Chapter 15

IMPROVEMENT OF METHODS OF DETECTION OF ANTIBIOTICS IN LIVESTOCK PRODUCTS

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INTRODUCTION

Livestock industry, in particular, poultry farming, is one of the largest consumers of antimicrobial drugs, a significant proportion of which are antibiotics[1, 2]. However, the majority of mutant strains and antibiotic-resistant microorganisms originate from livestock complexes. The use of antibiotics even for preventive purposes is not prohibited by law, but the situation may change in the near future.

In Ukraine, on the recommendation of the World Food and Agricultural Organization (FAO), a bill on veterinary medicine and animal welfare has been developed. After its adoption, the use of antimicrobial agents for prophylactic purposes will be prohibited by law.

In this regard, the assessment of the impact of antibiotics on the safety of products of animal origin is a relevant and important issue. In addition, it is important to develop laboratory capacity to detect residual amounts of antimicrobial substances in livestock products in order to assess their suitability for further safe use[3].

Both screening and confirmatory methods are used to determine antibiotics in products of animal origin. The main screening laboratory methods for determining the residual amounts of antibiotics are

[1] Палишнюк К. Ю., Ткачук С. А. Токсико-біологічна оцінка м'яса курчат-бройлерів експрес-методом з використання інфузорії Tetrachymena Pyriformis за застосування препарату Даноксан-50. *Проблеми зооінженерії та ветеринарної медицини* : збірник наукових праць Харківської державної зооветеринарної академії. Харків : РВВ ХДЗВА. 2016. Вип. 33. С. 167–170.

[2] Кучерук М. Д., Галабурда М. А. Потенційні ризики за органічного виробництва продукції птахівництва та способи їх запобіганню. *Науковий вісник ветеринарної медицини.* 2020. № 2. С. 28–38.

[3] Гаркавенко Т. О., Азиркіна І. М. Нормативно-законодавчі вимоги щодо визначення залишкових кількостей антимікробних препаратів у продукції птахівництва. *Ветеринарна біотехнологія*. 2015. (27). С. 96–104.

microbiological, enzyme-linked immunosorbent assay (ELISA)[4], high-performance liquid chromatography and the arbitration method – liquid chromatography using a mass spectrometric detector (LC/MS/MS)[5]. The use of modern methods of determining antibiotics will prevent residual amounts from entering products of animal origin and prevent negative consequences for human health[6].

It should be noted that residual amounts of antibiotics entering the body can lead to allergic reactions, dysbacteriosis, the development of microflora resistance, etc.[7].

Among the antibiotics that are often used for chemotherapy of bacterial infections in poultry farming are fluoroquinolones. They are molecules obtained by chemical synthesis and have a wide spectrum of action. Their bactericidal action is based on the ability to suppress the activity of both chromosomal and plasmid bacterial DNA gyrase and thereby cause a functional termination of DNA replication in bacteria[8, 9, 10].

It is important to identify antibiotics, in particular, enrofloxacin in livestock products. It is influenced by a number of factors, especially the appropriate selection of samples for the study, depending on the target tissues.

Therefore, the development and application of modern research methods, as well as monitoring, sanitary-hygienic assessment of broiler chicken

[4] Pauter K., Szultka-Młyńska M., Buszewski B. Determination and Identification of Antibiotic Drugs and Bacterial Strains in Biological Samples. *Molecules*. 2020. Vol. 25(11) Pp. 2556. doi: 10.3390/molecules25112556

[5] Li S., Zhang C., Tang H.X., Gu Yu, Guo A.-J., Wang K., Lian K.-Q. Determination of 24 sulfonamide antibiotics in instant pastries by modified QuEChERS coupled with ultra performance liquid chromatography-tandem mass spectrometry. J Food Drug Anal. 2023. Vol. 31(1). Pp. 73–84. https://doi.org/10.38212/2224-6614.3434

[6] Bajkacz S., Felis E., Kycia-Słocka E., Harnisz M., Korzeniewska E. Development of a new SLE-SPE-HPLC-MS/MS method for the determination of selected antibiotics and their transformation products in anthropogenically altered solid environmental matrices. *Science of The Total Environment*. 2020. Vol. 726, 138071. https://doi.org/10.1016/j.scitotenv.2020.138071

[7] Лінійчук Н. В., Якубчак О. М., Галка І. В. Особливості накопичення енрофлоксацину в організмі курчатбройлерів. *Науковий вісник Національного університету біоресурсів і природокористування України*. 2017. Вип. 273. С. 115–122.

[8] JiaYa., Zhao L. The antibacterial activity of fluoroquinolone derivatives: An update (2018–2021). *European Journal of Medicinal Chemistry*. 2021. Vol. 224. 113741. https://doi.org/10.1016/j.ejmech.2021.113741

[9] Bhatt S., Chatterjee S. Fluoroquinolone antibiotics: Occurrence, mode of action, resistance, environmental detection, and remediation – A comprehensive review. *Environmental Pollution*. 2022. Vol. 315. 120440. https://doi.org/10.1016/j.envpol.2022.120440

[10] Paton J. H., Reeves D. S. Fluoroquinolone antibiotics. Microbiology, pharmacokinetics and clinical use. Drugs. 1988. Vol. 36(2). Pp. 193–228. doi: 10.2165/00003495-198836020-00004

slaughter products for the use of enrofloxacin is relevant to guarantee the safety of poultry products[11].

1. Research materials and methods

The work was carried out during 2010–2020 at the Department of Veterinary and Sanitary Examination (now the Department of Veterinary Hygiene named after Professor A.K. Skorokhodko) of the National University of Life and Environmental Sciences of Ukraine. Separate studies were conducted on the basis of the State Research Institute for Laboratory Diagnostics and Veterinary-Sanitary Examination (Kyiv) in the research chemical-toxicology department: in the liquid chromatography laboratory, the ELISA-test laboratory and the determination of mycotoxins; in the research bacteriological department, in the educational laboratory of pathomorphology of the Department of Anatomy, Histology and Pathomorphology of Animals named after Academician V. G. Kasyanenko of the Faculty of Veterinary Medicine National University of Life and Environmental Sciences of Ukraine. When working with experimental animals, the "General Ethical Principles of Animal Experiments" adopted at the First National Congress on Bioethics (Kyiv, 2001) and the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Scientific Purposes" were followed[12].

The material for the study were broiler chickens of the American cross "Cobb 500" and their slaughter products, as well as the antimicrobial drug Baytril 10%, which contains the active ingredient enrofloxacin.

At the first stage of research, the method of enrofloxacin determination was validated using a liquid chromatograph using a "Waters" mass spectrometric detector.

At the second stage, an analysis of the results of the monitoring of residues of B1 antibacterial substances in Ukraine for 2013–2019 was carried out in accordance with the State Monitoring Plan for residues of veterinary drugs and other pollutants in live animals and unprocessed food products of animal origin.

At the next stage, the slaughter products of broiler chickens were investigated for safety and quality when using enrofloxacin, the residual amount of enrofloxacin in the muscles of broiler chickens, slaughter products, blood and droppings was determined.

[11]Лінійчук Н. В., Якубчак О. М. Токсико-біологічна оцінка м'яса курчат-бройлерів за застосування препарату «Байтрил 10 %». *Наукові доповіді НУБіП України*. 2018. № 3(73). С. 1–8.

[12]Європейська конвенція про захист хребетних тварин, яких використовують для експериментальних та наукових цілей [Електронний ресурс]. 1986. Режим доступу: http://zakon0.rada.gov.ua/laws/show/994_137

Two groups of 20-day-old broiler chickens were formed for the experiment: control and experimental (20 chickens each). The chickens of the experimental group were given the drug "Baytril 10%" at a dose of 0.1 ml/kg of body weight for 5 days, together with the daily norm of water. Drinking water was used for chickens in the control group. After drinking the veterinary drug, 5 broiler chickens from each group were slaughtered at the beginning of the withdrawal period (6 days after the last use of the drug), at the end of the withdrawal period (12 days), 14 days and 20 days after the last drinking. Broiler chickens of the control group were slaughtered similarly to the experimental group. In both the experimental and control groups, the clinical condition of the chickens was monitored daily, their activity and feed and water intake were monitored.

To determine the residual amount of enrofloxacin in the blood, blood sampling was performed starting from the second day of drinking the drug. Litter was collected from each group separately, starting from the third day. The selection was carried out from different places of the poultry house.

The content of the residual amount of enrofloxacin in muscles, internal organs, skin, blood, droppings was determined by liquid chromatography using a mass spectrometric detector "Waters".

Feeding of broiler chickens in all groups was carried out with complete ration compound feed in accordance with the norms according to the age periods of cultivation.

The live weight of the chickens was determined by weighing on the scales of the company "Ohaus" on the 1st, 5th, 11th, 17th, and 19th days of the experiment. Average daily growth, slaughter yield, carcass weight were determined according to the method of T.M. Polivanova (1988). Slaughter was carried out according to established technological instructions in compliance with the norms of bioethics.

Determination of chemical indicators in poultry meat was carried out on 2, 3, 4 days of storage after slaughter at 4–5 ºC. Evaluation of organoleptic indicators of meat, reaction with copper sulfate, reaction to peroxidase, to ammonia and ammonium salts was carried out in accordance with the regulatory documents in force in Ukraine. The pH value of the muscle extract was determined by the potentiometric method using the pH meter Seven Multi "Mettler Tolledo".

Determination of bacteriological indicators: mesophilic aerobic and facultatively anaerobic microorganisms (MAFAnM), bacteria of the group of *Escherichia coli* (BGCP) was carried out in accordance with DSTU ISO

4833:2006[13], DSTU ISO 8446:2015[14], bacteria of the genus *Proteus* – in accordance with DSTU ISO 7444:2013[15], *Salmonella*, *Listeria monocytogenes* – in accordance with DSTU ISO 6579:2006[16], DSTU ISO 11290–1.2003[17], *Staphylococcus aureus* – according to DSTU ISO 6888 – 1:2003[18].

Determination of relative biological value and toxicity was carried out by the express method of toxicological evaluation of meat, meat products and milk using *Tetrachymena piriformis* infusoria.

Muscles with the highest content of enrofloxacin were selected for histological examination: wing muscles, pectoral muscles, thigh muscles, and lower legs. Selected pieces of muscle for microscopic examination were fixed in 10% (pH 7.2) aqueous formalin solution, dehydrated in ethanol of increasing concentration (60°, 70°, 80°, 96°, 100°) and embedded in paraffin through chloroform. Sections, 7–10 μm thick, were made using a sled microtome. Sections were stained with Karatsi's hematoxylin and eosin. Histological preparations were studied under a microscope MS 100 LED ("Micros"), photographed using a photo attachment NDPL-2 (2X) and a Canon EOS 550D camera.

Variational and statistical processing of digital data was carried out using Microsoft Excel computer software packages. Probability was determined by Student's test[19]. taking into account the criterion of significance: $p<0.05$; $p<0.01$, $p<0.001$.

[13] ДСТУ ISO 4833:2006 Мікробіологія харчових продуктів і кормів для тварин. Горизонтальний метод підрахунку мікроорганізмів. Техніка підрахування колоній за температури 30 °C (ISO 4833:2003, IDT). Чинний від 01.10.2007.

[14] ДСТУ 8446:2015 Продукти харчові. Методи визначення кількості мезофільних аеробних та факультативно-анаеробних мікроорганізмів. Чинний від 01.07.2017

[15] ДСТУ 7444:2013 Продукти харчові. Методи виявлення бактерій родів Proteus, Morganella, Providencia. 01.07.2014.

[16] ДСТУ EN ISO 6579-1:2022 Мікробіологія харчового ланцюга. Горизонтальний метод виявлення, підрахунку та серотипування Salmonella. Частина 1. Виявлення Salmonella spp. Чинний від 31.12.2023.

[17] ДСТУ ISO 11290-1:2003 Мікробіологія харчових продуктів та кормів для тварин. Горизонтальний метод виявляння та підраховування Listeria monocytogenes. Частина 1. Метод виявляння (ISO 11290-1:1996, IDT). Чинний від 01.10.2004.

[18] ДСТУ ISO 6888-1:2003 Мікробіологія харчових продуктів та кормів для тварин. Горизонтальний метод підраховування коагулазо-позитивних стафілококів (Staphylococcus Aureus та інших видів). Частина 1. Метод з використовуванням агарового середовища Беард-Паркера (ISO 6888-1:1999, IDT).

[19] Mishra P., Singh U., Pandey C.M., Mishra P., Pandey G. Application of student's *t*-test, analysis of variance, and covariance. *Ann Card Anaesth*. 2019. Vol. 22(4). Pp. 407–411. doi: 10.4103/aca.ACA_94_19.

2. Evaluation of the suitability of the method for the determination of enrofloxacin in poultry muscles, blood and droppings by liquid chromatography using a mass spectrometric detector

Assessment of the suitability of the method (validation) was carried out in accordance with the Decision of the European Commission 2002/657/EC of August 12, 2002, which ensures the implementation of Council Directive 96/23/EC[20]. As a result, such basic operating parameters as average value, recovery, coefficient of variation, CCα, CCβ were determined.

During validation, 3 series of 6 aliquots of pure material enriched at 50 μg/kg, 100 μg/kg, and 150 μg/kg of enrofloxacin for muscles were studied. Calculations are made in accordance with BS ISO 5725-2:2019[21]. CCα is considered the main parameter for confirmatory methods. According to the calculations carried out for muscles CCα is 9.76. In order to include in the State Monitoring Plan of residues of veterinary drugs and pollutants in live animals and unprocessed food products of animal origin, taking into account the fact that enrofloxacin is not a prohibited substance and has a limit of permissible level (MDR), other calculations were carried out, according to which CCα is 118, 59, taking into account the error and uncertainty in measurements.

In further work and expansion of the method to other indicators of this group (quinolones), re-validation was carried out. Calculations were performed using InterVal Software (Germany).

CCα, CCβ were determined according to the calibration curve constructed by enriching the matrix with different concentrations of the standard. At the same time, CCα is equal to 128.45.

The validation data obtained by us during the conducted studies testify to the effectiveness of the method, accuracy, practicality, perspective, and this method meets the requirements of the European Union.

The validation of the method of antibiotic determination in blood and feces was carried out according to the same principle as in muscles. Since the determination of enrofloxacin in blood and feces is not regulated, its content was determined according to the sensitivity of the device and the method. To determine erofloxacin in the blood, aliquots were enriched at the following levels: 20 μg/kg, 40 μg/kg, 60 μg/kg. During the calculations, we obtained SSα, which is 42.85.

[20] Commission Decision of 14 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (notified under document number C (2002) 3044), text with EEA relevance 2002/657/ EC. Off. J. Eur. Union. 2002. L 221, 8–36.

[21] BS ISO 5725-2:2019. Accuracy (trueness and precision) of measurement methods and results Basic method for the determination of repeatability and reproducibility of a standard measurement method.

Aliquots with a concentration of 25 μg/kg, 50 μg/kg, and 100 μg/kg were prepared for the litter. According to the calculations, CCα is 55.65

Therefore, a method for determining enrofloxacin in products of animal origin and other biological substrates using the LC/MS/MS method has been developed, which meets the requirements of the European Union and is included in the State Monitoring Plan for residues of veterinary drugs and pollutants in live animals and unprocessed food products of animal origin. In turn, this method was adapted and validated for the determination of quinolones in blood and feces.

3. Analysis of the monitoring of the residual amount of antibacterial substances in Ukraine for 2013–2019

In Ukraine, as of 2019, it is mandatory to determine the residual amount of the following drugs: benzylpenicillin, amoxicillin, ampicillin, florfenicol, tetracyclines (tetracycline, chlortetracycline, oxytetracycline, doxycycline), aminoglycosides (kanamycin, apramycin, streptomycin, dihydrostreptomycin, neomycin, gentamicin, lincomycin, spectinomycin), fluoroquinolones (enrofloxacin, norfloxacin, ciprofloxacin, flumequin), tylosin, erythromycin, sulfonamide drugs (sulfathiazole, sulfadimethoxine, sulfaguanidine, sulfadiazine, sulfamerazine, sulfamethazine, sulfamethoxypyridazine, sulfamethoxazole, sulfanilamide, trimethoprim), cloxacillin, nystatin, colistin, cephalosporins (ceftiofur, cefquin, cephalexin).

According to the State Monitoring Plan as of 2020, the following matrices are being investigated: beef, pork, muscles of chickens, geese, rabbits, turkeys, aquaculture (fish); milk, chicken eggs, honey.

According to the results of the analysis of the implementation of monitoring for the years 2013–2019, 2 positive results were found in chicken eggs that contained a residual amount of fluoroquinolones. In 2016, enrofloxacin was detected in eggs in the amount of 60.86 μg/kg, in 2017 – 114.2 μg/kg (with SSα=14.01). The study was carried out by enzyme immunoassay (ELISA), confirming the results by liquid chromatography using a mass spectrometric detector (LC/MS/MS). At the same time, no exceedance of the MDR of enrofloxacin was detected in chicken muscles and other matrices, which meets the requirements of Commission Regulation (EU) No. 37/2010[22]. According to this Regulation, the content of enrofloxacin in eggs is not allowed, and in the muscles of poultry, cattle, pigs, sheep, fish, in milk can be no more than 100 μg/kg.

[22] Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin (Text with EEA relevance). http://data.europa.eu/eli/reg/2010/37(1)/oj

Table 1

Residual content of B1 antibacterial drugs in poultry products (meat and eggs) for 2013–2019

Antibacterial drugs	The number of samples within the maximum permissible levels	Relative to the total number of samples, %	Number of positive samples
Amoxicillin	704	30,2	-
Florfenicol	22	18,3	-
Gentamicin	24	14,5	-
Tylosin	548	23,5	-
Enrofloxacin	1753	75,2	2
Norfloxacin	378	16,2	-
Doxycycline	1260	54	-
Oxytetracycline	257	11	-
Tetracycline	236	10,1	-
Sulfathiazole	192	8,2	-

It should be noted that antimicrobial drugs with the active ingredient amoxicillin, florfenicol, gentamicin, tylosin, enrofloxacin, norfloxacin, doxycycline, oxytetracycline, tetracycline, sulfathiazole are widely used in poultry farming (Table 1). In addition, in 74.4% of cases, enrofloxacin was detected in the muscles of birds, which did not exceed the maximum permissible levels.

However, the presence of even a small amount of antibacterial substances in livestock products can have a negative effect on the human body.

4. Effect of antibiotic enrofloxacin on clinical and slaughter parameters of poultry

Effect of enrofloxacin on the clinical condition and performance of broiler chickens. According to the obtained results, it was established that drinking enrofloxacin does not affect the clinical condition of the birds of the experimental group. The clinical condition and behavior of birds in the control and experimental groups were similar.

At the beginning of the experiment, the live weight of broiler chickens in both the experimental and control groups did not differ significantly. After the use of enrofloxacin, the body weight of broiler chickens began to increase, compared to the control group. The tendency to increase the body weight of broiler chickens of the experimental group compared to the control group is monitored. However, according to the obtained results, no statistically significant difference was found between the body

weight indicators of broiler chickens of the experimental and control groups. Live weight and gains corresponded to the standard of broiler chickens of the American cross "Cobb 500".

After analyzing the obtained data, it was concluded that fluoroquinolones, namely enrofloxacin, do not affect the clinical condition of broiler chickens, but have a stimulating effect on the body of the bird, resulting in an increase in body weight. But one should not forget about the inadmissibility of uncontrolled use of antibacterial drugs and non-compliance with the rules for their use and compliance with the withdrawal period.

Analysis of pre-slaughter and post-slaughter examination of broiler chicken slaughter products. The pre-slaughter examination of the control and experimental groups showed that the broiler chickens moved actively, actively took feed and water, responded to external stimuli, had a natural body position, a clean feather cover, visible mucous membranes were pale pink, beaks were dry, there was no discharge from the eyes, the surfaces of the limbs are dry, without damage, breathing without wheezing, body temperature – in the range from 40.5 °C to 42 °C, the droppings are moderately thick. During the experiment, no cases of disease or death of experimental chickens were detected.

No pathological changes were detected during the examination of poultry slaughter products of the control and experimental groups. The chicken carcasses of the experimental groups had a general appearance similar to the poultry carcasses of the control group. The surface of the carcasses of broiler chickens of both groups was dry, the color of the skin on the entire surface in all groups was pale yellow, and the beak was glossy. The muscles of the carcasses were well developed, the subcutaneous fat was localized in the area of the lower part of the abdomen and on the back in the form of a strip. On cross-section, the muscles are slightly moist, elastic, and dense. The keel of the sternum is weakly distinguished, the shape of the sternum is rounded. The smell of carcasses is specific, characteristic of this type of meat.

When studying the effect of enrofloxacin on the body of broiler chickens, it was found that there was no statistically significant difference between the body weight indicators of chickens in the experimental and control groups. But there is a tendency to increase the body weight of broiler chickens from the experimental to the control group.

To assess the effect of fluoroquinolone on the body, the slaughter yield of carcasses was analyzed. The pre-slaughter live weight of broiler chickens of the experimental group on the 6th day after the last drinking of the drug exceeded the body weight of the birds of the control group by 14.89%, on the 12th day after drinking – by 16.24%, on the 14th day – by 17.6%.

The weight of half-fed carcasses at the beginning of the enrofloxacin elimination period exceeds the weight of carcasses of the control group by 18.66%, by 18.81% on the 12th day after drinking, and by 19.81% on the 14th day. The weight of the cartridge carcass of broiler chickens on the 6th day after drinking is 19.35%, on the 12th day – by 19.54%, on the 14th day – by 21.27% more than the mass of the cartridge carcass in the control group. According to the obtained data, an increase in the pre-slaughter weight, weight of half-carcass, and carcases of the experimental group was found, compared to the control group, both at the beginning and after the withdrawal period.

Organoleptic, chemical, microscopic parameters of the slaughter products of broiler chickens in case of ingestion of enrofloxacin. Organoleptic tests were carried out at room temperature in the range of 15–20 °C for 4 days in a well-lit room. The drying crust was pale pink, the muscles on the section were slightly moist, and did not leave a wet spot on the filter paper. The color of the meat is pale pink. When cut, the meat is dense, springy; the dimple formed when pressing with a finger quickly leveled off. The smell is pleasant, characteristic of this type of meat. The broth is transparent, aromatic, fat on the surface in the form of large drops.

The pH value of the muscles of broiler chickens in the control group was from 5.58 to 6.22, in the experimental group – from 5.72 to 6.32 for 2, 3, 4 days of storage in a refrigerated state, which is within the normal range ($p \leq 0,05$). The value of pH during the entire storage period and regardless of the day of slaughter was higher in the extract from the muscles of the experimental group. The use of enrofloxacin in a therapeutic dose increases the pH of meat of broiler chickens and the reaction changes in the alkaline direction.

According to the results of the reaction with copper sulfate, the broth prepared from the meat of broiler chickens of both the control and experimental groups 6, 12, 14 days after drinking the drug during storage for 2, 3, 4 days was fresh. But the meat taken on the 14th day after drinking the drug in the experimental group for the 4th day of storage at a temperature of 4–5°C was of questionable freshness.

The reaction to peroxidase in both groups was positive during 4 days of storage, which indicates the freshness of the meat.

According to the results of the reaction to ammonia and ammonium salts, the meat of broiler chickens of the experimental group is fresh during 3 days of storage. On the 4th day of storage, meat sampled on the 14th day after drinking enrofloxacin had a yellow color and slight turbidity was observed, which indicates questionable freshness.

During microscopy of smears-prints on the second day of storage, single microorganisms were detected in both groups, on days 3 and 4 – no more than 10 single microorganisms were detected, and on the 4th day of storage in the

meat of the experimental group, selected on the 14th day after the last drinking of the drug, up to 15 microorganisms were detected in the field of vision.

Therefore, according to chemical and microscopic indicators, the meat of both the control and experimental groups meets the requirements of a fresh product.

Residual amounts of enrofloxacin in the slaughter products of broiler chickens in case of enrofloxacin use. After drinking enrofloxacin, slaughter was performed on the 6th, 12th, 14th, 20th day. Determination of the active substance was carried out by the method of high-performance liquid chromatography using a mass spectrometric detector.

The obtained results indicate that the highest content of enrofloxacin was found in the muscles of the wings, chest, back, thigh, lower leg, skin, liver and stomach (Table 2).

Table 2

The residual amount of enrofloxacin in the slaughter products of broiler chickens after drinking enrofloxacin in a therapeutic dose, μg/kg, M±m, n=5

Carcass parts	6 days after stopping drinking	12 days after stopping drinking	14 days after stopping drinking	20 days after stopping drinking
Neck muscles	73.47±2.48	59.76±3.17*	61.94±2.12*	-
Wing muscles	441.39±6.56	151.25±7.31*	90.11±2.33*,**	-
Chest muscles	664.67±8.04	75.63±3.87*	46.57±1.61*,**	-
Back muscles	730.09±7.07	58.71±3.87*	63.06±1.79*,**	-
Thigh muscles	304.04±7.67	141.99±8.06*	45.70±1.32*,**	-
Muscles of the lower leg	685.68±13.11	71.62±3.60*	55.69±2.37*,**	-
Kidneys	55.82±2.64	48.76±4.01*	44.73±2.00*	-
Heart	40.34±1.45	41.04±2.60	30.96±1.00*,**	-
Liver	202.43±2.55	52.43±2.04*	37.25±1.71*,**	-
Lungs	17.39±0.65	44.26±2.59*	33.40±0.95*,**	-
Spleen	42.54±1.06	40.89±2.61	28.55±0.90*,**	-
Stomach	254.63±5.14	29.81±0.50*	29.53±1.04*	-
Skin	1016.5±55.02	742.23±14.78*	585.85±15.85*,**	90.12±23.04*,**

* – $p \leq 0.05$ compared to the indicators on the 6th day after stopping drinking the drug.

** – $p \leq 0.05$ compared to the indicators on the 12th day after stopping drinking the drug.

On the 6th day after stopping enrofloxacin drinking, the neck muscles and internal organs: kidneys, heart, lungs, spleen had enrofloxacin content below the maximum permissible levels. In turn, an excess of enrofloxacin was found in the muscles of the wings, pectorals, back, thigh, lower leg, muscle stomach, and liver. The highest content of the active substance was found in the skin, which is 10 times higher than the maximum permissible level.

The muscles of the wings and thighs have an excess – at the limit of the maximum permissible level. The skin on the 12th day after drinking the drug remains the most dangerous in terms of the content of enrofloxacin and has an excess, compared to the maximum permissible level, by 7.4 times.

On the 14th day after stopping drinking the drug, all examined skeletal muscles and internal organs are within the maximum permissible levels, except for the skin.

According to research results, even 12 days after the last use of enrofloxacin, an excess of its content was found in the muscles of the wings, thighs and skin. Only on the 14th day, the residual amount of enrofloxacin in the muscles is below the maximum permissible levels. The content of enrofloxacin in the skin within the maximum permissible levels was detected only on the 20th day (90.12±23.05) after the last intake of the drug. This indicates that the slaughter of poultry after the use of enrofloxacin must be carried out no earlier than 20 days after the last use of enrofloxacin. Therefore, the highest content of enrofloxacin after the withdrawal period was found in the skin, and according to the conducted risk assessment regarding the presence of enrofloxacin in the carcasses of broiler chickens, the need for a mandatory skin examination in case of drug use was proved.

Determination of the content of enrofloxacin in the blood and litter of broiler chickens in case of its use. To determine enrofloxacin in the blood, its selection was carried out from the second day of drinking the drug. Litter was collected from each group separately, starting from the third day (Table 3).

When determining the content of enrofloxacin in the blood by liquid chromatography with a mass spectrometric detector, the highest content was detected during the first days of use of the veterinary drug. The content of enrofloxacin decreased day by day. Despite the fact that antimicrobial agents are not regulated in the blood, the amount of enrofloxacin remains at a level above 100 μg/kg for 15 days from the start of its use.

The highest content of enrofloxacin in the litter was found in the first days of drug use, later its content decreased, but remained high for 17 days. Therefore, during the conducted study, a high content of enrofloxacin was observed in the blood and droppings, and a high content of enrofloxacin in the blood was detected up to the 16th day of the experiment, and in the droppings – up to the 18th.

Table 3

Enrofloxacin content in blood and litter of broiler chickens, M±m, n=15

Blood sampling, the day of the experiment	Residual amount of enrofloxacin, μg/kg	
	blood	feces
2	1091.56±13.91	-
3	1914.45±23.68	7431.70±85.74
4	1722.40±43.74	3203.75±47.28
5	1531.59±21.07	2181.18±57.57
6	981.82±10.22	1940.88±9.57
7	780.49±10.09	1117.75±21.03
8	458.69±6.92	945.05±5.13
9	304.54±3.71	699.77±13.17
10	218.41±2.76	619.48±6.23
11	166.75±3.37	509.49±11.65
12	145.99±4.53	432.32±7.04
13	123.57±1.99	401.81±2.3
14	119.05±2.09	254.11±3.03
15	105.36±2.26	184.88±3.48
16	96.32±1.82	131.15±3.23
17	72.89±1.52	104.08±1.31
18	62.30±2.94	96.44±1.64
19	46.87±1.05	79.71±0.55

So, if a significant amount of the drug is detected in individual muscles and skin even 12–14 days after drinking, then in the blood of the bird and, especially, in the droppings, enrofloxacin remains in significant quantities for much longer. This causes a significant negative impact on the environment, which must be taken into account when using the drug.

Muscle toxicity of broiler chickens under the conditions of enrofloxacin intake. The toxicological assessment was carried out according to the "Methodological instructions for the toxicological assessment of meat, meat products and milk using Tetrachymena piriformis ciliate (express method)". Taking into account the previous results of the study, pectoral muscles, thigh muscles, and lower legs were used for toxicological assessment (Table 4).

On the basis of the conducted studies, it was established that the meat of broiler chickens treated with enrofloxacin and the poultry meat of the control group already 6 days after the last drinking session are non-toxic to *Tetrachimena pyriformis* infusoria. In the broiler chickens of the experimental group, no death, retardation of movements, inhibition of growth, and pathological changes were detected.

During the study of the biological value of the muscles of broiler chickens, it was established that the meat of the experimental group on the 6th day after the last drinking of the drug has a slightly lower relative

biological value, compared to the corresponding muscles of the control group (by 0.56–0.96%).

On the 12th day, the biological value is lower by 0.03–0.19%, compared to the control group.

Table 4

Relative biological value of meat of broiler chickens, M ±m, n=5

Groups	Researched material	The number of cells in 1 ml of medium × 10^4	Biological value relative to control, %
Control	Pectoral muscles	48.661±0.154	100
	Thigh muscles	48.355±0.179	100
	Leg muscles	48.716±0.205	100
Experimental 6 days	Pectoral muscles	48.233±0.131	99.12
	Thigh muscles	48.082±0.186	99.44
	Leg muscles	48.250±0.181	99.04
Experimental 12 days	Pectoral muscles	48.649±0.108	99.97
	Thigh muscles	48.266±0.125	99.81
	Leg muscles	48.689±0.139	99.94
Experimental 14 days	Pectoral muscles	48.661±0.076	100
	Thigh muscles	48.333±0.108	99.95
	Leg muscles	48.711±0.160	99.59

On the 14th day after the last intake of enrofloxacin, the relative biological value of the pectoral muscles is the same in both experimental and control groups and is 100%, lower leg muscles – 0.05% lower, thighs – lower by 0.41%, compared to the control. Therefore, a trend towards an increase in the relative biological value of the meat of the experimental group was revealed, which is probably associated with the gradual elimination of the residual amount of the veterinary drug.

Microbiological indicators of muscles of broiler chickens in case of enrofloxacin use. Studies of microbiological indicators showed that the use of enrofloxacin in broiler chickens did not affect the level and species composition of the microflora in the studied muscles of the bird. Microbiological indicators in the research group met the requirements

of current legal acts. The amount of MAFAnM in the muscles of broiler chickens of the experimental group is smaller, compared to the control group. In turn, wing muscles from broiler chickens of the experimental group slaughtered on the 6th day after drinking have a 40% lower MAFAnM index compared to the control group, on the 12th day after drinking – by 32.4%. On the 14th day of slaughter, the muscles of broiler chickens of the experimental group also had a lower amount of MAFAnM compared to the control group. But at the same time, we can note that the amount of MAFAnM increases in all muscle groups, depending on the day of slaughter. The obtained results can be explained by the fact that bacteriological studies were carried out before the end of the withdrawal period and shortly after its end, as a result of which a certain amount of the residual amount of the antibiotic remained in the muscles of the bird, which caused a bactericidal effect, which affected the results of the study.

Microscopic changes in the muscles of broiler chickens with the highest content of enrofloxacin. The muscles of the wings, pectorals, thighs, and lower legs (biceps brachii, pectoralis major, biceps femoris, calf muscle) were used for the study.

During the histological examination, it was established that on the 6th day after the last use of enrofloxacin, microscopic changes were detected in all the skeletal muscles of the chickens we examined. Cells of altered muscle fibers were found in some parts of the muscles, the sarcoplasm of the muscle fiber was unevenly stained with eosin, on the transverse sections of the muscle fiber in its sarcoplasm, intensely red fragments of the myosymplast of a rounded shape were found. fibers

On the 12th day after the last use of the drug, changes were detected in the form of areas of altered muscle fibers, with partially destroyed myosymplast and in the form of atrophied muscle fibers.

On the 14th day after the last use of the drug, the microscopic changes differed from the changes detected on the 12th day. Previous changes were no longer recorded, except for atrophied muscle fibers, which indicates the correlation of microscopic changes in muscles with chemical parameters and residual amounts of enrofloxacin in them.

CONCLUSIONS

The applicability of the liquid chromatography method with mass spectrometric detection for determining the residual amounts of the antibiotic enrofloxacin in order to control the safety of broiler chicken slaughter products and poultry by-products was evaluated. At the same time, the following validation characteristics were obtained: for muscles

CCα=118.59 μg/kg, CCβ = 137.19 μg/kg, for blood – CCα = 42.85 μg/kg, CCβ=45.69, for litter – CCα= 55.65 μg/kg, CCβ=61.3.

A sanitary-hygienic and assessment of the slaughter products of broiler chickens in the case of the use of enrofloxacin in a therapeutic dose was carried out according to organoleptic, chemical, biochemical, microscopic, microbiological, histological indicators and residual amounts of the drug. The use of enrofloxacin in broiler chickens does not affect the clinical and slaughter parameters of birds that meet the cross standard.

According to organoleptic, chemical, biochemical, microscopic and microbiological indicators, the meat of broiler chickens, which were given enrofloxacin in a therapeutic dose, for 3 days of storage at a temperature of up to 4 °C meets the requirements of fresh meat, and on the 4th day, the meat has a questionable freshness. The highest content of enrofloxacin after administration of the drug to broiler chickens in a therapeutic dose (0.1 ml/kg) was found in the muscles of the wings, thighs and, especially, the skin. A decrease in the content of the drug in the skin to the maximum permissible level was detected only on the 20th day. A significant content of enrofloxacin was detected in the blood and droppings of broiler chickens in the case of its use in a therapeutic dose, which must be taken into account when monitoring its residues in slaughter products and poultry by-products. The use of slaughter products of broiler chickens, other than skin, 14 days after the last use of the drug is relatively safe, since the residual amounts of enrofloxacin do not exceed the maximum permissible level for slaughter products of poultry, established by Regulation of the European Union 37/2010.

According to the toxicological evaluation using the test culture of tetrachymena pyriformis ciliates, the muscles of broiler chickens 24 hours after the last use of enrofloxacin in a therapeutic dose for 5 days did not show a toxic effect and had a relative biological value characteristic of safe meat.

The use of enrofloxacin in broiler chickens in a therapeutic dose causes pathological changes in the microstructure of various muscle groups, which were characterized by areas of altered fibers: myosymplast defibrillation, lysis, destruction. The level of severity of these pathological changes in the microstructure of the muscles of broiler chickens decreased with an increase in the enrofloxacin withdrawal period.

SUMMARY

The first time in Ukraine developed and validated methodology for the determination of enrofloxacin in combination with other antibacterial substances of various groups in poultry muscles, blood and litter was improved and validated in accordance with Commission Decision 657/2002.

The residual amounts of antibacterial substances in Ukraine were monitored for 2013–2017. The two positive results were revealed in the eggs, which had a residual amount of enrofloxacin.

The meat of broiler chickens have been given the veterinary preparation Baitril 10% is non-toxic. Behind microbiological indicators, meat meets the requirements of regulatory legal acts. Histological examination revealed microscopic changes.

The highest concentration of enrofloxacin, when administered by a veterinary drug, was determined in the muscles of the wings, thigh and skin.

The high level of residual enrofloxacin in the blood and litter is observed during the study.

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