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Pharmacokinetic Evaluation of the Blood: Tissue Relationship in Poultry: Screening for Antibiotic Residues in Chicken Muscle

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PHARMACOKINETIC EVALUATION OF THE BLOOD: TISSUE RELATIONSHIP IN POULTRY: SCREENING FOR ANTIBIOTIC RESIDUES IN CHICKEN MUSCLE

PHARMACOKINETIC EVALUATION OF THE BLOOD: TISSUE RELATIONSHIP IN POULTRY: SCREENING FOR ANTIBIOTIC RESIDUES IN CHICKEN MUSCLE

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of the requirements for the degree of
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By

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ABSTRACT

During their life, humans are exposed to numerous substances that are naturally present in the environment or that are the result of man made processes, including heavy metals, persistent organic pollutants, pesticides and veterinary drugs. Some of these substances can leave residues in food products and possibly expose consumers to their potential toxic effects. To address these problems extensive regulatory efforts are devoted to prevent, detect and control these substances from reaching the food supply. However, given the vast diversity of food products that requires monitoring and the immense diversity of potential chemical contaminants, the need for an efficient and vigilant residue prevention program cannot be underestimated. Residue concentrations in animal food products are generally determined in samples collected from edible tissues (e.g. muscle, liver, skin, fat) which can lead to a loss of valuable product. Additionally, variations between different edible tissues and products require the development and use of specific analytical procedures. This dissertation presents different aspects related to the nature and origin of potential chemical residues in food products and the regulatory process used for their prevention and control. Finally, it discusses the possible use of body fluids, namely blood, as a predictive indicator of the concentrations of antibiotic residues in target tissues for monitoring purposes.

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PREFACE

Antibacterial drugs are among the most frequently used treatments in animal production (FDA, 2000; Botsoglou and Fletouris, 2001; Donoghue, 2003). These drugs have a critical role in the promotion of animal health, welfare and productivity. Additionally they are important in the prevention of the spread of pathogens from animals to humans, either by direct contact to producers or farm workers or indirectly via food products (Berkelman, 1994; von Essen and McCurdy, 1998). Yet, despite the benefits provided by these drugs, some of them can leave residues in the edible tissues of food producing animals (e.g. muscle, liver, kidney, skin or food products like eggs). In some cases, these residues can predispose consumers to health problems such as allergic or toxic reactions, or chronic conditions such as mutagenesis or carcinogenesis (Craigmill and Cortright, 2002; Anderson et al., 2003; Donoghue, 2003).

To protect consumers from potentially harmful concentrations of antibacterial residues, the Federal Government conducts extensive procedures to ensure the safe usage of these drugs in food producing animals (NRC, 1999; VICH-FDA, 2008; FDA, 2009a; 21CFR500.84, 2010; 21CFR514, 2010). During the preapproval process for veterinary drugs, studies are conducted to determine safe and efficient protocols for use. This process also includes specific studies on the characteristics of drug incorporation in the target animal including absorption, distribution, metabolism, storage and excretion (Riviere, 1991; Martín-Jiménez and Riviere, 1998). One of the most important results of these studies is the identification of the edible tissue in which the concentration of residues persists for the longest period of time at the highest concentration. This tissue, called the target tissue, is then used to monitor the safety of edible tissues from food

producing animals (Clement, 1995). Federal inspectors collect samples of the indicated target tissue to determine concentrations of residues in that edible tissue and then use results to estimate the concentration of residues in other tissues in the animal. Based on these determinations, it will be determined if the edible tissues of the animal are safe for human consumption.

Since the monitoring procedure requires collection of edible tissues for analysis (e.g. muscle), it can represent a monetary loss for the producer and therefore can limit the number of samples collected for each group of animals. From the inspector's perspective, the collection of samples from edible tissues at the processing plant can be a cumbersome process. Processing lines at poultry slaughter houses can move at speeds of approximately 70-160 birds per minute, making sample collection difficult (Berrang et al., 2007, 21CFR381, 2010). Furthermore, due to variations among the different types of tissues for monitoring purposes (e.g. muscle, liver, kidney, etc.), analytical procedures used to quantitate concentrations of residues must be developed for each specific tissue (Marazuela and Bogialli, 2009).

An alternative strategy to monitor residues in animals would be the use of blood as an indicator of the concentration of residues in the target tissue. This sampling strategy would simplify sample collection at the processing plant, reduce costs to producers due to product loss and expedite analytical procedures for residue quantitation. Few studies have been published examining this strategy to predict residue concentrations in edible poultry tissues. Because of the paucity of literature regarding this approach to monitoring, the review of literature will focus on occurrence of antibiotic residues in food animals, their significance to human health and the pre-approval and

post-approval monitoring process for these residues. Finally, pharmacokinetic data will be presented and discussed in two manuscripts regarding the ability to predict residues content in poultry muscle based upon blood concentrations.

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CHAPTER I

LITERATURE REVIEW

ANTIBACTERIAL RESIDUES IN ANIMAL FOOD PRODUCTS AND THEIR SIGNIFICANCE TO HUMAN HEALTH

1. INTRODUCTION

As the global population exceeds the 6.7 billion mark (U.S. Census Bureau, 2010), the demand for inexpensive, plentiful, wholesome and safe food products has never been greater (Tilman et al., 2002; OECD-FAO, 2009). Yet, at the same time, most consumers are concerned about the methods used for food production and the consequences of those agricultural practices on human animal and environmental health (Bruhn, 1999; Willis, 2000; Gorback, 2001; Tilman et al., 2002). One such practice that has attracted the public's attention is the use of veterinary drugs in agricultural settings and their potential presence as residues in food products (Bruhn, 1999; NRC, 1999; Resurreccion and Galvez, 1999; Willis, 2000; Verbeke et al., 2007). Results from a survey conducted in the U.S. by Nicholls and co-workers (1994) indicated that many consumers erroneously believe that exposure to chemicals in foods is one of the leading causes of death. Similarly, in the fall of 2005, the *Eurobarometer* survey showed that European consumers rank presence of chemicals, pesticides and toxic substances in food second after food poisoning as their main food related concerns (Becker, 2000; European Commission, 2006).

Unfortunately, the perception of risk that most consumers have about chemicals in foods is obscured by the lack of differentiation between broad categories of risks. Namely, consumers do not discriminate between various types of potential toxicants and thus they may perceive compounds like dioxins (organic compounds with very high toxicity) and residues of veterinary drugs (with low toxicity) as equally dangerous. Numerous authors have also reported that consumers may feel pessimistic about food

safety due to lack of confidence in the stringency of the safety and monitoring standards and protocols (Lofstedt, 2006; De Jonge et al., 2008, Cope et al., 2010).

2. USE OF ANTIBACTERIAL DRUGS IN FOOD PRODUCING ANIMALS AND THEIR RELEVANCE AS POTENTIAL SOURCES OF RESIDUES IN ANIMAL FOOD PRODUCTS

During the production cycle, food producing animals may be dosed with a variety of veterinary drugs (NRC, 1999; Botsoglou and Fletouris, 2001). These compounds are used to promote the health, welfare and productivity of food producing animals, reduce production costs and retail costs for the consumer (CAST, 1981; Langlois et al., 1986, Walton, 1986; Botsoglou and Fletouris, 2001; Donoghue, 2003). They are also important to reduce transmission of pathogenic agents from animals to humans (Berkelman, 1994; Von Essen and McCurdy, 1998).

Unfortunately, some antibacterial drugs and their metabolites may deposit in edible tissues which can be detrimental to consumers (Paige et al., 1997; Cerniglia and Kotarski, 1999; Donoghue, 2003). Antibacterial residues are characterized as pharmacologically active substances, whether active principles, excipients or degradation products and their metabolite(s) that persist in foodstuffs obtained from plants or animals that have been exposed to an antibacterial (Botsoglou and Fletouris, 2001; McGlinchey et al., 2008). Most of these residues are limited to specific tissues and tend to be stored and maintained for a short time and are innocuous to the host (VICH-FDA, 2010a). However, when present in edible tissues, consumers can be unknowingly exposed to these antibacterial residues. This is of particular importance in the case of antibacterials

that can induce health problems in consumers such as allergic or toxic reactions, or predispose to teratogenesis, carcinogenesis or mutagenesis (Craigmill and Cortright, 2002; Anderson et al., 2003; Donoghue, 2003). Thus it is important that producers, veterinarians and all involved in food production are aware and follow the approved labeling instructions for drugs used in animals to protect consumers from potentially harmful concentrations of residues in food products (Anadon and Martinez-Larrañaga, 1999; Willis, 2000).

2.1 ANTIBACTERIAL DRUGS

Antibacterials are defined as compounds that are capable, at low concentrations, of killing or inhibiting the growth of microorganisms (Botsoglou and Fletouris, 2001; Walsh, 2003). The term antibacterial includes two general classes of these drugs: antibiotics which are of natural origin and antibacterials which are synthetic or semi-synthetic compounds (Hagren et al., 2005; Marazuela and Boglialli, 2009). In general, the types of antibacterial drugs used for the control, prevention and treatment of diseases in animals are similar to those used to treat humans (Crosby, 1991; Prescott, 2008).

Antibacterials have been used in agriculture since the 1940s to improve animal wellbeing and production (Jones and Ricke, 2003). Currently, there are over 7,000 identified antibacterials and several hundred specific antibacterial applications are approved for use in the U.S. for animal production. It is estimated that a large proportion of the livestock and poultry in the U.S. and in the world, receive antibacterials regularly during their production cycle (FDA, 2000; Botsoglou and Fletouris, 2001; Donoghue, 2003). In the U.S. the Animal Health Institute estimated that annually over 8,000 metric

tons of these compounds are used in the production of the three major food producing animal species (swine, cattle and poultry), whereas the Union of Concerned Scientists estimated that quantity at over 11,000 metric tons per year (Mellon et al., 2001). In other parts of the world the use of antibacterials remains relatively unregulated and undocumented, therefore comprehensive reports on the quantity and type of antibacterials used for agricultural purposes in the world are unavailable (Katz and Ward, 2004).

a. Sulfonamides (e.g. sulfamethazine, sulfamethiozole, sulfadiazine, sulfamerzine, sulfanilamide, sulfadimethoxine, sulfapyridine and sulfaquinoxaline) are widely used in animal husbandry, for prophylactic and therapeutic purposes, particularly for respiratory conditions (Wang et al., 2006). Unfortunately, exposure to sulfonamides can induce adverse reactions in people including photosensitivity, thyroid toxicity (particularly caused by sulfadimidine), toxic epidermal necrolysis (Stevens-Johnson Syndrome and the severe form of the disease called Lyell Syndrome), urinary tract disorders, porphyria, hematopoietic disorders, onset of fetal hyperbilirubinemia and kernicterus during late pregnancy, as well as teratogenic effects (Dunn, 1964; Swarm et al., 1973; Peters et al., 1990; Mitchel, 1994; NRC, 1999; Slatore and Tilles, 2004; Wang et al., 2006). It has been reported that sulfonamides can induce hypersensitivity reactions in approximately 10% of the general population and about 60% of patients with human immunodeficiency virus (HIV; Dewdey et al., 1991; Dayan, 1993; Berends et al., 2001).

Sulfonamides have a number of characteristics that increase their potential for leaving residues in treated animals. For example, these drugs tend to be eliminated unchanged from the body and be disseminated to untreated animals through feed, water

or environmental contamination. Furthermore, as these drugs tend to persist for long periods of time in the environment this can lead to an underestimation of the exposure risk (Bevill, 1984, 1989; Van Dresser and Wilcke, 1989; McCaughey et al., 1990; Waltner-Toews and McEwen, 1994a, 1994b; Riviere and Spoo, 2001; Buur et al., 2006; Agwuth and MacGowan, 2006). Sulfonamides can also persist at injection sites for over 30 days after application, in contrast to other antibacterials that disappear from the injection site within 24 hours such as neomycin, tylosin and oxytetracycline (Galer and Monro, 1996; Van Donkersgoed et al., 1999; Reeves, 2005, 2007).

b. Penicillins, include antibacterials such as benzypenicillin (also called penicillin G), ampicillin, amoxicillin, cloxacillin, cephapirin, and ceftiofur (21CFR556, 2010). These antibacterials are classified as members of the group of the β-lactams. These antibacterials have been used widely in both human and veterinary medicine for over half a century (Wright, 1999). These compounds and their numerous metabolites can induce allergic reactions in several mammalian species. As a result, penicillins may elicit a variety of allergic reactions ranging from mild skin rashes to potentially fatal anaphylaxis (Adcock and Rodman, 1996; Jiminez et al., 1997). It has been estimated that approximately 10% of the population are allergic to these antibacterials, and that exposure to penicillins may account for up to 75% of deaths due to drug anaphylaxis in the United States (mainly caused by exposure during medical treatments; Idsoe et al., 1968; Delage and Irey, 1972).

Although there is a significant percentage of the population allergic to these compounds, it is very difficult to quantify the public health risk by β-lactam residues in

foods (Dewdney and Edwards, 1984). There are few identifiable cases of allergic reactions to penicillin caused by antibacterial residues in food. One report from 1984 indicated that a patient had developed pruritis, difficulty in swallowing and speaking within 20 minutes of eating a steak dinner. The meat was later found to contain penicillin (Schwartz and Sher, 1984). Another similar incident was reported after pork consumption (Tscheuschner, 1972). There has even been a report of anaphylaxis after the consumption of a β-lactam adulterated soft drink (how this occurred could not be determined; Wicher and Reisman, 1980). Interestingly, Lindemayr and co-workers (1981) reported that when penicillin allergic volunteers ate meat containing penicillin residues only a very small fraction of them developed allergic symptoms. The results of this experiment and the scarcity of verifiable incidents of adverse reactions to penicillin residues in foods suggest that allergic reactions caused by β-lactam residues in foods may be rare (Dewdney et al., 1991; Woodward, 1991; Dayan, 1993; McEwen and McNab, 1997; Woodward, 2005).

Similar to the sulfonamides, most β-lactams are excreted unchanged and thus can cause unintentional exposure to untreated animals. In the case of β-lactams that are metabolized before excretion, they retain their allergenic potential and thus still pose a danger for consumers (Assem and Vickers, 1974, 1975; Basomba et al., 1978; Pirmohamed et al., 1994; Gerber and Pichler, 2006; Roujeau, 2006). Some β-lactam metabolites have prolonged half lives. For example, amoxicilloic acid is one of the most allergenic metabolites of the antibacterial amoxicillin and has a longer half life then the parent compound (Reyns et al., 2008). Thus monitoring of these metabolites needs to be considered during the residue monitoring process.

c. Cephalosporins (e.g., cefalonium, cefalexin, ceftiofur, cefazolin, cefacetril) are an important class of antibacterial agents used for both human and animal medicine. To date, scientists have developed four generations of cephalosporins, all of which share the same β-lactam sub-structure first found in penicillin. With regards to animal production, most first and second generation cephalosporins are commonly used worldwide for the treatment of mastitis infections in dairy cattle, while the third and fourth generations of cephalosporins are primarily used for other veterinary purposes (Hornish and Kotarski, 2002). As such, these antibacterials are a common source of residues in milk and dairy products. This is a concern for both human health and also the manufacture of dairy products, such as cheese due to possible interference of the antibacterials with the microorganisms used to make them (Molina et al., 2003). Several on-farm residue screening tests are available to monitor treated cows to prevent residues from entering the food supply (Jones and Seymour, 1988; Kang et al., 2005).

Health concerns associated with these antibacterials are related to their allergenic potential, as about 5% of the general population appears to be allergic to these compounds (Botsoglou and Fletouris, 2001). Also, several cephalosporins, particularly, those classified as third generation (such as ceftiofur, cefixime, cefdinir and cefotaxime), can cause renal damage through hypersensitivity-induced interstitial nephritis or through direct toxicity of the renal tubules and thus represent a source of concern for already renally compromised patients (Fekety, 1990; Schliamser et al., 1991). Finally, these antibacterials have recently been associated with the apparent development of cephalosporin resistant pathogenic bacterial strains, consequently the extralabel use of

these antibacterials has been banned (FDA, 2008).

d. Tetracyclines (e.g., tetracycline, doxicycline, chlortetracycline and oxytetracycline) represent about half of all antibacterials used in animal production due to their low cost and are consequently one of the most commonly detected antibacterials in foods (Lynas et al., 1998; Kennedy et al., 2000; Oka et al., 2000; Guigere, 2006).

The tetracyclines are known for their ability to cause acute allergic reactions and thus are a major concern as residues in food products (Botsoglou and Fletouris, 2001). Also, it has been reported that some tetracycline residues can undergo heat degradation during cooking processes and produce toxic metabolites, which may have nephrotoxic properties (Fedeniuk, 1988; Rose et al., 1996; Moats, 1999). Tetracyclines are rapidly eliminated from edible tissues, thus in most cases a withdrawal period of only 24 hours is generally enough for concentrations to fall below the tolerance level (McEvoy et al., 1994; Lynas et al., 1998). In bone tissue, however, most tetracyclines bind almost irreversibly (Korner et al., 2001; Zakeri and Wright, 2008) and bonemeal used from treated animals can result in an unintentional source of residues in the animals.

e. Chloramphenicol has been included in the list of prohibited drugs for food producing animals because it may cause several health problems in consumers including cyanosis, respiratory failure (including the grey baby syndrome in newborns) and aplastic anemia (FDA, 1996; Kasten, 1999; Johnson, 2003; Hagren et al., 2005). Aplastic anemia is fatal in about 70% of cases and for those that survive; there is a sharp increase in the probability of developing leukemia (Fraunfelder, 1982; Fraunfelder et al., 1982;

Settepani, 1984; Page, 1991; NRC, 1999; Botsoglou and Fletouris, 2001). This disease appears in approximately 1:10,000 to 1:45,000 humans who receive chloramphenicol (Papich and Riviere, 2001).

An important characteristic of chloramphenicol's toxic effects is that they are not dose dependent, but rather related to the sensitivity of the individual (Hagren et al., 2005). Therefore, even low concentrations of this antibacterial via systemic (such as from food) or in topical exposure can cause toxic effects. Because there is no safe concentration for this drug, chloramphenicol is banned from all use in food animals (Black 1984; Settepani, 1984; Norcross and Post, 1990; Page, 1991; Cooper et al., 1998; NRC, 1999; Codex Committee, 2004; 21CFR530, 2010).

Because of these risks, surveillance for this compound in U.S. and imported food products is a high priority for regulatory agencies. Imported foods are a particular concern since chloramphenicol is used in many countries (e.g., widely used in China). Legal prosecution is vigorously pursued for violators, in accordance with 21U.S.C.331(a), through the FDA's Office of Criminal Investigations (Norcross and Post, 1990; Norcross and Brown, 1991; Botsoglou and Fletouris, 2001; FDA, 2002a). It is also important to mention that chloramphenicol is an antibiotic naturally produced by soil organisms of the genera *Streptomyces* and thus animals may unintentionally be exposed to small amounts of this compound (Wongtavatchai et al, 2005)

f. Nitrofurans and nitroimidazoles deserve special attention because they are carcinogenic (FDA, 2009b) and have been banned for use in the U.S. since the early 1990s and banned for extra-label use since 2002 (FDA, 2002b). In addition to being

carcinogenic some people may be hypersensitive to this product and liver damage during pregnancy and a syndrome similar to autoimmune hepatitis have been described (De Groot and Conemans, 1990; Peedikayil et al., 2006; Aksamija et al., 2009). These antibacterials form stable and irreversible deposits in the animals and thus, the use of these drugs in even young animals can cause residues at slaughter. Studies have shown that even topical (ocular) applications of nitrofurans may result in residues in the tissues and organs of the animals, including milk (FDA, 2002b).

Due to their potential toxic effects, the nitroimidazoles have been banned for use in production animals in both the U.S. and the European Union. Unfortunately, as in the case of chloramphenicol, these compounds are still used in other countries such as Burma, Thailand, Vietnam and China and are a significant source of contamination in shellfish and fish products imported from those countries (Hagren et al., 2005; FDA, 2009a).

g. Aminoglycosides (streptomycin, neomycin, gentamicin, lincomycin, bambermycin) are generally known for their ototoxic and nephrotoxic properties in both animals and humans (Drusano et al., 2007). Damage to the ear, primarily the vestibular and cochlear nuclei, has been particularly noted in the case of children exposed to these compounds during pregnancy (Al-Aloul et al., 2004; Matz et al., 2004; Selimoglu, 2007). The incidences of mild to severe renal effects in patients treated with these antibacterials have been reported at around 10-25% (Taber and Pasko, 2008). Additionally, allergic reactions associated to these compounds have been reported (Tinkelman and Bock, 1984; Faridah et al., 2004). The Federal government has imposed very low tolerance levels and

long withdrawal periods for these compounds in food products, particularly for neomycin, because these compounds tend to remain in the ear and kidneys for prolonged periods of time, sometimes months, after treatment (Stead, 2000; Gehring et al., 2005).

h. Quinolones or fluoroquinolones (oxolinic acid, nalidixic acid, flumequine, enrofloxacin, sarafloxacin, danofloxacin, orbifloxacin, marbofloxacin, gatofloxacin, grepafloxacin) have been widely used in animal production. These compounds are associated with potential damage to articular cartilages and tendons (particularly in children), as well as myalgia (Eisele et al., 2009) and neurological disorders (depression, confusion, anxiety; Takizawa et al., 1999a, 1999b; Ambrose et al., 2007; Kiangkitiwan et al., 2008). Some of these antibacterials, such as enrofloxacin, danofloxacin and orbifloxacin, have been prohibited from any use in food animals due to their possible role in the development of bacterial resistance (FDA, 2005a). Concerns regarding resistance associated with fluoroquinolone use in animal production were first expressed by Elam and co-workers (1951) and are an important factor in the regulation of these compounds (Swann Report, 1969; Piddock, 1996; NRC, 1999; Tollefson and Flynn, 2002; Angulo et al., 2004; Grugel and Wallmann, 2004; Turnidge, 2004; FDA, 2010; Sharfstein, 2010).

3. REGULATION OF VETERINARY DRUGS AS POTENTIAL SOURCES OF RESIDUES IN ANIMAL FOOD PRODUCTS

The federal regulatory oversight of the safety of veterinary drugs can be divided into two different stages: the premarket approval determination which establishes if a

drug is safe for use and the post-approval monitoring to ensure drugs are used correctly and to test food samples for violative residues (Botsoglou and Fletouris, 2001).

3.1. Pre-approval regulatory process regarding veterinary drug residues in animal food products

The premarket process is the responsibility of the Food and Drug Administration (FDA) through the Center for Veterinary Medicine (CVM). The approval process starts when the sponsor submits an investigational veterinary pharmaceutical product (IVPP) application to the Office of New Animal Drug Evaluation (ONADE) at the FDA-CVM, in accordance with the Title 21 of the Code of Federal Regulations (CFR), Parts 500 to 599. These CFR sections govern animal drugs, animal feeds and associated products; as well as specific regulations on registration, labeling and good manufacturing and laboratory practices (also included in Parts 200 to 299). Additionally, recent efforts for international harmonization of regulatory requirements for veterinary medical products have been published by the International Cooperation of Technical Requirements of Veterinary Medicinal Products (VICH) of the World Organization for Animal Health (OIE) and adopted by its members, including the U.S. and the European Union.

The FDA-CVM requires that the sponsor of all potential new veterinary pharmaceutical products conduct research in five main areas: 1) drug's efficacy, 2) safety of the drug for the target animal, 3) safety for the consumers of food products derived from those animals, 4) environmental impact and 5) quality of the manufacturing processes (NRC, 1999; VICH-FDA, 2008; FDA, 2009a; 21CFR514, 2010; 21CFR500.84, 2010).

As part of the evaluations on the food safety of products derived from medicated animals, the sponsor must consider the known pharmacologic and toxicologic properties of the active pharmaceutical ingredient (API), as well as conduct experiments to determine the acute and chronic toxic potential of the drug for each proposed application, (VICH-FDA, 2008; 21CFR500, 2010). Experimental animals are administered various doses of the compound to detect any toxic effects, such as the allergenic, carcinogenic, teratogenic or mutagenic properties of the drug (VICH-FDA, 2008). An important goal of these studies is to determine the dosing level that causes no harmful effects (Fink-Gremmels and van Miert, 1994; Donoghue 2003; Hurtt et al., 2003; FDA, 2009a). This level is called the no-observable adverse effect level (NOAEL or NOEL) and is used to establish a tolerance level for residues in edible tissues (Craigmill and Cortright, 2002). The tolerance level is established by taking the antibacterial dose at the NOAEL, reducing this dose by 100- or 1,000-fold (safety factor) and multiplying by the average daily intake of the edible tissue (e.g., poultry muscle) for a 60-kg adult. A safety factor of 100 is usually applied for antibacterials already in use (e.g., other animals, humans) with a known safety record. Otherwise, a safety factor of 1,000 is used (Donoghue, 2003).

The tolerance level is defined as the safe concentration of residues that are permitted in human food products (NRC, 1999; Paige et al., 1999b; Botsoglou and Fletouris, 2001; 21CFR530 and 556, 2010). Tolerance levels are determined for each individual edible product and are specific for each drug and animal species (Clement, 1995; Paige et al., 1999b; Botsoglou and Fletouris, 2001; 21CFR500.86, 2010; 21CFR556, 2010). If a tolerance level cannot be imposed on a drug due to potential toxic

effects to consumers, the antibacterial will be banned from use in food producing animals and any use would be considered illegal, as is the case with chloramphenicol (Hanekamp and Kwakman, 2004). Once the tolerance level has been determined, the FDA-CVM will provide the appropriate dosage protocols for the drug, and specific sampling procedures for monitoring of residues in these animals (21CFR514, 2010). This information must be included on the label of the product, or any written, printed or graphic material accompanying the product (21CFR514, 2010).

Studies are also conducted to evaluate the patterns of absorption, distribution, biotransformation and excretion of the drug, as well as the amounts, persistence and nature (parent drug and/or metabolites) of any drug derived residue in the edible tissues of the treated animal (Riviere, 1991; Martín-Jiménez and Riviere, 1998). The results of these studies and those from the total residue depletion study and metabolism studies are used to identify possible reservoir sites and determine the time required for the depletion of all drug related residues from the animal and its edible tissues (Clement, 1995; CVM, 2006; 21CFR500.86, 2010).

Based on the results of these safety studies, the FDA will impose and publish the conditions for drug usage for each approved animal species. If approved drugs are used properly and in compliance with label directions, there should be no violative residues (those that exceed the tolerance) in edible food products from treated animals. In several countries, the regulatory agencies have established tolerance limits for acceptable daily intake (ADI) and tolerance limits (also called maximal residue limits or MRLs in the European Union) for the amount of the drug and/or its metabolites that might be present

in animal products. Nonetheless, residues exceeding tolerances do occur requiring monitoring to detect and take remedial action to prevent its reoccurrence.

3.2 Post approval monitoring process for veterinary drug residues in animal food products

The government agencies primarily responsible for post approval monitoring of drug residues in food products are the USDA though the Food Safety and Inspection Service (USDA-FSIS) and the FDA. The USDA-FSIS is responsible for monitoring meat and poultry in federally inspected establishments, as well as and liquid eggs whereas the FDA monitors residue levels in milk and shell eggs (FDA, 1997; FDA, 2003; Botsoglou and Fletouris, 2000; FSIS, 2007). FSIS reports violative residues of drugs and both violative and non-violative residue levels of pesticides, veterinary drugs and other contaminants to the FDA (FDA, 2005b, 2005d). Since the late 1960s, the USDA-FSIS has administered the National Residue Program to collect data on chemical residues in domestic and imported meat, poultry and egg products. This program is designed to provide: 1) a structured process for identifying and evaluating compounds of concern by production class (including approved and unapproved compounds); 2) capability to analyze for compounds of concern; 3) appropriate regulatory follow-up of reports of violative tissue residues and 4) collection, statistical analysis and reporting of the results of these activities (FSIS-NRP, 2008).

Monitoring procedures are designed to detect and prevent potentially harmful concentrations of residues entering the food supply. The two federal agencies primarily responsible for monitoring veterinary drug residue in foods are the FDA and USDA.

These sister agencies cooperate to monitor and detect violative residues (concentrations of residues that exceed the imposed tolerance levels) or illegal residues (residues of drugs specifically prohibited from animal use; Craigmill and Cortright, 2002; Fajt, 2003). A current listing of drugs prohibited for use in animals is codified in 21CFR530 (2010)

The most obvious reason for the presence of violative residues is a failure to observe the recommended withdrawal period for the specific drug. This may be deliberate or accidental. In the first instance, farmers may deliberately send sick animals to slaughter to avoid condemnation of the carcass and monetary loss for the producer (Courtheyn et al., 2002; Morley et al., 2005). Furthermore, as the sampling procedure for drug residue testing is often nationwide and quite random, the chances of catching such deliberate acts are slim. This limited sampling can lead to a continuation of such practices by farmers. Farmers can also mistakenly give medicated feed to animals that should be on non-medicated feed. Thus, monitoring of residues in foods provides information on failure to follow good animal husbandry practices, misuse of drugs in medicated animal feeds, marketing of treated/medicated animals intended for rendering purposes, and inadequate animal identification and/or record keeping (Jones and Seymour, 1988). It can also indicate illegal sale of veterinary drugs, incorrect extra-label use (which includes inadequate pre-slaughter withdrawal period), cross-contamination of animal feeds at the feed mill or drug production errors due to incorrect implementation of Good Manufacturing Practices (GMPs; Brynes and Weber, 1996; 21CFR225 and 226, 2010).

Scheduled sampling plans consist of the random sampling of tissue from healthy appearing food animals in accordance to the available intelligence reports on the

prevalence and incidence of specific compounds in food products in a specific location (FSIS-NRP, 2008). These sampling plans are devised with the cooperation of members of the NRP-Surveillance Advisory team, that includes members from the Environmental Protection Agency (EPA), the Food and Drug Administration (FDA), the Center for Disease Control and Prevention (CDC), the Animal and Plant Health Inspection Service (APHIS), the Agricultural Marketing Service (AMS), the Agricultural Research Service (ARS) and members from the FSIS. Based on the recommendations of this committee, the NRP 1) proposes analytical procedures for identifying and evaluating drugs of concern by production class; 2) prioritizes drugs for monitoring; and 3) develops reports on the incidence of residue violations (FDA, 2005b).

In-plant generated sampling occurs when the public health veterinarian suspects that an animal or flock may have been treated with a pharmacologically active compound which might create residues. This decision to select and test a specific carcass is based on professional judgment and criteria outlined in FSIS Directives 10,800.1 and 10,220.3 (FSIS, 2007). These criteria may include animal disease signs and symptoms, producer history or results from random scheduled sampling. When an inspector generated sample is collected, the carcass is held pending the results of laboratory testing. If a carcass is found to contain levels of residues that exceed the imposed tolerance level for that product in that specific food product, the carcass is condemned (FSIS-NRP, 2008). In addition to being condemned, producer may be subject of legal sanctions (Dey et al., 2003). An open record of all notifications and incidents is posted on the websites of USDA-FSIS and the FDA and sent to the Residue Violation Information System (RVIS), the Microbiological and Residue Computer Information System (MARCIS) and the

Tissue Residue Information Management System (TRIMS). These systems facilitate the regulatory follow-up on violations and tracking of residue violators by both FSIS and FDA. Once violative residues are detected, the FDA will conduct an official inquiry, financed by the offending producer, which can lead to legal actions that range from monetary fines to criminal prosecution, if evidence of prior violations or intent can be documented (Guest and Paige, 1991; NRC, 1999; FDA, 2000; Botsoglou and Fletouris, 2001; FDA, 2005b; FSIS-NRP, 2008). A detailed description of these procedures can be found in the FDA's guidance document 7371.006 (FDA, 2005c).

An important aspect of residue monitoring is the development of analytical methods for specific drugs and their target tissues (e.g., Schneider and Donoghue, 2000; Chu et al., 2000; Heller et al., 2002; Schneider and Donoghue, 2002; Donoghue and Schneider, 2003; Schneider and Donoghue, 2004; Reyes-Herrera et al., 2005; Schneider et al., 2007; Reyes-Herrera and Donoghue, 2008). As part of the pre-market approval process, the sponsor has to develop a method to evaluate quantitatively and qualitatively the relationship between the parent drug and its metabolites in the target tissue in accordance to the guidelines for the approval of methods for analysis for residues (Clement, 1995; Donoghue, 2003; 21CFR500.86, 2010; VICH-FDA, 2010b). This method is known as the official analytical method and usually consists of surveillance and a confirmatory method. This method is published in the Code of Federal Regulations (Engel, 1994; NRC, 1999; 21CFR180, 2010; 21CFR556, 2010). A list of the official methods for the determination and confirmation of the specific drug residues are also included in the annual publications from the NRP and in the USDA-FSIS Chemistry Laboratory Guidebook (FSIS, 2008).

4. MONITORING OF RESIDUES IN FOOD PRODUCTS

After considering the vast diversity of chemicals that could possibly contaminate food products and their numerous potential consequences on the health of consumers, the magnitude, difficulties and importance of an efficient and vigilant residue prevention program are obvious. In the 2008 USDA-FSIS National Residue Program Scheduled Sampling Plans (Blue Book) there were 22 different types of production classes considered including different types of bovine, sheep and goat, swine and poultry, products. The Blue Book lists the veterinary drugs to be monitored and the number of samples to be collected from each class during the year. In brief, this program included approximately 23,000 samples in the domestic sampling plan, about 5,000 samples in the import sampling plan for a total of roughly 28,000 samples a year. This is in addition to samples collected from suspicious carcasses by the inspectors at the slaughterhouses and processing plants (FSIS-NRP, 2008). Similarly, the report of the FDA-Residue Monitoring Program for the fiscal years 2004-2006 included over 7,000 samples collected from food products such as grains and grain products, milk and dairy products, eggs, fish, shellfish and other aquatic products, fruits, vegetables, nuts and edible seeds, water, snack foods, as well as the feed used for food production animals. All of these tests are conducted in the raw ingredients and as part of the Total Diet Study (FDA-TDS, 2008, 2009).

It is important to consider that in most cases the complexity of the food matrices and the different physico-chemical characteristics of each possible contaminant make it difficult for the development of analytical methods appropriate for a great variety of

contaminant/food type combinations (Marazuela and Bogialli, 2009). Accordingly analytical procedures need to be developed or modified for the specific type of contaminant and food matrix to be analyzed. Additionally, to improve validation of analytical methods for most contaminants in foodstuffs there is a need for the development of certified reference materials and matrix blank materials for each of the different types of food matrices (Zeleny et al., 2006). As analytical methods have become more sensitive and accurate, sample preparation plays a more critical role in the analytical process. It has been estimated that approximately 50-70% of the time spent on residue analysis is used for sample preparation and this also accounts for a large part of the cost of analysis (Buldini et al., 2002; Hagren et al., 2005). For example, complex food sample matrices such as muscle also contains connective tissue, nerves and fat that needs to be homogenized before any analysis can be conducted. If the residues (usually composed of parent compound and metabolites) are present in a free form they can generally be separated from the homogenized food matrix with simple aqueous buffers or solvents, but if conjugated or bound residues are formed, then the additional use of solvents, proteolysis or hydrolysis steps may be required for accurate determination (such as is the case with nitrofurans and florfenicol; Kinsella et al., 2009). In some cases, the food matrices may possess intrinsic substances that interfere with detection of the compound, as it can occur with muscle and liver (McGlinchey et al., 2008). These difficulties must be considered for residue analysis.

In contrast to the difficulties of measuring residues in organs and tissues, most compounds can be easily quantitated from body fluids (blood, plasma, serum, urine, exudates) and only a simple dilution step may be required before analysis (Bacigalupo et

al., 1995; Elliot et al., 1996; Hagren et al., 2005). In humans, plasma, saliva or urine samples are used to monitor drug concentrations in therapeutic treatment or to determine drug abuse. This sampling strategy has been used for many years and is widely accepted as evidence of drug exposure and to estimate concentrations in the body (Dickson et al., 1994; Rivier, 2000; Hammet-Stabler et al., 2002; Kim et al., 2002; Rigamonti et al. 2005). For animals, several researchers have proposed the use of body fluids as predictive indicators of the concentration of residues in target tissues that are intrinsically more difficult to analyze (Ashworth et al., 1986; Crooks et al., 1998a, b; Peippo et al., 2005; Chiesa et al., 2006a; 2006b; Heller et al., 2006; Haasnoot et al., 2007; Schneider et al., 2007; 2009; Schneider and Lehotay, 2008). However, the number of studies in this area are limited.

A couple of studies on the disposition of sulfonamides in sheep identified similarities in the pharmacokinetic patterns of these antibacterials in different tissues and body fluids (Bevill et al., 1977a; 1977b). Subsequently, Ashworth and colleagues (1986) evaluated the use of blood samples as a means for pig producers to detect animals with possible violative or illegal residue concentrations of sulfamethazine before shipping the animals for slaughter. They reported that the concentration of residues in liver (the target tissue) would exceed the tolerance limit when concentration in blood exceeded the 45 ppb (Ashworth et al., 1986). In cattle, different studies explored the use of blood as a predictor of the concentration of antibacterial residues as a preslaughter test (Chiesa et al., 2006a; 2006b). In these studies, the researchers also measured concentration of residues in urine and reported that this measurement was highly variable depending on factors such as the time stored in the bladder and pH of the urine (Chiesa et al., 2006b).

In poultry only one study has examined the blood: tissue relationship of antibacterial residues. Haasnoot and co-workers (2007) evaluated the use of body fluids as markers for presence of different sulfonamides in different edible tissues (skin and fat, liver, muscle) of broiler chickens. These researchers reported that the blood: tissue ratio varied significantly depending on the tissue, with higher concentrations in blood and blood: tissue ratios of 6.2:1 in muscle. In an effort to evaluate the utility of using blood to monitor for residues in muscle, we conducted studies to determine the pharmacokinetic relationship between blood and muscle for two different antibacterials (enrofloxacin and oxytetracycline) in market aged broiler chickens.

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CHAPTER II

THE RELATIONSHIP BETWEEN BLOOD AND MUSCLE SAMPLES TO MONITOR FOR RESIDUES OF THE ANTIBIOTIC ENROFLOXACIN IN CHICKENS

Full-Length Paper prepared for the Processing, Products and Food Safety Section

The Relationship between Blood and Muscle Samples to Monitor for Residues of the Antibiotic Enrofloxacin in Chickens[1,2]

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ABSTRACT

In 2005, the United States Food and Drug Administration withdrew approval for use of enrofloxacin in poultry, thus effectively imposing a zero tolerance for residues of this antibiotic in poultry. Conventional residue monitoring for most antibiotics, including enrofloxacin, involves removing poultry carcasses from the processing line and collecting muscle tissues for analysis. Because of the loss of valuable edible products and difficulties and expense in sampling all the carcasses, only a small portion of carcasses are tested for violative residues. Unlike muscle tissue, blood is readily available from all birds at the beginning of processing and may be used to screen for illegal residues in all poultry carcasses. It is unknown, however, if enrofloxacin concentrations in blood are predictive of muscle concentrations. In an effort to evaluate this relationship, 156 broiler chickens, 5 weeks of age, were dosed with either 25 or 50 μg mL^{-1} enrofloxacin for 3 or 7 days, respectively, in the drinking water. Blood and muscle samples were collected at 0, 1, 3, 6, 12, 24 hours (n=6 birds/group) during the first dosing day, every 48 hours during the dosing period, and every 12 hours during withdrawal period for up to 60 hours post withdrawal. Enrofloxacin residues were determined in all blood and tissue samples during the dosing periods and most of the withdrawal period for both doses. These results support the potential to use blood to screen for illegal enrofloxacin residues in edible poultry tissues in an effort to protect the human food supply.

Key words: Antibiotic residues, Monitoring, Enrofloxacin, Blood: tissue ratio, Broiler

INTRODUCTION

Enrofloxacin is a fluoroquinolone class antibiotic that was developed and used exclusively in veterinary medicine. The United States Food and Drug Administration (FDA) approved different specific applications of this drug in 1996, including its use to treat bacterial infections in poultry (Anderson et al., 2003). In 2005, however, the FDA withdrew its approval for any use in avian species (including extra-label use; FDA, 2009; 21CFR530.41, 2010) due to information that linked the use of fluoroquinolones to the development of antibiotic resistant strains of *Campylobacter*, an important human food borne pathogen (Anderson et al., 2003; FDA, 2005; Gemer-Smidt and Whichard, 2008). Thus, after this ban it is illegal to dose poultry with any fluoroquinolone and there is a zero tolerance level for residues in poultry in the United States (FDA, 2005; FDA, 2009). Although withdrawn, it is still possible for there to be illegal use of this antibiotic, which could result in enrofloxacin residues in edible poultry tissues.

In an effort to protect consumers from potential toxic effects associated with concentrations of residues which exceed established tolerances in foods (e.g., antibiotic, pesticides or other chemicals), the U.S. Federal government has implemented specific programs to monitor for violative residues in food products and prevent their entrance into the food supply (FDA, 1997; Donoghue, 2003; Cerniglia and Kotarski, 2005; FSIS-NRP, 2008). In the specific case of monitoring of antibiotic residues in poultry meat products, it is the responsibility of the United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) to ensure the safety and wholesomeness of these products, in accordance to the Poultry Products Inspection Act (PPIA; 21 U.S.C. 451 et. seq.; FSIS, 2001; FDA, 2008; U.S. Code of Federal Regulations, 2010) and the

stipulations of the National Residue Program (USDA-FSIS-NRP). The USDA-FSIS inspectors collect samples at the poultry processing plants for monitoring purposes and the tissue selected for monitoring is called the target tissue. This is the tissue with the greatest antibiotic concentration and persistence, and for enrofloxacin in poultry, the imposed target tissue for residue monitoring is muscle (21CFR556.228, 2002) with the highest concentration in the breast muscle (Reyes-Herrera et al., 2005, Reyes-Herrera and Donoghue, 2008). Because the monitoring process for enrofloxacin requires the collection and destruction of the muscle tissue for analysis, and due to this loss of product and the large number of birds being processed, the number of samples evaluated per flock is limited (see the sampling protocols for monitoring purposes, FSIS-NRP, 2008). Alternatively, the monitoring of enrofloxacin residues in blood in the processing plant could provide a fast and effective means to screen for the presence and concentrations of this antibiotic in a large number of processed poultry. Blood samples are readily available from all birds at the beginning of processing, would be easy to collect, wouldn't interfere with the processing procedure or require removal of the parts of the carcass and have no economic value.

Previous work conducted to develop analytical methods to detect fluoroquinolones residues in chicken tissues (Schneider et al., 2007) indicated a potential relationship between blood and muscle concentrations. Therefore, the objective of this follow-up study is the first in a series of studies to evaluate if blood antibiotic concentrations (in this case enrofloxacin) are predictive of residue concentrations in muscle tissues. Because the target tissue for most antibiotics in poultry is muscle, these results may support the potential to use blood to screen for other antibiotic residues.

MATERIALS AND METHODS

Enrofloxacin Dosing Protocol

A total of 156 day-old male broiler chickens were obtained from a local hatchery and divided at random into two separate treatment groups. All birds had ad libitum access to a standard non-medicated broiler diet (starter and grower) and non-medicated (pre-dosing period) or medicated water (dosing period). Starting at day 33, the birds were dosed with enrofloxacin (Baytril®) in the drinking water, at either of the two different doses: 25μg mL^{-1} for 3 days or 50 μg mL^{-1} for 7 days, in accordance with the formerly FDA approved label directions for use of the antibiotic in poultry. Medicated water was prepared fresh daily.

Sample Collection and Preparation

Blood and breast muscle samples were collected from 6 birds per group at each collection point. Samples were collected immediately prior to initial dosing (controls, n=6 birds/2 pens); on day one of the dosing period samples were collected at 0, 1, 3, 6, 12, 24 hours, and then every 48 hours until the end of the dosing period. Finally, samples were collected every 12 hours for up to 60 hours post withdrawal. All samples were processed individually; blood samples were collected in sterile test tubes, and allowed to clot for approximately 30 minutes at room temperature and then centrifuged (1,500 x g, 10 min) to separate the serum fraction, which was then used for antibiotic determination. Serum and muscle samples were stored at -80°C until all the samples were collected. Muscle samples were homogenized using a standard tissue homogenizer (Omni

International). All samples were diluted 1:3 (wt/vol) with 1% phosphate buffer, pH 9.0 and centrifuged at 1,500 x g for 15 minutes at 5°C. The supernatant was decanted and stored at -80°C until assayed.

Sample Analysis

All serum and muscle samples were analyzed for fluoroquinolone residues using the quantitative bioassay as described previously by our laboratories (Schneider and Donoghue (2004) and Reyes-Herrera and Donoghue (2008). Briefly, on the day of the assay, Petri dishes (100 mm in diameter) were filled with 8 mL of Mueller-Hinton Agar inoculated with approximately $1x10^6$ cfu/mL of *Klebsiella pneumoniae* (ATCC 10031), as indicator organism, and then 6 penicylinders (8x10mm) were evenly placed on the agar. A standard curve was constructed by addition of known amount of enrofloxacin in buffer, with each standard concentration pipetted onto 3 plates; 3 alternate cylinders were filled with a known standard (200 μL each), and the other 3 cylinders were filled (200 μL each) with a reference concentration. Individual samples were assayed in a similar manner to standards except samples were assayed on one plate. Plates were incubated at 37°C for approximately 16 h. Plate averages for the standards and serum or muscle samples were corrected to the overall reference concentrations. The lower limit of assay sensitivity was approximately 15 ng g^{-1}. A best-fit regression line was calculated by the method of least squares using the diameter of the inhibition zones (mm) with a zone reader (Fisher-Lilly, Pittsburgh PA).

RESULTS

Fluoroquinolone residues were quantitated in all blood and muscle samples during the dosing periods and for up to 24 hours or 60 hours post-withdrawal for both dosing groups (Fig. 1-4). The concentration of fluoroquinolone residues in muscle were higher than those in blood at every sampling time, both during the dosing (Figures 1 and 2) and withdrawal periods (Figures 3 and 4), for both treatment groups (low dose at 25 μg mL^{-1} for 3 days and high dose at 50 μg mL^{-1} for 7 days). After the first hour of dosing and for the first 24 hours after drug withdrawal, fluoroquinolone residues were approximately twice the concentration in muscle than blood. Concentrations of residues peaked at day 3 of the dosing period for both treatment groups (for the low dose group 710±64 ng g^{-1} or 379±92 ng mL^{-1} for muscle versus blood, respectively, and for the high dose group 1538± 97 ng g^{-1} or 705±59 ng mL^{-1} for muscle versus blood, respectively).

DISCUSSION

In this study, fluoroquinolone residues were detectable in both muscle and blood samples during both the dosing (Figures 1 and 2) and most of the withdrawal periods (Figures 3 and 4) which suggests that testing blood may be an effective strategy to screen for illegal enrofloxacin concentrations in poultry. In addition, incorporation of data from the two tissues showed a similar kinetic update pattern of residue incorporation during the dosing and withdrawal periods, with quantifiable levels in muscle and blood starting at the first sampling period (1 hour) after dosing (Figures 1 and 2).

The similarities between the incorporation patterns for blood versus muscle support not only the ability to use blood to screen for residues in muscle but also to estimate muscle residue concentrations. Enrofloxacin has been described as having a

high tissue:blood concentration ratio (Garcia Ovando et al., 1999). In this experiment we observed that the tissue:blood correlation for fluoroquinolone residues between chicken muscle and blood was approximately 2:1 for both treatment groups (25 μg mL^{-1} for 3 days and 50 μg mL^{-1} for 7 days) during most of the dosing and withdrawal periods. In other words, if a concentration of 100 ng mL^{-1} is determined in the blood, the concentration of fluoroquinolone residues in muscle would be estimated to be approximately 200 ng g^{-1}. This approximate relationship is consistent with results obtained from therapeutic efficacy (Knoll et al., 1999) or liquid chromatography-fluorescence-mass spectrometry (HPLC/MS) methods development studies (Schneider et al., 2007) using incurred poultry blood and tissues samples.

The only times residues were detected in muscle but not blood was when concentrations were extremely low by 36 hours after drug withdrawal for birds receiving the lowest dose of enrofloxacin (25 μg mL^{-1} / 3days). Therefore, it is possible that at very low concentrations of enrofloxacin, evaluating blood would not accurately reflect illegal residues in muscle. However, the ability to screen a greater number of poultry carcasses using blood, which would still identify the majority of enrofloxacin contamination, should be preferable to sampling only a small portion of carcasses for muscle contamination. Furthermore, although the assay used in this study is considered reasonably sensitive (15 ng g^{-1}), the development of more sensitive methods which could detect residues in blood at even lower concentrations could resolve this issue.

The use of body fluids, such as blood, urine and kidney exudates, has also been proposed by several researchers to determine and monitor drug concentrations in live animals and in animal carcasses of swine (Ashworth et al., 1986); cattle (Lifschitz et al.,

1999; Chiesa et al., 2006a; 2006b; Heller et al., 2006); sheep (Bevill et al., 1977a, 1977b); elk (Clark et al., 2004) and poultry (Pant et al., 2005; Haasnoot et al., 2007; Schneider et al., 2007; 2009; Schneider and Lehotay, 2008). Furthermore, this type of analysis is widely used to determine concentrations of drug levels in humans (Dickson et al., 1994; Rivier, 2000; Hammet-Stabler et al., 2002; Rigamonti et al., 2005). With regards to poultry production, blood is available for sampling during standard activities at the processing plant and is a simple, inexpensive, relatively constant matrix to evaluate and does not require special, complicated or expensive preparations prior to analysis. Sampling blood may also eliminate many issues with specific tissue characteristics (e.g., muscle) which may complicate sample preparation and/or analysis (Okerman et al., 1998; Kubala-Drincic et al., 2003; Schneider et al., 2007).

The use of blood samples as an initial screening method to detect illegal drug residues in animal carcasses could represent a simple and inexpensive option to detect adulterated food products, thus reducing the number of target tissue samples that would have to be collected and analyzed with the approved monitoring method (e.g., HPLC/MS). Moreover, if the relationship between blood and the target tissue could be established for other antibiotics and veterinary drugs, this approach could increase the number of animals being tested at processing facilities and thus effectiveness of residue screening. Additional studies are currently underway to determine if tissue:blood relationships determined in the present study apply to other antibiotics used in poultry.

Figure legends

Figure 1. Concentrations of enrofloxacin residues determined during the dosing period (3 days) from blood and breast muscle tissue samples collected from broiler chickens dosed with 25 μg mL^{-1} of enrofloxacin (n=42 birds). Each time point represents the concentrations (average± SEM) from the samples collected from 6 birds.

Figure 2. Concentrations of enrofloxacin residues determined during the dosing period from blood and breast muscle tissue samples collected from broiler chickens dosed with 50 μg mL^{-1} of enrofloxacin for 7 days (n=54 birds). Each time point represents the concentrations (average± SEM) from the samples collected from 6 birds.

Figure 3. Concentrations of enrofloxacin residues determined during the withdrawal period (60 hours) from blood and breast muscle tissue samples collected from broiler chickens dosed with 25 μg mL^{-1} of enrofloxacin for 3 days (n=30 birds). Each time point represents the concentrations (average± SEM) from the samples collected from 6 birds.

Figure 4. Concentrations of enrofloxacin residues determined during the withdrawal period (60 hours) from blood and breast muscle tissue samples collected from broiler chickens dosed with 50 μg mL^{-1} of enrofloxacin for 7 days (n=30 birds). Each time point represents the concentration (average± SEM) from the samples collected from 6 birds.

Figure 1

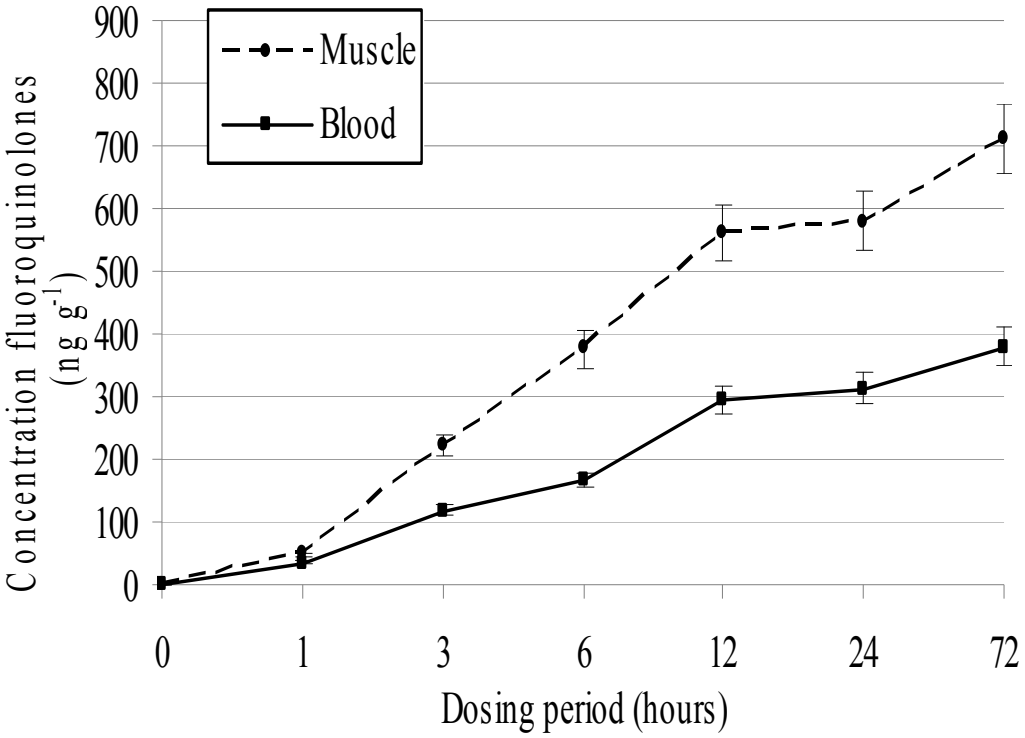

Muscle
Blood
Concentration fluoroquinolones (n g g-1)
900
800
700
600
500
400
300
200
100
0
0
1
3
6
12
24
72
Dosing period (hours)

Figure 2

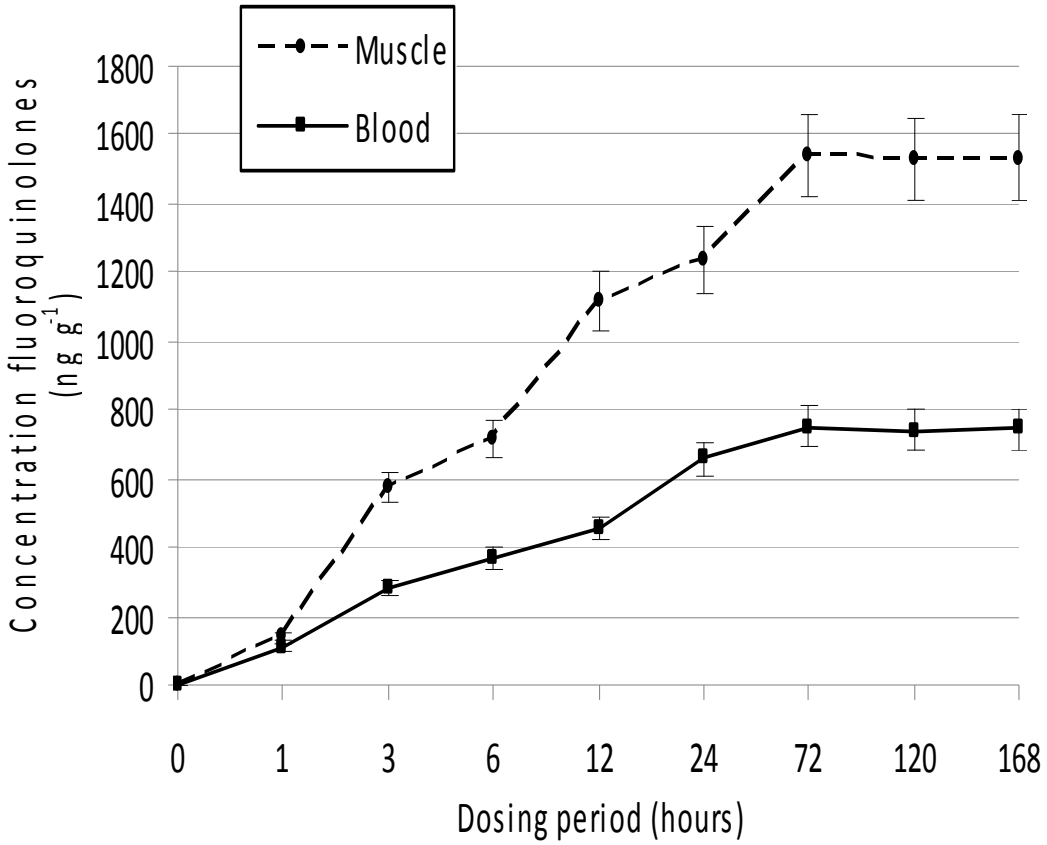

Muscle
Blood
Concentration fluoroquinolones (ng g-1)
1800
1600
1400
1200
1000
800
600
400
200
0
0
1
3
6
12
24
72
120
168
Dosing period (hours)

Figure 3

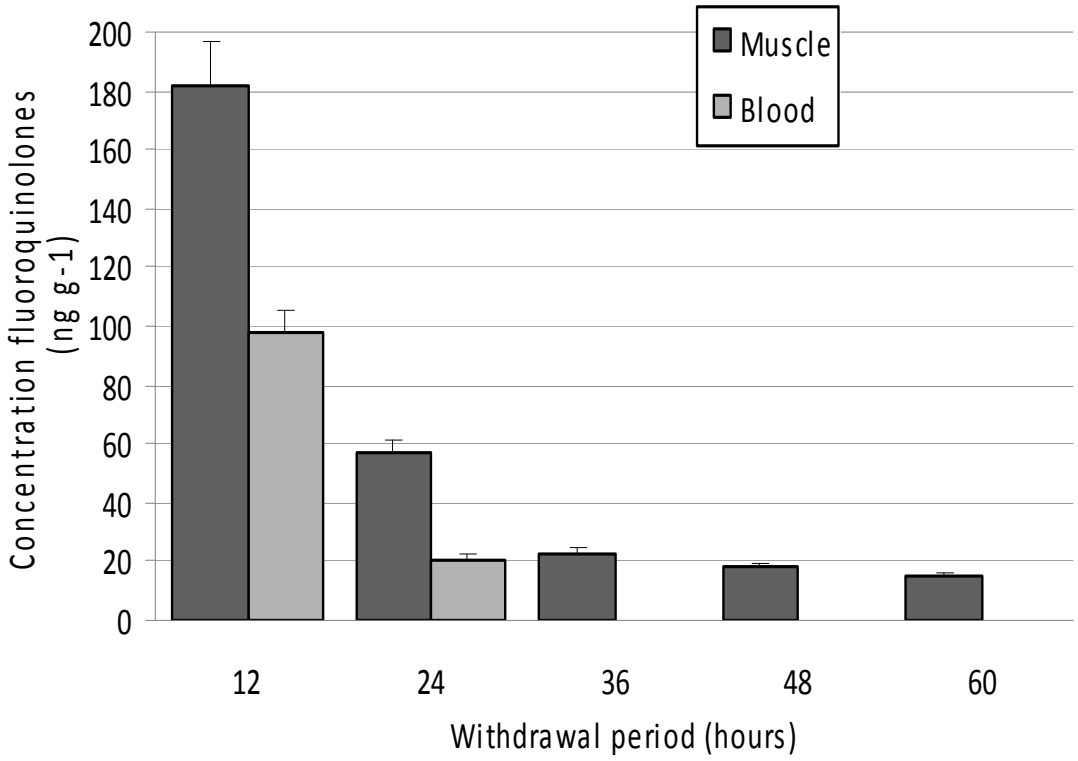
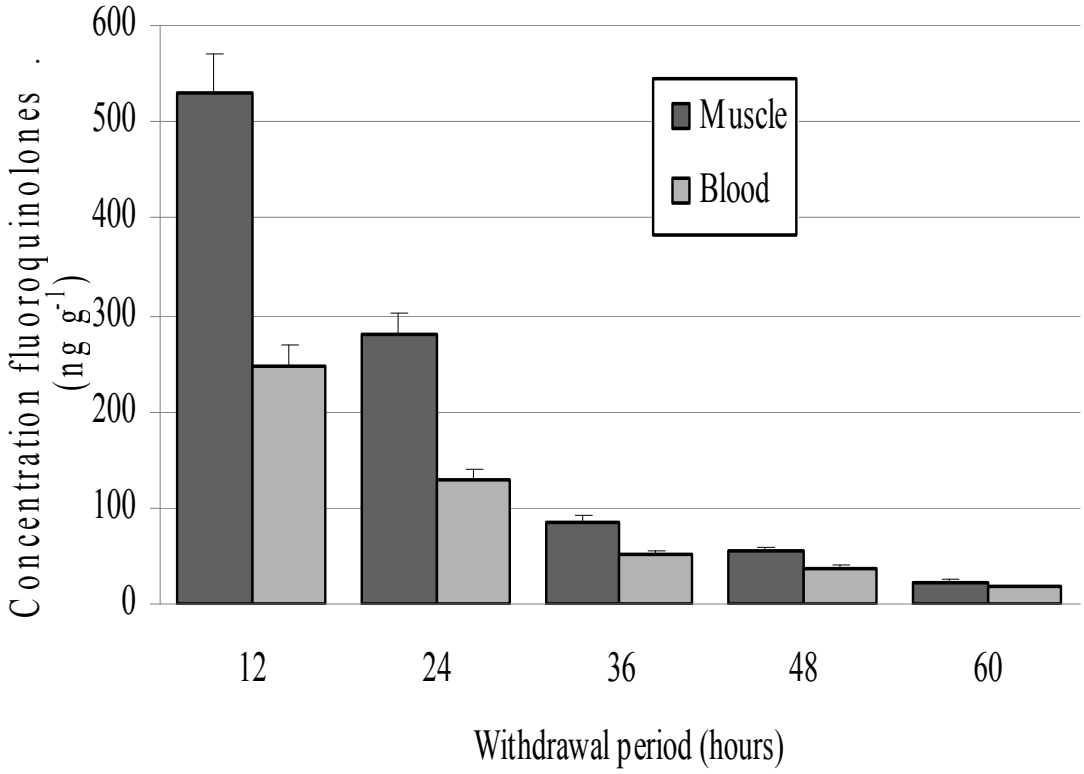

Muscle
Blood
Concentration fluoroquinolones (ng g-1)
200
180
160
140
120
100
80
60
40
20
0
12
24
36
48
60
Withdrawal period (hours)

Figure 4


600
500
400
300
200
100
0
Concentration fluoroquinolones . (ng g-1)
Muscle
Blood
12
24
36
48
60
Withdrawal period (hours)

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CHAPTER III

THE ABILITY TO PREDICT OXYTETRACYCLINE ANTIBIOTIC RESIDUES IN POULTRY MUSCLE TISSUES BY SCREENING BLOOD SAMPLES

The Ability to Predict Oxytetracycline Antibiotic Residues in Poultry Muscle Tissues by Screening Blood Samples.

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ABSTRACT

The presence of antibiotic residues in edible animal products is a human food safety concern. To address this potential problem, the government samples edible tissues, such as muscle, to monitor for residues. Due to loss of valuable product and analytical difficulties only a small percentage of poultry carcasses are tested. Alternatively, antibiotic residue concentrations could be screened in blood, which is readily available during carcass processing. To determine if blood concentrations are predictive of muscle concentrations, 252 market aged broilers were dosed with the antibiotic oxytetracycline (OTC) in water at three doses: the maximum OTC approved dose for broilers (800 mg/gal) or five or ten times that dose (4,000 or 8000mg/gal, respectively). Blood and muscle samples were collected prior to initial dosing (0 hour, controls), during the dosing period at 1, 3, 6, 12, 24, 48, 96 or 144 hours and at 12, 24, 36, 48 or 60 hours after drug withdrawal. Residues of OTC in blood and muscle were determined using a microbial inhibition method. Concentrations in both blood and muscle tissue followed similar time: concentration patterns, peaking 24 hours after initial dosing (396±9 vs. 557±37 ppb; 1,443±48 vs. 1,846±58 ppb or 2,447±67 vs. 3,210±36 ppb for the 1, 5 or 10x doses in blood vs. muscle respectively) and declined rapidly after withdrawal. These data suggest that blood samples may be used to predict OTC concentrations in muscle (by multiplying blood concentrations by 1.3) as a screening procedure for OTC residues in poultry.

Keywords: *Antibiotic residues, monitoring, oxytetracycline, blood: tissue ratio, chicken*

INTRODUCTION

The potential presence of antibiotic residues in food products is a major source of concern as they may induce adverse health effects in consumers (Bruhn, 1999; Gorbach 2001; Tilman et al., 2002). In an effort to prevent these problems, the Federal Government has developed strict protocols for the approval, use and monitoring of all veterinary drugs, particularly those used in food producing animals (Fink-Gremmels and van Miert 1994; Donoghue 2003; Hurtt et al., 2003; FDA, 2009).

Monitoring procedures are based on sample collection and analysis of edible tissues at slaughter and processing facilities around the country (FSIS, 2009; FSIS-NRP, 2009). These samples are collected from the edible tissue with the highest concentration and longest persistence of the residues in the body (called the target tissue), and used as an indicator of the concentration of residues in the rest of the carcass, as stipulated in the Code of Federal Regulations (Brynes, 2005, Federal Register, 2001).

Unfortunately, there are disadvantages using these procedures for sampling and analysis of samples for residue determination. First, sample collection usually involves the destruction of the edible tissue used for testing, with subsequent monetary loss for the producers (Clement, 1995). This is particularly true due to the large sample sizes which can be required for the determination of trace residues and contaminants in foods to improve the limits of detection of the assay (Ridgway et al., 2007). Second, as the processing lines for poultry move at speeds of approximately 70-160 birds per minute (Berrang et al., 2007, 21CFR381, 2010), sample collection can be difficult. Finally, due to the great variability between the different potential types of tissues used for monitoring purposes (e.g. muscle, liver, kidney, skin, fat, etc.), the development of specific

procedures for sample preparation and analysis is required (Okerman et al., 1998; Elliot, 2000; Andes and Craig, 2002; Berendsen and van Rhijn, 2006; Ridgway et al., 2007). In some cases, sample preparation can become a major hurdle for sample analysis as it may involve extensive sample extraction and/or the use of potentially toxic chemicals (e.g. solvents; Kubala-Drincic et al., 2003; Ridgway et al., 2007). Thus, the development of an initial screening strategy that could provide simple and reliable indication of the concentration of the residues in the target tissue without the need to undergo extensive extraction procedures or specific modifications due to matrix and antibiotic properties, could expedite sampling and screening activities. Additionally, such a method could also potentially reduce the effort, cost and time involved in this process and increase the efficiency of the monitoring procedures.

To overcome these problems several researchers have proposed the use of body fluids (e.g. bile, urine, kidney exudates, blood) to verify and monitor drug concentrations in live animals and carcasses at processing plants, such as those of swine (Ashworth et al., 1986); cattle (Mercer et al., 1977; Meijer et al., 1993; Lifschitz et al., 1999; Chiesa et al., 2006a; 2006b; Heller et al., 2006); sheep (Bevill et al., 1977; Delis et al., 2009); elk (Clark et al., 2004) and poultry (Dyer , 1988, 1989; Farrington et al., 1991; Moreno et al., 1996; Pant et al., 2005; Haasnoot et al., 2005; 2005b; Haasnoot et al., 2007; Schneider et al., 2007a; 2007b; 2009; Schneider and Lehotay, 2008). Additionally, this type of analysis is widely used to determine concentrations of drug levels in humans (Dickson et al., 1994; Rivier, 2000; Hammet-Stabler et al., 2002; Kim et al., 2002; Rigamonti et al., 2005).

Previous studies conducted in our laboratory evaluated the relationship between blood and muscle concentrations for the fluoroquinolone antibiotic, enrofloxacin, in broiler chickens (Schneider et al., 2007a; Reyes-Herrera et al., 2010). Blood is readily available at poultry processing plants and does not require removal or destruction of edible tissues from poultry carcasses. It was determined that the concentrations of fluoroquinolone residues in blood were a reliable predictor of the concentrations of residues in muscle (Reyes-Herrera et al., 2010). Thus, at least in the case of enrofloxacin in broiler chickens, blood samples could potentially be used as a tool for initial screening procedures to identify potentially adulterated carcasses that could then be tested with more specific monitoring procedures.

The objective of the current study was to evaluate if concentrations of oxytetracycline (OTC) concentrations in blood could provide a reliable estimate of residue concentrations in muscle (target tissue). Furthermore, the pattern of incorporation of OTC residues was compared with results previously obtained for enrofloxacin to evaluate if there are similarities between different classes of antibiotics. If there is a relationship, it may be possible to model (predict) residue concentrations for a wide variety of antibiotics. Oxytetracycline was evaluated in this study because tetracyclines are some of the most widely used class of antibiotics in the poultry industry and have been reported to produce violative residues in chicken muscle samples (De Wasch et al., 1998, Chopra and Roberts, 2001; Okerman et al., 2001; Oka et al., 2001; Okerman et al., 2004).

MATERIALS AND METHODS

A total of 252 day-old meat-type chickens were obtained from a local commercial hatchery and divided at random into three separate treatment groups. Birds had *ad libitum* access to a standard broiler diet and water during the entire experiment. Starting at day 33 of age, birds were dosed with oxytetracycline dihydrate (Sigma-Aldrich) in the drinking water at one of three different doses: 800 mg/gal (211 mg/L) the maximum approved dose (1X) for this antibiotic (FOI, 2005; 21 CFR520.1660, 2010), and then 5 times (5X) or 10 times (10X) the approved dose: 4,000 mg/gal (1,056 mg/L) or 8,000 mg/gal (2,113 mg/L) to investigate the kinetics of the drug at concentrations close to the imposed tolerance limit for this drug in the target tissue. Medicated water was prepared daily.

Blood and breast muscle samples were collected from 6 birds per group at each collection point. Samples were collected immediately before initial dosing (0 hour, controls) and during the dosing period at 1, 3, 6, 12, 24, 48, 96 or 144 hours and then at 12, 24, 36, 48 or 60 hours after drug withdrawal. All samples were processed individually as previously described (Reyes-Herrera et al., 2010). Blood samples were collected in sterile test tubes and then centrifuged (1,500 x g, 10 min) to separate the serum fraction, which was then used for antibiotic determination. Muscle samples were homogenized using a standard tissue homogenizer (Omni International). All samples were diluted 1:3 (wt/vol) with 1% phosphate buffer, pH 9.0 and centrifuged at 1,500 x *g* for 15 minutes at 5°C. The supernatant was decanted and stored at -80°C until assayed.

All blood and muscle samples were analyzed for oxytetracycline residues according to the approved method for oxytetracycline analysis from the USDA-FSIS,

Laboratory QA/QC Division (2007) with modifications (Donoghue et al., 1996). Briefly, on the day of the assay, Antibiotic Media No. 8 (Benton, Dickinson & Co.) was prepared, according to manufacturer instructions, and inoculated with the required quantity of *Bacillus cereus* spores (ATCC 11778; MEDTOX Diagnostics, Inc.) into the agar to make a final concentration of $5x10^3$ cfu/mL of indicator bacteria in the agar. The inoculated media was incubated for 45 minutes in a 48±2°C water bath before addition of 1.0 mL of penicillinase concentrate per 100 ml of seeded media (Becton, Dickinson & Co). Petri dishes (100 mm in diameter) were filled with 8 mL of inoculated media and then six penicylinders (8x10mm) were evenly placed on the agar. A standard curve was constructed by dilution of oxytetracycline in 0.01 N hydrochloric acid to produce a 1,000 ppm (parts per million or µg/ml) stock solution and further diluted using a 0.1M pH 4.5 phosphate buffer. Each standard concentration was pipetted onto three plates; three alternate cylinders were filled with a known standard (200 µL each), and the other three cylinders were filled with the overall reference concentration standard (200 µL each). The overall reference concentration falls within the range of the standard curve. Individual samples were assayed in a similar manner to that of the standard curve, except samples were assayed on only one plate. Plates were incubated at 29±1°C for approximately 16 hours. Plate averages for the standards, blood or muscle samples were corrected to the overall reference concentrations. A best-fit regression line was calculated by the method of Least Squares using the diameter of the inhibition zones (mm) with a zone reader (Fisher-Lilly, Pittsburgh PA). The assay detection limit was 68 ppb.

RESULTS

Oxytetracycline residues were detected in both blood and muscle tissue during all collection points during the dosing period [Figures 1, 2 and 3] and withdrawal period [Figures 4a and 4b], except for the withdrawal period of the 1X dosing group. In the 1X group, residues were detectable during the withdrawal period only in muscle (76±8 ppb) at 12 hours after drug withdrawal. Controls (samples collected prior to the beginning of the dosing period) were also negative for oxytetracyclines residues.

The residue concentrations of oxytetracyclines in muscle were higher than those in blood in all samples during the dosing and withdrawal period, except in the case of samples collected 1 hour after the beginning of the dosing when blood concentrations were slightly higher than those in muscle (212±13 vs. 177±10 ppb [Figure 1]; 327±21 vs. 251±18 ppb [Figure 2]; 726±42 vs. 317±51 ppb [Figure 3], for the 1X, 5X or 10X dosing groups, blood or muscle respectively). Oxytetracycline concentrations peaked in both blood and muscle at 24 hours during the dosing period for all treatment groups. The concentration of the antibiotic declined rapidly in both blood and muscle after drug withdrawal in all dosing groups. Oxytetracycline residues were not-detectable after 12 hours in the case of the birds treated with the approved dose of the oxytetracycline (1X), while the results for the other two treatments are shown in Figures 4a and 4b.

DISCUSSION

Results from this study support the potential use of blood to predict violative residues of oxytetracycline in muscle. By the third hour after oxytetracycline administration in the drinking water, there was a consistent relationship between blood

and muscle residue concentrations during the dosing period for birds receiving the maximum allowed dose for OTC in broiler chickens (800 mg/gal) or 5 or 10 times that dose. During the dosing period, the muscle concentrations were approximately 1.2 to 1.5 times higher than in blood for all three different dosing treatments (Figures 1, 2 and 3) and exceeded the FDA established tolerance in muscle (2,000 ppb) only for the chickens receiving the 10x dose (Figure 3). Upon drug withdrawal, oxytetracycline residue concentrations were undetectable in the 1x dosing group and dropped rapidly in the 5 and 10 x treatment groups (Figure 4). For oxytetracycline, the FDA established tolerance for residues in poultry muscle is 2,000 ppb (Arkin, 2005; FOI, 2005; 21CFR556.500b.1, 2010). Our results indicate that when blood concentrations approach or exceed 1,500 ppb, muscle residue concentrations are close to or in excess of the 2,000 ppb tolerance (Figures 2, 3). Thus, federal regulators could use blood samples to screen for oxytetracycline residues and any blood concentrations approaching or exceeding 1,500 ppb would trigger testing of carcasses for muscle residue determination.

These results are in agreement with previous studies conducted in our laboratory evaluating the relationship between blood and muscle concentrations for the fluoroquinolone antibiotic, enrofloxacin (Reyes-Herrera et al., 2010). In those studies, we also found that antibiotic concentrations in muscle were higher than in blood and blood concentrations were predictive of muscle concentrations. Although this relationship appears consistent for two different classes of antibiotics, it is possible that other antibiotic classes may behave differently. Factors such as specific tissue and protein binding affinities of an antibiotic and its lipophilicity, which determines its incorporation into the intracellular space, are important factors to consider (Ashworth et

al., 1986; Ryan et al., 1986). Depending on these factors the relationship between the concentration of residues in blood and the target tissue may vary. For example, due to their non-lipophilic nature, most β-lactams do not penetrate the cells and are confined to the extracellular fluids causing higher concentration of residues in blood than in tissue (namely muscle; Ryan et al., 1986; Li et al., 1995). Alternatively, the residue concentration may reach higher concentrations in muscle than in blood, as is the case of oxytetracyclines in poultry as shown in this study. Other antibiotics with higher concentrations in muscle are enrofloxacin (Knoll et al., 1999; Reyes-Herrera et al., 2010); flumequine (Haasnoot et al., 2007), and sulfonamides (Haasnoot et al., 1996; 2005a; 2005b). For instance, the blood concentrations of sulfamethoxazole and sulfadiazine are 6.2 and 8.7 times higher than those in muscle (Haasnoot et al., 2005b).

Moreover, for some antibiotics the blood:tissue relationship will vary significantly depending on the specific tissue. For example, even though the tetracyclines appear to behave as a two-compartment model with higher concentrations in muscle than in blood, the same antibiotic can almost irreversibly bind to bone and teeth (acting as a three or four compartment model) and thus blood levels will not provide an indication of antibiotic concentrations in these tissues (Anadón et al., 1985; Shish, 2009). This is of particular importance if bone meal from tetracycline treated animals is used to feed animals (still practiced in some countries). This practice has been identified as one potential source of unintentional exposure to tetracyclines for food producing animals (Kuhne et al., 2000; Korner et al., 2001). Another antibiotic group with similar complex compartmentalization patterns are the aminoglycosides (e.g. gentamicin, amikacin and neomycin), which tend to form irreversible binding to intracellular organelles in the

kidney cortex and cochlear tissues in the ear, and thus residues in these organs will persist for long periods of time even after they become non-detectable in blood levels (Nix et al., 1991; Chiesa et al., 2006a, 2006b). Consequently, in the case of these antibiotics, blood samples may not serve as reliable indicators of the concentration of these sequestered residues. These potential variations in the blood: tissue relations for different classes of antibiotics suggest the need for the study and characterization of each antibiotic of interest. However, since the study of the pharmacological profile of most veterinary drugs are conducted as part of the New Animal Drug Application approval process (including pharmacokinetic, pharmacodynamic and tissue distribution determinations; Clement, 1995; NRC, 1999; FDA, 2009), that information could be obtained and used by monitoring personnel.

The results from this study also demonstrate that oxytetracycline residues will not exceed the tolerance when the approved dose of this antibiotic (800 mg/gal) is used in broiler chickens (Figure 1). Yet, this class of antibiotic has been reported to produce violative residues in chicken muscle samples (De Wasch et al., 1998, Chopra and Roberts, 2001; Okerman et al., 2001; Oka et al., 2001; Okerman et al., 2004; FSIS, 2005; FSIS, 2008). These violations may be due to a failure to understand or follow label instructions when dosing food producing animals (Riviere, 1991, Brynes and Weber, 1996; FDA, 2005; FDA, 2009; FSIS-NRP, 2009). Because of these potential problems, monitoring is still necessary to ensure the safety of our food supply.

In conclusion, the use of blood samples as a potential initial screening method to detect harmful concentrations of antibiotic residues in poultry muscle could provide reliable estimations of the concentration of different antibiotic residues in target tissues

without the need for tissue collection and destruction for monitoring purposes. Although blood residues concentrations were predictive of muscle concentrations for both oxytetracycline and enrofloxacin (previous work), the blood:muscle relationships should be determined for other antibiotics of interest to determine the utility of blood to predict residues exceeding tolerances in edible tissues.

Figure legends

Figure 1. Concentrations of oxytetracycline residues determined during the dosing period from blood or breast muscle tissue samples collected from broiler chickens dosed with the approved dose for OTC for broiler chickens (800 mg/gal). Each time point represents the OTC concentrations (average± SEM) from 6 birds.

Figure 2. Concentrations of oxytetracycline residues determined during the dosing period from blood or breast muscle tissue samples collected from broiler chickens dosed with the five times (4,000 mg/gal) the approved dose for OTC for broiler chickens. Each time point represents the OTC concentrations (average± SEM) from 6 birds. The tolerance for OTC in muscle of broilers (2,000 ppb) is indicated by a dashed line.

Figure 3. Concentrations of oxytetracycline residues determined during the dosing period from blood or breast muscle tissue samples collected from broiler chickens dosed with ten times (8,000 mg/gal) the approved dose for OTC for broiler chickens. Each time point represents the OTC concentrations (average± SEM) from the samples collected from 6 birds. The tolerance for OTC in muscle of broilers (2,000 ppb) is indicated by a dashed line.

Figure 4. Concentrations of oxytetracycline residues determined during the withdrawal period from blood or breast muscle tissue samples collected from broiler chickens dosed with 5 (Fig. 4a) or 10 times Fig. 4b) the approved dose of OTC for broiler chickens

(4,000 mg/gal or 8,000 mg/gal in the drinking water, respectively). Each time point represents the OTC concentrations (average± SEM) from 6 birds.

Figure 1

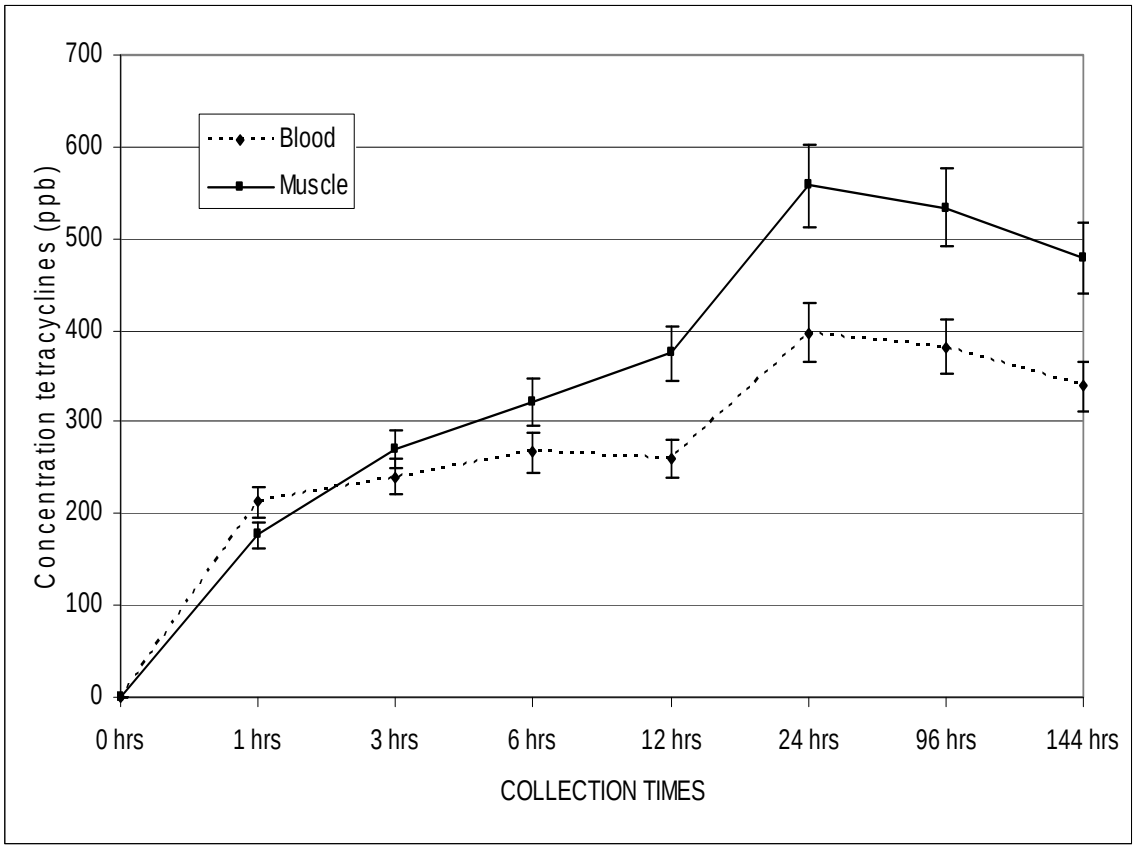

700
600
500
400
300
200
100
0
Concentration tetracyclines (ppb)
Blood
Muscle
0 hrs
1 hrs
3 hrs
6 hrs
12 hrs
24 hrs
96 hrs
144 hrs
COLLECTION TIMES

Figure 2

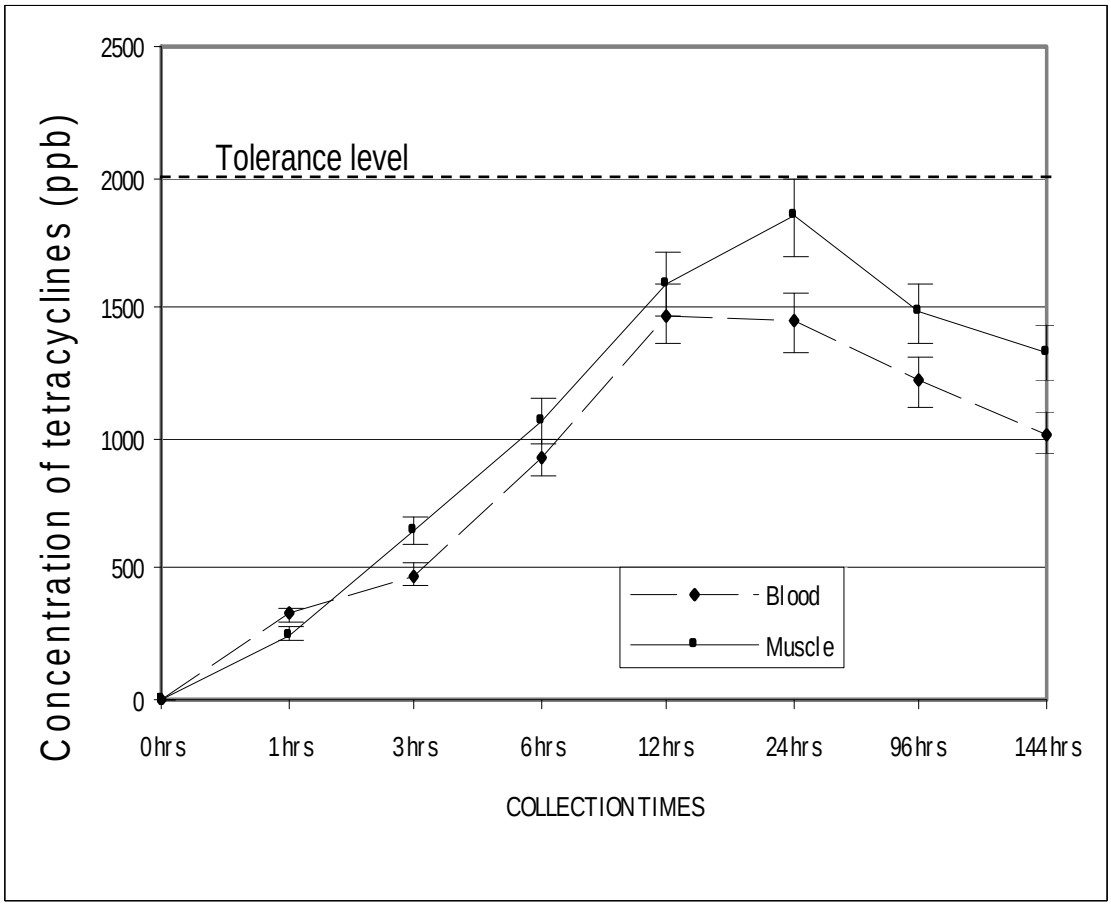

2500
2000
1500
1000
500
0
Tolerance level
Concentration of tetracyclines (ppb)
Blood
Muscle
0hrs
1hrs
3hrs
6hrs
12hrs
24hrs
96hrs
144hrs
COLLECTION TIMES

Figure 3

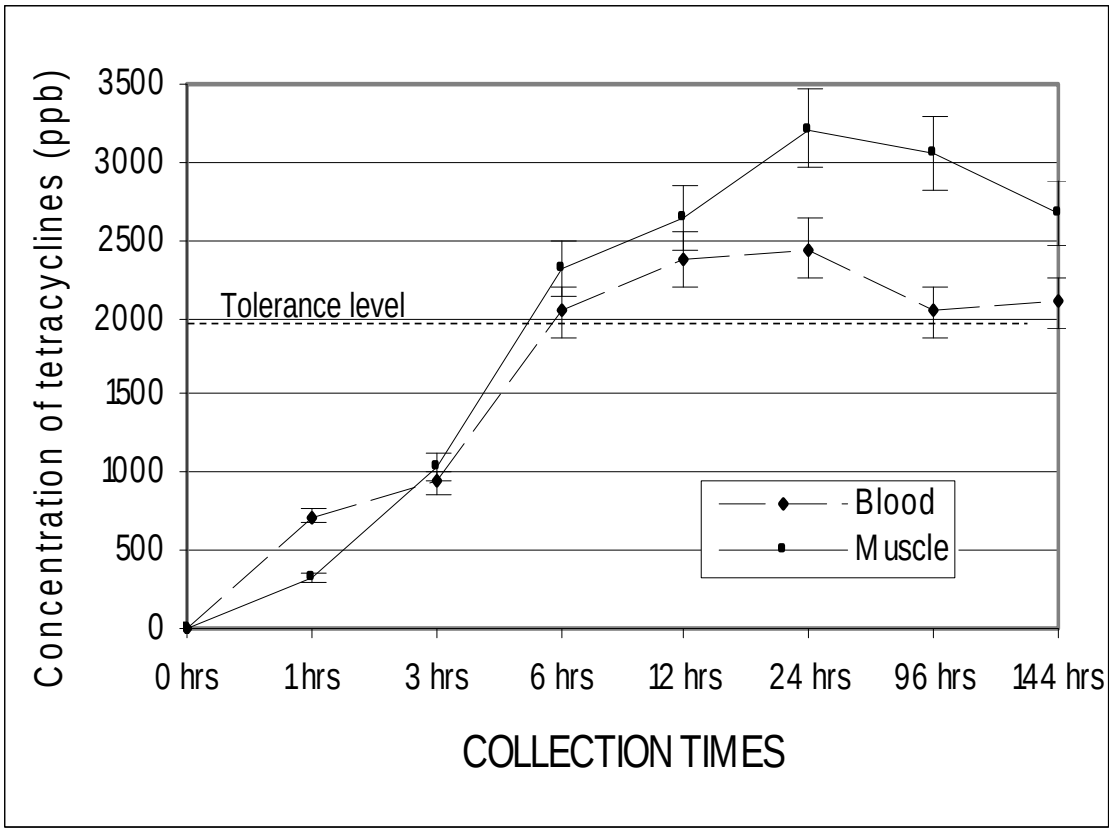
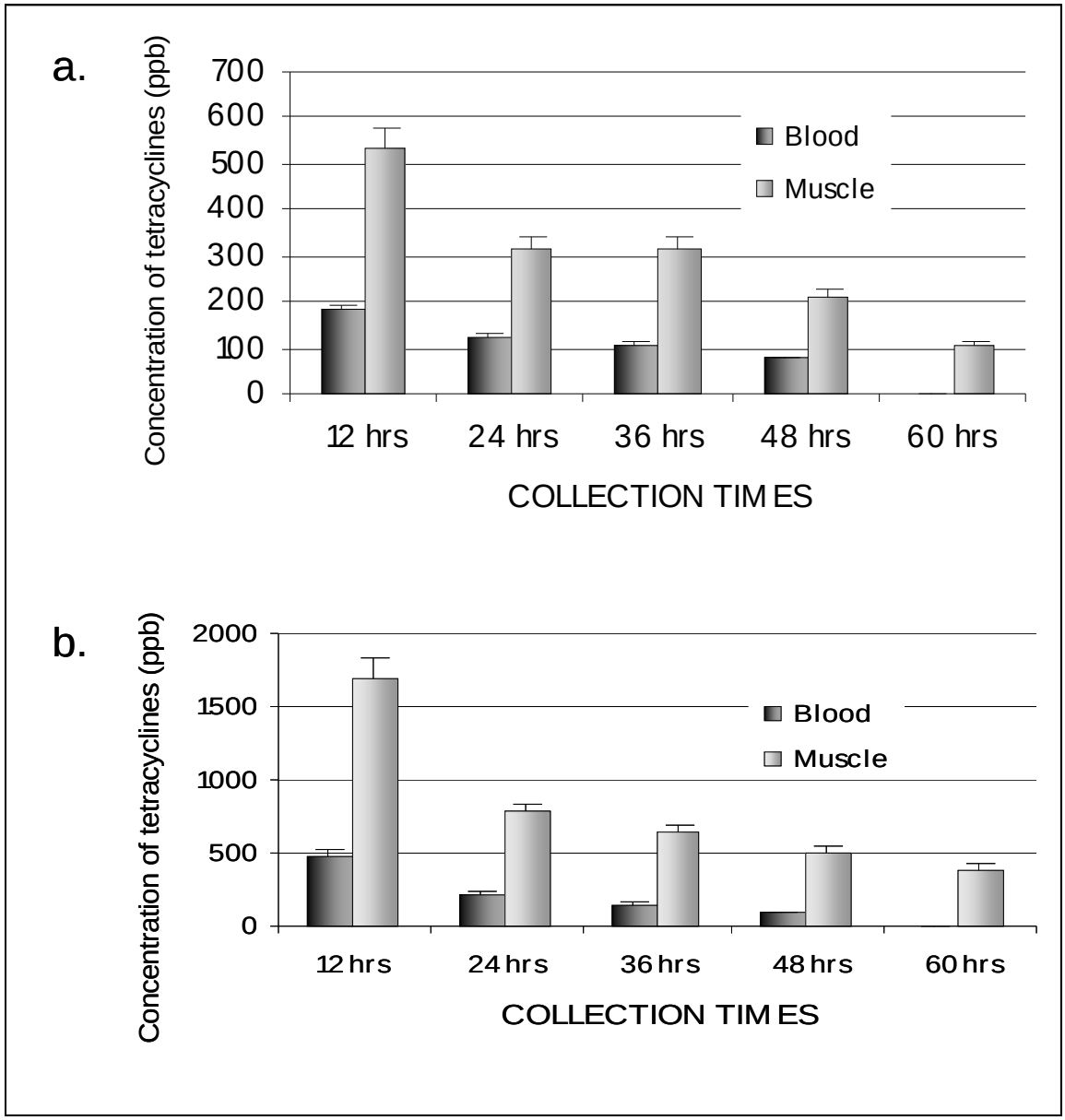

Concentration of tetracyclines (ppb)
3500
3000
2500
2000
1500
1000
500
0
Tolerance level
Blood
Muscle
0 hrs
1hrs
3 hrs
6 hrs
12 hrs
24 hrs
96 hrs
144 hrs
COLLECTION TIMES

Figure 4


a.
Concentration of tetracyclines (ppb)
700
600
500
400
300
200
100
0
Blood
Muscle
12 hrs
24 hrs
36 hrs
48 hrs
60 hrs
COLLECTION TIMES
b.
Concentration of tetracyclines (ppb)
2000
1500
1000
500
0
Blood
Muscle
12 hrs
24 hrs
36 hrs
48 hrs
60 hrs
COLLECTION TIMES

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CHAPTER IV

CONCLUSION

The potential use of blood samples as a screening tool for monitoring antibiotic residues in poultry edible tissues

The presence of antibiotic residues in edible animal products is a human food safety concern. To address this potential problem, the government samples edible tissues, such as muscle, to monitor for residues. Due to loss of valuable product and analytical difficulties only a small percentage of poultry carcasses are tested. Alternatively, antibiotic residue concentrations could be screened in blood, which is readily available during poultry carcass processing. To determine if blood concentrations are predictive of muscle concentrations, we conducted two different experiments to analyze the efficiency of a blood: tissue relationship in the estimation of residue concentration in edible tissues.

In the first experiment, we evaluated the blood:muscle relationship of the enrofloxacin (a fluoroquinolone type antibiotic) in broiler chickens. Enrofloxacin residues were determined in all blood and tissue samples during the dosing period and most of the withdrawal period for the two doses used (25 ppm and 50 ppm of enrofloxacin in drinking water). We observed that the tissue: blood correlation for fluoroquinolone residues between chicken muscle and blood was approximately 2:1 for both doses of enrofloxacin. In other words, the concentration determined in blood could be multiplied by two to estimate the concentration present in muscle. The use of blood samples could be an effective tool in the monitoring of antibiotics banned from use in poultry (such as enrofloxacin).

In the second experiment, we evaluated the blood:muscle relationship of the antibiotic oxytetracycline in broiler chickens. Results indicated that blood samples

may be used to predict oxytetracycline concentrations in muscle by multiplying blood concentrations by a coefficient of 1.3, as a screening procedure for monitoring oxytetracycline residues in broiler tissues.

The use of blood samples as a potential initial screening method to detect potentially harmful concentrations of antibiotic residues in poultry muscle could provide reliable estimations of the concentration of different antibiotic residues in edible tissues (as indicated for monitoring in the Code of Federal Regulations) without the need for tissue collection and destruction. However, even though blood residue concentrations were predictive of muscle concentrations for the two antibiotics evaluated in this work: enrofloxacin and oxytetracycline, the blood: muscle relationship should be determined for other antibiotics of interest to determine the utility of blood to estimate potentially harmful concentrations of antibiotic residues in poultry edible tissues.