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Screening Blood Samples to Estimate When Oxytetracycline Residues Exceed Regulatory Tolerances in Poultry Muscle

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Abstract: Presence of antibiotic residues in edible animal products is a human food safety concern. To address this potential problem, many governments sample edible tissues, such as muscle, to monitor for residues. 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 Oxytetracycline (OTC) in water at three doses: the maximum OTC approved dose for broilers (800 mg/gal) or five or ten times that dose. Blood and muscle samples were collected before initial dosing (0 hour, controls), during dosing at 1, 3, 6, 12, 24, 48, 96 or 144 hours and at 12, 24, 36, 48 or 60 hours after drug withdrawal. Concentrations in blood and muscle tissues followed similar time: concentration patterns, peaking 24 hours after initial dosing (396±9 vs. 557±37 ppb; 443±48 vs. 1846±58 ppb or 2447±67 vs. 3210±36 ppb for the 1, 5 or 10x doses in blood vs. muscle, respectively) and declined rapidly after withdrawal. These data suggest blood samples may be used to predict OTC concentrations in muscle as a screening procedure for OTC residues in poultry.

Key words: Antibiotic residues, monitoring, oxytetracycline, blood: tissue ratio, chicken

INTRODUCTION

The presence of violative antibiotic residues in food products is a major source of concern as they may adversely affect consumer health (Bruhn, 1999; Tilman *et al*., 2002). In an effort to prevent these problems, many countries around the world, including the US, Canada, Japan and the EU have 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; Health Canada, 2002; MHLW, 2003; Donoghue, 2003; Regulation (EC) No 470/2009 of the European Parliament and of the Council of 6 May, 2009). In general, monitoring procedures are based on sample collection and analysis of edible tissues at slaughter and processing facilities (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 in the US) and used as an indicator of the concentration of residues in the rest of the carcass, as stipulated in the US Code of Federal Regulations (US Federal Register, 2001).

For the antibiotic, oxytetracyline, the target tissue for poultry is muscle (US Federal Register, 2001). Unfortunately, there are a number of disadvantages in using muscle for determination of residue concentrations. First, sample collection requires destruction of the edible tissue used for testing, with subsequent monetary loss for the producers (Clement, 1995). Second, as the processing lines for broilers move at speeds of approximately 70-160 birds per minute (21CFR381, 2010), sample collection can be difficult. Finally, sample preparation may be expensive, time consuming and require the use of potentially toxic chemicals (e.g., solvents; Ridgway *et al*., 2007). Thus, providing a simple and reliable indication of the concentration of the oxytetracyline or other antibiotics in the target tissue without the need to undergo extensive extraction procedures or specific modifications due to the muscle matrix would expedite the sampling and screening activities.

To overcome these problems several researchers have proposed the use of body fluids (e.g., urine, blood) to verify and monitor drug concentrations in live animals and carcasses at processing plants, such as those of swine (Haasnoot *et al*., 1996); cattle (Chiesa *et al*., 2006; Schneider and Lehotay, 2008); sheep (Delis *et al*., 2009) and poultry (Schneider *et al*., 2007a, b, 2009). Additionally, this type of analysis is widely used to determine concentrations of drug levels in humans (Rivier, 2000; Rigamonti *et al*., 2005). Blood is an ideal candidate for screening residue concentrations since it is readily available at poultry processing plants and does not require destruction of edible tissues from

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poultry carcasses. Birds are exsanguinated at the start of processing and blood can simply be obtained from a collection tub.

Use of blood to screen for antibiotic residues in supported by previous studies from our laboratory evaluated the relationship between blood and muscle concentrations for the fluoroquinolone antimicrobial, enrofloxacin, in broiler chickens (Schneider *et al*., 2007a; Reyes-Herrera *et al*., 2010). 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, blood samples could be used as a tool for screening to identify adulterated carcasses.

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 exceeding the established tolerance, or maximum residue limit, for the drug (in the US the tolerance for this drug is 2000 ppb; 21CFR520.1660d, 2010).

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; Oka *et al*., 2001; Okerman *et al*., 1998, 2004). Water application was used since this is the most likely route for dosing sick poultry.

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 the maximum approved U.S. dose (1X) for this antibiotic (21CFR520.1660d, 2010) and then 5 times (5X) or 10 times (10X) the approved dose: 4,000 mg/gal or 8,000 mg/gal to investigate the kinetics of the drug at concentrations close to the U.S. 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 (1500 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 1500 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 (USDA-FSIS, 2007) with a 2 h sample preincubation to increase assay sensitivity (Donoghue *et al*., 1996). Briefly, on the day of the assay, Antibiotic Media No. 8 (Benton, Dickinson and 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 5×10^3 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 and Co). Petri dishes (100 mm in diameter) were filled with 8 ml of inoculated media and then six penicylinders (8 x 10 mm) 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 mg kg^{-1} stock solution and further diluted using a 0.1M pH 4.5 phosphate buffer. Each standard concentration (50-1600 ppb for the 1X and 5X samples or 200-3200 ppb for the 10X samples) 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 times during the dosing period (Fig. 1-3) and during the first 48 hours of the withdrawal period for the 5 and 10 x groups

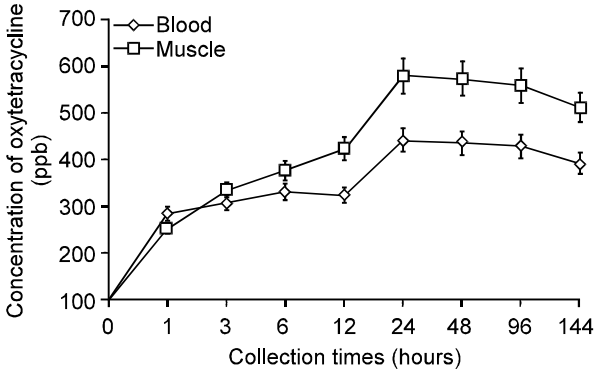

Fig. 1: Oxytetracycline residues (ppb) in blood or breast muscle collected during the dosing period from chickens dosed with 800 mg/gal OTC in the water

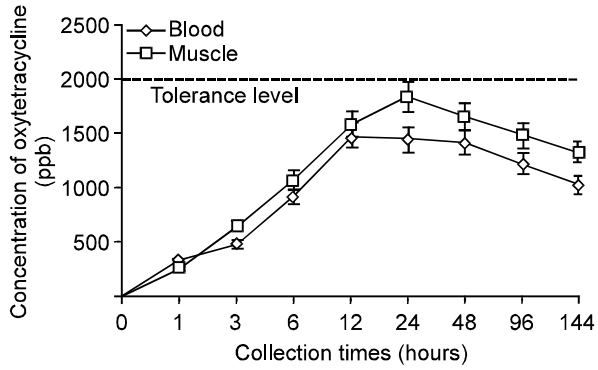

Fig. 2: Oxytetracycline residues (ppb) in blood or breast muscle collected during the dosing period from chickens dosed with 4000 mg/gal OTC in the water. The U.S. tolerance for OTC in muscle (2,000 ppb) is indicated by a dashed line

(Fig. 4a and b). In the 1X withdrawal group, residues were detectable only at 12 hours in muscle (76±8 ppb). 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 (Fig. 1); 327±21 vs. 251±18 ppb (Fig. 2); 726±42 vs. 317±51 ppb (Fig. 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

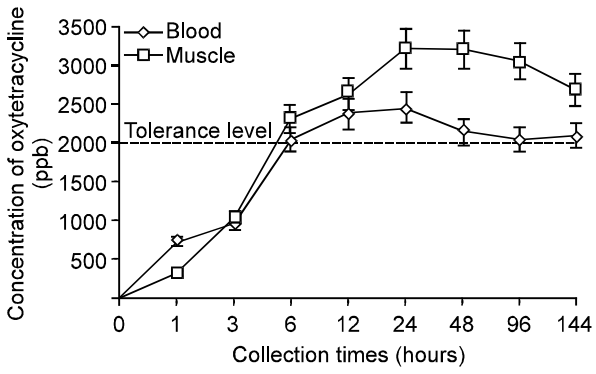

Fig. 3: Oxytetracycline residues (ppb) in blood or breast muscle collected during the dosing period from chickens dosed with 8000 mg/gal OTC in the water. The U.S. tolerance for OTC in muscle (2,000 ppb) is indicated by a dashed line

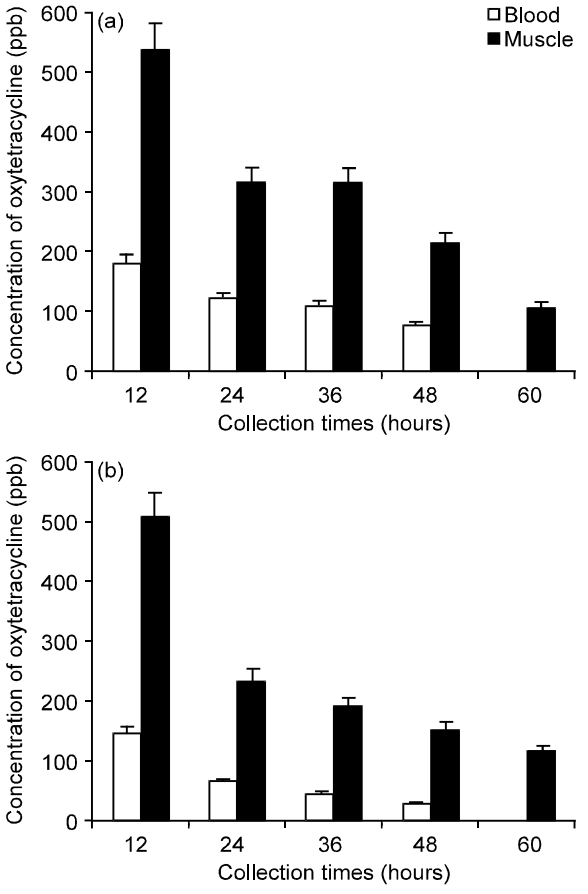

Fig. 4(a-b): Oxytetracycline residues (ppb) in blood or breast muscle collected during the withdrawal period after chickens were dosed with 4000 (Fig. 4a) or 8000 mg/gal (Fig. 4b) of OTC in the drinking water

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 Fig. 4a and b.

DISCUSSION

Results from this study support the potential use of blood samples to predict residue concentrations of oxytetracycline in muscle (the target tissue, i.e., the tissue with the highest drug concentration) that could be close to, or exceed, the U.S. tolerance, or maximum residue limit, for this antibiotic. 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 1.2-1.5 times higher than in blood for all three different dosing treatments (Fig. 1-3) and exceeded the U.S. established tolerance in muscle (2000 ppb) only for the chickens receiving the 10 x dose (Fig. 3). Upon drug withdrawal, oxytetracycline residue concentrations in the 1x (only detectable in the 12 hour muscle sample), 5 and 10 x treatment groups (Fig. 4). Our results indicate that when blood concentrations approach or exceed 1500 ppb, muscle residue concentrations are close to or in excess of the 2000 ppb U.S. tolerance during the dosing period (Fig. 2, 3). When sampled 12 h after drug withdrawal, however, the 10x dosed group had muscle concentrations approaching the tolerance level when blood concentrations were approximately 500 ppb (Fig. 4b). Therefore, it is proposed that blood OTC concentrations of 500 ppb constitute an “action level” for follow-up monitoring. Thus, federal regulators could use blood samples as a screening procedure for oxytetracycline residues and any blood concentrations approaching or exceeding 500 ppb would trigger follow-up testing of carcasses with a confirmatory method 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. Therefore, potential variations in blood: tissue relations for different classes of antibiotics needs to be evaluated for each class of antibiotics. Once determined, specific concentrations can be recommendations for each class of antibiotics when using blood as a predictor of illegal residue concentrations in edible tissues.

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 (Reyes-Herrera *et al*., 2010), 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.

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