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DOXYCYCLINE RESIDUES IN EDIBLE TISSUES OF PIGS

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Abstract

Doxycycline (DOX) is a variant of the tetracycline antimicrobial with similar properties, but with a longer action period. It is widely used in swine production. The presence of residues of antibiotics in food products of animal origin has a special toxicological interest due to their potential effects on human health.

Our aim was to evaluate the withdrawal time (WT) of DOX formulation (25%) in edible tissues of swine, after PO administration. Eighteen healthy young pigs (30-35 days old) were used. DOX was administered with drinking water during 5 days at 10 mg kg^{-1}. Two animals, as the control group, were not treated. Four animals per group were sacrificed by exsanguination 24 hours until 11 days post-treatment. Muscle, liver, kidney and skin/fat samples were obtained. DOX was determined by HPLC with UV detection. For muscle tissue, a WT of 4.3 days was determined. In other tissues, DOX concentrations were measured until 7-11 days post-administration. The WT was 7.2, 4.9 and 4.5 days for liver, kidney and skin/fat, respectively. After administration of DOX at 10 mg kg^{-1} for 5 days through medicated drinking water, a WT of 8 days must be set for safe consumption of medicated animals.

Introduction

Doxycycline (DOX) is a derivate of tetracycline with similar properties, but with a longer action period. It is the most active antibiotic of tetracycline group, so the minimum inhibitory doses are low. Is an antimicrobial widely used in swine production against Gram-positive and Gram-negative bacteria, including some anaerobes. Tetracycline agents are bacteriostatic antibiotics, which act by inhibiting the formation of proteins within the bacterial cell. Doxycycline is widely used in the treatment of respiratory and urinary tract infections in swine production. Doxycycline is more lipid soluble than the other tetracyclines, and after application it penetrates body tissues and fluids better. Long persistence of doxycycline in an animal's body could cause unacceptable concentration in animal tissues.

The European Commission has set maximum residue limits (MRLs) for oxytetracycline, tetracycline, and chlortetracycline as a sum of parent compounds with their corresponding 4-epimers. For the doxycycline constituent there is no 4-epimer, and an MRL for only the DOX compound is described. The MRLs are 100 ng g^{-1} for muscle, 300 ng g^{-1} for liver and skin plus fat, and 600 ng g^{-1} for kidney (EU 37/2010).

The antibiotic residue levels reached in organs and the rate of their depletion from tissues depend on the method of administration, animal species, as well as dose and the specific formulation of the drug given (Kung & Wanner, 1994). The differences in the antibiotic concentration and time of its depletion may be also influenced by the differences in the intake of drinking water by animals.

Residue studies of DOX have been conducted in several species, including chickens (Anadón *et al*., 1994), turkeys (Croubels *et al*., 1998), calves (van Dongen & Nouws, 1993) and pigs (Anadón *et al*., 1996). The purpose of this study was to determine the residues of DOX in edible tissues (kidney, liver, skin plus fat and muscle) of pigs after 5 days of oral medication *via* drinking water at 10 mg DOX 25% kg^{-1} body weight (BW) per day. The DOX concentrations in plasma and the stability of DOX in drinking water were also determined. Based on the residues in the tissues, a withdrawal time was calculated according to Guideline N° EMEA/CVMP/036/95 of the Committee for Veterinary Medicinal Products (EMEA, 1995).

Materials and Methods

Study Design Treatment and Administration

The study was conducted with 18 healthy Duroc Jersey pigs (30-35 days old). They were treated once a day with 10 mg kg^{-1} of an experimental formulation of DOX 25% water soluble powder, for five consecutive days through the drinking water. A solution was prepared by dilution of 400 g of the medicament in 1,000 mL of water.

The pigs were housed in four groups of four animals; one group of two pigs was managed as blanks. The animals were fed a pig feed free from antibiotics. Feed and water were available *ad libitum*. The drinking water system consisted of drinking nipples per box connected *via* plastic piping to a plastic storage tank of 100 L. The tanks were provided with a continuous stirring system and provisions for the measurement of daily water intake. After a controlled drug free period of 15 days, medicated drinking water was given for a period of five consecutive days at a daily dose of 10mg kg^{-1} of DOX 25%. Pigs were weighed daily and the water intake per group was recorded daily. Based on the mean daily water intake and the mean body weight per group of four the dosage of DOX was calculated every day.

The pigs treated with DOX were euthanized at 24 h, 3d, 7d and 11 d after the end of the drug administration (four animals at each time point). One whole kidney and about 300 g of liver, skin, fat and muscle were collected separately to avoid contamination and frozen at -20 °C pending analysis. All samples were analysed within two months after sampling. Two pigs used as controls were euthanized before experiment, and the same tissues samples were collected. This experimental animal protocol was accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (Federation of Animal Science societies -FASS).

Reagents
Doxycycline (DOX) standard was obtained from Sigma-Aldrich Chemical Company (USA). Acetonitrile and methanol were from JT Baker. Oxalic acid dehydrate, trichloroacetic acid and sodium sulphate anhydrous were from Fluka (USA). Solid-phase extraction (SPE) columns (Strata, C18, 100 mg, 1 mL) and analytical column (Luna C18) were obtained from Phenomenex (USA). Doxycycline was formulated as a 25% experimental water soluble powder (DOX 25 g, tartaric acid 5g and Lactose sqt 100 g).

Standard solutions
Stock standard solution (1 mg mL^{-1}), was prepared by weighing 10.0 ± 0.1 mg of standard substances and dissolving it in 10 mL methanol. The stock was stored at -20°C in amber glass, and was stable for six months. Secondary standard solutions (100 µg mL^{-1}, 10 µg mL^{-1}) prepared in methanol by diluting suitable aliquot of stock standard were stable for one month, stored at 2-8°C in amber glass. Working standard solutions in mobile phase were prepared on the day of analysis.

Extraction and clean-up
A portion of 0.4 g of tissue (incurred or spiked) was homogenized with 1.4 mL of McIlvaine buffer-EDTA, shaken at high speed, and centrifuged at 2,500 g at 4°C for 15 min. The upper layer (supernatant S1) was transferred into a new tube. The extraction was repeated three times. The supernatants S2, S3 and S4 were combined with the S1. The mixture was vortexed for 30 s, and centrifuged again for 10 min at 2,500 g at 4°C.

Clean-up
The supernatant mixture (S1-S2-S3-S4) was transferred to SPE C18 cartridges, which were preconditioned with 3 mL methanol and 2 mL ultrapure water. The tube reservoir mix supernatants were washed with 1 mL of McIlvaine buffer-EDTA and 1 mL water. After percolation of the whole solution, the columns were washed with these solutions (under vacuum). After drying for 2 min, the doxycycline was eluted with 4 mL methanol 0.01 M oxalic acid pH 2.0. The cleaned eluates were evaporated to dryness in nitrogen evaporator at 40°C. The dried residues were reconstituted in 200 µL mobile phase. Then after vortexing and centrifugation, 100 µL were injected into the chromatographic system.

LC-UV analysis
The instrumental analysis was performed using Gilson HPLC system, equipped with isocratic pump, autosampler, column oven, and UV/Vis detector (λ = 346 nm), controlled by Unipoint Workstation software. Chromatographic analyses were performed on Luna (Phenomenex) C18 column (5 µm, 150 mm x 4.6 mm) with mobile phase consisting of water-acetonitrile with 0.02 M oxalic acid and 0.5 mM EDTA (72:28, v/v) at 1.2 mL min^{-1} flow rate. The column oven temperature was controlled at 30°C.

Method validation
The following parameters were evaluated for the analysis of each matrix: linearity (concentrations of DOX ranging between 0.1 and 6.0 µg mL^{-1}), precision and accuracy, limit of quantitation, limit of detection and selectivity. The standard calibration curve was prepared by injection of standard solutions at seven concentration levels. The correlation coefficient and linearity ranges were evaluated. The detection limits (LOD) and limit of quantitation (LOQ) of the method were calculated. The accuracy was defined as the closeness of agreement between the true (spike) value and the mean result of a series of experiments (n = 6). It was determined by comparing the measured concentration to the spiked concentration. The mean accuracy (recovery %) should be within the range 85-115% and the variation in precision should be ≤ 20%. The limit of detection (LOD) was estimated through the analysis of 20 aliquots of control tissue (free of DOX). The noise of the base-line was measured; the average and the standard deviation were calculated. The LOD corresponds to the average plus three times SD (signal-to-noise ratio ≥ 3/1) and the limit of quantitation (LOQ) corresponds to the average plus ten times the SD (signal-to-noise ratio ≥ 10/1).

Withdrawal time
The withdrawal periods for edible tissues of pigs (muscle, liver, kidney and skin plus fat) were estimated by linear regression analysis of the log transformed tissue concentrations and determined at the time when the upper one-sided 95% tolerance limit for the residue was below the MRLs, with a confidence of 95% (EMEA, 2002). Doxycycline concentrations as a function

of time found in muscle, kidney, liver and skin/fat were plotted and analysed with the program WT version 1.4 in order to recommend a withdrawal time period for this experimental formulation.

Results

This method performed accurately and reproducibly over a range of 0.1 to 6.0 µg mL^{-1} for DOX. The linearity (r) was between 0.9913 to 0.9975 in all tissues assayed. The chromatographic analysis time was short: DOX eluted at 4 min as a sharp and symmetrical peak with no interfering peaks (Figure 1A and 1B).

The LODs were 0.020, 0.020, 0.030 and 0.026 µg g^{-1} for DOX in kidney, skin/fat, muscle and liver, respectively, while LOQs were 0.050, 0.136, 0.050 and 0.225 µg g^{-1} for kidney, skin/fat, muscle and liver, respectively.

The analytical method for tissue samples was thoroughly validated (Table 1), and was found specific for all samples with respect to interference from endogenous compounds. The validated analytical methodology showed satisfactory sensitivity, precision and accuracy that allow its use for the detection and quantification of DOX residues in pig tissue (Figure 2).

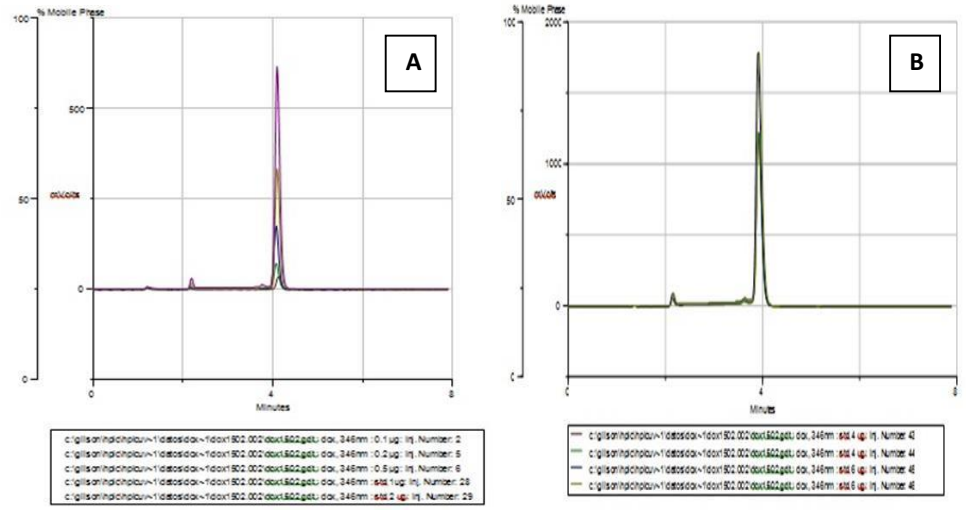

Figure 1. HPLC Chromatograms of DOX standard solution at seven concentrations: A) 0.1; 0.2; 0.5; 1.0 and 2.0, and B) 4.0 and 6.0 µg mL^{-1}.

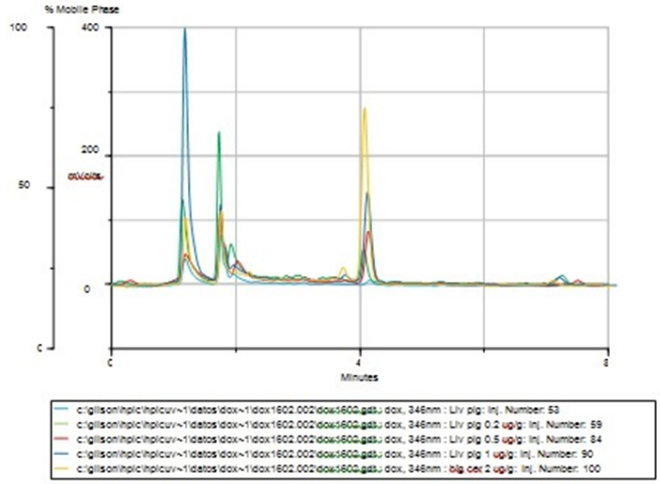

Figure 2. Chromatograms of blank liver and liver spiked at 0.2, 0.5, 1.0 and 2.0 µg g^{-1} DOX.

Doxycycline tissue concentrations

In Figure 3A, 3B, 3C and 3D, the mean tissue concentrations and their SD values for muscle, kidney, liver, skin +fat, respectively, at 1, 3, 7 and 11 days after cessation of medication are presented. Highest residues were found in liver, followed by kidney, muscle and skin + fat. The concentrations in all matrices were near or below the MRL at day 3 after treatment and below the respective LOQ at 11 days after the end of the treatment. In muscle sample, DOX was only detected at 24 h and day 3 post treatment.

Table 1. Analytical validation results for the analysis of DOX in pig tissues.

			Intra-day		Inter-day (over 3 days)	
matrix	r	µg g^{-1}	Accuracy (%), n=6	Precision (%), n=6	Accuracy (%)	Precision (%)
Muscle	0.995 (0.2-2.0 µg g^{-1})	0. 2	97.2	3.0	97.4	2.0
		0.5	92.2	1.2	91.4	1.7
		1.0	109	11.1	104.9	9.2
		2.0	97.9	3.0	97.5	2.5
Liver	0.994 (0.2-2.0 µg g^{-1})	0.2	88.4	4.1	89.5	5.7
		0.5	93.8	1.8	93.3	1.2
		1.0	111	3.0	109	2.4
		2.0	97.2	2.2	95.4	1.7
Kidney	0.998 (0.2-2.0 µg g^{-1})	0.2	93.4	2.1	92.8	2.8
		0.5	100	7.0	100	6.0
		1.0	103	1.2	103	2.5
		2.0	99.0	3.2	97.0	2.7
Skin/Fat	0.991 (0.2-2.0 µg g^{-1})	0.2	89.0	2.3	88.0	3.0
		0.5	97.7	6.2	91.2	7.5
		1.0	109	2.0	106	2.8
		2.0	97.2	2.1	93.6	3.4

Linear regression analysis of the logarithmic transformed data can be considered for the calculation of the withdrawal periods. Using this approach, the withdrawal time is determined as the time when the one-sided, 95% upper tolerance limit of the regression line with a 95% confidence level is below the MRL. The European Agency for the Evaluation of Medicinal Products Guideline recommends that values less than the LOQ should be set at one-half of the LOQ. Using this approach, the withdrawal time could only be calculated for muscle tissue: 4.33 days. In our study, taking into account the MRLs in pigs and considering that the marker residue is doxycycline, the calculated withdrawal times were 7.23, 4.87, 4.50 and 4.33 days for liver, kidney, skin + fat and muscle, respectively.

Discussion

Antibiotics are used in pig farms to enhance growth, feed efficiency and reduce diseases. Additionally, prophylactic treatment is common during periods of stress. Tetracyclines are the most commonly used antimicrobials in food-producing animals. In Argentina doxycycline is frequently used in pigs production; therefore, it is important to control their residues in edible tissues. Doxycycline given to pigs orally raise possibility for residues which, remain in edible tissues, particularly when the animals are slaughtered without respecting the withdrawal period. Such residues may pose public health hazards to consumers including toxicological, microbiological, immunological and pharmacological disorders depending on the type of food and the amount of residue present (Oka *et al*., 2000). Additionally, the use of antibiotics in related food may lead to resistance in bacterial populations that do not respond to treatment commonly used for human illnesses (Marchetti *et al*., 2012). Studies on tissue concentrations after different drug formulation administration are essential to recommend appropriate withdrawal times to secure control over the potential antibiotic residues in animal food products.

The data obtained after DOX administration with water shows that at the beginning after treatment, DOX reached high concentrations in all edible tissues. One day after administration of the last dose, DOX concentration rapidly decreased in all assayed tissues. The residue concentrations decreased gradually thereafter and only trace concentrations were detected on day 8.

Conclusions

This analytical method exhibited good linearity and reproducibility over the calibration range for DOX in edible tissues of pigs.

Our results demonstrate that oral administration of DOX at 10 mg kg^{-1} for five consecutive days through the drinking water require withdrawal time of 8 days in order to respect the fixed MRLs for edible tissues from treated pigs.

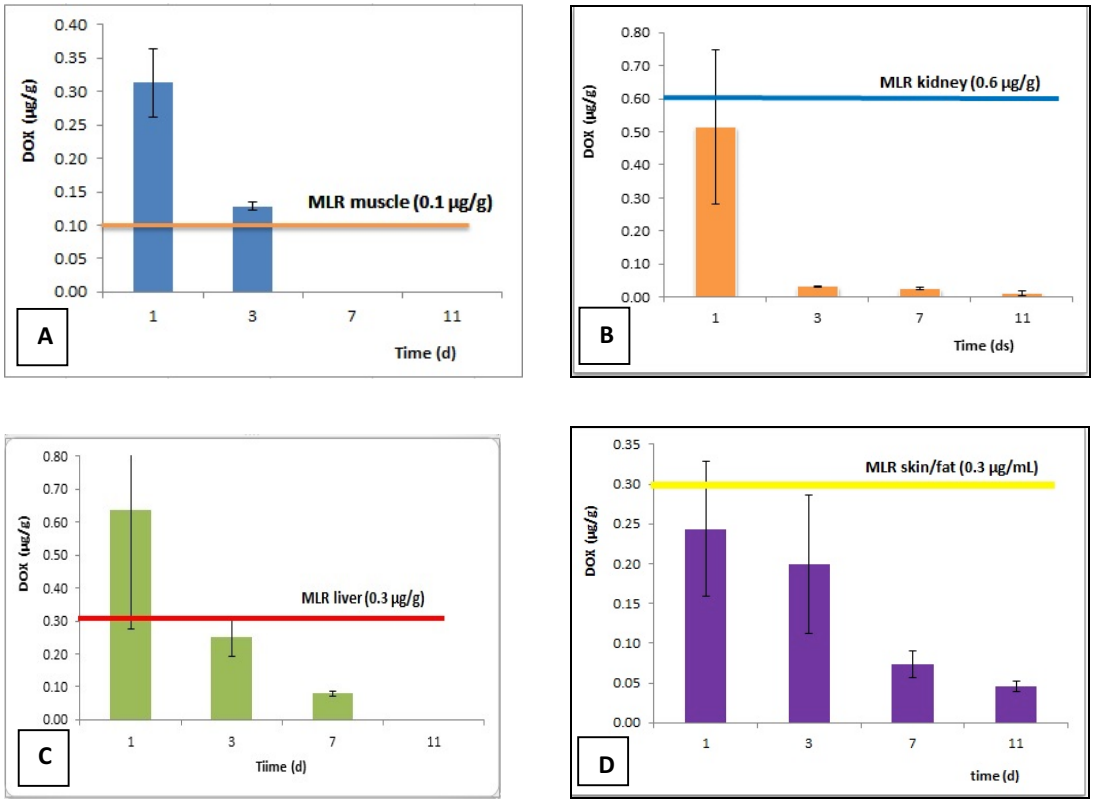

Figure 3. Mean tissue concentrations of doxycycline (A: muscle, B: kidney, C: liver and D: skin + fat) in pigs slaughtered 1, 3, 7 and 11 d after oral administration of DOX 25% (dose of 10 mg kg^{-1} body weight during 5 days).

Acknowledgements

Mr. Daniel Buldain is acknowledged for check the manuscript.

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