

LC-MS/MS analysis of doxycycline residues in chicken tissues after oral administration

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Abstract

For the purpose of quantitative determination of doxycycline (DC) residues in tissues, a sensitive liquid chromatography – tandem mass spectrometry (LC-MS/MS) method was developed. The method was used to determine DC residues in chicken tissues (breast and thigh muscle, liver and kidney) after oral administration with drinking water to five-week-old broiler chickens. The DC was administered for five consecutive days at a therapeutic dose of 10 mg/kg b.w. once a day. The tissues were collected after 6 h, 24 h, 7 d, and 8 d. The method was validated and the decision limit was established for muscle – 109.2 µg/kg, for liver – 326.1 µg/kg, and for kidney – 634.0 µg/kg. The detection limit was 2 µg/kg and the limit of quantification was 5 µg/kg. In a short period after ceasing the treatment, the detected concentrations of DC were much higher than the established maximum residue limit values. The highest residue concentrations of DC were observed in the kidney, followed by the liver and muscle. The lowest concentration of DC was determined in thigh muscle.

Keywords: chicken, doxycycline, residues, tissues.

Introduction

Doxycycline (DC) is a tetracycline derivate, widely used in the treatment of avian respiratory tract diseases. It shows a broad spectrum of antibiotic activity against Gram - positive and Gram - negative bacteria. This antibiotic is more lipid soluble than other tetracyclines and after application better penetrates body tissues and fluids (13). After oral administration, DC is rapidly and well absorbed from the gastrointestinal tract and widely distributed to the body with high affinity to tissues (14). The misuse of DC, illegal administration to animals, overdosing, and withdrawal period failure can lead to accumulation of this antibiotic residues in edible animal tissues. Because of persistence of DC residues in tissues, for consumer's health protection, the European Commission had set a maximum residue limits (MRLs) for this compound at 100 µg/kg in muscle, 300 µg/kg in the liver, and 600 µg/kg in the kidney (3). According to Commission Regulation (EU 37/2010) and EMA Summary Report Doxycycline 2, quantification of DC in animal tissues requires

a determination of only parent compound as the residue marker, without its 4-epimer, contrary to other tetracyclines in which case the MRLs are defined as a sum of parent drugs and their 4-epimers (3, 11).

DC residue studies have been performed in chickens (1), turkeys (8, 20), pigeons (9), and pigs (6, 7) with different doses and route of the drug administration. The antibiotics' 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 specific drug given (15). 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.

A suitable analytical method is an essential tool for determination of drugs in analysed matrix. Because DC is one of the most frequently used antibiotic in chickens, it was necessary to develop a suitable method for determination of this compound in chicken tissues. Many studies using analytical methods for determination of DC in animal tissues have been presented (4, 5, 8, 12, 17, 18, 21, 22). Most of them did not describe the application of methods in

the analysis of DC in samples collected from treated animals.

To determine DC residues in muscle, liver, and kidney, a sensitive and accurate LC-MS/MS method was developed. The method has been successfully validated according to the requirements of the European Decision 2002/657/EC (2). The presented method was applied for analysis of naturally incurred samples with DC. According to the control programme of antibiotic residues in food, the muscle and liver were collected. After oral administration of DC to broiler chickens at a therapeutic dose, and DC residue analysis in muscle, liver, and kidney, it was possible to verify the adequacy of the material collected as the target tissues for monitoring of the residues. Information about DC content and persistence in these tissues is important to ensure the safety of food supply. Additionally, the depletion of DC from muscle, liver, and kidney, and comparison of DC content in breast and thigh muscle was studied. The published information concerning deposition of DC in these two types of muscle in poultry is limited, so this data is necessary to select the most appropriate and target tissue for antibiotics monitoring.

Material and Methods

Animals. The experiment was conducted on 26 five-week-old chickens weighing 2.3 ± 0.3 kg, treated with 10 mg/kg b.w. of DC (Doxycycline 20%). The birds were kept in a special space designed for performing experiments on animals. The DC was given once a day, for five consecutive days. Administered product was prepared by dilution of 5 g of the medicament in 100 mL of water and the dose of 1 mL/kg b.w. was given to the crop of each chicken using syringe. Food and water were available *ad libitum*. Two chickens used as controls were euthanized before experiment, and muscle, liver, and kidney were collected and frozen at -20°C until analysis. The chickens treated with DC were euthanized 6 h, 24 h, 7 d, and 8 d after final drug administration (six animals at each time point) and muscle (breast and thigh), liver, and kidney were collected. All samples were kept separately at -20°C until analysis.

Reagents. Doxycycline (DC) and demeclocycline (DMC) standards were obtained from Sigma-Aldrich Chemical Company (USA). Acetonitrile and methanol were from J.T. Baker. Formic acid was from Fluka (USA). Oxalic acid dihydrate (ACS) and trichloroacetic acid (TCA) were from POCh (Poland). Water was purified using Milli-Q system. Strata X (33 μm , 100 mg, 6 mL) solid phase extraction (SPE) columns were obtained from Phenomenex (USA). Analytical column (Luna C18) was supplied by Phenomenex.

Standard solutions. Stock standard solution (1 mg/mL), was prepared by weighing 10.0 ± 0.1 mg of standard substances and dissolving in 10 mL of methanol. The stock was stored at the temperature below -18°C in amber glass, and was stable for six months. Working standard solutions (100 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$) prepared in acetonitrile by diluting suitable aliquot of stock standard were stable for three months, stored at $2-8^{\circ}\text{C}$ in amber glass. Working standard solutions in water were prepared on the day of analysis.

Extraction and clean-up. Two grams of muscle, liver, and kidney were homogenised and 100 μL of internal standard (IS) working solution, at the concentration of 2 $\mu\text{g/kg}$, was added to the samples.

Afterwards, the samples were mixed with 15 mL of 0.02 M oxalic acid buffer (pH 4.0), shaken for 10 min, and then centrifuged for 10 min at 2.500 rpm/min. The upper layer was transferred into a new tube. To the remaining matrix, an additional 10 mL of 0.02 M oxalic acid buffer (pH 4.0) was added and the samples were stirred and centrifuged for 10 min at 2.500 rpm/min. After double extraction, 3 mL of 20% trichloroacetic acid was added to the coupled extract solutions and the content of the tube was stirred and then centrifuged for 15 min at 2.500 g. The supernatant was transferred to SPE polymeric cartridges, which were preconditioned with 3 mL of methanol, 3 mL of 1 M hydrochloric acid, and 3 mL of water. After the percolation of the whole solution, the columns were washed with 3 mL of 0.02 M oxalic acid buffer (pH 4.0) and 2 mL of water, and dried (under vacuum) for 10 min. The TCs were eluted with 5 mL of methanol. The cleaned extracts were evaporated to dryness in nitrogen evaporator at 40°C . The dry residues were reconstituted in 1 mL of 0.1% formic acid in water and filtered through 0.45 μm PVDF filters.

LC-MS/MS analysis. Analyses were performed on an Agilent 1200 LC system consisting of a quaternary pump, autosampler, column heater (kept at 25°C), switching valve, automatic degasser (Agilent Technologies, USA), and API 4000 triple quadrupole mass analyser with a TurboIonSpray source (Applied Biosystems, Canada). The mass spectrometer was operated in electrospray positive ionisation mode (ESI+). MS data acquisition was performed in the MRM mode, selecting one precursor ion to two products ion transitions. The following mass spectrometer parameters were set: resolution Q1 and Q3 = unit, temperature = 500°C , curtain gas = 20 psi, nebulizer gas = 40 psi, collision gas = 3 psi, auxiliary gas = 50 psi, ion spray voltage = 5.500. Chromatographic analyses were performed on Luna C18 column (150 mm $\times$ 4.6 mm, 5 μm) with mobile phase consisting of A – 0.1% formic acid in water: B – 0.1% formic acid in acetonitrile with gradient mode at 0.2 mL/min flow rate. A gradient programme

was performed starting from 95% A and 5% B; at 1 min up to 12 min - 40% A and 60% B; at 12.01 min to 15.00 min - 10% A, 90% B; at 15.01 min to 20.00 - 95% A and 5% B. The injection volume was 30 μ L.

Validation. Samples of muscle, kidney, and liver were spiked with the DC working solution to levels corresponding to 0.5; 1; and $1.5 \times$ MRL. The recovery was determined by comparing peak area ratios (DC/internal standard) from fortified matrix samples with peak area ratios (DC/internal standard) from direct injections of equivalent quantities of standards. Six spiked samples with DC at three levels, within three different days were analysed. The precision (repeatability and reproducibility) of the method was determined. Linearity was tested by preparing matrix-matched calibration curve on six levels corresponding to 0.1; 0.5; 1.0; 1.5; 2.0; and $5.0 \times$ MRL. During the validation process, the decision limit ($CC\alpha$) and detection capability ($CC\beta$) were calculated. Additionally, in order to evaluate the limit of quantification (LOQ) of developed method, six samples were spiked at the concentration of 5 μ g/kg, which was the lowest point of matrix-matched calibration curve.

Results

In order to perform quantitative analysis of DC in muscle, liver and kidney, a liquid chromatography – tandem mass spectrometry method was chosen. In the presented method, the extraction was performed with oxalic acid 0.02 M, pH 4.0. For the clean up purposes, the polymeric Strata X cartridges were used. The use of polymeric SPE cartridges and double extraction of DC from tissues were found to be the most suitable with good recovery. The optimal conditions and transition parameters for the detection of DC are presented in Table 1. The dwell time for both transitions was 200 ms. Fig. 1 shows the MRM chromatograms of blank muscle sample and muscle sample spiked with DC. In the method, the separation

was made using C18 analytical column with mobile phase consisting of 0.1% formic acid in water and 0.1% formic acid in acetonitrile.

The developed procedure was sensitive with satisfactory precision and linearity. The limit of detection was established at 2 μ g/kg and the limit of quantification at 5 μ g/kg. The whole procedure was validated and the repeatability, reproducibility, recovery, linearity (calibration curve), decision limit, and detection capability were calculated. Obtained validation results are listed in Table 2.

After experimental administration of doxycycline to chickens at the dose of 10 mg/kg b.w. for five consecutive days, 6 h after treatment was completed, the highest content was found in the kidney and liver. The maximum determined concentration in the kidney was 8300 μ g/kg and in the liver - 7540 μ g/kg. In muscle, the detected values were lower, with maximum concentration of 4070 μ g/kg. Tissue concentration ranges of DC in breast and thigh muscle, liver, and kidney at 6, 24, 168, and 192 h after the last drug administration are presented in Table 3.

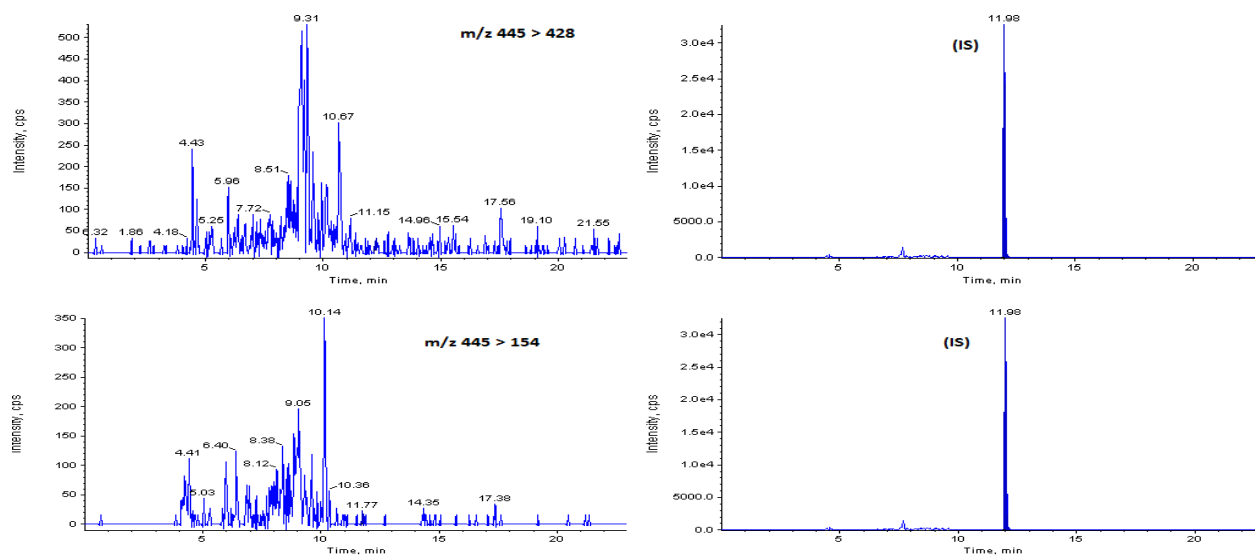
In the tissues collected after 24 h from final administration, a rapid decrease in DC concentration was observed. The DC content in muscle, liver, and kidney was much lower than that determined after 6 h, with the concentrations of DC slightly above imposed MRL values. After 1 d, the kidney contained 847 μ g/kg of DC, while in thigh muscle 243 μ g/kg of DC was detected. At 7 and 8 d after treatment was completed, the DC concentration was above LOQ of the method used. Additionally, the differences between breast and thigh muscle were observed. Higher concentrations in breast muscle comparing to thigh muscle were noted, just after the last administration, but after 8 d, the concentrations in both muscle were comparable. The depletion and mean DC concentrations with graphically presented standard deviation in muscle, kidney and liver are shown in Figs 2-5. Fig. 6 shows summarising comparison of determined DC content in the analysed tissues.

Table 1. Multiple reactions monitoring (MRM) mode for the detection of doxycycline

Analyte	RT(min)	Precursor (m/z)	Product (m/z)	DP (V)	CE (V)	CXP (V)
Doxycycline	10.47	445	428	55	25	15
			154	55	40	15
Demeclocycline (IS)	11.77	465	448	60	25	10

RT – retention time, DP – declustering potential, CE – collision energy, CXP – cell exit potential

a)



b)

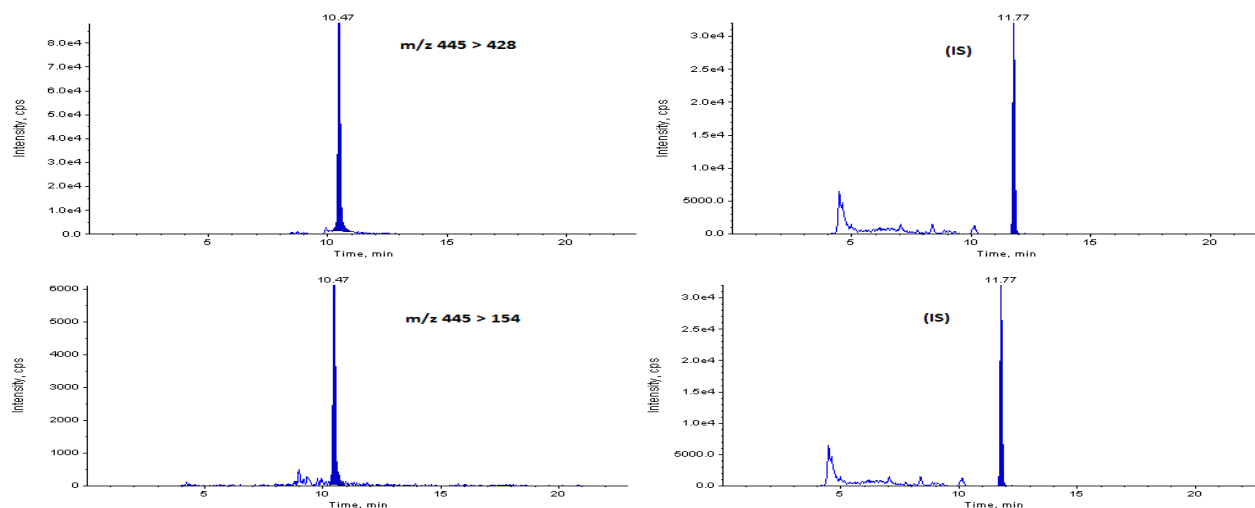


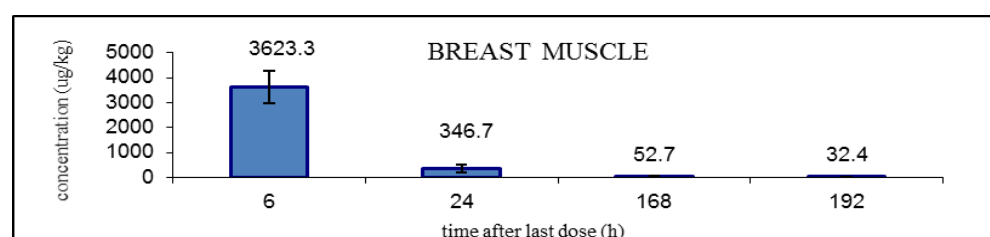
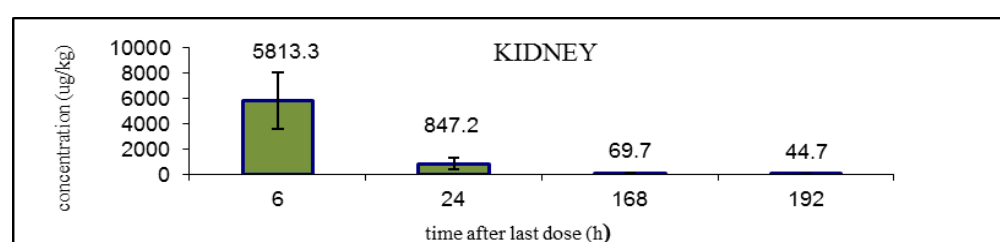
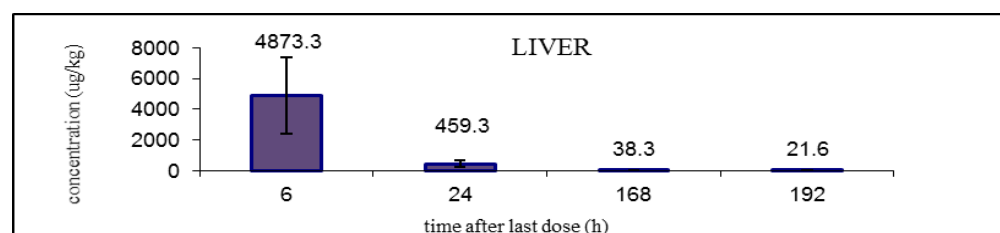
Fig.1. LC-MS/MS chromatograms of (a) blank muscle sample and (b) muscle sample spiked with DC at the concentration of 100 µg/kg

Table 2. Validation results of analytical procedure for the determination of doxycycline in muscle, liver, and kidney

Matrix	Level of spiked samples µg/kg	Repeatability CV%	Reproducibility CV%	Recovery %	Calibration curve	Working range µg/kg	CCα µg/kg	CCβ µg/kg
muscle	50	8.5	10.3	96.4	r = 0.9994	5-500	109.2	124.1
	100	3.9	7.3	100.1				
	150	3.2	7.1	102.6				
liver	150	8.5	11.3	100.5	r = 0.9976	5-1500	326.1	346.2
	300	4.2	14.3	98.1				
	450	5.9	15.8	99.7				
kidney	300	7.5	11.3	95.4	r = 0.9988	5-3000	634.0	659.5
	600	5.2	14.3	100.1				
	900	6.9	13.9	102.6				

Table 3. Tissue concentration ranges of doxycycline (10 mg/kg b.w.) after oral administration with water for five consecutive days

Time after last dose (h)	Tight muscle (n = 6)	Breast muscle (n = 6)	Kidney (n = 6)	Liver (n = 6)
	Concentration range (µg/kg)			
6	1223-3220	2900-4070	3940-8300	2620-7540
24	126-325	180-501	440-1370	236-618
168	36-50	36-63	52-100	29-49
192	19-26	26-37	36-56	15-27

**Fig. 2.** Depletion of doxycycline (mean values $\pm$ SD) in chicken breast muscle**Fig. 3.** Depletion of doxycycline (mean values $\pm$ SD) in chicken tight muscle**Fig. 4.** Depletion of doxycycline (mean values $\pm$ SD) in chicken kidney**Fig. 5.** Depletion of doxycycline (mean values $\pm$ SD) in chicken liver

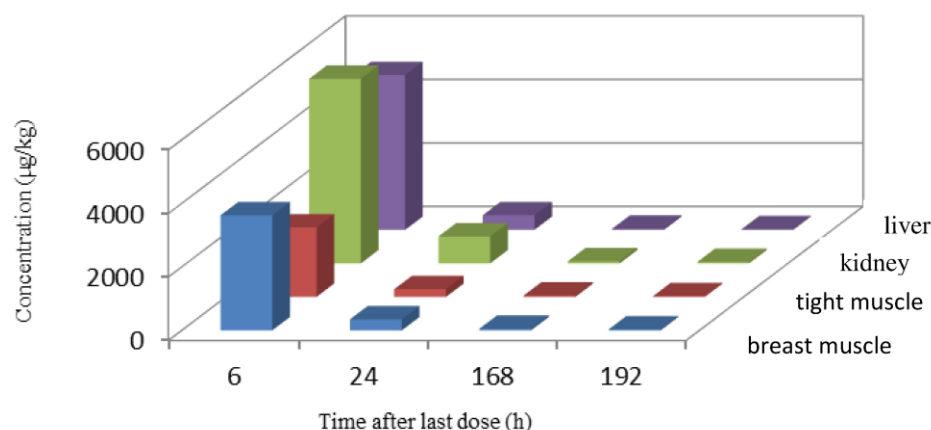


Fig. 6. Comparison of doxycycline concentrations in tissues

Discussion

Doxycycline is known as strong lipophilic compound, well absorbed after oral administration, and showing high tissue binding (13). The route of drug administration plays an important role in the effectiveness of the treatment, as well as in the distribution of antibiotics to tissues (16). In the presented study, the DC penetration and concentrations in muscle, liver and kidney after oral administration were analysed. Each bird was given the same dose of antibiotic, because the medicated water uptake can influence the antibiotic content in tissues. Studies on tissue concentrations after drug administration are significant in order to control antibiotic residues in food products, in references to the established MRLs values.

The results of conducted experiment show, that the developed method is suitable for quantitative determination of DC and depletion study in chicken tissues. The data obtained after DC administration with water shows, that at the beginning after treatment, the DC reached high concentrations in all collected tissues. The maximum DC concentrations during 1 and 8 d were significantly different. The elimination degree of DC was similar in the liver, kidney, and muscle. Twenty four hours after administration of the last dose, DC content rapidly decreased in all tested tissues. In the following days, the residues decreased gradually and only trace concentrations were observed on day 7. After 8 d, the DC concentration was less than one-half of MRL established.

Available literature shows some data regarding depletion of DC after experimental medication, but different doses and ways of administration were tested. The concentrations presented by Croubles *et al.* (8) in turkey's muscle and liver after administration of 25 mg of doxycycline HCl/kg b.w. in drinking water, determined at 12 h after last administration were similar to those achieved after 6 h in the present experiment. The DC contents, determined by Croubles *et al.* (7), 10 d

after treatment were as follows: 252 µg/kg in the liver and 151 µg/kg in muscle. The time necessary to reach concentrations below maximum residue limits imposed by EU were 12 d for the liver and 17 d for muscle, while in this experiment, DC residues were below MRL values after 7 d. Because of a higher dose of DC, the antibiotic persistence in tissues was longer. Croubles *et al.* (7) also determined the DC residues in edible tissues of pigs after administration with drinking water for 5 d at a dose of 10.5 mg/kg b.w. The concentrations in all matrices were below the MRL on 3 d after cessation of medication.

Anadon *et al.* (1) found the highest content of DC in the kidney and liver. After oral administration of 20 mg/kg of DC to broiler chickens for 4 d, 1920 µg/kg was determined in the kidney, 900 µg/kg in the liver, and 1180 µg/kg in muscle 24 h after the last administration. In our experiments, the DC content in muscle, kidney, and liver was lower after 24 h. Anadon *et al.* (1) found that 5 d were necessary to achieve mean concentration below the MRL. Tissue residue study presented by El-Gendi *et al.* (10) revealed that DC was still detected in chicken tissues 5 d after multiple oral administration of 20 mg/kg b.w. After intramuscular injection, its persistence for 9 d was recorded. Additionally, it was proven by El-Gendi *et al.* (10) that other drugs (diclazuril and halofuginone) induced higher doxycycline tissue residues.

Crivineanu *et al.* (6) demonstrated that after DC administration to pigs (60% Doxicol) at a dose of 200 g/1000 l water for 8 d, the antibiotic concentrations 24 h after the last dose were as follows: in muscle – 1042 µg/kg and in liver 2352 µg/kg. The described levels were higher than those obtained in our experiment. But after 8 d the residues of DC in tissues were comparable with results presented in this study.

Study regarding depletion and persistence of antibiotics in tissues collected from treated animals provide an accurate status of residues, which can occur in food of animal origin. The information about concentrations of antibiotics in various tissues can be

helpful in specifying, which type of matrix should be selected for residue monitoring. For DC residue monitoring in tissues, the muscle and liver are the target tissues. The site of drug deposition could vary between different antibiotics. Results obtained in the conducted experiment show that the greatest accumulation of DC was found in the kidney, so they are the most suitable for DC monitoring. However, because of a difficult access to the kidney in chickens, they are not included as material for DC monitoring. The comparison of DC concentrations in each tissue was presented in Fig. 6. As it was shown, the residues in the kidney are 2.6 times higher than those in muscle. In the described experiment, the thigh and breast muscle were collected separately. Our results showed higher residue concentrations in the breast *versus* thigh muscle tissues. The ratio concentration in the analysed muscle was 1:0.6 respectively. The similar observation was presented by Reyes-Herrera *et al.* (19) for fluoroquinolones, where the higher level of the drugs was found in breast muscle.

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