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# Investigation of doxycycline residues in bones after oral administration to broiler chickens

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## Abstract

**Introduction:** Chicken bones, a by-product of the poultry industry, can directly or indirectly enter the food chain. Bone meal and bone products could be sources of many contaminants. Considering the wide range of uses made of bones in the culinary and food industries, this material needs to be safe and antibiotic residue–free. To determine if such is the case, the concentration of doxycycline in chicken bones was investigated, this antimicrobial being one of the most commonly used in poultry production. **Material and Methods:** Ross 308 broilers were grouped into three experimental and one control group. Doxycycline was administered in drinking water at therapeutic and sub-therapeutic doses, as well as *via* spray treatment. The concentration of doxycycline in bones was determined post slaughter by ultra-high performance liquid chromatography–tandem mass spectrometry. **Results:** Doxycycline was quantified at 135 µg/kg 22 days after the last day of antibiotic administration at therapeutic doses; 2,285 µg/kg after sub-therapeutic treatment for 27 days and 9.62 µg/kg 22 days after the end of spray application. **Conclusion:** High concentrations and long persistence of doxycycline in bones were found in this study. Doxycycline can contaminate all bone-derived products in the food and fertiliser industries.

**Keywords:** doxycycline, bones, broilers, UHPLC-MS/MS.

## Introduction

Antibacterials have been widely used in livestock and poultry production to treat many diseases. The use of antimicrobial drugs in animals has recently become a significant public health issue, because drug residues can persist in animal tissues and products and may cause many harmful effects on consumers, such as allergic reactions or alteration of the intestinal microflora (3, 22). The improper use of substances, illegal application of unlicensed drugs, failure to allow withdrawal times and overdosing may lead to violative residues. Infections can also alter an antimicrobial's pharmacokinetics and drug metabolism and exacerbate the residue problem (5). Specific actions can prevent the occurrence of residues, such as selecting the right form of drugs, correct dosing, allowing the full drug withdrawal time, cleaning feeders, preventing drug carryover and flushing drinking water systems (34). Of the greatest importance for consumer safety is ensuring that there are no residues in animal-derived food.

The most needed food for humans is meat, and its consumption is increasing yearly. A consequence of this is that slaughterhouses produce vast amounts of beef, pork, poultry and fish bones as waste material. According to Regulation (EC) 1069/2009, animal by-products are classified into three categories, which indicate the human and animal health risk from their intake (17). Bones an certain other waste products fall into category 3 (low risk), but are a significant environmental challenge if improperly disposed of (17). Each year, the global slaughter industry generates about 130 billion kg of animal bone residues (23). More than 10% of this waste is produced by European countries (21).Waste from poultry slaughterhouses, including bones, is not suitable for direct human consumption (18). Bones are a highly nutritional and energetically valuable product. In some countries, like India, Iran and Hungary, the marrow is consumed (38). Bones are also boiled to make nutritious broth (41). The derivative products of animal bones are applied in multiple areas: nutrition, animal feed, condiments, the pharmaceutical industry and industrial raw materials (2). Poultry by-products as

a source of nanokeratin, collagen and bioapatite are exploited for biomedical purposes and are used in medicine capsules (32). Made from bones, gelatin is utilised in processed meat products and most sweets, ice cream and other frozen desserts as a stabiliser, as well as for clarifying beers and wines (4). Charcoal, the black, granular material produced from burnt bone, is used in the sugar industry as a refining and decolourising agent (turning naturally brown sugar white) (2). Additionally, bones are rich in calcium and phosphorus and are therefore used as ground soil and plant fertiliser (37). However, bone meal and bone products could be sources of drug residues, especially those of tetracyclines, which are known to be deposited in bones. Considering the wide range of uses bones are put to, this product needs to be accepted as safe and antibiotic-free, especially as many of the applications are in the culinary and food industries. Measures should be taken to ensure that this by-product has no drugs or residues and thereby to protect consumer health and prevent environmental pollution. Because of the possible risk of antimicrobials re-entering the food chain *via* poultry bones, the presence of doxycycline was investigated in this matrix, the selection of antimicrobial falling on doxycycline because it is one of the most frequently used antibiotics in poultry farming. The transfer to and concentrations in bones of antibiotics such as doxycycline after experimental administration to animals have not yet been studied. Broiler chickens were administered therapeutic and sub-therapeutic doses of doxycycline in drinking water and by spray application. The concentrations of the antibiotic were determined by ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS). The presented research offers data useful as a guide in deciding whether chicken bones can be used in a wide range of areas without any treatment or monitoring.

## Material and Methods

**Animal experiment.** The presented animal experiment was authorised by the Local Ethics Committee (Lublin, Poland, under Resolution No. 26/2018) and was conducted following sound ethical and animal welfare principles. A detailed description of the experiment can be found in our previous publication (20). Briefly, one-day-old Ross 308 broilers were divided into two experimental groups of 18, one experimental group of 8 and one control group of 4 birds. Fifteen days after rearing and acclimatisation, the 18 broilers in group 1 were given doxycycline in water (Doxycyclinum 20%, Vetos-Farma, Poland) at a therapeutic dose of 15 mg/kg b.w. with a syringe for five days. The 18 broilers from group 2 were sprayed with 5 mL of the doxycycline solution at the same strength, also for five days. The 8 chickens from group 3 were treated with a subtherapeutic dose of doxycycline at 1 mg/kg b.w. in drinking water for 27 days. As controls, the 4 birds in group 4 were not treated.

Slaughter of three broilers each from groups 1 and 2 took place on days 1, 4, 8, 15, 18 and 22 post treatment. Chickens from groups 3 and 4 were slaughtered at the last day of experiment (day 27) which correspond to 22 days after the final drug application in group 1 and 2, and the samples were collected Femurs, shank bones and wing bones were collected for this study. The bones were ground in a ball mill and stored at −20°C until being prepared and chromatographically analysed.

**Reagents and chemicals.** Doxycycline and demeclocycline as internal standards (IS) and trichloroacetic acid (TCA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile was supplied by J.T. Baker (Deventer, the Netherlands). Formic acid was from Fluka (St. Louis, MO, USA). Polyvinylidene fluoride syringe filters with 0.22 µm pore size were sourced from Restek (Bellefonte, PA, USA).

**Ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS).** The analysis of doxycycline from bones was based on a previous method used by the present researchers (15). The mass spectrometry was carried out with a Shimadzu Nexera X2 system (Shimadzu, Kyoto, Japan) coupled to a QTRAP 4500 triple-quadrupole mass spectrometer (Sciex, Framingham, MA, USA). Chromatographic analysis was carried out with a mobile phase gradient of phase A (acetonitrile) and phase B (0.5% formic acid). The gradient elution was as follows: 15% phase A from 0 to 2.0 min, 80% phase A from 2.01 to 3.30 min, and again 15% phase A from 3.31 to 4.0 min. The flow rate was 0.65 mL/min and the injection volume was 5 µL. A Luna C18 column of 2.0 mm diameter × 50 mm length and 3 µm particle size (Phenomenex, Torrance, CA, USA) was used at a temperature of 35°C. Mass spectrometry measurement was performed in positive electrospray ionisation and multiple reaction monitoring modes. The precursor ion for doxycycline was 445, with the fragment ions as 428 (ion 1) and 154 (ion 2). Precursor ion 465 and fragment ion 448 for IS were monitored.

**Sample preparation.** Two grams of ground bones were transferred to a 25 mL polypropylene centrifuge tube, and 50 µL of IS (2 µg/mL) was added and vortexed briefly. Then, 5 mL of 5% TCA was added to every sample and the mixture was shaken for 30 min on a rotary stirrer. Next, each sample was centrifuged at 3,060 × RCF and 4°C for 10 min. After centrifugation, 1 mL of supernatant was transferred into amber vials by polyvinylidene fluoride syringe filter.

**Validation.** The linearity, repeatability, reproducibility and recovery calculations were performed following Commission Regulation (EU) 2021/808 (13). The limit of quantification (LOQ) and limit of detection (LOD) were calculated based on EUR 28099 EN (14). Matrix calibration curves at 10–500 µg/kg and 500–5,000 µg/kg were generated for linearity. In the estimation of calibration curve linearity, the concentration levels used for the experimental purposes were determined by

the F test (lack of fit) with a P-value of >0.05. Repeatability was estimated by arranging for six samples fortified with doxycycline at 10, 100 and 1,000 µg/kg to be analysed on the same day by the same person. The reproducibility and repeatability were verified by having two additional sets at the same concentrations analysed by different operators on two different days. For precision (repeatability and reproducibility), relative standard deviation was calculated as a percentage. Recovery was estimated by comparison of the mean measured content with the particular concentration of a fortified sample. The lowest point of the calibration curve was set as the LOQ of the method, establishing the minimum concentration at which the analyte could be reliably quantified. The LOD was set as 1/10 of the LOQ value.

**Statistical analysis:** All statistics were performed using Microsoft Excel 2016 (Microsoft Corporation, Redmond, WA, USA) and one-way analysis of variation. Differences were considered significant when the P-values were <0.05.

## Results

The UHPLC-MS/MS method for analysing doxycycline concentrations in chicken bones was validated as adequate for this experiment, by virtue of its validation parameters conforming to the criteria established for quantitative analytical methods (18). The correlation coefficient ($R^2$) of the matrix calibration curve linearity was set as 0.998, showing sufficient linear response. The requirements for linearity were met for the obtained data. In the selectivity analysis, no interfering peak was observed at the retention time of doxycycline. The relative standard deviations for repeatability (8.2 ± 2.6%) and laboratory reproducibility (12.1 ± 3.9%) were satisfactory. Achieving an LOQ of 10 µg/kg and an LOD of 1 µg/kg, the method was sensitive. Calculation of recovery produced values in a range of 98.5–102.3%. The extracted ion chromatograms of a blank bone sample, of a sample fortified with doxycycline at 100 µg/kg and of a real bone sample are presented in Fig. 1.

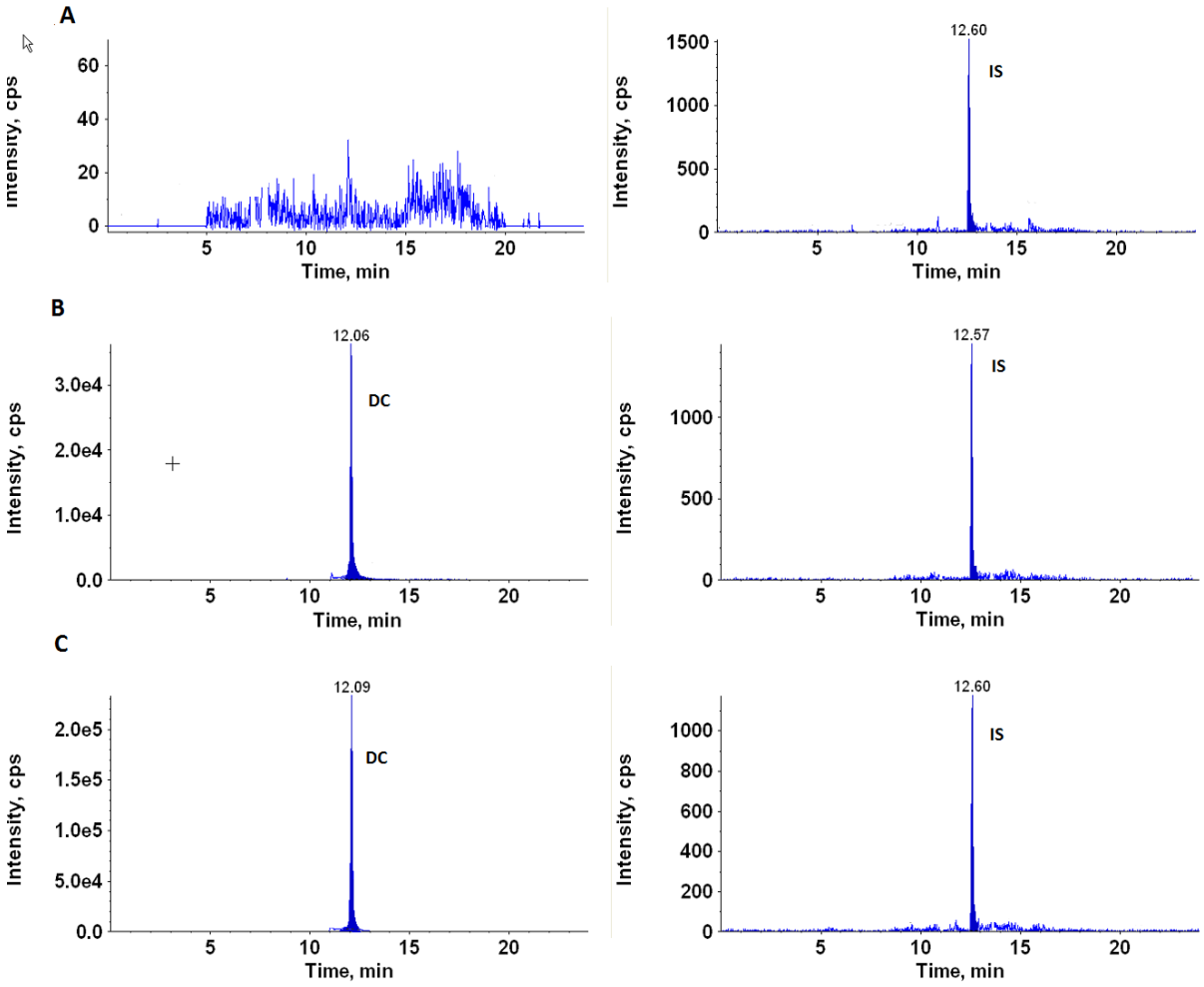


**Fig. 1.** Extracted ion chromatograms of A – blank bone sample; B – doxycycline (DC)-spiked bone sample fortified at 100 µg/kg; C – bone sample after administration of a veterinary medicinal product taken 15 days post-treatment with doxycycline concentration at 610 µg/kg
cps – counts per second; IS – internal standard

**Table 1.** Concentration of doxycycline in broiler bones following different routes of drug administration

| Post-treatment day | Age of birds (days) | Group 1<br>DC mean concentration<br>(± SD, µg/kg) | Group 2<br>DC mean concentration<br>(± SD, µg/kg) | Group 3<br>DC mean concentration<br>(± SD, µg/kg) |
|---|---|---|---|---|
| 1 | 20 | 5,840 ± 843 | 132 ± 38 | - |
| 4 | 24 | 2,860 ± 410 | 83.1 ± 21 | - |
| 8 | 28 | 1,760 ± 322 | 45.5 ± 15 | - |
| 15 | 35 | 610 ± 150 | 41.6 ± 13 | - |
| 18 | 38 | 168 ± 84 | 16.4 ± 5.4 | - |
| 22 | 42 | 135 ± 51 | 9.62 ± 2.1 | 2285 ± 362 |

DC – doxycycline; SD – standard deviation

The highest concentrations in bones were found (5,840 ± 843 µg/kg after the therapeutic dose in drinking water and 132 ± 38 µg/kg after spray treatment) on the first day after the cessation of treatment with doxycycline. Gradual decreases in antibiotic concentrations were observed in both groups during the experiment, but much higher levels were observed in group 1 (the group which received a therapeutic doxycycline dose in drinking water) at each sampling point. On day 8, which is the day after the end of the withdrawal time for veterinary drugs used in chickens, the concentrations of doxycycline in the bones of birds from group 1 considerably exceeded the Maximum Residue Limit values for poultry tissues (100 µg/kg for muscle, 300 µg/kg for liver and 600 µg/kg for kidney). In birds from group 2, the levels of antibiotics were already below 100 µg/kg on day 4. Doxycycline was quantified for 22 days post treatment in each experimental group. On the last day of the conducted study (day 22), high concentrations were still detected in group 1 (135 ± 51 µg/kg), but in group 2 they were much lower (9.62 ± 2.1 µg/kg). After administration of doxycycline at a sub-therapeutic dose to group 3, the concentrations found on the last day of sampling were high (2,285 ± 362µg/kg). There were no significant differences between experimental groups with the same route of drug administration. Between the groups to which doxycycline was administered in different ways, concentrations in bones were observed which were significantly different, however (P-value < 0.05). The mean doxycycline content in bones in chickens from groups 1, 2 and 3 are shown in Table 1.

## Discussion

The EU ban on using meat waste to feed animals for human consumption has created a bone utilisation problem (39). Bones are one of the components of meat and bone meal (MBM), produced mainly from cattle, poultry and swine slaughterhouse leftovers. Meat and bone meal contains abundant minerals, protein and amino acids, contributing to a nutrient-rich diet for poultry and swine. Limiting the use of MBM in some areas, such as in feed for animals except pets, has led to a considerable increase in the amount of MBM to be disposed of (1, 7, 10). As waste disposal is a major global environmental issue, by-product-based alternative fertilisers, including bones, have been used to recycle high-value nutrients of animal wastes in soil application (27, 29). Bones are rich in calcium and phosphorus and can be used as ground soil and plant fertiliser (24, 37). However, when some fertiliser ingredients are contaminated with antibiotics, these substances can be released into the soil, potentially threatening all microorganisms in this environment and causing antibiotic resistance development in them (11). In soil, antibiotics can be subjected to many processes, such as degradation and transformation, and can be taken up by plants or transported into the groundwater (6, 12, 31). The rates of degradation of pharmaceuticals in the soil differ much, steroids having half-lives of a few weeks or shorter, for example. A study by Cycoń *et al.* (11) showed the long-term persistence of doxycycline in soil and established a half-life of 578 days for the drug.

Besides for reasons of chicken bone doxycycline residues' environmental impact, their investigation is also significant for food safety. It is because many products derived from bones are used by the food industry that the distribution and accumulation of doxycycline in bones was investigated in the presented study. According to EU reports, the most commonly detected antibacterial in poultry muscles is doxycycline, which is one of the tetracyclines (14, 15). Additionally, based on the list of antibiotic groups by incidence (%) established by the World Organisation for Animal Health, tetracyclines are the most frequently applied antibacterials, reaching 87.1% (35). Roblez-Jimenez *et al.* (35) presented the residues of antibiotics in animal tissues and other animal products such as milk and eggs and also presented them in the environment, wastewater and soil, following a very extensive literature review. The highest concentrations of tetracyclines emerged as being in Europe, and the sales of these antibiotics were the largest proportion by antimicrobial class (32.8%). These data are the primary reason for choosing tetracycline antibiotics in our experimental work. Additionally, specific properties of doxycycline that make it prone to form complexes with calcium ions cause this compound to accumulate in the bones (36). Because of this binding of tetracyclines to bones, as well as tetracyclines' frequent use in animal

husbandry, high concentrations can be found in the bones of slaughtered animals. Bound doxycycline residues in bones will contaminate both mechanically deboned meat and MBM, as well as all derivative products of animal bones.

Most papers on chemical residues in bones concern elements like lead (40). A study conducted on chicken bone broth–based foods showed they contained more lead than tap water (28). Very limited data for antibiotic residues exist in the literature regarding poultry by-products. Some publications reported the depletion and persistence of oxytetracycline and doxycycline in feathers (9, 19). Others presented the bioaccumulation of oxytetracycline, florfenicol and doxycycline in chicken claws (8, 20, 33). Among these studies, doxycycline was found at the highest concentrations and with the longest persistence times in feathers and claws. The concentration of doxycycline in feathers one day after treatment was 1,050 ± 596 µg/kg, while in claws, doxycycline was found at the level of 6,370 ± 922 µg/kg (19, 20). In the present research, the determined concentration of doxycycline in bones was 5,840 ± 843 µg/kg one day after ceasing treatment. Doxycycline was detectable for 22 days at different levels. Compared to findings of our previous research describing residues of doxycycline in feathers and claws, the concentration of antibiotic in bones at the 22-day point post treatment at 135 ± 51 µg/kg was lower than that in chicken claws (223 ± 77 µg/kg) but higher than that in feathers (58 ± 17 µg/kg) (19, 20). In this study, the accumulation of doxycycline in bones was analysed after therapeutic treatment and after spray and sub-therapeutic application. After spray treatment, doxycycline was quantified during the entire sampling period, but the concentrations were lower than in chickens given therapeutic-concentration treatment in drinking water. After sub-therapeutic administration, the highest doxycycline content was found on day 22 at 2,285 ± 362 µg/kg. These results show the persistence of this antibiotic in the tested matrix, exceeding the corresponding MRLs for edible tissues at withdrawal time. In the same experiment as the one researching residues in feathers and claws, the muscle and liver were also collected, and the results of doxycycline presence in these tissues were described in the resultant publication (19). Much lower concentrations were recorded in muscle and liver. One day after the end of therapeutic administration in drinking water, doxycycline was determined at 161 µg/kg in muscle and 610 µg/kg in liver, and was possible to quantify until the 15$^{th}$ day. After spray treatment, 23.9 µg/kg in muscle and 9.1 µg/kg in liver were found at this time, and doxycycline was not detectable from day 8. As described in this report, doxycycline persisted at a significantly higher level. The strong propensity of doxycycline to form complexes with calcium and other metal ions and doxycycline's good distribution, high bioavailability and longer elimination half-life result in high bioaccumulation of this antibacterial in bones and derived matrices.

Studies describing the residues of antibiotics in bones are scarce. One of them presents the incidence of tetracycline, chlortetracycline and oxytetracycline residues in the bones of pigs, turkeys, chickens, ducks and calves (26). The tetracyclines were determined visually by yellow fluorescence intensity measurement using a UV lamp as well as by high-performance liquid chromatography with a UV detector. The concentrations of tetracyclines found in that study in random samples from different species ranged from 0.14 to 50.0 mg/kg. In our study, the more advanced and accurate UHPLC-MS/MS technique, providing reliable concentration measures, was used to determine antibiotic concentration. A second extant antibiotic bone residue research report concerns the behaviour of bound residues of chlortetracycline during *in vitro* and *in vivo* digestion uptake (25). It was found that hens fed with MBM containing chlortetracycline could release bound tetracycline residues. The cytotoxic effects of oxytetracycline residues in the bones of broiler chickens after therapeutic oral administration were also described in other research by Odore *et al.* (30), who found them to have the potential to induce biological responses in pets and human consumers. They noted the level of oxytetracycline and 4-epi oxytetracycline to be as high as 1,286 µg/kg on day 10 post treatment. The concentration of doxycycline in our study 8 days after treatment was 1,760 µg/kg.

## Conclusion

The residue of antibiotics such as doxycycline in bones can contaminate food industry products including mechanically deboned meat, and can be a source of antibiotics in all bone-derived products. For food and environmental safety, as well as in terms of the warning they may give of increasing resistance, residues of doxycycline in bones should not be ignored. Studies concerning the persistence of antibiotics in samples from treated animals of material which could be a slaughter by-product precisely indicate the possible residue situation. The potential risks to human and animal health from doxycycline residues in the bones of treated livestock entering the food chain should be considered.

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