”, but important in the livestock production of Northern Italy. Scarce data exist about the usage of antimicrobial drugs in turkey and even less is known about their efficacy. As the limited number of medicinal products authorized in this species, antimicrobial therapy is frequently carried out with the few products authorized or with drugs “extra-label” used with the consequence of increases of selective pressure and also with the possibility of cross-resistance within the same pharmacological group of compounds. In this regard, the aim of the study is the revision of dosages and schemes of treatment of two fluoroquinolones.

The first step was to optimize and validate a fast, sensitive, and specific liquid chromatography-mass spectrometry (LC-MS) method suitable for the detection of a wide range of concentrations of different fluoroquinolones from several biological matrices as those occurring in pharmacokinetic and residue depletion studies (first trial). The developed method was then used for the quantification of fluoroquinolones in plasma and tissue samples of turkeys derived from pharmacokinetic studies (second and third trials).

In the pharmacokinetic trials, three types of oral treatment were considered: single oral gavage, that is considered ideal for the pharmacokinetic profiles, but is not feasible infield conditions, five days of 10 hours pulsed water medication and five days of 24 hours continuous water medication, which are commonly used in farms. Pulse administration can be a viable choice for concentration-dependent antimicrobials, as fluoroquinolones, because it allows to reach higher concentration levels in systemic circulation in a shorter time compared to continuous water medication.

In order to evaluate and eventually recommend a different route of administration than the oral one, the subcutaneous administration was also carried out.

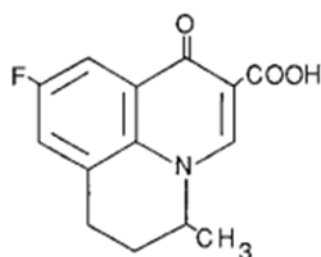
To revise the recommended dosage, the EU authorized dose and the doubled dose were examined. The double dose was chosen to assess if better results can be achieved with the dosage schemes considered.

The kinetic parameters obtained from the above described trials were correlated with MIC of *E.Coli* field strains, in order to obtain the PK/PD parameters for doses optimization. *E.Coli* is a Gram-negative bacterium responsible of different local and systemic diseases of poultry, such as colibacillosis. Mostly, it acquires resistance quite easily, in fact it is now resistant to many antibiotics that are used as the first line treatment of colibacillosis. The fluoroquinolones are among the most effective antimicrobial compounds for poultry pathogens and for colibacillosis, in particular, but being the drug of choice for many human bacterial diseases, these antimicrobial agents must be considered as last choice product in avian medicine.

The drugs under consideration were **flumequine** and **enrofloxacin**.

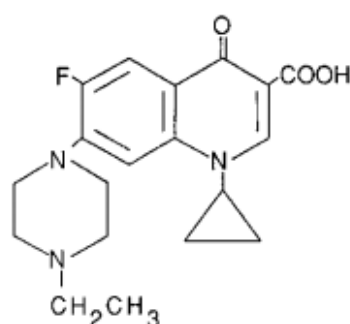
Flumequine is a 1<sup>st</sup> generation quinolone, that is no longer used in humans because similar congeners with broader activity and better tissue distribution are available (Crumplin, 1988). However, it is still used in food-producing species to control infection caused by various Gram-negative bacteria. It is employed in several animal species, including avian species and turkeys, due to its relative low cost and good tolerability.

Figure 1: **Chemical structure of flumequine** (Jacobs-Reitsma et al., 1995).



Enrofloxacin is a 2<sup>nd</sup> generation quinolones, approved only in veterinary medicine, with an extended spectrum of antibacterial activity and used in food and companion animals to control infection caused by various Gram-negative bacteria. Ciprofloxacin, its primary active metabolite is also a very potent antibacterial used mainly in human medicine. In European Countries, enrofloxacin was approved in the 1990s and is still extensively used in poultry for colibacillosis treatment, due to its unique effectiveness against multidrug-resistant avian pathogenic *Escherichia coli* (Lutful Kabir, 2010).

Figure 2: **Chemical structure of enrofloxacin** (Jacobs-Reitsma et al., 1995).



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## **CHAPTER 3**

# **Development and validation of an LC-MS/MS/MS method for the quantification of fluoroquinolones in several matrices from treated turkeys**

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### 3. Development and validation of an LC-MS/MS/MS method for the quantification of fluoroquinolones in several matrices from treated turkeys

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#### 3.1 Abstract

The study presents a sensitive and reliable confirmatory method for the extraction, identification, quantification of five fluoroquinolones (FQ) namely enrofloxacin, ciprofloxacin, difloxacin, sarafloxacin and flumequine, in plasma, liver, kidney, muscle, skin þ fat, lung and intestinal content from turkeys.

For the extraction and matrix clean-up of FQ residues from all biological matrices, the Quick EasyCheap Effective Rugged Safe (QuEChERS) methodology was adopted; only for plasma samples acetonitrile was used.

The analyses were performed by liquid chromatography with mass spectrometry detection (LC-MS).

LC separation was performed on a C<sub>18</sub> Kinetex column (100x2.1 mm, 2.6 µm, Phenomenex, CA, USA) with gradient elution using ammonium acetate solution (10 mM, pH 2.5) and methanol containing 0.1% formic acid. Mass spectrometric identification was done using an LTQ XL ion trap (Thermo Fisher Scientific, CA, USA), with a heated electrospray ionization probe, in positive ion mode.

The method was validated according to the European Legislation (decision 2002/657/EC) and EMA guideline (EMA/CVMP/VICH/463202/2009); selectivity, linearity response, trueness (in terms of recovery), precision (within-day repeatability and within-laboratory reproducibility), limit of detection, limit of quantification, decision limits, detection capability, absolute recovery and robustness were evaluated using turkey blank matrices. All data were within the required limits established for confirmatory methods except for flumequine which presented a recovery value slightly higher than 110% in muscle and intestinal content. For all FQs, all the extraction rates were greater than 70% and limits of quantification ranged from 1.2 µg kg<sup>-1</sup> to 118.8 µg kg<sup>-1</sup>.

This fast and robust method was suitable for the identification and quantification of FQ residues in tissues, plasma and intestinal content as confirmed by data

obtained from incurred samples of turkeys treated at farm for therapeutic purposes.

### 3.2 Introduction

In EU, fluoroquinolones (FQs) have been authorized for several veterinary species for the treatment of gastrointestinal and respiratory infections caused by gram positive and negative bacteria (Barnes, Nolan, & Vaillancourt, 2008; Riviere & Papich, 2009; Webber & Piddock, 2001).

In USA, in 2005, ENRO was banned in poultry due to the wide spread of resistance in *Campylobacter* spp., a commensal microorganism or poultry but a pathogen for human (FDA, 2005). In EU, the drug is still authorized and largely used in poultry (EMA, 2006), despite monitoring plans indicate the increase of resistant microorganisms in poultry farms (EFSA, 2010; Piccirillo Dotto, Salata, & Giacomelli, 2013; Russo et al., 2012; Walsh & Fanning, 2008). Recently, in the North East of Italy, from the surveillance of medication protocols in poultry farms, resulted that pulsed water medication was more frequently used than the authorized continuous water medication to treat the birds in the sheds. When different dosage, treatment interval or administration route are adopted, the residue monitoring on animal carcasses becomes determinant to guarantee food safety and high through-put analytical methods are required to process large numbers of samples. Moreover, to ensure a prudent use of antimicrobial drugs in veterinary medicine, the restriction on drug usage in food producing animals cannot be sufficient and the optimal dosage regimen to minimize bacterial resistance should always be assessed for an effective treatment (AliAbadi & Lees, 2000; Martinez, McDermott, & Walzer, 2006; McKellar, Sanchez Bruni, & Jones, 2004). In this context, it is very important to have a selective, sensitive and rapid method for the determination of FQ concentrations in food-producing animals.

An important and fundamental step for all analytical procedures is the sample preparation, especially when complex matrix as animal tissues composed of lipids, carbohydrates, proteins, vitamins, phenolic compounds and organic acids are used. Several extraction strategies were described in the literature for FQs detection in food of animal origin: solid phase extraction (SPE) (Garcés, Zerzanová, Kucera, Barrón, & Barbosa, 2006; Hermo, Barrón, & Barbosa, 2006; Toussaint, Chedin, Bordin, & Rodriguez, 2005; Verdon, Couedor, Roudaut, & Sandérs, 2005), liquid to liquid extraction (LLE) (García, Sarabia, Ortiz, & Aldama, 2005), solid-phase micro-extraction (SPME) (Huang, Lin, Yu, & Feng, 2006) and supercritical fluid extraction (SFE) (Shim, Lee, Kim, Lee, & Kim, 2003), mostly laborious and time consuming techniques with poor extraction efficiency and relatively low recoveries (Huan et al., 2012). Recently, more innovative FQ extraction technique from different matrices, were applied:

pressurized liquid extraction(PLE) from enfant food product (Rodríguez, Navarro-Villoslada, Moreno-Bondi, & Marazuela, 2010), microwave assisted extraction(MAE) with in situ LLE clean-up from chicken breast muscle (Xu et al., 2011), accelerated solvent extraction (ASE) from muscle, liver, kidney of swine, bovine, chicken and fish (Huan et al., 2012),dispersive liquid-liquid micro-extraction (DLLME) from chicken liver (Moema, Nindi, & Dube, 2012) or fish muscle (Tsai et al., 2009)and molecularly imprinted polymers (MIP) from chicken muscle or eggs (Blasco & Picò, 2012; Qiao & Sun, 2010) and QuEChERS technology. The QuEChERS (QUick, Easy, CHEap, Effective, Rugged and Safe)extraction and clean-up approach, attracted great interest in the last few years because it allowed to reduce and simplify the time needed to complete the processes; initially applied to the analysis of pesticides (Anastassiades, Lehotay, Stajnbaher, & Schenck, 2003)was subsequently extended to veterinary drug residues extraction from different biological matrices (Lopes, Reyes, Romero-González,Frenich, & Vidal, 2012; Stubbings & Bigwood, 2009).QuEChERS technique was adopted for the extraction of FQs from milk (Karageorgou, Myridakis, Stephanou, & Samanidou, 2013;Lombardo-Agüí, Gámiz-Gracia, Cruces-Blanco, & García-Campaña,2011), eggs (Capriotti, Cavaliere, Piovesana, Samperi, & Laganà,2012; Garrido Frenich, Aguilera-Luiz Mdel, Martínez Vidal, &Romero-González, 2010), honey (Lombardo-Agüí, García-Campaña,Gámiz-Gracia, & Cruces-Blanco, 2012; Wang & Leung, 2012), chicken muscle (Lopes et al., 2012), bovine muscle and swine muscle (Nakajima et al., 2012).The objective of the study was to optimize and validate a fast, simple, sensitive, and specific LC-MS/MS/MS method suitable for the detection of a wide range of concentrations of FQs as those occurring in pharmacokinetic and residue depletion studies from several matrices. In the present study, five FQs (enrofloxacin, ENRO;ciprofloxacin, CIPRO; difloxacin, DIFLO; sarafloxacin, SARA; flumequine,FLUME) were extracted from plasma, lung, intestinal content, muscle, liver, kidney, skin þ fat from turkeys, applying one single LLE to plasma samples and QuEChERS clean-up procedure to the other matrices. For the validation purposes, all the five FQs above reported were used and the biological matrices were obtained from healthy never treated turkeys; all the incurred samples were obtained from turkeys experimentally administered with ENRO and FLUME via pulsed medicated water as reported in previous studies by Ferraresi et al. (2013) and Cagnardi et al. (2014).

### 3.3 Materials and methods

#### 3.3.1. *Animals and treatments*

The study was conducted according to Italian law (D.L.116/1992) and was ethically approved by the Italian Health Ministry (Animal Welfare Unit, 2009R4KM4F\_002). Thirty-two female turkeys (breed B.U.T.6) 63±9 days old, weighing about 4.6 kg and determined to be healthy by a thorough physical examination, were used. Turkeys were randomly assigned to 4 groups of 8 animals to be subjected to treatments with the FQs: groups 1 and 3 were repeatedly treated for 5 days via drinking water in a 10-h pulsed scheme administration with ENRO (Baytril oral solution 10%, BAYER, Milano, Italy) at the dose of 20 mg kg<sup>-1</sup> b.w. while groups 2 and 4 were treated for 5 days via drinking water in a 10-h pulsed scheme administration with FLUME (Flumechina 40% DOXAL) at the dose of 30 mg kg<sup>-1</sup> b.w. (Cagnardi et al., 2014; Ferraresi et al., 2013). The doses selected were double the recommended doses of ENRO (10 mg kg<sup>-1</sup> b.w.) and FLUME (15 mg kg<sup>-1</sup> b.w.) in poultry. Plasma and tissue samples used as blank matrices were collected from healthy, never treated animals from an organic farm. For groups 1 (ENRO) and 2 (FLUME), blood samples were collected on days 1 and 5, immediately before the treatment, at 1, 3, 6, 9 h during the 10-h treatment, and at 1, 2, 4, 8, 14 h after the withdrawal of medicated water. Plasma was separated by centrifugation at 2000 rpm for 10 min and stored at -20 °C pending analysis. Three and 5 days after the last treatment, turkeys of group 1 and 2 respectively, were sacrificed and liver, kidney, muscle (breast), skin & fat, were collected and stored at -80 °C before analysis. Animals of groups 3 and 4 were sacrificed 24 h after the last treatment and lung and intestinal content were collected and stored at -80 °C before analysis.

#### 3.3.2. *Chemical and reagents*

Enrofloxacin (ENRO, purity: 99.0%), ciprofloxacin (CIPRO, purity: 99.9%), difloxacin (DIFLO, purity: 99.8%), sarafloxacin (SARA, purity: 97.2%) flumequine (FLUME, purity: 99.7%) and norfloxacin (internal standard, NOR, purity: 99.7%) were obtained from Sigma-Aldrich (Steinheim, Germany). Acetonitrile (ACN) and methanol (MeOH) were from Carlo Erba Reagents. Formic acid (FA, 98%), ammonium acetate (98%), potassium phosphate monobasic KH<sub>2</sub>PO<sub>4</sub> were from Sigma-Aldrich (Steinheim, Germany). All reagents were of analytical grade. Ultrapure water generated by the Milli-Q system (Millipore) was used. SampliQ Quick Easy Cheap Effective Rugged Safe (QuEChERS) EN buffered extraction kits and SampliQ QuEChERS dispersive-SPE 2 ml tube for drug residue in meat (containing 25 mg of C18 and 150 mg of anhydrous MgSO<sub>4</sub>) were used for the analysis of FQs in turkey matrices (liver,

kidney, muscle, skin þ fat, lung, intestinal content) and were purchased by Agilent (Santa Clara, CA, USA). Phenex-RC (Regenerated Cellulose) syringe filters 0.22 µm (Phenomenex, Torrance, CA, USA) were used to filter the extracts before the injection in the LC-MS system.

### *3.3.3. Standards and stock solutions*

Individual stock solutions of ENRO, CIPRO, DIFLO, SARA, FLUME, NOR (IS) were prepared at a concentration of 1000 µg ml<sup>-1</sup> by dissolving the proper quantity of each compound, exactly weighted, in methanol with 10% (v/v) of NaOH into volumetric flasks. These solutions were stored at 4 °C in amber glass and prepared fresh every 6 months. Working solutions (containing all FQs except of the IS) used to spike blank samples of turkey, were prepared by appropriate dilutions of the concentrated stock standard solutions with mobile phase (10 mM ammonium acetate pH 2.5:0.1% formic acid in methanol, 80:20). From IS stock solution, different dilutions were prepared to spike matrices: IS at 3 µg ml<sup>-1</sup> for plasma, IS at 160 µg ml<sup>-1</sup> for kidney and liver, IS at 100 µg ml<sup>-1</sup> for muscle, lung, skin þ fat and intestinal content.

### *3.3.4. Instrumentation*

All analyses were performed by liquid chromatography with mass spectrometry detection (LC-MS). The chromatographic separation was achieved using an Accela600 HPLC pump with CTC automatic injector (Thermo Fischer Scientific, San Jose, CA, USA) equipped with a C-18 Kinetex (100x2.1 mm, 2.6 µm) analytical column by Phenomenex (Torrance, CA, USA). The mass detection was achieved with an LTQ XL ion trap (Thermo Fischer Scientific, San Jose, CA, USA), equipped with a heated electrospray ionization (HESI-II) probe. The system was controlled by the X-calibur software (version 2.1), that was also used for the data acquisition and analysis.

### Chromatographic and mass spectrometric conditions

Gradient elution was applied using a 10 mM ammonium acetate adjusted at pH 2.5 with formic acid as solvent A and methanol with 0.1% formic acid (v/v) as solvent B. The mobile phase composition (A:B, v/v) was: 80:20 at 0 min, 50:50 at 10 min, 10:90 at 13 min and kept unchanged until 14 min, 0:100 from 14.50 min to 16 min and 80:20 from 17 min to 20 min to re-equilibrate the system. The sample trays were maintained at 4°C and the flow rate was set on 200 µl min<sup>-1</sup>. Standard solutions at 1 µg ml<sup>-1</sup> of each FQ were infused directly via syringe pump with 20 µl min<sup>-1</sup> flow rate to the mass spectrometer in order to find fragmentation patterns, tuning parameters and MS<sup>3</sup> parameters for each analyte. Precursor ions, product ions, collision energies and retention times are shown in Table 1.

Due to the presence of the amino group in most FQs that is easily protonated in acidic medium, the ESI source was used in positive mode. The mass analyzer was set on the full scan monitoring mode. The following optimum tuning parameters were common for all FQs: sheath gas flow 40 arbitrary units, auxiliary gas flow 5 arbitrary units; ion spray voltage 3.5 kV; capillary temperature 300 °C; capillary voltage 26 V; tube lens 80 V.

Retention time windows for each analyte were checked daily with a mixture of the five FQs in mobile phase. Confirmation was achieved by examination of the relative ion intensities of two major MS<sup>3</sup> product ions.

Table 1: Instrument acquisition data for the analysis of FQs by LC-MS/MS/MS; <sup>a</sup> product ion used for quantification; Rt: retention time.

Analyte	Precursor ion (m/z)	Fragmentation pattern	Collision energy (%)	Rt (min)
Enrofloxacin	360	360 > 316	46	5.6
		316 > 288, <b>245<sup>a</sup></b>	23	
Ciprofloxacin	332	332 > 288	22	5.3
		288 > <b>268<sup>a</sup></b> , 245	30	
Difloxacin	400	400 > 356	30	6.2
		356 > 336, <b>299<sup>a</sup></b>	20	
Sarafloxacin	386	386 > 342	30	6.6
		342 > <b>322<sup>a</sup></b> , 299	30	
Flumequine	262	262 > 244	40	12.5
		244 > <b>202<sup>a</sup></b> , 176	25	
Norfloxacin (IS)	320	320 > 276	36	4.9
		276 > <b>256<sup>a</sup></b> , 233	30	

### 3.3.5. Sample preparation

The plasma samples purification was performed as reported by Ferraresi et al. (2013) whereas QuEChERS technology, which consists of two steps, a salting-out extraction and a dispersive SPE clean-up, was adopted and used for the extraction of FQs from all turkey tissues (Núñez, Gallart-Ayala, Martins, & Lucci, 2012; Stubbings & Bigwood, 2009).

Before proceeding with the extraction, IS solution (10 µl) was added to plasma samples to obtain IS at 150 µg l<sup>-1</sup> final concentration.

Turkey matrices (liver, muscle, kidney, skin + fat, lung, intestinal content) were first chopped into small pieces and homogenized; 2 g of samples (1 g for intestinal content) were placed into 50 ml centrifuge tubes and added with 50 ml of the different IS solutions reported above (see Section 3.3.3.), to obtain IS final concentration at 4 µg g<sup>-1</sup> in liver and kidney and at 2.5 µg g<sup>-1</sup> in muscle, lung, skin + fat, and intestinal content, respectively.

An 8 ml volume of 30 mM of KH<sub>2</sub>PO<sub>4</sub> buffer pH 7.0 were added and the tubes were agitated for 1 min. To each tube, a 10 ml volume of 5% formic acid in ACN was added and the tubes were shaken for other 1 min. Then, an Agilent SampliQ

QuEChERS EN extraction salt packet was added to each tube and the sample tubes were capped tightly and shaken vigorously for 3 min. After centrifugation at 4000 rpm for 5 min, a 1 ml aliquot of the upper ACN layer was transferred into an Agilent SampliQ QuEChERS dispersive-SPE 2 ml tube and the samples were vortexed for 1 min and centrifuged at 13,000 rpm for 5 min with a micro-centrifuge.

The supernatant (700 µl) was transferred to a 15 ml tube and evaporated to dryness under a stream of air at 50°C with a TurboVap evaporator (Zymark, Hopkinton, MA, USA). The residue obtained was dissolved in 700 µl of mobile phase (10 mM ammonium acetate pH 2.5:0.1% formic acid in methanol, 80:20), vortex, mixed, sonicated for 10 min and filtered through a Phenex-RC (Regenerated Cellulose) syringe filter 0.22 µm (Phenomenex, Torrance, CA, USA) before LC-MS/MS/MS analysis.

Fluoroquinolone concentrations of all incurred and spiked samples were quantified with a daily calibration curve prepared in matrix.

### *3.3.6. Method validation*

Prior to application to incurred samples, the method was validated according to the European Commission Decision 2002/657/EC for the residue depletion study in liver, kidney, skin + fat and muscle, and to the EMA guidelines (EMA/CVMP/VICH/463202/2009) for the pharmacokinetic and distribution study in plasma, lung and intestinal content. Blank biological matrices from different untreated turkeys were used.

Aliquots of blank samples (200 µl for plasma, 2 g for liver, kidney, lung, muscle, skin + fat and 1 g for intestinal content) were transferred into polypropylene tubes and spiked with 50 µl of IS and with proper amounts of working solutions of FQs to obtain fortified samples at different concentrations (for intestinal content, working solutions containing only ENRO, CIPRO and FLUME were used). The mixtures were shaken and then the samples were allowed to stand in the dark for 30 min at room temperature to permit the interaction between FQs and tissues before proceeding with the extraction described in Section 3.3.5.

The following analytical performance parameters were assessed: specificity, linearity response, trueness, precision (within-day repeatability and within-laboratory reproducibility), limit of detection and quantification, decision limits, detection capability, matrix effect, absolute recovery and robustness. Confirmation of the identities of the FQs was carried out by comparison of the chromatographic peak area of two prominent product ions in MS<sup>3</sup>, with the calibration standard at comparable concentrations. Identification was considered reliable if the ratio was within the criteria laid down in the European Commission Decision.

### Specificity

To verify specificity, a representative number of blank biological matrix samples of different origin (n = 10-20) were analyzed to check the absence of potential matrix interference peaks at the retention time of the target FQs.

### Linearity

Method linearity was evaluated by preparing six different calibration curves on six different days by spiking each of the seven biological matrices from untreated turkeys (blank samples) with different FQ mixed standard solutions, before proceeding with the extraction. Final concentrations of FQs were different in plasma, lung and intestinal content: 2.5, 5, 10, 25, 50, 100, 200  $\mu\text{g l}^{-1}$ , 6.2, 12.5, 25, 50, 125, 250, 500, 1000  $\mu\text{g kg}^{-1}$  and 12.5, 25, 50, 100, 250, 500, 1000, 2000  $\mu\text{g kg}^{-1}$ , respectively.

For each FQ, a different range of concentrations in liver, kidney, muscle and skin + fat, was adopted and final concentrations were reported in Table S1 (see Supplementary data). Calibration lines were constructed by plotting the ratio of the standard area to internal standard area versus the added concentrations and carrying out linear regression analysis. The linearity was considered acceptable when the coefficient of correlation was above 0.990 and the evaluation of residual was lower than 20%.

### Limit of detection (LOD) and limit of quantification (LOQ)

For plasma, lung and intestinal content, limit of detection (LOD) and limit of quantification (LOQ) were determined as follows:  $\text{LOD} = 3.3 \times \text{SD}/S$ ;  $\text{LOQ} = 10 \times \text{SD}/S$ , where SD is the standard deviation of y-intercepts and S is the average slope obtained from the different calibration curves prepared for each matrix (Ribani, Collins, & Bottoli, 2007). For kidney, muscle, liver and skin + fat LOD values were determined as described above, whereas LOQ for each FQ was defined as the smallest measured content of the identified analyte that can be quantified with an acceptable precision and trueness (EMA/CVMP/VICH/463202/2009) in agreement with the limits reported by European Commission Decision.

### Decision limit ( $\text{CC}\alpha$ ) and detection capability ( $\text{CC}\beta$ )

The Commission of the European Communities, to ensure food safety, has established MRLs legally permitted and accepted in liver, kidney, muscle, skin + fat for ENRO, CIPRO, DIFLO, FLUME (Council Regulation 2377/90/EEC). For these FQs, the decision limit ( $\text{CC}\alpha$ ) and detection capability ( $\text{CC}\beta$ ) were calculated. As no MRL has been set for SARA in muscle and kidney,  $\text{CC}\alpha$  and  $\text{CC}\beta$  for this FQ were calculated only for liver and skin + fat.



These values were determined by analyzing blank samples fortified around the permitted limit in equidistant steps (the calibration curve procedure).  $CC\alpha$  was calculated as the mean measured concentration at the MRL of each compound plus 1.64 times the standard deviation of the within-laboratory reproducibility at this concentration;  $CC\beta$  was calculated as  $CC\alpha$  plus 1.64 times the standard deviation of the within-laboratory reproducibility at  $CC\alpha$  (Verdon, Hurtaud-Pessel, & Sanders, 2006).

Table 2: Linearity evaluation and sensitivity data for the FQs detected in this study in the different biological matrices (plasma, liver, kidney, muscle, skin + fat, lung and intestinal content): linear determination coefficient ( $R^2$ ), limit of detection (LOD) and limit of quantification (LOQ).

Plasma				Liver			
analyte	$R^2$	LOD ( $\mu\text{g l}^{-1}$ )	LOQ ( $\mu\text{g l}^{-1}$ )	analyte	$R^2$	LOD ( $\mu\text{g kg}^{-1}$ )	LOQ ( $\mu\text{g kg}^{-1}$ )
ENRO	0.9999	0.8	2.5	ENRO	0.9999	2.6	12.5
CIPRO	0.9998	0.5	1.4	CIPRO	0.9998	5.7	12.5
DIFLO	0.9995	1.5	4.6	DIFLO	0.9993	43.8	118.8
SARA	0.9998	0.6	1.8	SARA	0.9997	3.3	6.3
FLUME	0.9996	0.9	2.5	FLUME	0.9997	29.3	50.0
Kidney				Muscle			
analyte	$R^2$	LOD ( $\mu\text{g kg}^{-1}$ )	LOQ ( $\mu\text{g kg}^{-1}$ )	analyte	$R^2$	LOD ( $\mu\text{g kg}^{-1}$ )	LOQ ( $\mu\text{g kg}^{-1}$ )
ENRO	0.9999	9.8	18.8	ENRO	0.9995	5.2	12.5
CIPRO	0.9999	4.1	18.8	CIPRO	0.9995	2.0	12.5
DIFLO	0.9999	9.7	37.5	DIFLO	0.9990	13.0	37.5
SARA	0.9998	1.7	6.3	SARA	0.9992	0.5	1.3
FLUME	0.9996	25.1	62.5	FLUME	0.9988	8.3	50.0
Skin + fat				Lung			
analyte	$R^2$	LOD ( $\mu\text{g kg}^{-1}$ )	LOQ ( $\mu\text{g kg}^{-1}$ )	analyte	$R^2$	LOD ( $\mu\text{g kg}^{-1}$ )	LOQ ( $\mu\text{g kg}^{-1}$ )
ENRO	0.9942	4.8	12.5	ENRO	0.9998	2.7	8.2
CIPRO	0.9998	8.8	12.5	CIPRO	0.9996	2.9	8.9
DIFLO	0.9969	16.7	50.0	DIFLO	0.9997	2.2	6.8
SARA	0.9972	0.9	1.2	SARA	0.9998	0.9	2.7
FLUME	0.9978	22.7	31.2	FLUME	0.9997	1.8	5.4
Intestinal content							
analyte	$R^2$	LOD ( $\mu\text{g kg}^{-1}$ )	LOQ ( $\mu\text{g kg}^{-1}$ )				
ENRO	0.9997	3.1	9.5				
CIPRO	0.9997	1.1	3.4				
FLUME	0.9994	4.5	13.8				

Table 3: Validation results obtained from plasma and lung ( $C_N$ : Nominal Concentration; CV=Coefficient of Variation; REC: absolute recovery; ME: matrix effect).

<b>PLASMA</b>						
<b>analyte</b>	<b><math>C_N</math> <math>\mu\text{g l}^{-1}</math></b>	<b>TRUENESS (%)</b>	<b>Within-day Repeatability (CV %)</b>	<b>Within-Laboratory Reproducibility (CV %)</b>	<b>REC% <math>\pm</math> SD</b>	<b>ME</b>
<b>ENRO</b>	<b>2.5</b>	96.0	3.4	10.6	101.6 $\pm$ 5.9	1.5 $\pm$ 4.0 $\times 10^{-2}$
	<b>10</b>	99.0	3.9	5.5	108.5 $\pm$ 6.1	0.9 $\pm$ 2.0 $\times 10^{-2}$
	<b>50</b>	103.8	5.5	7.1	97.3 $\pm$ 2.6	0.9 $\pm$ 5.0 $\times 10^{-2}$
<b>CIPRO</b>	<b>2.5</b>	92.0	8.8	12.6	99.5 $\pm$ 7.9	1.0 $\pm$ 1.0 $\times 10^{-2}$
	<b>10</b>	98.0	6.6	7.2	103.7 $\pm$ 14.3	1.0 $\pm$ 3.0 $\times 10^{-2}$
	<b>50</b>	100.6	6.7	5.7	110.1 $\pm$ 11.2	0.9 $\pm$ 3.0 $\times 10^{-2}$
<b>DIFLO</b>	<b>2.5</b>	96.0	2.2	10.3	105.6 $\pm$ 10.9	1.2 $\pm$ 5.0 $\times 10^{-2}$
	<b>10</b>	101.0	10.5	9.8	101.7 $\pm$ 11.0	0.9 $\pm$ 3.0 $\times 10^{-2}$
	<b>50</b>	103.4	2.9	10.1	102.9 $\pm$ 10.9	0.9 $\pm$ 2.0 $\times 10^{-2}$
<b>SARA</b>	<b>2.5</b>	100.0	8.7	8.0	101.0 $\pm$ 9.2	1.0 $\pm$ 1.0 $\times 10^{-2}$
	<b>10</b>	99.0	7.1	7.2	102.0 $\pm$ 5.6	0.9 $\pm$ 6.0 $\times 10^{-2}$
	<b>50</b>	96.6	3.6	5.3	99.0 $\pm$ 10.0	0.9 $\pm$ 5.0 $\times 10^{-2}$
<b>FLUME</b>	<b>2.5</b>	104.0	4.7	12.2	112.8 $\pm$ 1.9	1.2 $\pm$ 5.0 $\times 10^{-2}$
	<b>10</b>	104.0	2.7	5.9	105.6 $\pm$ 14.4	0.9 $\pm$ 2.0 $\times 10^{-2}$
	<b>50</b>	96.8	3.0	6.2	112.2 $\pm$ 10.9	0.9 $\pm$ 3.0 $\times 10^{-2}$
<b>LUNG</b>						
<b>analyte</b>	<b><math>C_N</math> <math>\mu\text{g kg}^{-1}</math></b>	<b>TRUENESS (%)</b>	<b>Within-day Repeatability (CV %)</b>	<b>Within-Laboratory Reproducibility (CV %)</b>	<b>REC% <math>\pm</math> SD</b>	<b>ME</b>
<b>ENRO</b>	<b>12.5</b>	96.8	7.9	12.9	95.2 $\pm$ 10.5	1.3 $\pm$ 9.0 $\times 10^{-2}$
	<b>50</b>	100.4	4.4	7.1	87.9 $\pm$ 5.2	1.0 $\pm$ 1.2 $\times 10^{-1}$
	<b>500</b>	96.2	7.9	5.6	87.4 $\pm$ 6.6	1.3 $\pm$ 5.0 $\times 10^{-2}$
<b>CIPRO</b>	<b>12.5</b>	94.4	2.7	10.2	70.6 $\pm$ 5.4	1.3 $\pm$ 2.1 $\times 10^{-1}$
	<b>50</b>	97.6	5.6	5.5	69.1 $\pm$ 3.7	1.1 $\pm$ 1.5 $\times 10^{-1}$
	<b>500</b>	98.2	2.6	3.4	77.9 $\pm$ 11.8	1.4 $\pm$ 7.0 $\times 10^{-2}$
<b>DIFLO</b>	<b>12.5</b>	103.2	4.7	13.1	102.4 $\pm$ 9.5	1.3 $\pm$ 5.0 $\times 10^{-2}$
	<b>50</b>	104.2	5.1	5.5	88.8 $\pm$ 6.6	1.0 $\pm$ 1.0 $\times 10^{-1}$
	<b>500</b>	95.8	3.6	5.2	95.7 $\pm$ 10.5	1.1 $\pm$ 6.0 $\times 10^{-2}$
<b>SARA</b>	<b>12.5</b>	93.6	5.9	6.6	92.3 $\pm$ 5.8	1.2 $\pm$ 3.0 $\times 10^{-2}$
	<b>50</b>	99.4	3.9	7.3	86.6 $\pm$ 6.2	1.1 $\pm$ 1.3 $\times 10^{-1}$
	<b>500</b>	99.5	4.5	4.9	91.4 $\pm$ 9.4	1.1 $\pm$ 5.0 $\times 10^{-2}$
<b>FLUME</b>	<b>12.5</b>	104.8	6.3	9.7	89.4 $\pm$ 9.0	1.1 $\pm$ 6.0 $\times 10^{-2}$
	<b>50</b>	101.6	8.5	11.2	78.9 $\pm$ 6.2	1.0 $\pm$ 1.3 $\times 10^{-2}$
	<b>500</b>	96.5	5.1	6.0	89.8 $\pm$ 8.9	1.0 $\pm$ 3.0 $\times 10^{-2}$

### Precision and trueness

Precision and trueness of the method were determined by performing tests on three sets of blank samples fortified with FQs at three different concentrations (six replicates each): for plasma, lung and intestinal content, the levels considered were 2.5, 10, 50  $\mu\text{g l}^{-1}$ , 12.5, 50, 500  $\mu\text{g kg}^{-1}$  and 25, 100, 1000  $\mu\text{g kg}^{-1}$  respectively. The matrices liver, kidney, muscle and skin + fat, for which an MRL has been set (see Table S2, Supplementary data), were fortified with FQ concentrations at 0.5, 1, 1.5 times each respective MRLs. Blank samples of muscle and kidney were fortified with SARA at 5, 10, 15  $\mu\text{g kg}^{-1}$  and 50, 100, 150  $\mu\text{g kg}^{-1}$  respectively. For each matrix, samples were analyzed on three different days in the same laboratory, with the same instrument but by three different operators,

corresponding to a total number of 54 samples. The precision of the method has been calculated either in terms of within-day repeatability, the variability of independent test results obtained on the same day, with the same method on identical test items in the same laboratory by the same operator using the same equipment, or in terms of within-laboratory reproducibility, the variability of independent test results obtained by different operators in different times as unique difference from above (Karageorgouet al., 2013; Muscarella, Lo Magro, Palermo, & Centonze, 2007). For the matrix intestinal content, due to the limited availability of blank material, only within-day repeatability was evaluated.

Precision was expressed in terms of imprecision and calculated as the variation coefficient (CV %) of measured concentrations at each level:  $CV \% = (\text{standard deviation} / \text{mean measured concentration}) \times 100$ . The CV % values for repeatability are acceptable if they are below two third of the value calculated from the Horwitz equation, whereas for reproducibility, they are acceptable if they are below the values calculated from the Horwitz equation (23% if concentration is between 100 and 1000  $\mu\text{g kg}^{-1}$  and 16% if the concentrations are higher than 1000  $\mu\text{g kg}^{-1}$ ). The Horwitz equation is not applicable to concentrations below 120  $\mu\text{g kg}^{-1}$ , and the values of repeatability and within-laboratory reproducibility are considered acceptable if they are below 14.7% and 22% respectively, as suggested by Thompson (2000).

The trueness, as no certified reference materials for FQs in the turkey tissues are available, was evaluated by the recovery of the known amount of FQs added to the blank matrices. It was calculated by dividing the mean measured value by the fortification level and multiply by 100 to express the result as a percentage. According to 2002/657/EC, the trueness should be between 70 and 100% for fortification levels between 1.0 and 10.0  $\mu\text{g kg}^{-1}$ , and between 80 and 110% for fortification levels  $\geq 10.0 \mu\text{g kg}^{-1}$ .

#### Absolute recovery and matrix effect

The absolute recovery of all analytes from all biological matrices was determined by comparing the analytical results of extracted FQs from fortified samples (FQs and IS were added before the extraction procedure) with unextracted standards added at the same concentrations in blank extracts representing 100% recovery.

Matrix effects were evaluated by calculating the peak area of the analytes in the presence of matrix (analytes added to blank matrix after extraction), to the peak area in absence of matrix (pure solution of the analyte at the same concentration).

Absolute recovery and matrix effect for each analyte were evaluated at three different levels (the same concentrations considered for the evaluation of precision and trueness), depending on the target biological matrix and FQ ( $n=6$ ). Three sets of samples were used for determination, one consisting of neat

standards (set1), one prepared in a blank matrix extract and spiked after extraction (set 2) and one spiked before extraction (set 3). Absolute recovery (REC %) and matrix effect (ME) were calculated using the formulas:

$$\text{REC (\%)} = \text{set3}_{\text{area}} / \text{set2}_{\text{area}} \times 100;$$

$$\text{ME} = (\text{set2}_{\text{area}} / \text{IS}_{\text{area}}) / (\text{set1}_{\text{area}} / \text{IS}_{\text{area}}).$$

Table 4: Validation results obtained from liver, kidney and muscle ( $C_N$ : Nominal Concentration; CV = Coefficient of Variation; REC: absolute recovery; ME: matrix effect).

<b>LIVER</b>						
analyte	$C_N$ $\mu\text{g kg}^{-1}$	TRUENESS (%)	Within-day Repeatability (CV %)	Within-Laboratory Reproducibility (CV %)	REC% $\pm$ SD	ME
ENRO	100	94.3	3.6	4.6	82.9 $\pm$ 4.8	0.9 $\pm$ 3.0 $\times 10^{-2}$
	200	97.3	7.9	6.5	87.1 $\pm$ 4.6	0.9 $\pm$ 3.0 $\times 10^{-2}$
	300	100.3	3.2	3.8	100.0 $\pm$ 4.8	1.1 $\pm$ 1.6 $\times 10^{-1}$
CIPRO	100	94.2	6.9	6.9	70.0 $\pm$ 6.5	1.0 $\pm$ 2.0 $\times 10^{-2}$
	200	98.9	5.7	7.8	75.1 $\pm$ 2.0	1.1 $\pm$ 5.0 $\times 10^{-2}$
	300	98.4	5.2	6.0	84.2 $\pm$ 3.7	1.2 $\pm$ 1.5 $\times 10^{-1}$
DIFLO	950	97.7	2.2	2.9	86.8 $\pm$ 3.8	0.8 $\pm$ 8.0 $\times 10^{-2}$
	1900	106.9	2.7	4.7	84.8 $\pm$ 3.3	1.0 $\pm$ 5.0 $\times 10^{-2}$
	2850	98.6	3.0	3.9	93.1 $\pm$ 4.3	1.2 $\pm$ 1.3 $\times 10^{-1}$
SARA	50	96.2	10.3	8.1	84.4 $\pm$ 7.5	0.7 $\pm$ 1.0 $\times 10^{-2}$
	100	104.1	6.2	6.5	88.5 $\pm$ 1.7	0.9 $\pm$ 4.0 $\times 10^{-2}$
	150	100.9	4.6	3.4	105.7 $\pm$ 2.8	1.1 $\pm$ 8.0 $\times 10^{-2}$
FLUME	400	91.9	4.7	7.3	95.0 $\pm$ 2.2	0.7 $\pm$ 4.0 $\times 10^{-2}$
	800	98.9	2.6	5.5	97.1 $\pm$ 3.3	0.8 $\pm$ 2.0 $\times 10^{-2}$
	1200	102.1	2.4	2.6	102.5 $\pm$ 4.4	0.9 $\pm$ 5.0 $\times 10^{-2}$
<b>KIDNEY</b>						
analyte	$C_N$ $\mu\text{g kg}^{-1}$	TRUENESS (%)	Within-day Repeatability (CV %)	Within-Laboratory Reproducibility (CV %)	REC% $\pm$ SD	ME
ENRO	150	98.7	1.8	2.9	94.7 $\pm$ 3.4	1.0 $\pm$ 3.0 $\times 10^{-2}$
	300	96.2	1.2	2.4	100.8 $\pm$ 11.8	0.9 $\pm$ 3.0 $\times 10^{-2}$
	450	98.3	1.2	1.3	106.1 $\pm$ 9.8	0.9 $\pm$ 2.0 $\times 10^{-2}$
CIPRO	150	99.6	1.6	4.5	81.3 $\pm$ 7.3	0.8 $\pm$ 4.0 $\times 10^{-2}$
	300	100.3	1.7	5.0	85.9 $\pm$ 8.7	0.8 $\pm$ 2.0 $\times 10^{-2}$
	450	100.4	1.1	2.3	88.8 $\pm$ 8.1	0.7 $\pm$ 4.0 $\times 10^{-2}$
DIFLO	300	99.6	2.1	2.7	99.9 $\pm$ 5.7	0.9 $\pm$ 5.0 $\times 10^{-2}$
	600	99.7	1.9	1.9	102.5 $\pm$ 6.7	0.9 $\pm$ 4.0 $\times 10^{-2}$
	900	101.3	1.8	1.8	108.8 $\pm$ 7.2	0.8 $\pm$ 3.0 $\times 10^{-2}$
SARA	50	99.8	5.2	6.3	108.1 $\pm$ 6.5	1.0 $\pm$ 8.0 $\times 10^{-2}$
	100	101.4	2.0	3.1	103.2 $\pm$ 9.1	1.0 $\pm$ 8.0 $\times 10^{-2}$
	150	100.1	1.8	2.4	101.2 $\pm$ 5.9	0.9 $\pm$ 6.0 $\times 10^{-2}$
FLUME	500	97.9	2.0	3.4	102.6 $\pm$ 2.6	0.9 $\pm$ 4.0 $\times 10^{-2}$
	1000	97.8	2.2	2.8	102.5 $\pm$ 7.8	1.1 $\pm$ 8.0 $\times 10^{-2}$
	1500	98.0	1.1	3.2	106.2 $\pm$ 7.3	0.9 $\pm$ 7.0 $\times 10^{-2}$
<b>MUSCLE</b>						
analyte	$C_N$ $\mu\text{g kg}^{-1}$	TRUENESS (%)	Within-day Repeatability (CV %)	Within-Laboratory Reproducibility (CV %)	REC% $\pm$ SD	ME
ENRO	50	90.0	10.0	6.9	92.9 $\pm$ 2.7	0.9 $\pm$ 2.0 $\times 10^{-2}$
	100	103.4	3.7	7.0	99.6 $\pm$ 2.3	1.1 $\pm$ 5.0 $\times 10^{-2}$
	150	104.3	4.7	4.8	98.8 $\pm$ 3.0	1.1 $\pm$ 5.0 $\times 10^{-2}$
CIPRO	50	92.4	4.7	5.2	83.0 $\pm$ 5.0	0.9 $\pm$ 1.0 $\times 10^{-2}$
	100	100.7	4.0	5.6	80.4 $\pm$ 3.2	1.2 $\pm$ 4.0 $\times 10^{-2}$
	150	100.9	4.3	5.6	81.3 $\pm$ 3.9	1.2 $\pm$ 4.0 $\times 10^{-2}$
DIFLO	150	86.1	5.8	7.0	97.9 $\pm$ 1.2	0.9 $\pm$ 2.0 $\times 10^{-2}$
	300	102.8	5.1	7.7	105.0 $\pm$ 1.7	1.1 $\pm$ 3.0 $\times 10^{-2}$
	450	105.0	3.7	9.3	102.3 $\pm$ 4.2	1.1 $\pm$ 6.0 $\times 10^{-2}$
SARA	5	92.0	12.8	10.3	98.1 $\pm$ 11.3	0.9 $\pm$ 6.0 $\times 10^{-2}$
	10	99.0	9.8	9.9	99.9 $\pm$ 7.1	1.1 $\pm$ 9.0 $\times 10^{-2}$
	15	100.7	7.3	9.9	104.5 $\pm$ 6.8	1.0 $\pm$ 5.0 $\times 10^{-2}$
FLUME	200	95.7	6.9	7.5	104.9 $\pm$ 2.3	0.9 $\pm$ 6.0 $\times 10^{-2}$
	400	111.7	6.9	6.5	109.0 $\pm$ 1.9	1.2 $\pm$ 6.0 $\times 10^{-2}$
	600	113.1	4.5	4.8	105.8 $\pm$ 2.7	1.1 $\pm$ 2.0 $\times 10^{-2}$

## Robustness

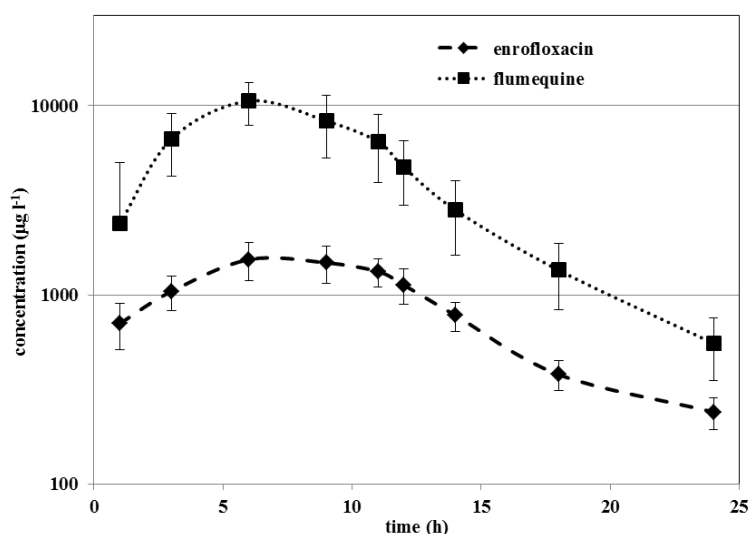
The robustness of the method was assessed according to the Youden and Steiner approach (Youden & Steiner, 1975). For this purpose, seven reasonable variables were chosen in the sample preparation procedure (volume and pH of dilution buffer; shaking, centrifugation and sonication time; formic acid percentage in acetonitrile and evaporation temperature of the final extract) and slightly modified with respect to the standard procedure. For each factor two different conditions were adopted. Eight experiments were carried out for the evaluation of the seven selected factors by using eight spiked turkey liver samples at the MRL. The effect of each factor was calculated by subtracting the mean result obtained with the variable at high level and the mean result achieved with the factor at low level. The standard deviation of the differences has been calculated and compared with the values obtained under within-laboratory reproducibility conditions.

## **3.4 Results and discussion**

### *3.4.1. Method validation*

The specificity was assessed by comparing the chromatograms of blank samples with those of the corresponding spiked samples to test for endogenous interference; no significant endogenous interferent peaks were evident at the retention time of the five FQs. The linearity of the calibrations curves in matrix was checked at 6 different days after calculating slopes and intercepts of each individual curve. Good linearity was observed within the concentrations range for all FQs in all matrices since the calculated determination coefficients  $R^2$  was always  $>0.99$  (Table 2) and residual in the range 10-20%. The slopes of the different calibration curves did not vary considerably and the intercepts were near to theoretical zero value, demonstrating good constancy of the measuring system.

Figure 1: ENRO (+ CIPRO) and FLUME plasma concentration–time profiles at the 5<sup>th</sup> day of 10-h oral pulsed administration. Mean values ( $\pm$  SD) of 8 turkeys.



The LOQs for all FQs in plasma, lung and intestinal content were set according to method sensitivity and by far lower than the FQs concentrations in matrices from treated turkeys, confirming the method suitability for distribution study. The LOQ set in liver, muscle, kidney, skin + fat for all FQs, is significantly lower than the respective half MRL: the values were from 5 to 16 times below these limits (Table S2, Supplementary data). Considering the aim of this work and the MRL in these matrices, the LOQs were considered acceptable although, based on the performance of the analytical method used and on the basis of signal-to noise ratio, it was possible to define even lower LOQ values.

In Table S2 of Supplementary data, the  $CC\alpha$  values with an error of 5% (probability of false non-compliance  $\leq$  5%) and the  $CC\beta$  values with an error of 5% (probability of falsely compliant samples  $\leq$  5%) are reported. The decision limit ( $CC\alpha$ ) and detection capability ( $CC\beta$ ) take into account the variability of the method and the statistical risk of making a wrong decision, and allow the assessment of the critical concentrations above which the method reliably distinguishes and quantifies a substance (European Decision no.657/2002/EC). These parameters were established for ENRO, CIPRO, DIFLO, FLUME, in liver, kidney, muscle and skin + fat; for SARA,  $CC\alpha$  and  $CC\beta$  values were calculated only for liver and skin + fat, because there is no fixed MRL in kidney and muscle. For each matrix, the precision of the method was evaluated at three different levels of fortification by calculating the CV % of the FQ concentrations under within-day repeatability conditions (calculated from six replicated samples analyzed on one day), and under within-laboratory reproducibility conditions (calculated from batches of 18 samples analyzed on three different days by different operators). The results, listed in Tables 3-5, reveal that all CV % values, for within-day repeatability and within-laboratory reproducibility, were

acceptable, ranging from 1.1 to 14.2% and from 1.3 to 13.1% respectively, for all concentrations.

The trueness of the developed method, expressed as relative recovery, ranged from 86.1 to 106.9% for all FQs (Tables 3-5) in agreement with the limits reported by Commission Decision 2002/657/EC. The only exception was FLUME in muscle with a recovery of 111.7% and 113.1% at 1 and 1.5 MRL respectively, and of 111.3% at 25  $\mu\text{g kg}^{-1}$  in intestinal content; thus, an overestimation of this FQ in muscle and intestinal content could be expected.

The QuEChERS-based extraction procedure adopted for FQs recovery, from different matrices, did not require further clean-up step. For all analytes, the absolute recovery ranged from 69.1% to 112.8%, with CV % lower than 14.4%, all of this confirms the good reproducibility of the method.

The matrix effects ranged from 0.70 to 1.50, indicating that the analytes are only slightly influenced by the matrix of the tissues and by plasma extract as a consequence of optimized samples clean-up procedures, optimized chromatography conditions and dilution of extracts that allowed to minimize the matrix effect due to the different biological matrices considered in this study.

Results of robustness test indicated that the method was not affected by slight variations of some critical factors in the sample preparation procedure and can be considered acceptably robust.

Figure 2: ENRO, CIPRO and FLUME concentrations in plasma, lung and intestinal content after oral pulsed administration for 5 days. Mean values ( $\pm$  SD) of 8 turkeys sacrificed at 24 h after the last treatment.

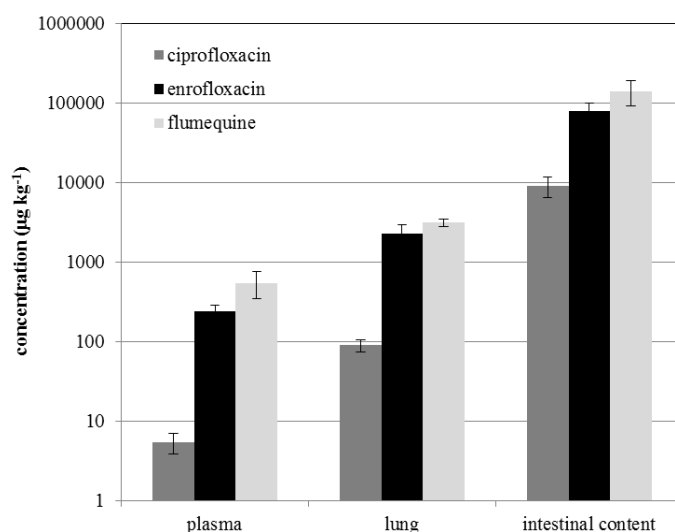




Figure 3: ENRO concentrations in muscle, kidney, liver and skin + fat from 8 turkeys (T1-T8) after oral pulsed administration for 5 days. Animals were sacrificed after three days from the end of treatment. CIPRO concentrations higher than LOD (5.7 µg kg<sup>-1</sup>) were detected only in liver.

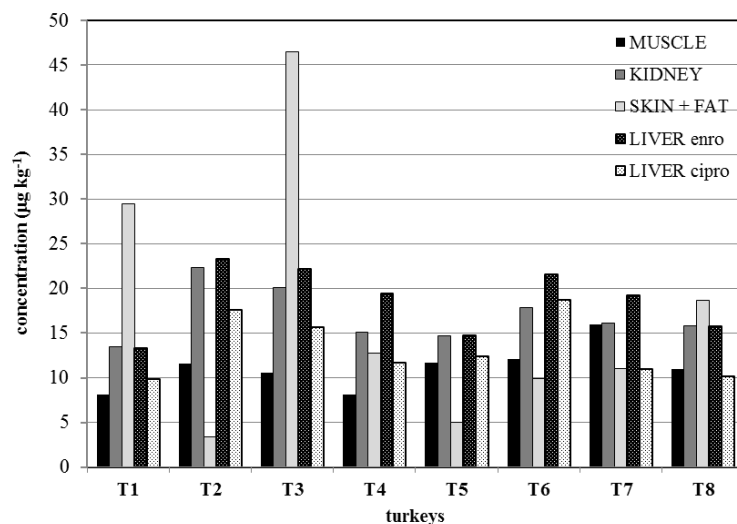


Table 5: Validation results obtained from skin + fat and intestinal content (CN: Nominal Concentration; CV=Coefficient of Variation; REC: absolute recovery; ME: matrix effect).

SKIN + FAT						
analyte	C <sub>N</sub> µg l <sup>-1</sup>	TRUENESS (%)	Within-day Repeatability (CV %)	Within-Laboratory Reproducibility (CV %)	REC% ± SD	ME
ENRO	50	102.2	2.5	10.9	109.6 ± 10.1	0.9 ± 5.0 x 10 <sup>-2</sup>
	100	102.8	5.2	8.2	97.1 ± 4.9	0.9 ± 3.0 x 10 <sup>-2</sup>
	150	101.7	5.9	6.3	99.9 ± 3.6	0.9 ± 2.0 x 10 <sup>-2</sup>
CIPRO	50	99.6	3.8	8.4	102.1 ± 2.4	0.8 ± 1.0 x 10 <sup>-2</sup>
	100	100.7	8.1	5.7	84.2 ± 8.8	0.9 ± 4.0 x 10 <sup>-2</sup>
	150	102.8	9.6	8.9	82.1 ± 5.5	0.9 ± 3.0 x 10 <sup>-2</sup>
DIFLO	200	100.4	3.6	5.2	111.7 ± 6.3	0.8 ± 7.0 x 10 <sup>-2</sup>
	400	97.3	2.5	2.9	101.7 ± 7.9	0.9 ± 6.0 x 10 <sup>-2</sup>
	600	95.8	2.6	2.6	95.2 ± 7.7	1.0 ± 6.0 x 10 <sup>-2</sup>
SARA	5	91.0	14.2	11.0	110.6 ± 4.2	0.9 ± 6.0 x 10 <sup>-2</sup>
	10	102.3	9.3	6.8	107.7 ± 4.9	0.8 ± 6.0 x 10 <sup>-2</sup>
	15	102.8	9.5	7.4	98.1 ± 5.6	0.9 ± 7.0 x 10 <sup>-2</sup>
FLUME	200	101.7	2.4	4.4	110.5 ± 4.4	0.9 ± 6.0 x 10 <sup>-2</sup>
	400	99.1	5.1	4.8	103.1 ± 13.3	0.9 ± 4.0 x 10 <sup>-2</sup>
	600	105.5	1.7	5.0	99.1 ± 3.2	1.0 ± 6.0 x 10 <sup>-2</sup>
INTESTINAL CONTENT						
analyte	C <sub>N</sub> µg l <sup>-1</sup>	TRUENESS (%)	Within-day Repeatability (CV %)	REC% ± SD	ME	
ENRO	25	104.2	2.4	96.7 ± 11.3	1.1 ± 3.0 x 10 <sup>-2</sup>	
	100	102.6	3.6	85.2 ± 2.2	1.2 ± 6.0 x 10 <sup>-2</sup>	
	1000	97.5	3.3	87.4 ± 5.2	1.1 ± 2.4 x 10 <sup>-1</sup>	
CIPRO	25	99.6	7.0	79.3 ± 6.5	1.0 ± 1.0 x 10 <sup>-2</sup>	
	100	102.6	1.9	100.2 ± 2.2	0.9 ± 1.0 x 10 <sup>-1</sup>	
	1000	97.4	2.1	99.3 ± 4.9	0.8 ± 1.3 x 10 <sup>-1</sup>	
FLUME	25	111.3	6.8	80.6 ± 2.4	1.0 ± 1.0 x 10 <sup>-1</sup>	
	100	106.5	2.8	92.7 ± 1.7	0.9 ± 1.6 x 10 <sup>-1</sup>	
	1000	97.7	3.0	94.5 ± 5.4	0.9 ± 1.6 x 10 <sup>-1</sup>	

### *3.4.2. Analysis of samples from treated turkeys*

The validated method allowed to detect ENRO, CIPRO and FLUME concentrations in plasma and in the biological matrices obtained from turkeys orally treated via 10-h pulsed medicated water for 5 consecutive days with ENRO and FLUME.

ENRO and CIPRO were determined separately but, for pharmacokinetic analysis, tissue distribution and depletion study, the sum of ENRO þ CIPRO was always considered. The plasma concentration-time profiles of ENRO and FLUME at day 5 of pulsed administration are reported in Fig. 1. The FQ distribution in target tissues reported in Fig. 2 confirmed the ability of FQs to diffuse freely in lungs reaching concentration higher than in plasma together with the importance of biliary elimination route for ENRO and FLUME as indicated by the great concentrations of the two FQs in intestinal content at the last day of treatment.

ENRO concentrations in muscle, kidney and skin + fat at 3 days after treatment, were always lower than the corresponding MRL and, in several turkeys, lower than the LOQ values (Fig. 3).

With the exception of skin + fat, no large variability of ENRO concentrations was observed in the different tissues from treated birds. As reported by San Martín, Cornejo, Iragüen, Hidalgo, and Anadón (2007), quinolones accumulate in follicles and feathers can become a long lasting reservoir; thus, the variability observed in skin + fat can be related to the accidental occurrence of a few small feathers.

No figures are reported for flumequine as after 5 days of withdrawal time, its concentrations were always lower than LOQ (average concentration:  $10.8 \mu\text{g kg}^{-1}$ ) despite the double dosage administered with medicated water.

### **3.5. Conclusions**

An LC-MS/MS/MS method was developed and validated for rapid and simultaneous determination of the five FQs ENRO, CIPRO, DIFLO, SARA and FLUME in incurred plasma, liver, kidney, muscle, skin + fat, lung and intestinal content from treated turkeys.

For the first time, the QuEChERS technology was successfully applied for the extraction of FQs from matrices such as the lung, skin + fat, kidney and intestinal content.

The method proved to be simple, fast, efficient, stable, precise, accurate and robust, providing good validation parameters, such as linearity, limits of quantification, precision, trueness and recovery in all the matrices considered.

The applicability of the method and its good performances were confirmed in all the different approach of the study, plasma kinetics, target tissue distribution and residue depletion in liver, kidney, muscle, skin + fat, thus making an effective and reliable determination of the target FQs in real samples.

### **3.6 Acknowledgments**

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### **3.7 Appendix A. Supplementary data**

Supplementary data related to this article are placed after references

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### 3.9 Supplementary data

Table S1: Concentrations of FQs considered for the evaluation of linearity in the different tissues and MRL values established for each analyte (there is no MRL for SARA in kidney and muscle).

<b>LIVER</b>		
analyte	Concentrations ( $\mu\text{g kg}^{-1}$ )	MRL ( $\mu\text{g kg}^{-1}$ )
ENRO	12.5, 25, 50, 125, 250, 500, 1000, 2000	<b>200</b>
CIPRO	12.5, 25, 50, 125, 250, 500, 1000, 2000	<b>200</b>
DIFLO	118.8, 237.5, 475, 1187.5, 2375, 4750, 9500, 19000	<b>1900</b>
SARA	6.2, 12.5, 25, 62.5, 125, 250, 500, 1000	<b>100</b>
FLUME	50, 100, 200, 500, 1000, 2000, 4000, 8000	<b>800</b>
<b>KIDNEY</b>		
analyte	Concentrations ( $\mu\text{g kg}^{-1}$ )	MRL ( $\mu\text{g kg}^{-1}$ )
ENRO	18.8, 37.5, 75, 187.5, 375, 750, 1500, 3000	<b>300</b>
CIPRO	18.8, 37.5, 75, 187.5, 375, 750, 1500, 3000	<b>300</b>
DIFLO	37.5, 75, 150, 375, 750, 1500, 3000, 6000	<b>600</b>
SARA	6.2, 12.5, 25, 62.5, 125, 250, 500, 1000	--
FLUME	62.5, 125, 250, 625, 1250, 2500, 5000, 10000	<b>1000</b>
<b>MUSCLE</b>		
analyte	Concentrations ( $\mu\text{g kg}^{-1}$ )	MRL ( $\mu\text{g kg}^{-1}$ )
ENRO	12.5, 25, 50, 125, 250, 500, 1000	<b>100</b>
CIPRO	12.5, 25, 50, 125, 250, 500, 1000	<b>100</b>
DIFLO	37.5, 75, 150, 375, 750, 1500, 3000	<b>300</b>
SARA	1.2, 2.5, 5, 10, 25, 50, 100	--
FLUME	50, 100, 200, 500, 1000, 2000, 4000	<b>400</b>
<b>SKIN + FAT</b>		
analyte	Concentrations ( $\mu\text{g kg}^{-1}$ )	MRL ( $\mu\text{g kg}^{-1}$ )
ENRO	12.5, 25, 50, 125, 250, 500, 1000	<b>100</b>
CIPRO	12.5, 25, 50, 125, 250, 500, 1000	<b>100</b>
DIFLO	50, 100, 200, 500, 1000, 2000, 4000	<b>400</b>
SARA	1.2, 2.5, 5, 12.5, 25, 50, 100	<b>10</b>
FLUME	31.2, 62.5, 125, 312.5, 625, 1250, 2500	<b>250</b>

Table S2: MRL of FQs established in liver, kidney, muscle and skin + fat and  $CC\alpha$  and  $CC\beta$  calculated expressed in  $\mu\text{g kg}^{-1}$  (there is no MRL for SARA in kidney and muscle).

<b>LIVER</b>				<b>KIDNEY</b>			
analyte	MRL ( $\mu\text{g kg}^{-1}$ )	$CC\alpha$	$CC\beta$	analyte	MRL ( $\mu\text{g kg}^{-1}$ )	$CC\alpha$	$CC\beta$
ENRO	<b>200</b>	226.4	252.7	ENRO	<b>300</b>	313.2	326.3
CIPRO	<b>200</b>	232.5	265.5	CIPRO	<b>300</b>	320.0	339.6
DIFLO	<b>1900</b>	2067.4	2234.7	DIFLO	<b>600</b>	624.2	648.4
SARA	<b>100</b>	109.4	118.8	SARA	--	--	--
FLUME	<b>800</b>	908.2	1016.4	FLUME	<b>1000</b>	1040.2	1080.4
<b>MUSCLE</b>				<b>SKIN + FAT</b>			
analyte	MRL ( $\mu\text{g kg}^{-1}$ )	$CC\alpha$	$CC\beta$	analyte	MRL ( $\mu\text{g kg}^{-1}$ )	$CC\alpha$	$CC\beta$
ENRO	<b>100</b>	114.8	129.5	ENRO	<b>100</b>	124.2	125.1
CIPRO	<b>100</b>	108.0	115.7	CIPRO	<b>100</b>	109.0	118.0
DIFLO	<b>300</b>	343.9	387.8	DIFLO	<b>400</b>	439.1	478.1
SARA	--	--	--	SARA	<b>10</b>	12.2	14.2
FLUME	<b>400</b>	465.9	531.8	FLUME	<b>250</b>	305.8	361.6



## **CHAPTER 4**

# **Pharmacokinetic/pharmacodynamic evaluation of the efficacy of flumequine in treating colibacillosis in turkeys**

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## 4. Pharmacokinetic/pharmacodynamic evaluation of the efficacy of flumequine in treating colibacillosis in turkeys

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### 4.1 Abstract

Flumequine (FLU) is used in the treatment of systemic bacterial infections in poultry, including colibacillosis, which is a common disease in turkeys. The pharmacokinetic (PK) behavior of FLU administered to 32 healthy turkeys as an oral bolus via gavage or as 10-h pulsed administration in drinking water were compared, using the authorized dose of 15 mg/kg and the double dose of 30 mg/kg. The minimum inhibitory concentrations (MIC) of 235 *Escherichia coli* field strains isolated from poultry were determined for pharmacodynamics (PD) to develop a PK/PD model. Blood samples were collected at established times over 24 h, and the obtained plasma was analyzed using a liquid chromatography tandem mass spectrometry method that was validated in-house. A monocompartmental model and a noncompartmental model were applied to the data to obtain the PK results. For both types of administration and both dosages, the ratios of the maximum concentration ( $C_{max}$ )/MIC<sub>50</sub> and the area under the plasma concentration-time curve (AUC)/MIC<sub>50</sub> achieved were considerably lower than the fluoroquinolone breakpoints usually adopted for efficacy. The  $C_{max}$ /MIC<sub>50</sub> and AUC<sub>0-24</sub>/MIC<sub>50</sub> ratios were, respectively,  $0.67 \pm 0.09$  and  $4.76 \pm 0.48$  and  $1.18 \pm 0.35$  and  $7.05 \pm 2.40$  for the 15 and 30 mg/kg bolus doses, respectively. After 10-h pulsed administration of 15 mg/kg, values of  $C_{max}$ /MIC<sub>50</sub>,  $0.19 \pm 0.02$  on d 1 and  $0.30 \pm 0.08$  on d 5 of therapy were obtained, the AUC/MIC<sub>50</sub> ratios were  $2.09 \pm 0.29$  and  $3.22 \pm 0.93$  on d 1 and 5, respectively. Higher values were obtained with the doubled dose of 30 mg/kg: the  $C_{max}$ /MIC<sub>50</sub> ratios were  $0.49 \pm 0.11$  on d 1 and  $0.69 \pm 0.18$  on d 5; the AUC/MIC<sub>50</sub> ratios were  $5.15 \pm 1.15$  and  $6.57 \pm 1.92$  on d 1 and 5, respectively. Based on these results, FLU administration should be adopted when specific diagnostic findings indicate its efficacy, and revising the dosage scheme to comply with the prudent and responsible use of antimicrobials in veterinary medicine is advisable.

## 4.2 Introduction

The turkey (*Meleagris gallopavo*), although generally considered a minor species, is an extremely important livestock in northern Italy. Scarce data exist about the usage of antimicrobial drugs in this species, and even less is known about their efficacy in this species (Sanders, 2001; Bywater, 2005).

Because of the limited number of authorized medicinal products for turkeys, antimicrobial therapy is frequently performed using a few authorized products. A frequent drawback of this approach is the onset of resistant bacterial strains that often exhibit cross-resistance for pharmacological groups of compounds. Fluoroquinolones (FQ) are widely used to treat pulmonary and enteric diseases in poultry (Papich and Riviere, 2009); among these, colibacillosis is considered the main cause of economic loss for turkey breeding (Webber and Piddock, 2001; Barnes et al., 2008). Flumequine (FLU), a second-generation fluoroquinolone drug, is useful in the treatment of systemic *Escherichia coli* infections and possibly other infections that are caused by gram-negative bacteria in poultry. Despite the availability of newer FQ, FLU is still employed because of its relatively low cost and good tolerability, especially in minor species such as turkey, for which the cost of therapy is relevant. In avian species, drinking water is the most common route of administering mass medication; the treatments can be conducted following 2 schemes: continuous administration during the entire light period or pulsed administration for a limited period between 4 and 10–12 h (Charleston et al., 1998). Individual therapy is reserved for valuable animals and breeders for practical reasons, although it represents a prudent use of antimicrobials for the limitation of antibiotic resistance in bacteria. Mass therapy is reportedly one of the main causes of the development of microbial resistance in veterinary species (EMA, 2006; Lohren et al., 2008); an increase in the number of FQ-resistant strains of *E. coli*, *Campylobacter*, and *Salmonella* spp. has been frequently reported in recent years (Walsh and Fanning, 2008; EFSA, 2010).

Several scientific and health institutions have expressed serious concern over the emergence of FQ resistance, manifesting the need for risk management intervention regarding the use of FQ in humans and animals. Due to the development of FQ resistance in *Campylobacter* strains of poultry origin, the Food and Drug Administration has banned the use of FQ for the treatment of poultry infections since 2005 (FDA, 2005). In the European Union (EU), risk evaluation is ongoing, and according to the guidelines for risk management, FQ should be reserved for the treatment of clinical conditions that have responded poorly to other classes of antimicrobials (EMA, 2006). In addition, better dosage regimens

should be determined based on pharmacokinetic/pharmacodynamic (PK/PD) integration (Martinez et al., 2006).

The available data on FLU have mainly concerned species other than turkeys and generally involved intravenous, intramuscular, or oral-bolus administration (Mevius et al., 1990; Villa et al., 2005). Therefore, pharmacokinetic parameters specific for turkeys given medicated water are still lacking, which may result infrequent improper dosages.

To improve the availability of PK data about FLU in turkeys, the present study first aimed to evaluate 2 different oral treatments (a single oral gavage and 5 d of repeated 10-h pulsed water medication) and 2 different doses of FLU (the EU authorized dose, 15 mg/kg, and double the EU authorized dose, 30 mg/kg). Oral administration by gavage allows precise control of the predetermined dose intake, and 10-h pulsed water medication is a frequent dosage scheme in avian clinical practice that is easily handled by farmers.

Finally, to evaluate the effectiveness of FLU against *E. coli*, the most common zoonotic avian pathogen, a PK/PD approach was implemented, correlating the PK results after gavage or 10 h-pulsed administration with the MIC determined for the 235 *E. coli* strains isolated from poultry in Italy.

## 4.3 Materials and methods

### 4.3.1. Birds

Thirty-two female turkeys (breed B.U.T. 6) that were 63 to 79 d old, weighed approximately 4 to 6 kg, and were determined to be healthy by a thorough physical examination were selected from a farm belonging to an industrial group. The birds were housed according to the requirements of the European Union (Council of Europe, 2007) and were kept in 4 groups of 8 individuals, housed in 4 boxes of 5 m<sup>2</sup> on wood shavings at 20°C and 65% RH and receiving 16 h of light/day. Commercial diets and water were provided ad libitum. Before the experiments, the birds did not receive any pharmacological treatment.

After an acclimatization period of 8 d, the birds were weighed and individually marked for identification. The study was conducted according to Italian law (D.L. 116/1992) and received ethical approval by the Italian Health Ministry (Animal Welfare Unit, 2009R4KM4F\_002).

### 4.3.2. Experimental Design

Flumequine was orally administered to turkeys via gavage as a single bolus or via 10-h pulsed medicated water for 5 consecutive days at the target dose of 15 mg/kg of BW and at the doubled dose of 30 mg/kg of BW.

Food and water were withdrawn 8 h before administration to reduce any variability in the absorption due to drug-feed interaction and over dilution of the drug. The turkeys were randomly assigned to 4 groups of 8 animals each, indicated as groups 1, 2, 3, and 4.

Groups 1 and 2 received FLU (Flumechina 40%DOXAL, Sulbiate MB, Italy) as a single oral dosage via gavage at the doses of 15 and 30 mg/kg of BW, respectively, whereas groups 3 and 4 were treated via drinking water at the same respective doses. The water intake over a period of 10 h was measured for 3 d before the treatment. The FLU was added to the water based on the birds' mean weight and mean daily water intake. The medicated water was provided in a pulsed scheme for 10 h/d, from 8.00 to 18.00 for 5 d, and was then replaced with fresh water. The daily water consumption was measured at the end of the pulse period of each day to calculate the mean antibiotic intake.

For groups 1 and 2 (oral gavage), blood samples were collected from ulnar or metatarsal veins in heparinized tubes before treatment and at 0.25, 0.5, 1, 2, 4, 8, 12, and 24 h post treatment. For groups 3 and 4 (10-h pulsed medicated water given for 5 consecutive days), blood samples were collected on d 1 and 5 of treatment. Samples were taken immediately before treatment, at 1, 3, 6, and 9 h during the 10-h treatment, and then at 1, 2, 4, 8, and 14 h after the withdrawal of medicated water, which was h 11, 12, 14, 18, and 24 after the onset of the treatments. Plasma was separated by centrifugation at  $1,500 \times g$  for 10 min at  $20^{\circ}\text{C}$  and was stored at  $-20^{\circ}\text{C}$ , pending analysis.

#### *4.3.3. Liquid Chromatography–Mass Spectrometry Analysis*

The plasma sample purification was performed as reported by Samanidou et al. (2005) with slight modifications: 200  $\mu\text{l}$  of plasma was spiked with 10  $\mu\text{l}$  of the internal standard (IS) Norfloxacin (3  $\mu\text{g}/\text{ml}$ ) to have a final concentration of 0.15  $\mu\text{g}/\text{ml}$ ; afterward, 3 ml of acetonitrile was added. The tubes were briefly vortexed and centrifuged at  $3,082 \times g$  for 10 min. The supernatant was transferred to another tube and then evaporated to dryness at  $50^{\circ}\text{C}$ . The residue was dissolved in 200  $\mu\text{l}$  of the mobile phase and filtered through a 0.22- $\mu\text{m}$  pore-size membrane, and 10  $\mu\text{l}$  was injected into the HPLC system after an appropriate dilution.

An Accela 600 HPLC pump with a CTC automatic injector was used (Thermo Fischer Scientific, San Jose, CA). Chromatographic separation was achieved using a C<sub>18</sub> Kinetex column (100  $\times$  2.1 mm, 2.6  $\mu\text{m}$ , Phenomenex, Torrance, CA) with guard column. The mobile phase consisted of (A) ammonium acetate solution (10 mM, pH 2.5) and (B) 0.1% formic acid in methanol. The mobile phase composition (A:B, vol/vol) was: 80:20 at 0 min, 50:50 at 10 min, 10:90 at 13 min and unchanged until 14 min, 0:100 from 14.50 to 16 min, 80:20 from 17 to 20 min to re-equilibrate the system. The sample tray was maintained at  $4^{\circ}\text{C}$ .

Mass spectrometric analysis (Garces et al., 2006) was performed using a LTQ XL ion trap (Thermo Fisher Scientific, San Jose, CA) equipped with a heated electrospray ionization probe (HESI-II) operating in the positive-ion mode under the following conditions: sheath and auxiliary gas flow: 40 and 5 arbitrary units, respectively; ion spray voltage: 3.5 kV; capillary temperature: 300°C; capillary voltage: 26 V; and tube lens: 80 V. The collision energies that were determined to be necessary for fragmentation in MS<sup>2</sup> and MS<sup>3</sup> of the molecules of interest, precursor ions, product ions, and collision energies are shown in Table 1. The Xcalibur (version 2.1) data acquisition software from ThermoFisher Scientific was used.

Calibration curves were constructed using pooled turkey plasma obtained from untreated animals. The blank plasma was spiked with 10 µL of IS and with FLU to obtain a concentration range of 2.5 to 200 ng/ml. Quantification was based on the ratios of the peak areas of the analyte to that of IS, and a least-squares linear regression analysis was performed to calculate the calibration curves. Flumequine (>99% pure) and norfloxacin (>99% pure) were purchased from SigmaAldrich (Steinheim, Germany). Other reagents and solvents were purchased from Carlo Erba-Reagenti (Milano, Italy).

Prior to being routinely applied, the method was validated in-house using a set of parameters [linearity, within-run and between-run accuracy and precision, limit of quantification (LOQ), limit of detection (LOD), and selectivity] that were in compliance with the recommendations defined by the European Community (Commission Decision 2002/657/EC, 2002) and with the reference guidelines defined in other EU and FDA documents (VICH GL 49, 2011). The calibration curves were constructed using matrix-matched calibrator samples (concentration range: 2.5–200 ng/ml), and the correlation coefficient was always  $r > 0.99$  for 6 replicates.

The within-day precision (repeatability) and accuracy were determined by analyzing blank samples that were spiked with 2.5 (n = 6), 10 (n = 6), or 50 (n = 6) ng/ml on the same day. The between-day precision and accuracy were determined by analyzing quality control samples [concentration levels: 2.5 (n = 18), 10 (n = 18), and 50 (n = 18) ng/ml] with each batch of analytical samples on 3 different days. The following mean values were obtained: within-run accuracy  $2.10 \pm 0.12$ ,  $9.8 \pm 0.6$ , and  $52.5 \pm 2.6$  ng/ml and between-run accuracy 2.5, 10.2, and 50.5 ng/ml. The results fell within the accepted ranges for precision (within-run precision: 5.9, 6.03, and 4.9% for 2.5, 10, and 50 ng/ml, respectively; between-run precision: 12.2, 5.9, and 6.2% for 2.5, 10, and 50 ng/ml, respectively).

An LOQ value of 2.5 ng/ml was obtained. None of the values below the LOQ were included in the plasma concentration-time curves or in the pharmacokinetic analysis. The LOD was defined as the concentration corresponding to a signal-

to-noise ratio of 3 and was found to be 0.5 ng/ml. The specificity of the method was demonstrated because no interference from endogenous compounds was observed in the 20 blank samples tested.

Table 1: Characteristics obtained using mass spectrometry (MS) analysis

Compound	Precursor ion [M-H] <sup>+</sup> (m/z)	Collision energy MS <sup>2</sup> (%)	Precursor ion MS <sup>2</sup> (m/z)	Collision energy MS <sup>3</sup> (%)	Product ion MS <sup>3</sup> (m/z)
Flumequine	262	40	244	25	202
Norfloxacin (internal standard)	320	36	276	30	256, 233

#### 4.3.4. Antimicrobial Susceptibility Testing

The minimum inhibitory concentrations (MIC) of FLU for 235 *E. coli* strains isolated from poultry in Italy were determined using the broth microdilution method according to CLSI (2008) guidelines.

#### 4.3.5. Pharmacokinetics and Statistical Analysis

The pharmacokinetic parameters were deduced from the plasma concentration-time data using the Win-NonLin 6.1 software (Pharsight Corporation, MountainView, CA), which allows both compartmental and noncompartmental analyses of experimental data. Minimum information criterion estimation (Yamaoka et al.,1978) was used to choose the best-fitting model for the data. All of the data points were weighted by the inverse square of the fitted value. The plasma concentrations after a single oral bolus were fitted to a standard monocompartmental model, and also noncompartmental analyses were conducted. The kinetics after the 10-h pulsed administrations was determined at d 1 and 5 using a noncompartmental analysis (Gibaldi and Perrier,1982).

The peak concentrations,  $C_{max}$ , and the time to peak,  $T_{max}$ , were obtained from the experimentally observed data. The elimination half-life was calculated as  $\ln 2/\lambda_n$ , and the mean residence time (MRT) was determined using the following equation:  $MRT = AUMC/AUC$ , where AUMC is the area under the moment curve and AUC is the area under plasma concentration-time curve.



The pharmacokinetic parameters are reported as the mean values ( $\pm$ SD). The harmonic mean values and pseudo-SD were calculated for the half-lives using a jack-knife technique (Lam et al., 1985). The normality of the kinetics data was assessed using the Kolmogorov–Smirnov test. The differences between the 2 bolus gavage doses (group 1 vs. 2), the two 10-h pulsed doses (group 3 vs. 4) and the 2 administrations methods (groups 1 vs. 3 and 2 vs. 4) were compared using a 2-tailed unpaired *t*-test;  $P < 0.05$  was considered statistically significant (GraphPad Prism version 4.00 software, San Diego, CA).

The following PK/PD indices were calculated as predictors of the success or failure of the therapy: the  $C_{\max}/\text{MIC}$  and  $\text{AUC}/\text{MIC}$  ratios (Toutain et al., 2002; McKellar et al., 2004). The breakpoint values of  $C_{\max}/\text{MIC}_{50} = 8\text{--}10$  and  $\text{AUC}/\text{MIC}_5 = 100$  h were considered representative of the therapeutic efficacy of this antimicrobial class to prevent the development of resistant bacterial strains in poultry (Anadon et al., 2001; Dimitrova et al., 2007; Ozawa et al., 2010).

## 4.4 Results

### 4.4.5. Single Bolus Gavage Administration (Groups 1 and 2)

The mean plasma concentrations +SD of FLU at the various sampling times after oral gavage administration of both doses adopted are shown in Figure 1. The individual plasma concentration-time profiles were similar, and a low inter individual variability was observed, particularly in group 1, whereas a larger inter individual variability was observed when the double dose was administered (group 2). The maximum concentrations were reached at approximately 2 h in both groups; the drug concentrations rapidly decreased, but the drug was still detectable at 24 h after administration, with mean concentrations of  $0.26 \pm 0.11$   $\mu\text{g/ml}$  (group 1) and  $0.72 \pm 0.74$   $\mu\text{g/ml}$  (group 2). The pharmacokinetic parameters obtained from the mono- and noncompartmental analyses are presented in Table 2.

Figure 1. Mean values + SD in the plasma concentration–time profiles of flumequine in group 1 (filled triangles) and group 2 (filled squares), which were given the dosages of 15 mg/kg b.w. or 30 mg/kg b.w., respectively, via gavage.

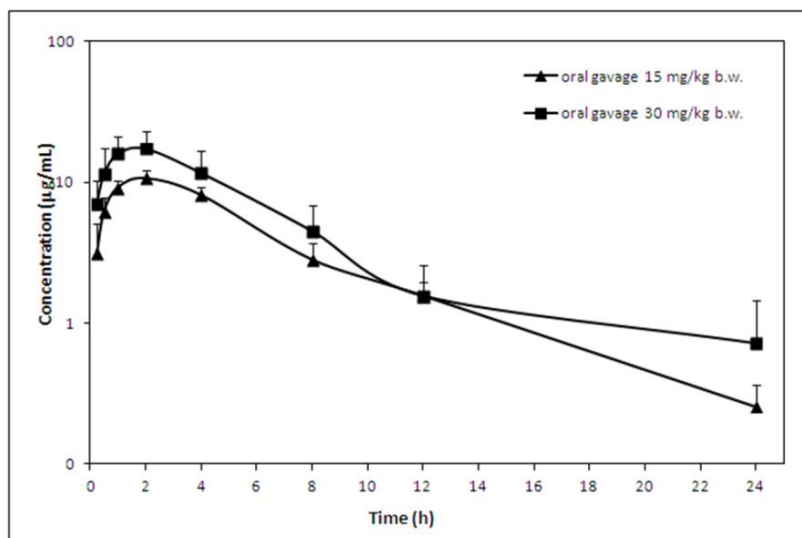


Table 2: Pharmacokinetics parameters in turkeys<sup>1</sup>

Parameter	Group 1 (oral gavage 15 mg/kg)	Group 2 (oral gavage 30 mg/kg)	Group 3 (oral pulsed 15 mg/kg; d 1)	Group 3 (oral pulsed 15 mg/kg;d 5)	Group 4 (oral pulsed 30 mg/kg;d 1)	Group 4 (oral pulsed 30 mg/kg;d 5)
T <sub>max</sub> (h)	1.88±0.35‡,§	1.75±0.46#,\$	5.75±2.71€	6.00±1.60€	6.75±2.12†	6.38±1.92†
C <sub>max</sub> (µg/ml)	10.76±1.50‡,§, †	18.93±5.65#,\$	3.05±0.36€,#	4.81±1.30€,\$	7.89±1.73‡,†,\$	10.84±2.92†,§, #
AUC <sub>0-∞</sub> (h*µg/ml)	74.10±7.98†	123.07±48.72€	—	—	—	—
AUC <sub>0-24</sub> (h*µg/ml)	76.14±7.69‡,§, †	112.73±38.40€	33.46±4.65€,\$,#	51.54±14.87‡,€ ,\$	82.33±18.40‡	105.18±30.71§
AUMC <sub>0-24</sub> (h*h*µg/ml)	418.04±64.65 ‡	545.94±275.35 #	257.60±39.23€, \$,#	418.88±100.6‡ ,\$	676.31±153.6‡ ,\$†	878.45±309.29 §
t <sub>1/2</sub> elim (h)	4.01±0.57* ‡,§	4.25±1.60* #	—	—	—	—
MRT <sub>0-24</sub> (h)	5.48±0.48 ‡,§	4.62±1.26#,\$	7.69±0.34€	8.28±0.64€	8.23±0.62†	8.27±1.00†
MIC <sub>50</sub> (µg/ml) for <i>Escherichia coli</i>	16 (0.015 to >256)					
C <sub>max</sub> /MIC <sub>50</sub>	0.67±0.09†	1.18±0.35#,\$	0.19±0.02# <sup>1)</sup>	0.30±0.08\$	0.49±0.11‡,†,\$	0.68±0.18†,§,#
AUC/MIC <sub>50</sub>	4.76±0.48‡,§,†	7.05±2.40€	2.09±0.29§,#	3.22±0.93‡,€,\$	5.15±1.15‡	6.57±1.92§

<sup>1</sup>Tmax = observed time for Cmax; Cmax = maximum concentration; AUC<sub>0-∞</sub> = area under the concentration versus time curve from 0 to infinity; AUC<sub>0-24</sub> = area under the concentration versus time curve from 0 to 24 h; AUMC<sub>0-24</sub> = area under moment curve; t<sub>1/2</sub> elim. = elimination half-life; MRT<sub>0-24</sub> = mean residence time; MIC<sub>50</sub> = minimum inhibitory concentration 50%; \*harmonic mean ± pseudo SD.

‡Significantly different (P < 0.05) from group 3, d 1.

§Significantly different (P < 0.05) from group 3, d 5.

†Significantly different (P < 0.05) from group 2.

#Significantly different (P < 0.05) from group 4, d 1.

\$Significantly different (P < 0.05) from group 4, d 5.

€Significantly different (P < 0.05) from group 1.

#### 4.4.6 10-h Medicated Water Administration for 5 Consecutive Days (Groups 3 and 4)

In groups 3 and 4, the medicated water concentration was adjusted daily based on water intake; nevertheless, the daily measurements of water intake showed that the mean dose received by the turkeys was lower than the targeted doses of 15 and 30 mg/kg of BW (Table 3). The mean FLU concentration-time profiles following repeated oral administrations of medicated water at the 2 dosages are shown in Figures 2 and 3, respectively; the data refer to d 1 and 5 of therapy. In both trials, the highest concentrations were achieved on d 5 of treatment. The pharmacokinetic parameters obtained in the noncompartmental analysis are summarized in Table 2.

Table 3: Mean values  $\pm$  SD of doses in turkeys after 10-h pulsed water administration

Item	Actual dose
Target dose:15 mg/kg of BW	
d1	12.53
d 2	7.56
d 3	9.94
d 4	14.25
d 5	15.34
Mean $\pm$ SD	11.92 $\pm$ 3.18
Target dose:30 mg/kg of BW	
d 1	15.24
d 2	14.76
d 3	20.48
d 4	26.50
d 5	23.68
Mean $\pm$ SD	20.13 $\pm$ 5.15

Figure 2. Mean values + SD in the plasma concentration–time profiles of flumequine in group 3 at days 1 (filled triangles) and 5 (filled squares) following 10-h oral pulsed administration of an average dosage of 11.92 mg/kg b.w. for 5 days.

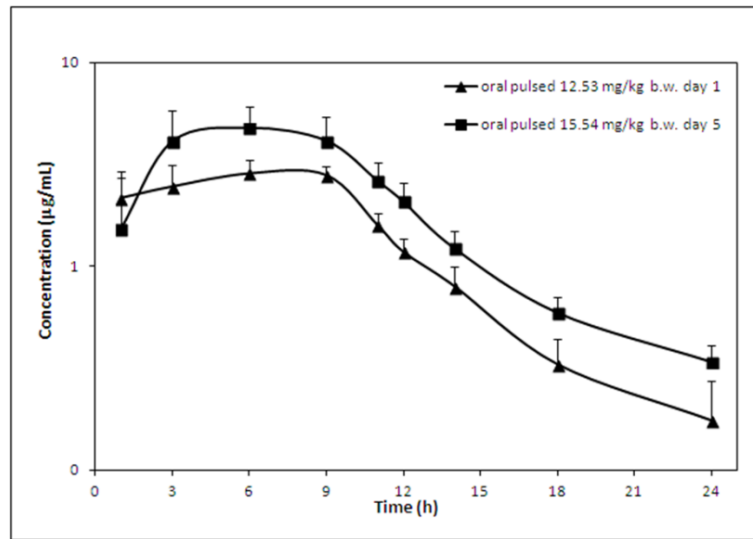
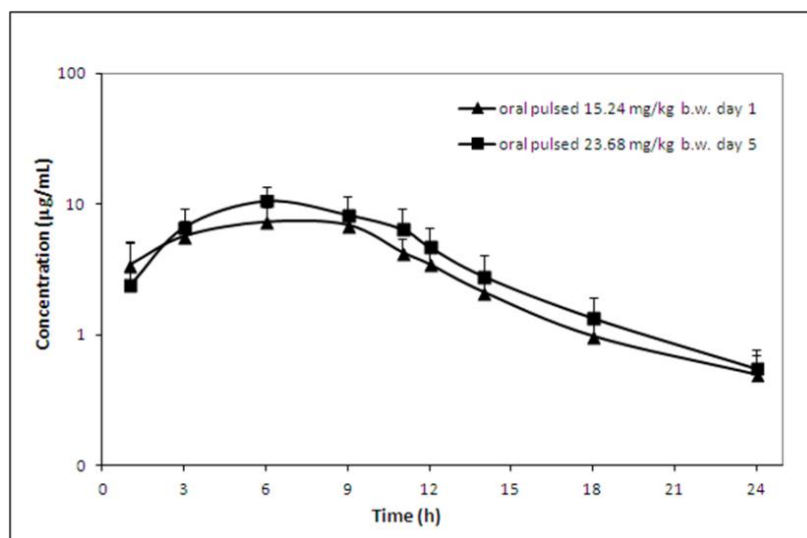


Figure 3. Mean values + SD in the plasma concentration–time profiles for flumequine in group 4 at days 1 (filled triangles) and 5 (filled squares) following 10-h oral pulsed administration of an average dosage of 20.13 mg/kg b.w. for 5 days.



#### 4.4.7. MIC Determination and PK/PD Integration

The range of FLU MIC for the 235 poultry-derived *E. coli* isolates was 0.016 to >256 µg/ml. Only 29.8% of the strains were susceptible (n = 70) to FLU, whereas 70.2% were classified as resistant (n = 165). The MIC<sub>50</sub>, defined as the minimum inhibitory concentration at which 50% of the isolates were inhibited, was 16 µg/ml. Based on the PK parameters and the MIC<sub>50</sub> value, the PK/PD integrations were calculated for the different forms of administration; the values are presented in Table 2. Significant differences in the C<sub>max</sub>/MIC<sub>50</sub> and AUC/MIC<sub>50</sub> ratios were observed when the dose was doubled for the gavage and 10-h pulsed administrations and between those at d 1 and 5 of 10-h pulsed administration (Table 2).

## 4.5. Discussion

When given by oral gavage, FLU was very rapidly absorbed, presenting a  $T_{max}$  of approximately 2 h, in contrast to those reported for other FQ, such as enrofloxacin and danofloxacin, for which higher values have been recorded ( $T_{max}$ :  $6.33 \pm 2.5$  h for enrofloxacin and  $6.0 \pm 3.29$  h for danofloxacin; Haritova et al., 2006; Dimitrova et al., 2007). In our birds, the half-life proved to be rather short ( $t_{1/2}$  elim:  $4.01 \pm 0.57$  and  $4.25 \pm 1.60$  h), and as expected, was not dependent on the dose given. After gavage administration of the 2 different doses of FLU, the increase in the  $C_{max}$  and AUC values was related to the increase in the dose, whereas the concentration-time profiles were similar (Figure 1).

Oral administration by gavage is not easily practicable in intensive turkey farming due to the high population density in the sheds, the need for trained farmers to individually handle the birds, and the occurrence of bird stress, despite the higher  $C_{max}$  and  $AUC_{0-24}$  values obtained compared with those obtained using medicated water, for which a prolonged intake of lower drug concentrations occurs (Table 2).

The pulsed administration trials showed that the  $AUC_{0-24}$  was increased from d 1 to d 5 in group 3 ( $AUC_{0-24}$ :  $33.46 \pm 4.65$  to  $51.54 \pm 14.87$  h\* $\mu$ g/ml); and that the  $C_{max}$  in group 4 likewise increased ( $C_{max}$ :  $7.89 \pm 1.7$  to  $10.84 \pm 2.92$   $\mu$ g/ml). This slight increase can be explained by the different intakes of medicated water and do not support an accumulation of the drug. Lower intake of the drug was observed at the first day of the trial, and medicated water concentrations were thus adjusted based on the water intake of the previous administrations. Most likely due to the poor palatability of the medicinal veterinary product, the targeted doses were never reached using a 10-h pulsed administration. It is likely that the availability of unmedicated water during the remaining 6 h of the light period affected the animals behavior and that the birds drank less medicated water while waiting for the unmedicated water. It is known that increasing the concentration of a drug can affect the intake of an adequate amount of the drug due to its limited solubility in water and alteration of the water's palatability; moreover, the photoperiod is also a very important parameter that can have repercussions for the water uptake and thus on the amount of drug intake (Vermeulen et al., 2002).

Administering the 2 different doses as medicated water allowed a significant increase in the  $C_{max}$ ,  $AUC_{0-24}$ , and  $AUC_{0-24}$ , but not in the  $T_{max}$  and MRT with the higher dosage; thus a correlation with the increased dosage was observed, although the targeted dose of 30mg/kg was not reached.

In agreement with many previous studies on *E. coli* resistance to FQ (EFSA, 2010; Ozawa et al., 2010; Russo et al., 2012), a very high percentage of the isolated strains proved to be resistant to FLU. According to our MIC results,

70.2% of the *E. coli* strains tested were resistant. This result suggests that FQ should be used in turkeys only when a susceptibility test clearly indicates the efficacy of the drug, as proscribed by EMA (2006) and WHO (2011) in the last sets of guidelines on the prudent use of antimicrobials.

Although specific breakpoints have not been defined for avian colibacillosis, several studies on FQ in poultry (Anadon et al., 2001; Dimitrova et al., 2007; Ozawa et al., 2010) adopted a  $C_{\max}/\text{MIC}$  ratio of 8 or 10 and an  $\text{AUC}/\text{MIC}$  ratio of 100 as the minimal dose required to prevent the selection of resistant bacteria. As reported in Table 2, after gavage administration, the mean  $C_{\max}/\text{MIC}_{50}$  and  $\text{AUC}_{0-24}/\text{MIC}_{50}$  ratios were, respectively,  $0.67 \pm 0.09$  and  $4.76 \pm 0.48$  and  $1.18 \pm 0.35$  and  $7.05 \pm 2.40$  for the 15 and 30 mg/kg doses, respectively. After the administration of 10-h pulsed medicated water with the dosage regimen of 15 mg/kg, lower values of  $C_{\max}/\text{MIC}_{50}$ ,  $0.19 \pm 0.02$  on d 1 and  $0.30 \pm 0.08$  on d 5 of therapy were obtained; the  $\text{AUC}_{0-24}/\text{MIC}_{50}$  ratios were  $2.09 \pm 0.29$  and  $3.22 \pm 0.93$  on d 1 and 5, respectively. The following slightly higher values were obtained with the doubled dose: the  $C_{\max}/\text{MIC}_{50}$  ratios were  $0.49 \pm 0.11$  on d 1 and  $0.69 \pm 0.18$  on d 5; the  $\text{AUC}_{0-24}/\text{MIC}_{50}$  ratios were  $5.15 \pm 1.15$  and  $6.57 \pm 1.92$  on d 1 and 5, respectively. The breakpoints values were not reached with either type of administration or dosages, and the PK/PD correlation yielded very unsatisfactory results.

These findings are discouraging but not completely surprising, as FLU was licensed for use in food animals at the beginning of the 1980s and has been widely used for the mass medication of poultry and because the adoption of 10-h pulsed medication is conflicting with its rapid clearance from the blood.

Based on these results, the EU-authorized dosage of 15 mg/kg may be ineffective to achieve adequate drug plasma concentrations. Furthermore, the 10-h pulsed doses of medicated water did not allow reaching plasma concentrations that were efficacious in controlling *E. coli*, due to the long periods with unmedicated water. Medicated water should always be provided continuously, as reported in the leaflets of the commercial products. To improve treatment efficacy and comply with the prudent and responsible use of antimicrobials in food-producing species, our results suggest that FLU administration should be adopted when specific diagnostic results indicate its efficacy and that a revision of the dosage scheme is advisable.

#### 4.6. Acknowledgments

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## CHAPTER 5

# Enrofloxacin against *Escherichia coli* in turkeys: which treatment scheme is effective?

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## 5. Enrofloxacin against *Escherichia coli* in turkeys: which treatment scheme is effective?

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### 5.1 Abstract

The efficacy of enrofloxacin (ENRO) was evaluated against multidrug-resistant avian pathogenic *Escherichia coli* correlating the minimum inhibitory concentrations (MIC) of 235 *E. coli* field strains with its pharmacokinetics (PK) in 50 healthy turkeys (5 groups) with a PK/pharmacodynamic approach. The treatments were as follows: *a*) single oral gavage and *b*) single subcutaneous (SC) treatment at the recommended dose of 10 mg/kg; *c*) single oral gavage, *d*) 5 d of 10-h pulsed water medication, and *e*) 5 d of 24-h continuous water medication at the doubled dose of 20 mg/kg. Blood samples were collected at established times over 24 h. Plasma was analyzed using a liquid chromatography tandem mass spectrometry method that was validated in house. A monocompartmental and a noncompartmental model were applied to the data to obtain the PK results. After gavage administration, the mean maximum concentration  $C_{\max}/MIC_{50}$  and area under the curve  $AUC_{0-24}/MIC_{50}$  ratios were, respectively,  $3.07 \pm 0.62$  and  $7.01 \pm 1.03$  and  $25.48 \pm 3.04$  and  $57.2 \pm 3.73$  for the 10 and 20 mg/kg doses, respectively. After SC administration of 10 mg/kg,  $C_{\max}/MIC_{50}$  and  $AUC_{0-24}/MIC_{50}$  ratios were  $3.45 \pm 0.75$  and  $33.96 \pm 7.46$ , respectively. After the administration of 10-h pulsed or 24-h continuous medicated water at 20 mg/kg, lower values of  $C_{\max}/MIC_{50}$  (10-h pulsed:  $3.45 \pm 0.7$ ; 24-h continuous:  $3.05 \pm 0.48$ ) and  $AUC_{0-24}/MIC_{50}$  (10-h pulsed:  $42.42 \pm 6.17$ ; 24-h continuous:  $53.32 \pm 5.55$ ) were obtained. Based on these results, the European Union-recommended dosage of 10 mg/kg seems ineffective to achieve adequate drug plasma concentrations and even the 20 mg/kg by 10 h pulsed or continuous medicated water administration did not reach completely efficacious concentrations in plasma against colibacillosis. Although the results obtained were not completely encouraging, the medicated water should preferably be provided continuously. To conclude about the efficacy of ENRO treatment against colibacillosis, target tissue concentration should be extensively considered.

## 5.2 Introduction

Enrofloxacin (ENRO) is an antimicrobial agent of the fluoroquinolone group approved only in veterinary medicine, with a broad antimicrobial spectrum and high bactericidal activity (Walker et al., 1992). In European Countries, ENRO was approved in the 1990s and is still extensively used in poultry for colibacillosis treatment, due to its unique effectiveness against multidrug-resistant avian pathogenic *Escherichia coli* (Bass et al., 1999; Lutful Kabir, 2010).

In avian species, the low economic value of individual birds makes single therapy cost-prohibitive and drinking water is the most common route of administering mass medication because sick birds continue to drink. For practical reasons, individual therapy by oral or parenteral route is reserved for high value breeders or small flocks.

In commercial turkeys, colibacillosis requires a prompt and efficacious antimicrobial treatment, preferably via the drinking water. The treatments can be conducted following 2 schemes: continuous administration during the entire light period or pulse administration for a limited period between a minimum of 4 and a maximum of 12 h (Charleston et al., 1998). In Europe, turkeys are considered a minor species and the cost of the therapy influences strongly the choice of the drug.

In recent years, notwithstanding the scarce information on pharmacokinetics (PK) of antimicrobial drugs in turkeys, the ENRO use was increased in this species as a result of its effectiveness and the availability of generic products. However, an indiscriminate use of these drugs may both select for a resistant bacterial population and reduces their clinical efficacy. Mass therapy, frequently adopted to cure large numbers of animals, is one of the main causes for the development of microbial resistance in veterinary food producing species (EMA, 2007; Lohren et al., 2008). In poultry, an increase in the number of fluoroquinolone resistant strains of *E. coli*, *Campylobacter* spp., and *Salmonella* spp. has been frequently reported in recent years (Walsh and Fanning, 2008; EFSA, 2010). Several scientific and health institutions have serious concern over the emergence of fluoroquinolone-resistance, manifesting the need for risk management intervention regarding the use of fluoroquinolones in humans and animals (EFSA, 2010).

During the last 10 years, particular attention has been devoted to a correct evaluation of efficacious dosages for a more prudent and targeted use of antimicrobials in animal species. The correlation between minimum inhibitory concentrations (MIC) in field isolates and the PK behavior of antimicrobials in target species, known as PK/pharmacodynamic (PD) model is the best tool for a prudent and targeted use of antimicrobials (Martinez et al., 2006).

The aims of the present study were to evaluate 3 different oral treatments (a single oral gavage, 5 d of 10-h pulsed water medication, and 5 d of 24-h continuous water medication) and single parenteral (subcutaneous; **SC**) treatment using 2 different doses of ENRO (i.e., the EU authorized dose, 10 mg/kg, and double the EU recommended dose, 20 mg/kg). The effectiveness of different treatment schemes against *E. coli* was evaluated by a PK/PD approach, correlating the PK results with the MIC determined for 235 *E. coli* strains isolated from poultry in Italy reported by Vanni et al. (2014).

## 5.3 Materials and methods

### 5.3.1. *Birds*

Fifty female turkeys (commercial breed, British United Turkeys, B.U.T.6, Aviagen Turkeys, Cremona, Italy) 62 to 83 d old, weighed between 3.4 and 6.9 kg and determined to be healthy by a thorough physical examination, were selected from a commercial farm. The turkeys were housed according to the requirements of the European Union (Council of Europe, 2007) and were divided into 5 groups of 10 individuals kept into 5 pens of 5 m<sup>2</sup> on wood shavings. The birds were housed at 20°C and 65% RH and received 16 h of light/day. Standard commercial diet and water were supplied ad libitum in feeders and drinkers. After an acclimatization period of 8 d, the turkeys were weighed and individually marked for identification.

The study was conducted by the Animal Production Research and Teaching Centre of the University of Milan (Lodi, Italy) according to Italian law (D.L. 116/1992) and was ethically approved by the Ethical Committee of University of Milan (Opinion n. 31/11).

### 5.3.2 *Experimental Design*

Enrofloxacin was orally administered to turkeys via gavage as a single bolus at the dose of 10 mg/kg of BW or at the doubled dose of 20 mg/kg of BW, or via 10-h pulsed medicated water, or via continuous administration for 5 consecutive days at the doubled dose of 20 mg/kg of BW. Parenteral administration was a single SC injection at 10 mg/kg of BW.

Food and water were withdrawn 8 h before administrations to reduce any variability in the absorption due to drug-feed interaction and over dilution of the drug and treatments were carried out at the beginning of the light period. One hour after single treatments (oral and SC), fresh water and feed were supplied, whereas for repeated water medications only feed was supplied after 1 h.

The turkeys were randomly assigned to 5 groups of 10 birds each, indicated as groups 1, 2, 3, 4, and 5.

Groups 1 and 2 received ENRO (Baytril oral solution 10%, Bayer, Milano, Italy) as single oral dosage via gavage at the doses of 10 and 20 mg/kg of BW, respectively. Groups 3 and 4 were repeatedly treated via drinking water at the dose of 20 mg/kg of BW for 5 d with ENRO (Baytril oral solution 10%, Bayer). The water intake over a period of 10 or 16 h was measured for 3 d before the treatment. Enrofloxacin was added to the water based on the birds' mean weight and mean daily water intake. In group 3 the medicated water (ENRO mean concentration for 5 d:  $179.7 \pm 48.7$  mg/ml) was provided in a pulsed scheme for 10 h/d from 0700 to 1700 h for 5 d and was then replaced with fresh water; in group 4 the medication (ENRO mean concentration for 5 d:  $147 \pm 1.5$  mg/ml) was provided for 24 h and renewed every morning at 0700 h. The daily water consumption was measured at the end of the pulsed period (group 3) or before the each day renewal (group 4) to calculate the mean antibiotic intake. Birds in group 5 were administered SC at the base of neck with ENRO (Baytril injectable solution 5%, Bayer) at the dose of 10 mg/kg. Blood samples (maximum 1 ml) were collected from ulnar or metatarsal veins in heparinized tubes in all groups as indicated in Table 1. Plasma was separated by centrifugation at  $1,500 \times g$  for 10 min and stored at  $-20^{\circ}\text{C}$  pending analysis.

Table 1: Treatments, doses and sampling times in all Groups of turkeys

Group	Treatment	Dose (mg/kg)	Sampling times (h)
1	single oral gavage	10	0, 0.25, 0.5, 1, 2, 4, 8, 12, 24
2	single oral gavage	20	0, 0.25, 0.5, 1, 2, 4, 8, 12, 24
3	5 d 10-h pulsed medicated water	5 d actual dose: $15.06 \pm 3.33$	d 1 and 5: 0, 1, 3, 6, 9, 11, 12, 14, 18, 24
4	5 d 24-h continuous medicated water	5 d actual dose: $21.9 \pm 2.31$	d 5: 0, 2, 6, 9, 12, 15, 18, 20, 24
5	single subcutaneous injection	10	0, 0.25, 0.5, 1, 2, 4, 8, 10, 24



### 5.3.3 *Liquid Chromatography–Mass Spectrometry Analysis and Method Validation*

The plasma samples purification was performed as reported by Lucatello et al. (2013, 2014). Mass spectrometric (MS) analysis was performed on a LTQ XL ion trap (Thermo Fischer Scientific, San Jose, CA), equipped as indicated by Lucatello et al. (2013, 2014). The collision energies that were necessary for fragmentation of the parent compounds (ENRO and ciprofloxacin, CIPRO) into precursor ions (MS/MS) and product ions (MS/MS/MS) are shown in Table 2. Calibration curves were constructed using pooled turkey plasma obtained from untreated birds. Blank plasma was spiked with 10  $\mu$ l of internal standard (IS) norfloxacin (3  $\mu$ g/ml) and with ENRO and CIPRO to obtain a concentration range of 2.5 to 200 ng/ml. Quantification was based on the ratios of the peak areas of the analyte to that of IS and a least squares linear regression analysis was performed to calculate calibration curves. The method was in-house validated using a set of parameters [linearity, within-run and between-run accuracy and precision, limit of quantification (LOQ), limit of detection (LOD), and selectivity] that were in compliance with the recommendations defined by the European Community (European Commission, 2002) and with the reference guidelines defined in other European Union and FDA documents (VICH GL 49, 2012). The calibration curves were constructed using matrix matched calibrator samples (concentration range: 2.5–200 ng/ml) and the correlation coefficients was always  $r > 0.99$  for 6 replicates. Within-day precision (repeatability) and accuracy were determined by analyzing blank samples that were spiked with both compounds at 2.5 ( $n = 6$ ), 10 ( $n = 6$ ), and 50 ( $n = 6$ ) ng/ml on the same day. The between-day precision and accuracy were determined by analyzing quality control samples, concentration level: 2.5 ( $n = 18$ ), 10 ( $n = 18$ ), and 50 ( $n = 18$ ) ng/mL, with each batch of analytical samples on 3 different days. The validation results are reported in Table 2 and fell within the accepted ranges for validation. All values below the LOQ were not included in the plasma concentration-time curves and the pharmacokinetic analysis.

Table 2: Characteristics obtained using mass spectrometry analysis and validation results<sup>1</sup>

COMPOUND	Precursor ion [M-H] <sup>+</sup> (m/z)	Collision energy MS/MS (%)	Precursor ion MS/MS (m/z)	Collision energy MS/MS/M S (%)	Product ion MS/MS/MS (m/z)
Enrofloxacin	360	46	316	23	245
Ciprofloxacin	332	22	288	30	245, 268
Norfloxacin (IS)	320	36	276	30	256, 233
VALIDATION					
Concentration	Within-run precision(n. 6)		Between-run precision(n. 18)		
g/mL					
2.5	ENRO: 7.2%; CIPRO: 11.9 %		ENRO: 10.6%; CIPRO: 12.6		
10 ng/mL	ENRO: 6.1%; CIPRO: 5 %		ENRO: 5.5%; CIPRO: 7.2		
50 ng/mL	ENRO: 5.5%; CIPRO: 4.7%		ENRO: 7.1%; CIPRO: 5.7		
	LOQ		LOD		
	ENRO: 2.47 ng/mL; CIPRO: 1.4 ng/mL		ENRO: 0.82 ng/mL; CIPRO: 0.46 ng/mL		

1MS= mass spectrometry; ENRO= enrofloxacin; CIPRO= ciprofloxacin; IS= internal standard; LOQ=limit of quantification; LOD= limit of detection.

### 5.3.4 PK and Statistical Analysis

The PK parameters were deduced from plasma concentration-time data using the WinNonLin Prof 6.1 software (Pharsight Corporation, Mountain View, CA), which allows both compartmental and noncompartmental analyses of experimental data. Minimum information criterion estimation (Yamaoka et al., 1978) was used to choose the best fitting model for the data. All of the data points were weighted by the inverse square of the fitted value. Plasma concentrations after single oral bolus, SC, and continuous administration were fitted to a standard monocompartmental model and also a noncompartmental analysis was carried out. The kinetics after the 10-h pulsed administrations was determined at d 1 and 5 using a noncompartmental analysis (Gibaldi and Perrier, 1982). The peak concentrations,  $C_{max}$ , and time to peak  $T_{max}$  were obtained from the experimentally observed data. The elimination half-life was calculated as  $\ln 2/\lambda_n$ ; mean residence time (MRT) was determined from the following equation:  $MRT = AUMC/AUC$ , where AUMC is the area under the moment curve and AUC is the area under plasma concentration-time curve.

Pharmacokinetic parameters are reported as the mean values ( $\pm$ SD). The harmonic means and pseudostandard deviations were calculated for half-lives using a jack-knife technique (Lam et al., 1985). The normality of the kinetics data was assessed using the Kolmogorov–Smirnov test. The differences between the 2 gavage doses (group 1 vs. 2), and between the 10 mg/kg gavage and the 10 mg/kg SC (group 1 vs. 5) were compared by a 2-tailed unpaired *t*-test. The water

medication by 10-h pulsed scheme at d 1 and 5 were compared by a 2-tailed unpaired *t*-test; the same test was used to compare the d 5 of 10-h pulsed and continuous administration. A *P*-value < 0.05 was considered statistically significant (GraphPad Prism version 4.00, San Diego, CA).

The following PK/PD indices were calculated as predictors of the success or failure of the therapy:  $C_{\max}/\text{MIC}$  and  $\text{AUC}/\text{MIC}$  ratios.  $\text{MIC}_{50}$ , defined as minimum inhibitory concentration at which 50% of isolates tested are inhibited, is generally used for PK/PD correlation (Toutain et al., 2002; McKellar et al., 2004). The breakpoint values of  $C_{\max}/\text{MIC}_{50} = 8\text{--}10$  and  $\text{AUC}/\text{MIC}_{50} = 100$  h were considered representative of the therapeutic efficacy of fluoroquinolones to prevent the development of resistant bacterial strains in poultry.

## 5.4 Results

The CIPRO concentrations were low in all samples from all birds reaching approximately 3 to 4% of the parent compound. Therefore, the mean plasma concentration-time profiles and PK parameters were reported as the sum of ENRO and its metabolite.

### 5.4.1 Single Gavage Administration (Groups 1 and 2)

Mean plasma concentrations  $\pm$ SD of ENRO of both doses are shown in Figure 1. Following oral gavage at the dose of 10 mg/kg (group 1) or 20 mg/kg (group 2), ENRO reached the maximum concentrations at approximately 2 h; subsequently, drug levels decreased rapidly, but were still detectable at 24 h after administration with a mean concentration of  $0.09 \pm 0.02$   $\mu\text{g}/\text{ml}$  (group 1) and  $0.16 \pm 0.03$   $\mu\text{g}/\text{ml}$  (group 2). The pharmacokinetic parameters obtained by monocompartmental and noncompartmental analysis are presented in Table 3. The  $C_{\max}$  for ENRO after gavage ( $1.53 \pm 0.31$   $\mu\text{g}/\text{ml}$  and  $3.51 \pm 0.55$   $\mu\text{g}/\text{ml}$  in group 1 and 2, respectively) were attained at  $1.88 \pm 0.33$  (group 1) and  $1.88 \pm 0.99$  h ( $T_{\max}$ ; group 2). The mean  $\text{AUC}_{0-24}$  and half-lives were  $12.74 \pm 1.52$  h $\cdot\mu\text{g}/\text{ml}$  and  $5.27 \pm 0.67$  h in group 1 and  $28.60 \pm 2.00$  h $\cdot\mu\text{g}/\text{ml}$  and  $4.99 \pm 0.32$  h in group 2.

Figure 1. Mean values + SD in the plasma concentration–time profiles of enrofloxacin in all groups of treated birds: group 1 (open triangles, solid line), oral gavage at 10 mg/kg; group 2 (filled triangles, solid line), oral gavage at 20 mg/kg; group 5 (filled circles, solid line), subcutaneous (SC) administration at 10 mg/kg; group 3 at d 1 (open square, dotted line) and 5 (open squares, dashed line) following 10-h oral pulsed administration of an average dosage of 15.06 mg/kg of BW for 5 d and in group 4 (cross, dotted line) at d 5 after continuous water medication of 20 mg/kg for 5 d.

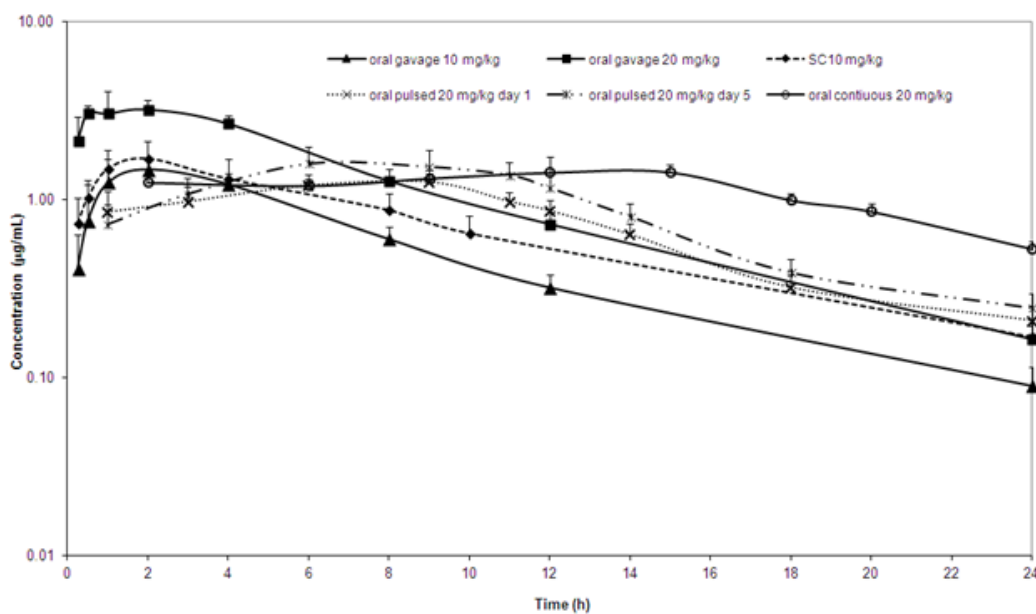


Table 3: Pharmacokinetics parameters in turkeys after oral gavage at 10 (group 1) or 20 mg/kg (group 2) or subcutaneous (SC) administration at 10 mg/kg (group 5)<sup>1</sup>

Parameter (Unit)	Group 1 Oral gavage 10 mg/kg	Group 2 Oral gavage 20 mg/kg	Group 5 SC 10 mg/kg
T <sub>max</sub> (h)	1.88±0.33	1.88±0.99	1.87±0.35
C <sub>max</sub> (µg/ml)	1.53±0.31 <sup>‡</sup>	3.51±0.55 <sup>†</sup>	1.73±0.44
AUC <sub>0-∞</sub> (h*µg/ml)	13.13±1.40 <sup>‡, §</sup>	29.17±1.97 <sup>†</sup>	18.10±3.33 <sup>†</sup>
AUC <sub>0-24</sub> (h*µg/ml)	12.74±1.52 <sup>‡, §</sup>	28.60±2.00 <sup>†</sup>	16.82±4.05 <sup>†</sup>
AUMC <sub>0-24</sub> (h* <sup>2</sup> µg/ml)	83.43±9.69 <sup>‡, §</sup>	179.25±19.85 <sup>†</sup>	114.88±36.83 <sup>†</sup>
t <sub>1/2</sub> elim. (h)	5.27±0.67 <sup>*</sup>	4.99±0.32 <sup>*</sup>	6.22±1.36 <sup>*</sup>
MRT <sub>0-24</sub> (h)	6.57±0.53	6.26±0.48	6.73±1.32
MIC <sub>50</sub> (µg/ml) for <i>Escherichia coli</i>	0.5		
C <sub>max</sub> /MIC <sub>50</sub>	3.07±0.62 <sup>‡</sup>	7.01±1.03 <sup>†</sup>	3.45±0.75
AUC/MIC <sub>50</sub>	25.48±3.04 <sup>‡, §</sup>	57.20±3.73 <sup>†</sup>	33.96±7.46 <sup>†</sup>

<sup>1</sup>T<sub>max</sub> = observed time for C<sub>max</sub>; C<sub>max</sub> = maximum concentration; AUC<sub>0-∞</sub> = area under the concentration vs. time curve from 0 to infinity; AUC<sub>0-24</sub> = area under the concentration vs. time curve from 0 to 24 h; AUMC<sub>0-24</sub> = area under moment curve; t<sub>1/2</sub>elim. = elimination half-life; MRT<sub>0-24</sub> = mean residence time; MIC<sub>50</sub> = minimum inhibitory concentration 50%.

\* harmonic mean ± pseudo SD

<sup>‡</sup> significantly different (P<0.05) from Group 2

#### 5.4.2 10-h Medicated Water Administration (Group 3)

During pulse scheme trials the drug water concentration was adjusted daily based on water intake, the measurement of water at the end of 10-h treatment indicated that the dose received by turkeys was lower than the targeted 20 mg/kg of BW, reaching a value of 14.18 and 16.67 mg/kg at d 1 and 5, respectively; the mean dose received by the group was  $15.06 \pm 3.33$  mg/kg. The ENRO mean concentration–time profiles following 10-h administration of medicated water are shown in Figure 1, the data refer to d 1 and 5 of therapy. The mean kinetic parameters obtained by noncompartmental analysis are resumed in Table 4;  $C_{\max}$  were attained at about 8 h and the highest concentrations were achieved on d 5 of treatment ( $1.28 \pm 0.14$   $\mu\text{g/ml}$  and  $1.72 \pm 0.37$   $\mu\text{g/ml}$  at d 1 and 5, respectively). The mean  $\text{AUC}_{0-24}$  at d 1 and 5 were  $17.28 \pm 2.03$  and  $21.21 \pm 3.30$   $\text{h}\cdot\mu\text{g/ml}$  ( $P < 0.05$ ), respectively.

#### 5.4.3 24-h Continuous Medicated Water Administration (Group 4)

During continuous treatment the drug water concentration was adjusted daily based on water intake. The measurement of water at the end of 24-h treatment indicated that the dose received by turkeys was between 24.73 and 18.39 mg/kg at d 1 and 5, respectively; the mean dose received by the group was  $21.9 \pm 2.31$  mg/kg. The ENRO mean concentration–time profile at d 5 following 24-h administration of medicated water for 5 consecutive days is shown in Figure 1, together with the data from all the other scheme of administration. The mean kinetic parameters obtained by mono- and noncompartmental analysis are resumed in Table 4;  $C_{\max}$  was attained at about 12 h with a mean value of  $1.53 \pm 0.24$ . The mean  $\text{AUC}_{0-24}$  and elimination half-life were  $26.66 \pm 2.77$   $\text{h}\cdot\mu\text{g/ml}$  and  $9.78 \pm 1.40$  h, respectively.

Table 4: Pharmacokinetics parameters in turkeys after oral water medication following 10-h (group 3) or continuous administration (group 4) at 20 mg/kg

Parameter (Unit)	Group 3 Oral pulsed 20 mg/kg (d 1)	Group 3 Oral pulsed 20 mg/kg (d 5)	Group 4 Oral continuous 20 mg/kg (d 5)
T <sub>max</sub> (h)	7.88±1.55	8.50±2.33 <sup>§</sup>	12.02±2.67 <sup>‡</sup>
C <sub>max</sub> (µg/ml)	1.28±0.14 <sup>‡</sup>	1.72±0.37 <sup>†</sup>	1.53±0.24
AUC <sub>0-∞</sub> (h*µg/ml)	-	-	37.37±3
AUC <sub>0-24</sub> (h*µg/ml)	17.28±2.03 <sup>‡</sup>	21.21±.3.3 <sup>†, §</sup>	26.66±2.77 <sup>‡</sup>
AUMC <sub>0-24</sub> (h*h*µg/ml)	160.07±17.87 <sup>‡</sup>	201.72±28.92 <sup>†, §</sup>	309.32±31.33 <sup>‡</sup>
t <sub>1/2</sub> elim. (h)	-	-	9.78±1.40*
MRT <sub>0-24</sub> (h)	9.27±0.22	9.53±0.44 <sup>§</sup>	11.61±0.23 <sup>‡</sup>
MIC <sub>50</sub> (µg/ml) for <i>Escherichia coli</i>	0.5		
C <sub>max</sub> /MIC <sub>50</sub>	3.07±0.62 <sup>‡</sup>	3.45±0.70 <sup>†</sup>	3.05±0.48 <sup>‡</sup>
AUC/MIC <sub>50</sub>	25.48±3.04 <sup>‡</sup>	42.42±6.71 <sup>†, §</sup>	53.32±5.55 <sup>‡</sup>

<sup>†</sup>T<sub>max</sub> = observed time for C<sub>max</sub>; C<sub>max</sub> = maximum concentration; AUC<sub>0-∞</sub> = area under the concentration vs. time curve from 0 to infinity; AUC<sub>0-24</sub> = area under the concentration vs. time curve from 0 to 24 h; AUMC<sub>0-24</sub> = area under moment curve; t<sub>1/2</sub>elim. = elimination half-life; MRT<sub>0-24</sub> = mean residence time; MIC<sub>50</sub> = minimum inhibitory concentration 50

\* harmonic mean ± pseudo SD.

<sup>‡</sup> significantly different (P<0.05) from Group 3 day 5

<sup>§</sup> significantly different (P<0.05) from Group 4

<sup>†</sup> significantly different (P<0.05) from Group 3 day 1

#### 5.4.4 Single SC Administration (Group 5)

Mean plasma concentrations + SD of ENRO after single SC administration at 10 mg/kg are shown in Figure 1. A low inter individual variability was observed in all birds. The ENRO reached the maximum concentrations at approximately 2 h; subsequently, drug levels decreased rapidly, but were still detectable at 24 h after administration with a mean concentration of 0.17 ± 0.03 µg/ml.

The pharmacokinetic parameters obtained by mono and noncompartmental analysis are presented in Table 3. The C<sub>max</sub> of 1.73 ± 0.44 µg/ml for ENRO after SC administration was attained at 1.87 ± 0.35 h (T<sub>max</sub>). The mean AUC<sub>0-24</sub> and half-life were 16.82 ± 4.05 h·µg/ml and 6.22 ± 1.36 h, respectively.

### 5.4.5 PK/PD Integration

The PK/PD integrations were calculated for the different trials, based on PK parameters and MIC<sub>50</sub> value; the values are presented in Tables 3 and 4. The MIC<sub>50</sub>, defined by the broth microdilution method for 235 avian *E. coli* strains isolated in Italy and reported by Vanni et al. (2014), resulted in 0.5 µg/ml, and this value was used for PK/PD correlation. Statistical differences ( $P < 0.05$ ) in the  $C_{\max}/MIC_{50}$  and  $AUC/MIC_{50}$  ratio were observed when the dose was doubled for the gavage and only in the  $AUC/MIC_{50}$  ratio between oral gavage and SC administration. For the 10-h pulsed administrations, the  $C_{\max}/MIC_{50}$  and  $AUC/MIC_{50}$  ratio were significantly different at d 1 and 5, whereas comparisons with continuous administration reported differences for  $AUC/MIC_{50}$  between 10-h pulsed at d 5 and continuous administration (Table 4).

## 5.5 Discussion

Unlike in other animal species, biotransformation of ENRO into its active metabolite CIPRO is low in poultry (Carreras et al., 2004; Dimitrova et al., 2007), as also confirmed by the very low amounts of CIPRO recovered (approximately 3–4% of ENRO) in this study. After gavage administration of 2 different doses of ENRO, the increase of  $C_{\max}$  and AUC was related to the dose increase and the concentration-time profiles were similar (Figure 1). Enrofloxacin was rapidly absorbed,  $T_{\max}$  approximately 2 h, in contrast to what was reported for ENRO by Dimitrova et al. (2007) and for danofloxacin by Haritova et al. (2006), where higher values were recorded ( $T_{\max}$ :  $6.33 \pm 2.5$  h for enrofloxacin and  $6.0 \pm 3.29$  h for danofloxacin). Conversely, similar results were obtained for flumequine by the same group of authors ( $T_{\max}$ : 2 h; Ferraresi et al., 2013). The half-life proved to be rather short ( $t_{1/2 \text{ elim}}$ :  $5.27 \pm 0.67$  and  $4.99 \pm 0.32$  h, groups 1 and 2, respectively), and as expected, was not dependent on the dose given.

After SC treatment similar results were obtained,  $T_{\max}$  approximately 2 h and  $t_{1/2 \text{ elim}}$   $6.22 \pm 1.36$  h, the maximum concentrations were also comparable,  $C_{\max}$   $1.73 \pm 0.44$  µg/ml (group 5) versus  $1.53 \pm 0.31$  µg/ml (group 1).

The  $C_{\max}$  and  $AUC_{0-24}$  obtained, which were higher compared with those with medicated water (Tables 3 and 4), may indicate that individual treatments by oral gavage or SC administration are suitable for efficacious therapy. However, they are not easily practicable in intensive turkey farming due to the high bird density in the sheds, the need for a high number of trained personnel to individually handle the birds, and the stress caused to the birds. This administration route should be preferably adopted in small groups of birds or for breeders because these have an important genetic impact on the progeny, are expected to live longer, and have an high economic value in the flock. In group 3, the pulsed administration trial showed an  $AUC_{0-24}$  and  $C_{\max}$  increase at d 5 ( $AUC_{0-24}$ : from

17.28 ± 2.03 to 21.21 ± 3.30 h·µg/ml;  $C_{\max}$ : from 1.28 ± 0.14 to 1.72 ± 0.37 µg/ml, respectively). These results can be explained by an increase in ENRO concentrations in medicated water due to the low intake of the drug observed at the first day of the trial. In fact, medicated water concentrations were adjusted based on the water intake of previous administrations. The achievement of the targeted dose of 20 mg/kg was never obtained, likely due to the poor palatability of the product and due to the availability of unmedicated water in the remaining 6 h of light period. It is known that drug intake can vary dramatically due to both bird factors (hierarchy, flock size, sex, age, weight, species, breed, health status, and so on) and environmental factors (temperature, humidity, feed and water availability, photoperiod, and so on; Vermeulen et al., 2002). Comparing these results with those by Russo et al. (2012), who assessed the PK of ENRO at the 10 mg/kg via medicated water in healthy and colisepticemic turkeys, a dose proportional increase of  $C_{\max}$  and AUC was observed, whereas  $T_{\max}$  was not affected by dosage. In group 4, the drug concentration in water was adjusted according to water intake and the dose received by the turkeys (21.90 ± 2.31 mg/kg) was close to the targeted dose of 20 mg/kg. Notwithstanding the likely poor palatability of the water, the lack of fresh water forced the birds to drink all the available water and take the targeted dose of ENRO. Compared with pulsed administration at d 5, the 24-h continuous administration of ENRO for 5 d resulted in a longer  $T_{\max}$  12.2 ± 2.67 h and MRT of 11.61 ± 0.23 h and higher  $AUC_{0-24}$ , whereas  $C_{\max}$  was similar.

In agreement with several studies on *E. coli* strains isolated from food-producing animals, a high percentage of strains has proved to be resistant to ENRO (EFSA, 2010; Ozawa et al., 2010). As reported in the co-authored paper by Vanni et al. (2014), 38.7% of *E. coli* tested was resistant, a percentage lower than that observed with old generation fluoroquinolones (70.2% with flumequine), but confirming an increasing trend since the introduction of fluoroquinolones in poultry. The increase in the prevalence of antimicrobial-resistant pathogenic bacteria in farm animals requires reevaluation of treatment options. As prescribed by EMA (2007) and WHO (2011) in the last sets of guidelines on the prudent use of antimicrobials, fluoroquinolones should be used in turkeys only when a susceptibility test clearly indicates the efficacy of the drug. It was widely accepted that fluoroquinolone dosage regimens that lead to high PK/PD indices as  $AUC/MIC >125$  and  $C_{\max}/MIC >8$  resulted in less frequent selection of resistant mutants (McKellar et al., 2004). Although specific breakpoints have not been defined for avian colibacillosis, several studies on fluoroquinolones in poultry (Anadon et al., 2001; Dimitrova et al., 2007; Ozawa et al., 2010; Ferraresi et al., 2013) adopted a  $C_{\max}/MIC$  ratio of 8 or 10 and an  $AUC/MIC$  ratio of 100 as the minimal values required to prevent the selection of resistant bacteria. As reported in Tables 3 and 4, neither type of administration reached the breakpoint



values and the PK/PD correlation yielded unsatisfactory results. After gavage administration, the mean  $C_{\max}/MIC_{50}$  and  $AUC_{0-24}/MIC_{50}$  ratios were, respectively,  $3.07 \pm 0.62$  and  $7.01 \pm 1.03$ , and  $25.48 \pm 3.04$  and  $57.2 \pm 3.73$  for the 10 and 20 mg/kg doses, respectively. After SC administration  $C_{\max}/MIC_{50}$  and  $AUC_{0-24}/MIC_{50}$  ratios were  $3.45 \pm 0.75$  and  $33.96 \pm 7.46$ , respectively. After the administration of 10-h pulsed (group 3) or 24-h continuous (group 4) medicated water with the dosage regimen of 20 mg/kg lower values of  $C_{\max}/MIC_{50}$  (group 3:  $3.45 \pm 0.70$ ; group 4:  $3.05 \pm 0.48$ ) and  $AUC_{0-24}/MIC_{50}$  (group 3:  $42.42 \pm 6.17$ ; group 4:  $53.32 \pm 5.55$ ) were obtained. These results were similar to those obtained by Russo et al. (2012). Conversely, data published by Dimitrova et al. (2007) supported the efficacy of 10 mg/kg administered via drinking water to turkeys, but PK/PD results were obtained correlating the kinetic parameters with a value of MIC first reported in 1996 and significantly lower than those adopted in the present study (0.06 versus 0.5  $\mu\text{g}/\text{ml}$ ).

Based on the present results, the EU-recommended dosage of 10 mg/kg might be ineffective to achieve adequate drug plasma concentrations. Also the 20 mg/kg by 10-h pulsed doses of medicated water did not reach plasma concentrations that were completely efficacious in controlling *E. coli*, the scenario could be even worse when considering the long period with unmedicated water. Indeed, Santos et al. (1997) showed the influence of the photoperiod on the PK of drugs during drinking water administration in turkeys. The eating and drinking patterns can be altered by the light scheme (Classen et al., 1994; Watteyn et al., 2013), which could have a huge influence on the uptake of drinking water medication. Although the results obtained were not completely encouraging, the medicated water should always be provided continuously, as an increase of PK/PD indices was achieved for AUC/MIC.

*Escherichia coli* is generally located in the intestine and active ENRO concentrations undergo a biliary excretion; thus, plasma concentration does not reflect the same magnitude order of intestinal environment. A similar scenario should be foreseen for pulmonary infections, against which ENRO is frequently used. Indeed, plasma concentrations might not be a good predictor of efficacy, as ENRO concentrations and AUC are reported to be higher in lungs than in plasma (Tang et al., 2007). In addition, interesting results were obtained during the validation process of the LC-MS analytical method by detecting ENRO and CIPRO in lung tissue and intestinal content from turkey treated with ENRO (Lucatello et al., 2014). Both CIPRO and ENRO concentration in lung and intestinal content were much higher than in plasma in turkeys killed after ENRO treatment. Therefore, target tissue concentrations need to be evaluated to define the efficacy of ENRO treatment against colibacillosis. All the treatment scheme evaluated in this study, based on plasma concentration, were not completely

satisfactory against *E. coli*, supporting the unsuitability of the ENRO-recommended dosage scheme. Thus, to improve treatment efficacy and comply with the prudent and responsible use of fluoroquinolones in poultry species, a revision of the ENRO dosage scheme, which includes an extensive distribution study in the target tissues (i.e., intestine and lung), is advisable for a real efficacy evaluation against colibacillosis.

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# **CHAPTER 6**

## **General Discussion**

## 6. General discussion

The emergence of antimicrobial resistance in commensal and pathogenic bacteria is a growing concern in both human and veterinary medicine and it is primarily correlated with the use of antimicrobials in humans and animals.

In human and veterinary medicine the use of antimicrobial agents is often correlated and many antimicrobials used in humans are often authorized for companion and farm animals. This correlation is worrying for the repercussions on resistance phenomenon. The deep comprehension of antimicrobial effects and the rise of antimicrobial resistance in food producing species and in humans, encouraged international agencies to review the use of authorized antimicrobial drugs in veterinary and human practice. In human and veterinary medicine many studies have been carried out to define precise dosages, to improve drug activity and reduce selection of resistance in antimicrobial therapy. Although considered ideal, the individual administration is not a viable treatment in poultry breeding due to the high number of animals bred and the low cost of individual animals compared to the high management cost of individual therapy. Therefore oral mass medication is still used and preferred. In poultry the preferred method of oral mass treatment is drinking water medication, because ill birds usually tend to stop feeding but not drinking. Drinking water therapy can vary dramatically in response of efficacy as a function of several factors, such as photoperiod schema, chemical characteristics of water and preparation of medical solution. Consequently, the underdosing of the drug is likely, and such risk must be considered and limited as much as possible (i.e. accurate weighting of the animals to determine the correct drug dosage, correct consideration of water intake, etc.).

Another important aspect to consider in order to optimize antimicrobial therapy, is the assessment of the bacterial susceptibility (MIC or antibiogram) aimed at knowing whether the selected drug will be effective or not. The potential for rapid spread of disease in avian species often needs empirical and rapid treatment prior to the results of bacterial culture and susceptibility testing.

The studies reported in this thesis aimed to evaluate the use of fluoroquinolones in turkey to maintain the efficacy and reduce the spread of resistance against *E.Coli*, in accordance with a prudent use of this drugs as suggested by the European Guidelines (EMA, 2011).

In the trials, the pulsed water medication has highlighted the failure to achieve the target dose intake by animals, likely due to the poor palatability of the products and due to the availability of “clean water” in the remaining light period. This is certainly an important aspect that can promote the underdosing with consequent increase of selective pressure leading to an increase in the

antimicrobial resistance. Therefore the medicated water should always be provided continuously during the entire light period.

The data obtained by oral treatments at two different doses (15 mg/kg and 30 mg/kg) with flumequine showed how the breakpoints values ( $C_{\max}/\text{MIC}$  ratio and AUC/MIC ratio) were not reached. Despite its use suggests that clinical efficacy is still achievable, the PK/PD correlation yielded very unsatisfactory results, especially considering the high MIC values that indicate a widespread of antimicrobial resistance. These findings are not totally unexpected, since flumequine was licensed for use in food animals over 20 years ago and has been widely used for the mass medication of poultry. Following these results, the continuous administration with flumequine was not tested. In tissue samples higher concentrations were achieved and this result are more encouraging and can justify the repeated use of this antimicrobial in breeding. It would be desirable, since the low sensitivity and the possibility of cross-resistance against other fluoroquinolones, that flumequine would be used more prudently in farms. The oral treatments and the single parenteral treatment using two different doses of enrofloxacin (10 mg/kg and 20 mg/kg) were not completely satisfactory against *E. coli*. To improve the efficacy of the treatment and to comply with the prudent and responsible use of fluoroquinolones in poultry species, a revision of the enrofloxacin dosage scheme is advisable for a real efficacy evaluation against colibacillosis.

Fluoroquinolones concentrate in the some tissues, as lung and intestine, as confirmed by the results of this thesis. These tissues are target tissue of the antimicrobial effects of fluoroquinolones. So as for flumequine, also for enrofloxacin this account for the efficacy of the treatment, notwithstanding the unsatisfactory results of the PK/PD correlation with plasma results. Based on these aspects, further studies on the drug concentrations profile in liver, lung and intestinal content would be helpful to allow the integration of PK/PD data, in order to evaluate the real efficacy of flumequine and enrofloxacin against colibacillosis.

It should be underlined that the determination of bacterial susceptibility is one of the two mainstay for the PK/PD correlation for the evaluation of antimicrobial efficacy. Thus the re-examination of susceptibility of bacteria with specific values of MIC that become breakpoints for determining “susceptibility”, “intermediate” or “resistance” of specific bacteria can help to set realistic values for decision making of the correct choice of antimicrobial therapy. The CLSI-VAST subcommittee has been setting breakpoints for the new and old veterinary drugs, this is an important step forward in the evaluation of antimicrobial therapy, as realistic breakpoints are necessary in order to avoid ineffective administration of antibiotics that only serve to select for other resistant bacteria and contaminate the environment (CLSI, 2013).

Finally, the reported studies allowed to highlight important aspects about the usage and efficacy of antimicrobial drugs in turkey, that were still lacking. A further diffusion of this type of studies is even more relevant for the so called “minor species”. It is possible to conclude that fluoroquinolones, and enrofloxacin in particular, represent an useful tools for the treatment of various infections in turkey, but their efficacy should be accurately monitored due to the spreading of resistance in zoonotic bacteria. Moreover, any lack of efficacy should be reported by pharmacovigilance and, if possible, the causes should be deeply investigated. This can represent an important instrument for monitoring drug resistance in the target species and to retain the potential efficacy of fluoroquinolones for critically dangerous bacteria.



# CHAPTER 7

## Summary



## 7. Summary

The importance of prudent and rational use of antimicrobial is important, not only to safeguard the efficacy of these drugs in humans and veterinary medicine but, even more so, to prevent the emergence and spread of undesirable resistance phenotypes in zoonotic pathogens as well as in commensal bacteria that can be transmitted between animals and humans.

Even more importance and attention is now given to the prudent use of medically important antimicrobial drugs, referring to those drugs for human therapeutic use.

The fluoroquinolones belong to this category. These are very potent antimicrobials and active against a wide range of pathogenic organisms and are well distributed in the body after administration. This class of antimicrobials has a therapeutic effect on most infections in different organs or tissues. Although it is rare that fluoroquinolones are the only available agent for treatment of a specific infectious disease, fluoroquinolones are important alternative medicinal products for a veterinarian to have as option for treatment. Fluoroquinolones have a unique mechanism of action not related to conventional antimicrobials, and therefore their efficacy should be retained as long as possible.

The avian production increased enormously in the last 50 years and the European Union (EU) is one of the world's top producers in poultry meat and a net exporter of poultry products. In this production, turkeys that is considered a “minor species”, but it is important in the livestock production of Italy. Scarce data exist about the usage of antimicrobial drugs in turkey and even less is known about their efficacy. As the limited number of medicinal products authorized in this species, antimicrobial therapy is frequently carried out with the few products authorized or with drugs “extra-label” used with the consequence of increases of selective pressure and also with the possibility of cross-resistance within the same pharmacological group of compounds.

The studies reported in this thesis aimed to revise the use of fluoroquinolones in turkey to maintain the efficacy and reduce the spread of resistance against *E.Coli*, the most common zoonotic avian pathogen. Pharmacokinetic(PK)/pharmacodynamics (PD) models are the best tool in order to select optimal dosage regimen. To confirm dosages used at farms and allow the integration of PK/PD data, the plasma concentrations in blood from healthy animals collected during treatment with flumequine and enrofloxacin, were determined.

The first step was to optimize and validate a fast, simple, sensitive, and specific liquid chromatography-mass spectrometry (LC-MS)/MS/MS method suitable for the detection of a wide range of concentrations of fluoroquinolones as those occurring in pharmacokinetic and residue depletion studies from several

matrices. The first trial presents a sensitive and reliable confirmatory method for the extraction, identification, quantification of five fluoroquinolones. For the extraction and matrix clean-up of fluoroquinolones residues from all biological matrices, the Quick Easy Cheap Effective Rugged Safe (QuEChERS) methodology was adopted; only for plasma samples acetonitrile was used. The analyses were performed by LC-MS. LC separation was performed on a C<sub>18</sub> Kinetex column (100x2.1 mm, 2.6 μm, Phenomenex, CA, USA) with gradient elution using ammonium acetate solution (10 mM, pH 2.5) and methanol containing 0.1% formic acid. Mass spectrometric identification was done using an LTQ XL ion trap (Thermo Fisher Scientific, CA, USA), with a heated electrospray ionization probe, in positive ion mode. The method was validated according to the European Legislation (decision 2002/657/EC) and EMA guideline (EMA/CVMP/VICH/463202/2009); selectivity, linearity response, trueness (in terms of recovery), precision (within-day repeatability and within-laboratory reproducibility), limit of detection, limit of quantification, decision limits, detection capability, absolute recovery and robustness were evaluated using turkey blank matrices. All data were within the required limits established for confirmatory methods except for flumequine which presented a recovery value slightly higher than 110% in muscle and intestinal content. For all fluoroquinolones, all the extraction rates were greater than 70% and limits of quantification ranged from 1.2 μg/kg to 118.8 μg/kg. This method was suitable for the identification and quantification of fluoroquinolones in plasma samples of turkeys treated for the purpose of second and third trials.

In the second trial, the PK behavior of flumequine administered to 32 healthy turkeys as an oral bolus via *gavage* or as 5 days of 10-hours pulsed administration in drinking water were compared, using the EU authorized dose of 15 mg/kg and the double dose of 30 mg/kg. The MIC of 235 *Escherichia coli* field strains isolated from poultry were determined for PD to develop a PK/PD model. Blood samples were collected at established times over 24 h, and the obtained plasma was analyzed using the LC-MS/MS/MS method previously described. A monocompartmental model and a noncompartmental model were applied to the data to obtain the PK results. The maximum concentration ( $C_{max}$ )/MIC<sub>50</sub> and the plasma concentration-time curve from 0 to 24 hours ( $AUC_{0-24}$ )/MIC<sub>50</sub> ratios were, respectively,  $0.67 \pm 0.09$  and  $4.76 \pm 0.48$  and  $1.18 \pm 0.35$  and  $7.05 \pm 2.40$  for the 15 and 30 mg/kg bolus doses, respectively. After 10-hours pulsed administration of 15 mg/kg, values of  $C_{max}$ /MIC<sub>50</sub>,  $0.19 \pm 0.02$  on day 1 and  $0.30 \pm 0.08$  on day 5 of therapy were obtained, the AUC/MIC<sub>50</sub> ratios were  $2.09 \pm 0.29$  and  $3.22 \pm 0.93$  on d 1 and 5, respectively. Higher values were obtained with the doubled dose of 30 mg/kg: the  $C_{max}$ /MIC<sub>50</sub> ratios were  $0.49 \pm 0.11$  on day 1 and  $0.69 \pm 0.18$  on day 5; the AUC/MIC<sub>50</sub> ratios were  $5.15 \pm 1.15$  and  $6.57 \pm 1.92$  on d 1 and 5, respectively. For both types of administration and both

dosages, the  $C_{\max}/MIC_{50}$  and the  $AUC/MIC_{50}$  ratios achieved were significantly lower than the fluoroquinolones breakpoints usually considered for efficacy.

The last trial involving 50 healthy turkeys, was conducted to evaluate the efficacy of enrofloxacin. As in the previous study, the effectiveness of different treatment schemes against *E. coli* was evaluated by a PK/PD approach, correlating the PK results with the MIC determined for 235 *E. coli* strains.

In this study, 3 different oral treatments (a single oral gavage, 5 days of 10-hours pulsed water medication, and 5 days of 24-hours continuous water medication) and single parenteral (subcutaneous; SC) treatment using 2 different doses of enrofloxacin (i.e., the EU authorized dose, 10 mg/kg, and double the EU recommended dose, 20 mg/kg) were evaluated. Blood samples were collected at established times over 24 h. Plasma was analyzed using a LC-MS/MS/MS that was validated in house. A monocompartmental and a noncompartmental model were applied to the data to obtain the PK results. After gavage administration, the mean maximum concentration  $C_{\max}/MIC_{50}$  and area under the curve  $AUC_{0-24}/MIC_{50}$  ratios were, respectively,  $3.07 \pm 0.62$  and  $7.01 \pm 1.03$  and  $25.48 \pm 3.04$  and  $57.2 \pm 3.73$  for the 10 and 20 mg/kg doses, respectively. After SC administration of 10 mg/kg,  $C_{\max}/MIC_{50}$  and  $AUC_{0-24}/MIC_{50}$  ratios were  $3.45 \pm 0.75$  and  $33.96 \pm 7.46$ , respectively. After the administration of 10-h pulsed or 24-h continuous medicated water at 20 mg/kg, lower values of  $C_{\max}/MIC_{50}$  (10-h pulsed:  $3.45 \pm 0.7$ ; 24-h continuous:  $3.05 \pm 0.48$ ) and  $AUC_{0-24}/MIC_{50}$  (10-h pulsed:  $42.42 \pm 6.17$ ; 24-h continuous:  $53.32 \pm 5.55$ ) were obtained. Based on these results, the European Union-recommended dosage of 10 mg/kg seems ineffective to achieve adequate drug plasma concentrations and even the 20 mg/kg by 10 h pulsed or continuous medicated water administration did not reach completely efficacious concentrations in plasma against colibacillosis. Although the results obtained were not completely encouraging, the medicated water should preferably be provided continuously. To conclude about the efficacy of enrofloxacin treatment against colibacillosis, target tissue concentration should be extensively considered.

