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Original article

Investigation of tylosin and tilmicosin residues in meat by high-performance liquid chromatography method

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Abstract

In this study, the presence and level of macrolide group antibiotics (tylosin and tilmicosin) were analyzed by the High-Performance Liquid Chromatography (HPLC) method in a total of 126 raw meat samples, including 42 chicken breast and 84 beef neck, available for consumption in the Burdur province (Turkey). The method demonstrated good linearity ($R^2 > 0.999$) over the assayed concentration range (0.10-10 μg/mL). Intra-day and inter-day recoveries were used to express the accuracy of the method at three different levels of 0.5, 1, 2.5 μg/mL. Intraday recoveries and relative standard deviation values ranged from 97.270 (0.054)% to 98.643 (0.061)%, and inter-day recoveries and relative standard deviation values ranged from 97.057 (0.070)% to 98.197(0.042)% for tylosin. Intraday recoveries and relative standard deviation values ranged from 96.360 (0.065)% to 98.153 (0.046)%, and inter-day recoveries and relative standard deviation values ranged from 96.050 (0.058)% to 97.053 (0.096)% for tilmicosin. The limit of detection (LOD) value was calculated as 0.473 μg/kg for tylosin, and 0.481 μg/kg for tilmicosin; the limit of quantification (LOQ) value was calculated as 1.561 μg/kg for tylosin, and 1.587 μg/kg for tilmicosin. In general, tylosin and tilmicosin were determined in the range of 8-256 μg/kg and 30-447 μg/kg, respectively, in chicken breast meat samples; also, they were detected in the range of 36-1209 μg/kg and 30-1102 μg/kg, respectively, in beef neck meat samples. It was also found that the residues of tylosin and tilmicosin in chicken and beef meats from the market were at a much higher level than the acceptable limits specified in the regulations. This creates serious problems in terms of the ecosystem, food technology, and public health, and causes significant economic losses.

Key words: meat, HPLC, tilmicosin, tylosin

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Introduction

Antibiotics are natural, semi-synthetic, and synthetic drugs used in human and veterinary medicine to treat diseases caused by bacteria. Antibiotics act by killing bacteria or preventing their reproduction. In addition, they are also used outside of control in veterinary medicine as growth promoters for farm animals (Landová and Vávrová 2017, Tasci et al. 2021). Tylosin and tilmicosin, which are from the macrolide group antibiotics, act against gram-positive and gram-negative bacteria. They are used in the treatment of cattle, sheep, pigs, and poultry (Lewicki et al. 2009, Elsayed et al. 2014, Kolanovic et al. 2014, Lemli et al. 2018, Zhu et al. 2018). Tylosin is a natural antibiotic that contains tylosin A (>80%), tylosin B (desmycosin), tylosin C (macrosin), and tylosin D (relomycin) (Lewicki et al. 2009, Song et al. 2016). Tilmicosin is a semisynthetic broad-spectrum antibiotic synthesized from tylosin (Lemli et al. 2018, Liu et al. 2022). Tylosin and tilmicosin show their antibacterial effects by binding to the 23S rRNA component of the 50S subunit of ribosomes, thereby preventing protein synthesis (Ammar et al. 2016, Arsic et al. 2018). Although the use of antibiotics to improve the growth and performance of all animal species has been banned in the European Union since 2006 (EC 2005), they are still used in poultry, pigs, and cattle in some countries (Lewicki et al. 2009, Kolanovic et al. 2014). The administered drugs are excreted from the body in the form of inactive metabolites or unchanged through urine, feces, and then spread to waters and the environment (Landová and Vávrová 2017). Macrolide antibiotics are not sensitive to biodegradation, and therefore their long stay in the environment causes concern for the environment and public health (Harris et al. 2012).

In parallel with the increase in the world population, the demand for animal protein is increasing. This, in turn, leads to an increase in animal production and the use of more drugs such as antibiotics (Khaniki et al. 2018, Manyi-Loh et al. 2018). Currently, it is observed that 70-80% of the total antibiotic consumption in many countries is in the livestock sector. There is very little data on the amount of antibiotics used in the livestock sector outside of Europe (Burki 2018). High amounts, continuous or illegal use of veterinary drugs cause the accumulation of residues in meat, milk, eggs, honey, and all edible tissues of animals. The consumption of animal source foods containing antibiotics causes various dangerous health problems such as allergies, super-infection, changes in the small and large intestinal bacterial flora, development of bacteria resistant to antibiotics, leads to mutagenic, carcinogenic, teratogenic effects and affects the starter cultures used in the food industry, thus causes losses in the quality of fermented foods (Martinez 2009, Babapour et al. 2012, Elsayed et al. 2014, Manaia 2017, Arslanbaş et al. 2018, Qiao et al. 2018, Ben et al. 2019, Falowo and Akimoladun 2019, Trott et al. 2021). Therefore, to identify or anticipate problems, it is necessary to constantly monitor and periodically assess the risks of antibiotics and take appropriate measures for public health (Khaniki et al. 2018). Biosensor, enzyme-linked immunosorbent assay (ELISA), high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), liquid chromatography-tandem mass spectrometry (LC-MS/MS), and ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) are widely used in various analytical techniques for the detection of antimicrobial residues in animal source foods (Jayalakshmi et al. 2017, El Tahir et al. 2021, Tasci et al. 2021). Of these techniques, the HPLC method has many important properties such as repeatability, selectivity, resolution, high recovery, and ease of application, and is frequently used (Ghanjaoui et al. 2020, Treiber and Beranek-Knauer 2021).

Antibiotics bring additional burdens to the country's economy as well as harm to humans, animals, and the environment. In the scope of this research, the presence and level of tylosin and tilmicosin residues in meats of animals raised for food purposes were investigated by the High-Performance Liquid Chromatography (HPLC) method to determine the risk of using antibiotics.

Materials and Methods

Material

In this study, a total of 126 meat samples, including 42 chicken breast and 84 beef neck, were randomly collected from retail outlets in Burdur province at different times. Each sample was collected into sterile bags and brought to the laboratory under a cold chain. Afterward, the samples were stored at -20°C until their analysis was performed.

Chemicals and reagents

All chemicals and solvents were used with analytical purity. Analytical standards of tylosin and tilmicosin (>95% purity) were obtained from Sigma-Aldrich (St. Louis, MO, USA), also methanol and acetonitrile (HPLC >95% purity), orthophosphoric acid (H_3PO_4 85%) from Merck (Darmstadt, Germany), and Disodium ethylenediaminetetraacetate (Na_2EDTA) from Sigma Aldrich (St. Louis, MO, ABD). Ultrapure water

Table 1. Confirmation parameters of antibiotic analyzes in meat samples.

Antibiotics	Calibration equation	R^2	LOD (µg/kg)	LOQ (µg/kg)	Linear range (µg/mL)	Retention time (min)
Tylosin	y=20494x+9337.1	0.9993	0.473	1.561	0.1-10	4.1
Tilmicosin	y=2175x+6971.4	0.9995	0.481	1.587	0.1-10	3.4

Explanations; R^2: Correlation Coefficient; LOD: Limit of Detection; LOQ: Limit of Quantification

was obtained using the H2OPRO-VF-T/Arium Ultrapure (Sartorius, Germany) device.

Preparation of stock solutions and calibration standards

The stock standard solutions of tylosin and tilmicosin were prepared by dissolving them in 10 µg/mL acetonitrile. The stock standard solutions were placed in amber glass bottles and stored at -20°C.

Sample extraction

In this study, the extraction method proposed by Chico et al. (2008) was used. For extraction, 5 grams of meat samples were taken in 50 mL polypropylene tubes, 10 mL of 70% methanol, and 200 µL of 0.1 M EDTA were added to them. The prepared mixture was homogenized with vortex for 1 minute and then centrifuged for 15 minutes at 5000 rpm. The resulting supernatant (500 µL) was taken into polypropylene tubes and mixed by adding 2 mL of pure water to it. Then, it was filtered through a 0.45 µm filter and taken in vials in an amount of 1.5 mL and 100 µL of which was injected into the HPLC system.

Device and operating conditions

Chromatographic separation of antibiotics was performed with modifying HPLC technique proposed by Yaneva et al. (2015). The analysis of tylosin and tilmicosin in the meat samples was performed using the HPLC device (Shimadzu, Japan) with Photo Diod Array (PDA) detector. The column was a InertSustain C18 (5 µM, 4.6 x 250 mm) (GL Sciences, Japan). The mobile phase consisted of a mixture of ACN and 0.1M H_3PO_4 (60:40, v/v). The buffer pH was adjusted to 2.5 with H_3PO_4. The operation was isocraic with a chromatogram monitored at the wavelength of 280 nm. The stock standard solutions of tylosin and tilmicosin were prepared by dissolving them in 10 µg/mL acetonitrile. Working standard solutions were prepared daily by dilution with mobile phase. The volume injected into the HPLC column was 100 µL. The flow rate was 0.8 mL/minute and the column temperature was maintained at 30°C. Elution time was 30 minute.

Validation

The method was validated, also the correlation equation, correlation coefficient (R^2), the limit of detection (LOD), the limit of quantification (LOQ), recovery rate, intraday, and interday precisions were determined as quality parameters (ICH 2005). Working standards, diluted at different concentrations (0.10 to 10 µg/mL) from standard stock solution were prepared for calibration graphic, and each concentration level was injected three times. The sensitivity of the method was evaluated by determining the limit of detection (LOD) and the limit of quantification (LOQ). The Signal-to-noise (S/N) ratio of LOD was taken as 3 and the signal-to-noise (S/N) ratio of LOQ was taken as 10. For recovery, the known amounts of the analyzed standards were added to the sample at different concentrations (0.5-2.5 µg/mL) and the mixture was extracted again, and this process was repeated three times to calculate the recovery rates (R) and relative standard deviation (RSD).

Statistical analysis

Evaluation of data was performed using descriptive statistics through Minitab Version 16.1 statistical software.

Results

The residues of tylosin and tilmicosin in chicken breast and beef neck meat originating from retail outlets in Burdur were analyzed by the HPLC method, and the correlation equation, correlation coefficient (R^2), limit of detection (LOD), the limit of quantification (LOQ), recovery rate, intraday and interday precision results determined as quality parameters for antibiotics are given in Table 1 and Table 2. In this study, reference standards were prepared between various concentrations (0.10 to 10 µg/mL), and the calibration equation obtained in the corresponding peak areas was obtained. It was found that the calibration curves showed good linearity, characterized by a high correlation coefficient (R^2>0.999). Intra-day and inter-day recoveries were used to express the accuracy of the method at three dif-

Table 2. Intra- and inter-day precisions for tylosin and tilmicosin in meat samples.

Spiked level (μg/mL)	Intra-day R (%, n = 3) RSD (%, n = 3)	Inter-day R (%, n = 3) RSD (%, n = 3)
Tylosin		
0.5	98.553 (0.086) 97.567 (0.058) 98.643 (0.061)	97.410 (0.047) 97.437 (0.041) 97.307 (0.057
1	98.040 (0.071) 98.603 (0.056) 97.513 (0.031)	98.197 (0.042) 97.860 (0.047) 97.057 (0.070)
2.5	97.270 (0.054) 98.113 (0.036) 97.677 (0.046)	97.783 (0.036) 98.080 (0.031) 97.087 (0.053)
Tilmicosin		
0.5	96.360 (0.065) 96.420 (0.062) 96.767 (0.053)	96.057 (0.052) 96.100 (0.045) 96.050 (0.058)
1	97.053 (0.067) 97.133 (0.043) 96.993 (0.053)	96.500 (0.052) 96.460 (0.055) 96.327 (0.078)
2.5	97.183 (0.036) 98.153 (0.046) 97.027 (0.072)	97.053 (0.096) 96.923 (0.051) 96.847 (0.057)

R: Recovery Rate; RSD: Relative Standard Deviation

ferent levels of 0.5, 1, 2.5 μg/mL. Intraday recoveries and relative standard deviation values ranged from 97.270 (0.054)% to 98.643 (0.061)%, and inter-day recoveries and relative standard deviation values ranged from 97.057 (0.070)% to 98.197(0.042)% for tylosin. Intraday recoveries and relative standard deviation values ranged from 96.360 (0.065)% to 98.153 (0.046)%, and inter-day recoveries and relative standard deviation values ranged from 96.050 (0.058)% to 97.053 (0.096)% for tilmicosin. The limit of detection (LOD) value was calculated as 0.473 μg/kg for tylosin, 0.481 μg/kg for tilmicosin; the limit of quantification (LOQ) value was calculated as 1.561 μg/kg for tylosin, and 1.587 μg/kg for tilmicosin.

The residual levels of tylosin and tilmicosin in chicken breast and beef neck meat samples and results of meat samples exceeding the maximum residue limits according to national and international legal regulations were shown in Table 3. At the same time, distribution of antibiotics in meat samples were given in Table 4 and Table 5. In general, tylosin and tilmicosin were determined in the range of 8-256 μg/kg and 30-447 μg/kg, respectively, in chicken breast meat samples; also, they were detected in the range of 36-1209 μg/kg and 30-1102 μg/kg, respectively, in beef neck meat samples.

Discussion

In this study, the HPLC method demonstrated good linearity ($R^2 > 0.999$) over the assayed concentration range (0.10-10 μg/mL). The LOD and LOQ values were below the maximum residue limits (MRL) specified in national and international legal regulations. It was concluded that these results were sufficiently low and sensitive to detect the presence of antibiotics below MRL. The method used in this study is characterized by high precision, high linearity, predictive LOD and LOQ values.

Tylosin and tilmicosin are widely used in the treatment of various diseases of animals raised for food purposes, in particular, gastrointestinal infections, and respiratory infections. Oral products containing tylosin and tilmicosin have been approved for use in poultry and oral and injectable applications have been approved for use in cattle (EFSA 2021). The high rate of antibiotic residues detected in meat samples in this study poses a serious risk in terms of food safety, and this situation reveals the need for a comprehensive residue screening. Antibiotic residues were investigated by different methods in meat belonging to different species in the world and Turkey. Very few studies have been conducted on this issue in Turkey. When the conducted studies were examined, Akar (1994) found that tylosin was at the level of 0.2-0.3 ppm in 2 (1.14%) of 175

Table 3. Residue levels of tylosin and tilmicosin in meats and evaluation according to national and international regulations.

	Beef neck meat	Chicken breast meat
Tylosin		
Analyzed sample n	84	42
Number of positive samples n (%)	49 (58.33)	23 (54.7619)
Median (µg/kg)	326.0	98
Mean ± RSD (µg/kg)	382.3± 313.1	104.3±59.0
Range (µg/kg)	36-1209	8-256
Exceeding legal limits according to EC and TFC n (%)	36 (73.47)	11 (47.83)
Exceeding legal limits according to CAC n (%)	36 (73.47)	11 (47.83)
Exceeding legal limits according to FDA n (%)	28 (57.14)	1 (4.35)
Tilmicosin		
Analyzed sample n	84	42
Number of positive samples n (%)	75 (89.2857)	39 (92.8571)
Median (µg/kg)	206.0	127.0
Mean ± RSD (µg/kg)	236.3±103.5	172.9±126.6
Range (µg/kg)	30-1102	30-447
Exceeding legal limits according to EC and TFC n (%)	72 (96)	28 (71.79)
Exceeding legal limits according to CAC n (%)	65 (86.67)	17 (43.59)
Exceeding legal limits according to FDA n (%)	65 (86.67)	(-)

Explanations; n: number of samples; RSD: Relative Standard Deviation; (-): Not specificed

Tablo 4. Distribution of antibiotics in chicken meat samples.

Antibiotics in chicken meat	Total sample	Positive sample	Range of antibiotic concentration (µg/kg)			
	n	n (%)	n (%)			
			≤75	>76-100	>101-200	>201
Tylosin	42	23 (54.76)	10 (43.48)	2 (8.70)	10 (43.48)	1 (4.35)
Tilmicosin	42	39 (92.86)	11 (28.21)	4 (10.26)	11 (28.21)	13 (33.33)

n: Number of samples

Tablo 5. Distribution of antibiotics in cattle meat samples.

Antibiotics in cattle meat	Total sample	Positive sample	Range of antibiotic concentration (µg/kg)			
	n	n (%)	n (%)			
			≤50	>51-100	>101-200	>201
Tylosin	84	49 (58.33)	2 (4.08)	11 (22.45)	8 (16.33)	28 (57.14)
Tilmicosin	84	75 (89.29)	3 (4)	7 (9.33)	26 (34.67)	39 (52)

n: Number of sample

chicken meat by thin-layer chromatography/bioautography method in Ankara; Arslanbaş et al. (2018) determined tylosin at the level of 105.4-109.2 µg/kg in 2 (0.6%) of 300 chicken meats using the HPLC method. Yipel et al. (2018) collected 25 chicken meat from Hatay, Adana, Gaziantep, Mersin, and Osmaniye provinces and analyzed them with LC-MS/MS; as a result, no tylosin and tilmicosin were detected in any of the samples. Tylosin and tilmicosin were not found in 9 chicken meats, but were detected in 1 of 36 beef analyzed by biochip array-based immunoassay technique in Bursa by Caycı et al. (2019). It was determined that although the tilmicosin level (196.1 µg/kg) exceeded 75 µg/kg MRL, the tylosin level (72.5 µg/kg) did not exceed 100 µg/kg MRL. Studies in various countries, it was stated that there are different rates of tylosine and tilmicosin residues in chicken and beef meat. Tao et al. (2012) analyzed swine and bovine tissue samples (muscle, liver, kidney) with LC-MS/MS in China, and ultimately detected tylosin in 2 pig livers and tilmicosin in 1 pig liver. It was determined that the average amount of tylosin was less than 38.8 µg/kg, tilmicosin was 54.1 µg/kg which did not exceed MRL (100 µg/kg). Kolanović et al. (2014) determined by the ELISA method that 6.27% of 646 meat samples contained tylosin in the range of 0.06-49.7 µg/kg. Meat samples obtained by Yamaguchi et al. (2015) from slaughterhouses and retail outlets in Vietnam were analyzed by LC-MS/MS method and tilmicosin was detected in 10 chicken meat samples in the range of 150-450 µg/kg. El Tahir et al. (2021) found an average of 50.67 µg/kg of tylosin in 100 chicken breast meats and an average of 150.33 µg/kg of tylosin in 100 liver analyzed by the ELISA method in Oman. When compared to other studies (Akar 1994, Tao et al. 2012, Kolanovic et al. 2014, Arslanbaş et al. 2018, Yipel et al. 2018, Cayci et al. 2019, El Tahir et al. 2021), tylosin and tilmicosin residue levels and ratios determined in this study were found to be much higher in chicken and beef; also they were found to be similar to those obtained by Yamaguchi et al. (2015). High levels of tylosin and tilmicosin determined in present study in meat samples indicated that antibiotics are widely used, also foods are put on the market for consumption without waiting for adequate disposal time after the use of antibiotics for therapeutic purposes in animals raised for food purposes, which poses a great risk to public health.

To prevent or reduce the high toxicity and health risks of antibiotics, maximum residue limits have been determined in national and international legal regulations (EC 2009, TFC 2017, CAC 2018, FDA 2020). The maximum tylosin residue limit that can be found in chicken meat and beef is 100 µg/kg according to the Turkish Food Codex (TFC 2017), European Commission (EC 2009), Codex Alimentarius Commission (CAC 2018) regulations, and 0.2 ppm (200 µg/kg) according to the United States Food and Drug Administration (FDA 2020). In this study, according to the legislation of the European Commission and the Turkish Food Codex, the rate of tylosin exceeding MRL was found to be 47.83% in chicken meat, and 73.47% in beef. The maximum tilmicosin residue limit that can be found in chicken meat is 75 µg/kg according to the Turkish Food Codex (TFC 2017), European Commission (EC 2009), and 150 µg/kg according to Codex Alimentarius Commission (CAC 2018) regulations, and no value has been specified by the United States Food and Drug Administration (FDA 2020). In this study, according to the European Commission and the Turkish Food Codex, the rate of tilmicosin exceeding MRL in chicken meat was found to be 71.79%. The maximum tilmicosin residue limit that can be found in beef is 50 µg/kg according to the Turkish Food Codex (TFC 2017), European Commission (EC 2009), and 100 µg/kg according to Codex Alimentarius Commission (CAC 2018) and the United States Food and Drug Administration (FDA 2020). In this study, the level of tilmicosin exceeding MRL in beef according to the legislation of the European Commission and the Turkish Food Codex was found to be 96%. The results obtained in this study indicated that the antibiotic was administered at a high dose because it was well above the maximum residue limits specified in national and international legal regulations, or animals were slaughtered without waiting for the excretion period of the drug before slaughter. Various studies have been conducted on the duration of excretion, that is, becoming harmless after the administration of tylosin and tilmicosin. Dimitrova et al. (2012) have recommended a 28-day period of excretion after subcutaneous administration of tilmicosin to ruminants. Liu et al. (2013) have stated that the excretion time of tilmicosin phosphate for pigs should be 12 days. Elsayed et al. (2014) have suggested that chickens should not be slaughtered before 4 days from the application of tilmicosin, Soliman and Sedeik (2016) have reported that chickens should not be slaughtered for human consumption 6 days after the last oral tylosin administration. Tylosin and tilmicosin are included in the group of "critically important antimicrobial agents" from a veterinary point of view (OIE 2019). In cases where there are no clinical signs in animals, they should not be added to feed or water and used as a preventive treatment. They should not be used as a first-line treatment unless justified, but when used as a second-line treatment, it should ideally be based on the results of bacteriological tests. Off-label use should be limited (OIE 2019). In this study, determining the presence and level of tylosin and tilmicosin residues in meats will contribute to the residue monitoring program and taking precautions.

Conclusion

Although antibiotics play an important role in the treatment of diseases, they cause a residue problem in animal source foods such as meat, milk, eggs, honey due to their unconscious or uncontrolled use, and foods containing antibiotic residues pose the public health hazards. When the data obtained in this study were evaluated, it was found that the level of tylosin and tilmicosin in chicken and beef samples from the market was much higher than the acceptable levels specified in national and international legislation. This creates serious problems in terms of food technology, public health and causes significant economic losses. Antibiotics should be administered in the recommended doses and under the supervision of a veterinarian, and an adequate period of excretion should be obeyed after the use of antibiotics. The meat and edible tissues of animals slaughtered at enterprises should be checked for antibiotic residues seriously and effectively. Antibiotics should be avoided to be administered by people other than veterinarians and used in animal feed. To reduce the need for antibiotics, especially in the veterinary field, hygiene standards should be obeyed; Antimicrobial substances derived from plants, probiotics, prebiotics, and phage treatments should be used as an alternative to antibiotics. Animal breeders and society should be educated about the harms of antibiotic-containing foods, and awareness about antibiotic applications should be raised.

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