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Determination of the residual content of veterinary drugs in raw materials of animal origin by enzyme immuno assay method

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Abstract

Background and Aim. The article deals with the problem of the use of residual veterinary drugs in animal husbandry and poultry farming. In the modern world, the use of antibiotics in animal husbandry is a common problem. However, their use is often uncontrolled and can lead to contamination of food products of animal origin with medicines. Within the framework of this work, the contamination of raw materials of animal origin and food products sold on the territory of Kazakhstan has been studied.

Materials and Methods. The studies were conducted by enzyme-linked immunoassay (hereinafter referred to as ELISA) to determine the permissible levels of residual content of veterinary drugs. The studies took into account the content of residual amounts of pollutants such as chloramphenicol, sulfamethazine, nitrofuran 3-amino-5morpholinomethyl-2oxazolidinone (AMOZ), bacitracin in raw materials of animal origin: horse meat, pork, poultry, beef, lamb. The use of the ELISA method for the analysis of the content of antibiotics in meat allows for accuracy and reliability in determining the residues of veterinary drugs. This is necessary to comply with regulatory requirements and ensure the safety of meat products consumption.

Results. Our results show that the residual levels of antibiotics in animal raw materials are within the permissible limits. This is an important conclusion for food safety.

Conclusion. Based on the studies conducted in this work, monitoring of antibiotic residue in animal raw materials, it can be concluded that, their use in livestock and food production should be monitored continuously.

Keywords: chloramphenicol; ELISA; meat; nitrofuran; sulfamethazine.

Introduction

Among a number of substances that can contaminate food raw materials and foodstuffs, veterinary drugs used both for the treatment of animals and as growth stimulants occupy an important place. Antibiotics remain the most potent drugs used in veterinary medicine [1].

Antibacterial drugs are widely used in the cultivation of productive animals, so it is necessary to monitor their content in food at all stages of production. For these purposes, many laboratories use the method of immuno-enzyme analysis.

The term "antibiotics" covers a wide range of chemicals that are produced naturally, semi-synthetically and synthetically and are used to inhibit bacterial growth or destroy them [2].

It should be noted that antibiotics with a similar chemical structure have a similar antimicrobial spectrum [3].

The systematic intake of antibiotics into the human body with food is extremely harmful, most often they can cause various allergic reactions, dysbacteriosis, metabolic disorders, impaired kidney function, suppress the activity of certain enzymes, and inhibit the intestinal microflora.

The possibility of toxic, teratogenic and mutagenic effects is also not excluded [4].

Conjugates of antigens and antibodies with various proteins, synthetic polymers, enzymes and their substrates and cofactors are used in ELISA [5].

The method of enzyme immunoassay continues to develop. On the one hand, the range of research objects is expanding, and on the other, analysis methods are deepening and improving. This leads to simplification of the procedure, reduction of reagent consumption and reduction of analysis time. The development of the enzyme immunoassay method is also influenced by the chemistry of high-molecular compounds, cellular and genetic engineering, which changes the technology of obtaining reagents for this method. To determine antibiotics, a solid – phase enzyme immunoassay is used, based on the competition of a free antibiotic from the sample and an immobilized antibiotic in the solid phase during reaction with specific antibodies. After separating the unbound reagents, the quantity of antibodies interacting with the immobilized antigen is determined, using secondary antibodies labeled with peroxidase. The amount of secondary antibody conjugate bound to the antibodies is measured using a substrate and chromogen mixture. The antibiotic concentration in the sample is inversely proportional to the optical density measured from the enzymatic reaction product [6, 7].

To confirm the results obtained, an instrumental method of highperformance liquid chromatography with mass selective detection (HPLC MS/MS) is used.

According to the study of Arsene M.M.J and others (2021), antibiotics are the most important compounds in the field of veterinary medicine and animal husbandry. They are substances that can destroy or inhibit the growth of bacteria. Their use is almost inevitable in the treatment of bacterial infections in both animals and humans [8].

According to Arsène M.M.J. et al (2021), maintaining a waiting period and conducting physico-chemical tests are necessary to ensure that residue levels of antibiotics or their analogues do not exceed the acceptable limits (MRL) before food is released for sale. This measure is extremely important for public health, since antibiotic residues in food and the rise of antibiotic resistance are a serious problem [9].

According to Busch G. et al (2020), with the increasing use of antibiotics in agriculture, the focus is on their safety and effectiveness. This is of great importance as it aims to protect consumers from serious infections that can be transmitted to humans through contact with infected animals, consumption of contaminated food or spread in the environment [10].

On the territory of Kazakhstan, the residual content of antibacterial drugs in raw materials of animal origin is regulated by regulatory documents, such as:

Technical Regulations of the Customs Union "On food safety" (TR CU 021/2011) [11].

Technical regulations of the Customs Union (TR CU 034/2013) "On the safety of meat and meat products" [12].

Technical Regulations of the Eurasian Economic Union (EAEU TR 051/2021) "On the safety of poultry meat and processed products" [13].

"Unified sanitary-epidemiological and hygienic requirements for products (goods subject to sanitary-epidemiological supervision (control)" Approved by the Decision of the Commission of the Customs Union dated May 28, 2010 No. 299 [14].

The purpose of this work was to determine the content of the residual amount of antibiotics in food products of animal origin sold on the territory of the Republic of Kazakhstan.

The scientific novelty of the work consists in the fact that by the authors conducted monitoring studies to study the extent of distribution of raw materials and animal products containing residual amounts of antibiotics such as chloramphenicol, sulfamethazine, bacitracin, nitrofurans AMOZ.

Meat containing residual antibiotics should be sent to the manufacture of canned meat and vegetable meat, with the exception of canned food for baby food.

Materials and Methods

The study was conducted at the testing center of the National Veterinary Reference Center for Veterinary Control and the Supervision Committee of the Ministry of Agriculture of the Republic of

Kazakhstan (NVRC). This center is accredited in the accreditation system of the Republic of Kazakhstan in accordance with the requirements of GOST ISO/IEC 17025-2019 "General requirements for the competence of testing and calibration laboratories". The object of the study was animal samples received by the NVRC to determine the content of the residual amount of antibiotics in them. The study considered the residual content of pollutants such as chloramphenicol, sulfamethazine, nitrofuran (3-amino-5-morpholinomethyl-2-oxazolidinone, AMOZ), and bacitracin in animal-derived raw materials, including horse meat, pork, poultry, beef, and lamb.

Sample preparation and result processing were conducted according to standards MG–4.1.1912-04, MG 4.1.2158-07, MP No. KZ 06.03.00127-2021, and ST RK 2.638-2019.The determination of the residual content of pollutants in raw materials of animal origin was carried out in accordance with the methodology and instructions for use by the ELISA method, using such commercial test systems: "Algimed Techno" LLC, manufactured in the Republic of Belaarus; Elabscience Biotechnology Inc, manufactured in China; I'screen, Eurofins Technologies, manufactured in Hungary.

One of the main stages of the analysis can be distinguished: sample preparation and processing of the obtained results. For example, the sample preparation of bacitracin.

The progress of the study from sample preparation to calculation is reflected in Table №1.

Table 1- Bacitracin sample preparation

Sample preparation
Bacitracin
Meat samples without adipose tissue were crushed on a homogenizer, 1gram sample of homogenized sample was taken into a 15 ml centrifuge tube with a screw cap, 2.0 ml of 75% methanol solution was added and stirred on a vortex at maximum speed for 15 minutes. The test tube was centrifuged on a Thermo centrifuge at an acceleration of 4000 rpm at room temperature (20-25 °C) for 10 minutes. Aliquots of 100 µl of the supernatant were taken with a dispenser and transferred to 2ml test tubes. In test tubes with aliquots of the supernatant, we add 400 µl buffer to dilute the samples mixed on a vortex for 1 minute. For the analysis, 100 µl were used for each well of the plate.
Preparation of a microplate photometer (according to the operating instructions)
Application of standards and test samples. In two parallel wells of the microtitration plate, we dispense 100-µl of each standards in ascending concentration order. In corresponding wells, we place parallel samples of each test sample
Incubation of the Plate. Cover the plate with a film, incubate it at 20-25 °C for 60 minutes, then remove the film and quickly invert the plate to discard the liquid.
Rinsing the plate uncover the sealer carefully remove the liquid in each well. Immediately add 250µl wash buffer to each well and wash. Repeat wash procedure for 3 times, 30s intervals/time. Invert the plate and pat it against think absorbent paper.
Adding a substrate Solution for staining. Immediately after washing, add 100 µl of TMB substrate solution to each well and mix the contents with gentle circular motions on the plate surface.
Subsequent incubation. Cover the plate with a film, incubator it at 20-25 °C for 20minutes in a dark place
Completion of the staining reaction. Immediately after the end of the incubation time, 100 µl of stop reagent is added to each well to stop the enzymatic reaction

Continuation of table 1

OD Measurement: determine the optical density (OD value) of each well at 450 nm with a microplate reader. This step should be finished in 10 min after stop reaction.

Processing measurement results

Kit specification

Detection limit: 9.00 ppb.
Dilution ratio: 15, Cross–reaction rate - bacitracin-100%, zinc bacitracin -100%

Calculation

The result of measuring the optical density is expressed as a percentage of the optical density of the well with a zero standard (% absorption) according to the formula:

Absorption (%) = B/B0*100%

B – Average absorbance of standard or sample;

B0 – Average absorbance of 0 ppb Standard. Calibration curves are plotted on semi-logarithmic paper based on the relative absorption values calculated for standard solutions and the corresponding known values of antibiotic concentrations of mcg/kg (mcg/l), which should be linear in the main data range.

The concentration of the antibiotic in the studied samples is determined by the calibration curve of the measured relative optical density, respectively, Fig. 1,2.

Results

Study results. In 2023, 4,286 samples, of which beef meat - 2,109, horse meat -372, lamb-804, poultry - 498, pork - 503, were analyzed. Of these, 837 samples were examined in Astana, 850 samples in Akmola region, 662 samples in Pavlodar region, 581 samples in Karaganda region, 671 samples in North Kazakhstan region, and 685 samples in Kostanay region. Based on the results of laboratory studies within the framework of monitoring studies "Veterinary measures and food safety".

In Kazakhstan, the residual content of veterinary drugs in animal derived raw materials is regulated by the technical regulations "On Food Safety" (TR CU 021/2011) and "On the Safety of Meat and Meat Products" (TR CU 034/2013). The permissible level and limit of detection of veterinary drugs in raw materials of animal origin by the ELISA method are shown in Table No. 2.

Table 2 - Permissible level and limit of detection of veterinary drugs in raw materials of animal origin by the ELISA method

The studied samples	Name of the study	Permissible the level of antibiotics, mg/kg according to TR CU 021/2011, 034/2013	Limit of detection of veterinary drugs
Meat	Bacitracin	not allowed (< 0,02 mg/kg)	9 ppb
	Chloramphenicol	not allowed (< 0,0003 mg/kg)	0,0125 ppb
	Nitrofuran AMOZ	not allowed < 0,1 mg/kg	0,05 ppb
	Sulfamethazine	< 0,1 mg/kg	0,5 ppb

Table 2 presents the detection limits for bacitracin, chloramphenicol, nitrofuran, and sulfamethazine in animal-derived raw materials. In accordance with the requirements of TR CU 021/2011, 034/2013, the permissible level of antibiotics in meat is strictly regulated. According to the regulations TR CU 021/2011 and 034/2013, the content of bacitracin in meat is not allowed above the permissible level. The ELISA method allows the detection of bacitracin at a detection limit of 9 ppb (parts per billion). The maximum permissible level of bacitracin in meat is less than 0.02 mg/kg.

In accordance with regulations, the content of chloramphenicol in meat is not allowed < 0,0003 mg/kg. The ELISA method has a detection limit of 0.0125 ppb for chloramphenicol. The presence of nitrofurans in meat is not allowed < 0,1 mg/kg. The ELISA method can detect AMOZ with a limit of 0.05 ppb. The allowable level of sulfamethazine in meat is below 0.1 mg/kg. The detection limit of this substance by the ELISA method is 0.5 ppb.

Discussion and Conclusion

In a comparative aspect, the sensitivity of enzyme immunoassay methods to determine the presence of antibiotics has been studied. The calibration curve for bacitracin is shown in Figure 1. The results of the optical density of graduated solutions for bacitracin are shown in Table -3.

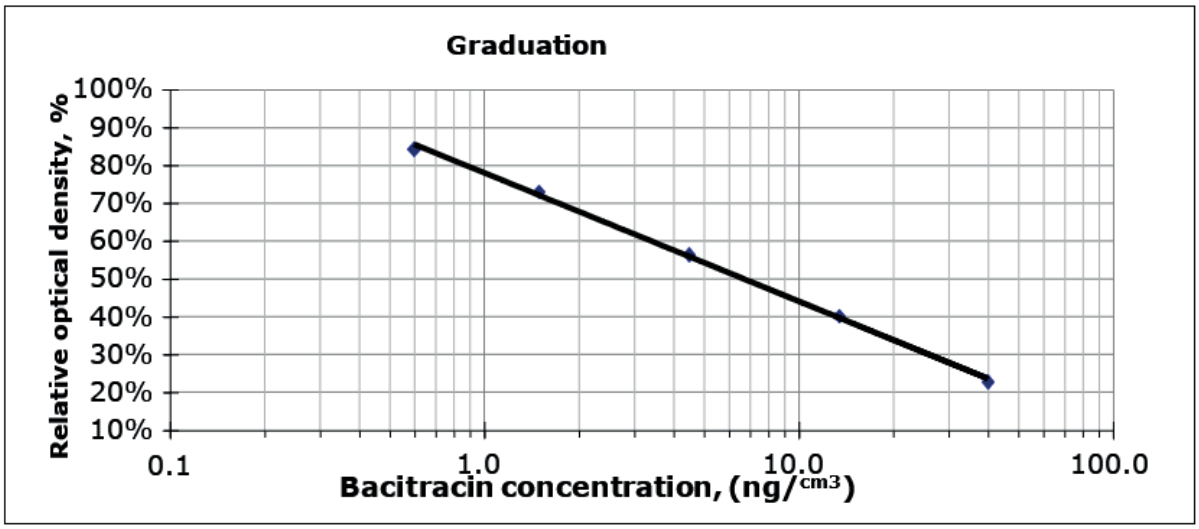

Figure 1- Calibration curve for bacitracin

Table 3 - Optical density of graduated solutions for bacitracin

Std. (ng/ml)	O.D.1	O.D.2	Bi	C.V. (%)	B/Bo (%)
0	2.797	2.913	2.855	2.86%	100.00
0.6	2.377	2.422	2.399	1.34%	84.04
1.5	2.037	2.156	2.097	4.01%	73.44
4.5	1.668	1.683	1.676	0.63%	58.69
13.5	1.075	1.073	1.074	0.13%	37.62
40.0	0.649	0.699	0.674	5.27%	23.61

As can be seen from Table 3, concentration and optical density as the concentration of bacitracin increases, there is a decrease in optical density (O.D.), which indicates an inverse relationship between the concentration and the measured optical density. The coefficients of variation for each standard are within acceptable values, which indicates good repeatability of measurements. B/Bo (%) shows how the optical density of each standard correlates with the density of the zero standard, which is key for constructing a calibration curve.

These data can be used to construct a calibration curve that will accurately determine the concentrations of bacitracin in samples with unknown concentrations. An accurate and repeatable methodology for measuring optical density using ELISA ensures reliable determination of bacitracin residues in animal raw materials.

Table 4 - Average content of antibacterial drugs in meat

The studied samples	Antibiotic	The permissible level of antibiotics, mg/kg according to TR CU 021/2011, 034/2013	The average content in all samples, mg/kg
Beef	NitrofuranAMOZ	< 0.1 mg/kg	< 0.0000115
	Chloramphenicol	< 0.0003mg/kg	< 0.0000125
	Bacitracin	< 0.02 mg/kg	0.0014
	Sulfamethazine	< 0.1 mg/kg	< 0.0005
Horse meat	Nitrofuran AMOZ	<0.1 mg/kg	< 0.00005
	Bacitracin	<0.02 mg/kg	< 0.009
	Chloramphenicol	< 0.0003 mg/kg	< 0.000075
	Sulfamethazine	<0.1 mg/kg	< 0.0005
Lamb	Nitrofuran AMOZ	< 0.1 mg/kg	< 0.00005
	Chloramphenicol	<0.0003mg/kg	< 0.000013
	Bacitracin	<0.02 mg/kg	0.0029
	Sulfamethazine	<0.1 mg/kg	0.0005
Pork	Nitrofuran AMOZ	< 0.1 mg/kg	0.00005
	Chloramphenicol	<0.0003mg/kg	< 0.000056
	Sulfamethazine	<0.1 mg/kg	0.0005
	Bacitracin	<0.02 mg/kg	< 0.009
Poultry	Chloramphenicol	<0.0003mg/kg	0.000013
	Nitrofuran AMOZ	< 0.1 mg/kg	0.00005

It can be seen from Table 4 that the studied samples of the residual content of antibacterial drugs are contained within the permissible limits. In the beef samples studied, the average content of all antibiotics except bacitracin is significantly below permissible levels. Bacitracin is present in amounts not exceeding acceptable levels.

In horse meat samples, the average content of all studied antibiotics is significantly below permissible levels. The average content of all antibiotics in lamb samples is below permissible levels, and some antibiotics are present in trace amounts. The average content of nitrofuran AMOZ in pork is below permissible levels, but they are present in quantities close to the detection limit.

In the studied poultry samples, all the studied antibiotics are present in trace amounts, significantly below permissible levels. The average content of antibiotics in meat samples of various animal species is within the permissible levels established by regulatory acts. These results indicate compliance with veterinary standards and a safe level of residual antibiotics in meat.

Authors' Contributions

AB and MM: Conceptualized and designed the study, conducted a comprehensive literature search, analyzed the gathered data and drafted the manuscript. ShG, EY and AH: Conducted the final revision and proofreading of the manuscript. All authors have read, reviewed, and approved the final manuscript.

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