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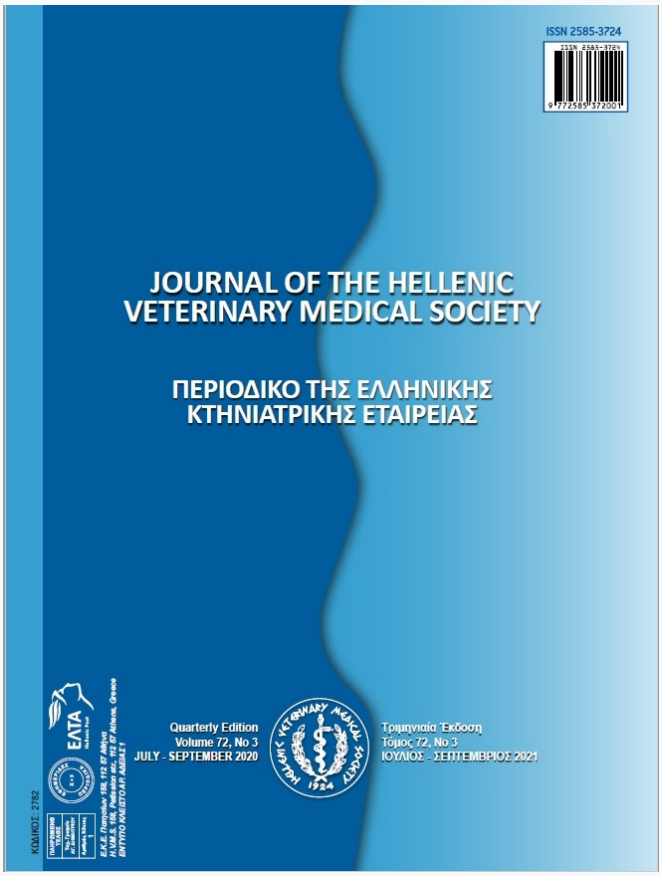

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Research article
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Assessment for the passage of tylosin into the milk of Anatolian buffaloes

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ABSTRACT: Tylosin is a broad-spectrum macrolide antibiotic commonly employed in veterinary medicine to treat bacterial infections. The present study assessed the milk-passage patterns of tylosin up to the 16th milking after a single intramuscular injection at the dose of 10 mg/kg/b.w. to Anatolian buffaloes. The residue levels of tylosin in milk samples of each animal were analysed by LC-MS/MS. The detection and determination limits of the employed method were 0.19 μg/kg and 0.64 μg/kg, respectively. The highest level of tylosin was found to be at the second milking. At the ninth milking, tylosin residue level decreased under the maximum residue limit of 50 μg/kg. Additionally, the employed LC-MS/MS method is used to assess tylosin residue in 40 milk samples and all of the samples were found to be tylosin free. In conclusion, this study determined the milk passaged levels of tylosin into milk of Anatolian buffaloes using an LC-MS/MS method.

***Keywords*:** Anatolian buffaloes, tylosin, milk, LC-MS/MS.

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INTRODUCTION

Macrolide antibiotics are commonly employed in veterinary medicine to treat several diseases such as enteric infections and respiratory diseases in sheep, cattle, poultry and swine due to their effects on both gram-positive and some gram-negative bacteria (Draisci et al., 2001).

Milk is an indispensable part of human nutrition due to its valuable ingredients. It provides a high diversified composition with essential roles in metabolism such as minerals, fat and lactose, proteins and vitamins. In addition, milk is a source of significant trace elements i.e. zinc, copper, iron and manganese, which are vital in various physiological functions of human body (Acaroz et al., 2019). On the other hand, Buffalo milk as the second most consumed milk after the cow milk is an inevitable source of nourishment in many regions of the world and this milk type is widely used in the production of different milk products. Also, Turkey is one of the major buffalo milk producers with Italy and Bulgaria in Europe (Acaroz et al., 2020; Pasquini et al., 2018).

Milk provides many beneficial health effects; however, it can contain some hazardous substances such as antibiotic residues due to improper usage or misuse of antibiotics to livestock animals (Cháfer-Pericás et al., 2010; Vishnuraj et al., 2016). The presence of antibiotic residues in milk should be taken into consideration due to its negative impacts on human health including transiently disturbing intestinal flora or inducing allergic reactions which may be ended up even with anaphylaxis. In addition, the development of antibacterial resistance may be caused by antibiotic residues. Lastly, the production of fermented milk products such as yoghurt and cheeses could be inhibited by means of these residues (Graham et al., 2014; Quintanilla et al., 2018). In case of macrolide antibiotics, although negative effects on human health were rarely reported for these antibiotics, they have been also associated with serious adverse effects for pregnancy by increasing risk of cardiovascular malformation, miscarriage, major malformations in observational studies (Fan et al., 2019).

To minimize possible food-associated health risks of a macrolide antibiotic tylosin, a maximum residue limit for milk at the level of 50 µg/kg was established by European Union (EU No37/2010). To control the residues of tylosin in many foodstuffs, different methods such as immunoassay (Burkin and Galvidis, 2012), quantum dot-based immunoassay (Le et al., 2015), HPLC (Dudriková et al., 1999), LC-MS/MS (Wang et al., 2006) were established. LC-MS/MS is considered as one of the most promising methods because of its ability to quantify and confirm macrolides at trace levels (Wang and Leung, 2007).

Some studies were carried out to assess the milk passage of macrolide antibiotics of different animal species including cows (Avci and Elmas, 2014), ewes (Al-Wabel, 2008) and goats (Quintanilla et al., 2018). However, there is no available information on residual patterns of tylosin in Anatolian buffalo milk. Therefore, this study aimed to evaluate the milk passage of tylosin into the milk of Anatolian buffaloes using a LC-MS/MS method.

MATERIAL AND METHODS

Tylosin was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and reagents of analytical grade were obtained from commercial sources.

Healthy female Anatolian buffaloes (n=5) that were weighing 400-500 kg were employed for the study. The Anatolian buffaloes were provided by Afyon Kocatepe University, Veterinary Faculty Research and Application Farm. Additionally, the ethical approval was given by Afyon Kocatepe University Animal Experiments Local Ethics Board with an approval number of 49533702/95. Experimental animals were given standard ration and water under similar conditions. Each buffalo was intramuscularly injected with tylosin at the dose of 10 mg/kg (Tylan 200, Elanco). Then, milk samples were taken for 8 consecutive days at the 0, 12, 24, 36, 48, 60, 72, 84, 96, 108, 120, 132, 144, 156, 168, 180, 192 hours and stored stored at -20°C for further residue analysis Also, a blank sample was collected before applying the drug to each experimental animal.

Forty buffalo milk samples were collected from Afyonkarahisar province of Turkey from September to December in 2017. The samples were purchased from local markets and producers and then directly transported to the laboratory under cold chain conditions. The samples were kept at -20°C for further residue analysis.

The stock solution of tylosin was prepared in methanol at the concentration of 1 mg/mL. The respective stock solution was diluted in milk to produce calibration curve (0.5, 1, 2, 5, 10, 20, 50 ng/ml) and calibration standard samples of tylosin.

Methods

The extraction of tylosin from milk was carried out in line with Dudriková et al. (1999). Briefly, 30 ml of homogenized buffalo milk sample was spiked with the related concentration of tylosin and then fat layer was separated by a centrifugation step at 3000 rpm, 10 min and 4°C. 10 ml of the skim layer was mixed with 20 ml of acetone in another centrifuge tupe. After this step, a centrifugation at 3400 rpm, for 10 min at 4°C was performed. The obtained extract was diluted with water to 100 ml and a solid-phase extraction step was done with a Sep-Pak C18 cartridge. Firstly, the activation of the cartridge was carried out with methanol (2 ml) and water (5ml). Then, the extract applied to SPE cartridge and filtered under vacuum. The elution was done with 0.1M ammonium acetate solution in methanol (2x2 ml).The obtained eluate was given to 6 ml phosphate buffer (of 0.01M, pH 6.0) in a separatory funnel. This mixture was shaken for 5 min after the addition of dichloromethane (20 ml). The organic layer was evaporated using a vacuum evaporator. The remaining residue was dissolved in mobile phase (2 ml) and transferred to HPLC vials. Then, the analysis of milk samples was performed with Agilent Technologies 1200 series (Waldbronn, Germany), attached with a binary high-pressure gradient pump. LC separation was carried out by Zorbax Eclipse XDB-C8 (150 mm × 4.6 mm, 1.8 µm Agilent Technologies) at 45°C. The mobile phases consisted of solvent A (aqueous solution of 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid). The gradient profile was performed as follows: 0.0 min, A/B (100/0); 1.0 min, A/B (100/0); 3.0 min, A/B (20/80); 7.50 min, A/B (5/95); 8.10 min, A/B (100/0), 12.00 min, A/B (100/0). The flow rate of mobile phase was 0.6 ml/min and the injection volume was 10 µl. Mass spectrometry analysis was conducted on Agilent 6460 LC/MS Triple Quadrupole instrument equipped with an ESI (Waldbronn, Germany). The employed nitrogen generator (Balston, Haverhill, MA, USA) produced nebulizer and drying gas (350°C). MS parameters including nebulizer gas, capillary voltage, sheath gas temperature, and flow were as 40 p.s.i., 4000 V, 400°C, and 10 l/min, respectively. MS analysis was performed on positive ion mode. The retention time of tylosin was detected as 5.608. Molecular weight, precursor ions (m/z), and product ions (m/z) of tylosin were 916.5, 174.1, 101.2 respectively. The method validation was done by spiking milk samples. The quality parameters were as follows; intra- and inter-day precisions recovery, limit of detection (LOD), limit of quantification (LOQ) and linearity range. The residual levels of tylosin in milk samples were determined by means of the calibration curve for which a series of standard solutions (0.5, 1, 2, 5, 10, 20, 50 ng/ml) were prepared and calculated. To establish the equation of calibration, data fitted on a line and the obtained equation was emplyed to determine the level antibiotic residue in unknown samples. Additionally, the strength of linear regression was expressed by the coefficient of determination (r^2). The lowest concentration that the analytical process can confidingly differentiate from background levels (signal-to-noise ratio≥3) was defined as LOD whereas lowest concentration that can be quantified (signal-to-noise ratio ≥10) was decribed as LOQ.

RESULTS

The chromatogram of tylosin was presented in Figure 1. In addition, as the validation parameters of the used LC-MS/MS method, accuracy, recovery, linearity, precision, LOQ and LOD were employed. The calibration curve for tylosin was given in Figure 2. The linearity of related curve was in the range from 0.5 to 50 µg/kg and showed good the coefficient of determination (r^2=0.997). Additionally, the sensitivity of the method was quite high and LOD and LOQ parameters were presented in Table 1. Relative standard deviation (RSD%) was employed for the overall precision of the method. These values were found to be lower than 5.89 %. The accuracy was expressed by intra-day and inter-day recoveries at the concentrations of 45, 90, 135 µg/kg. Recovery and RSD values were presented in Table 1.

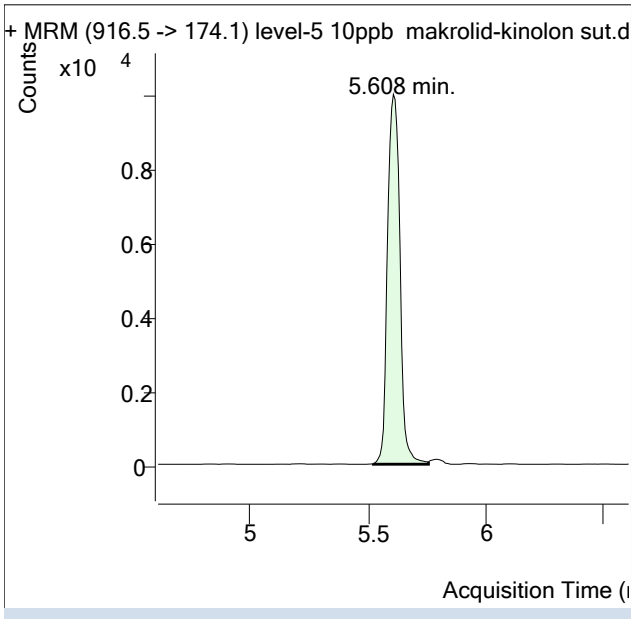

Figure 1. Chromatogram for tylosin standard.

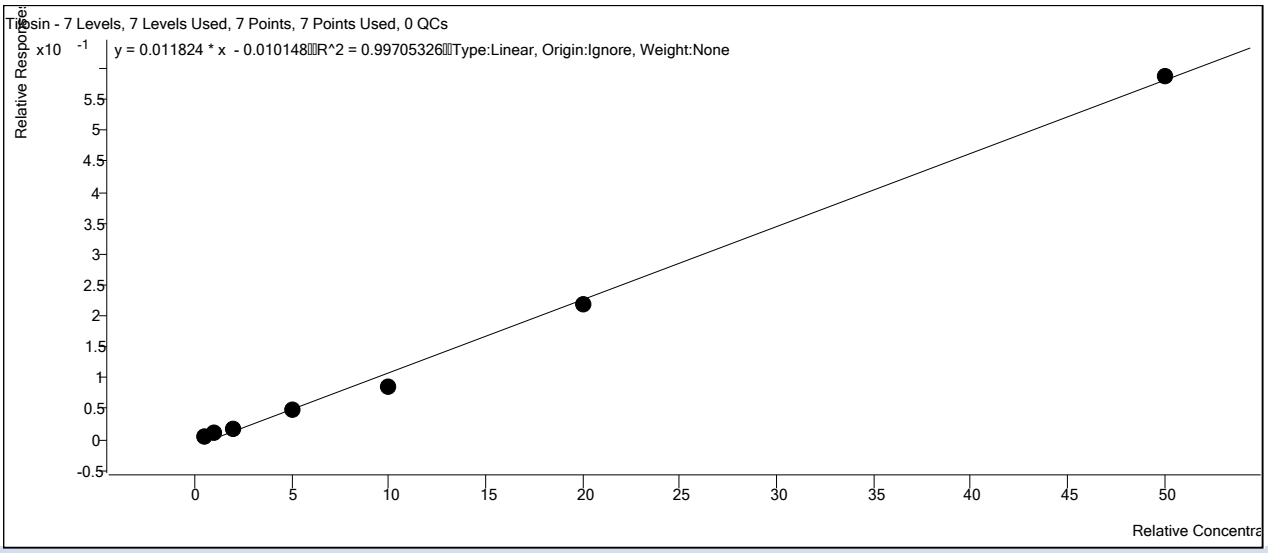

Figure2. Calibration curve for tylosin

Table 1. Intra- and inter-day precisions for tylosin in buffalo milk samples.

Spiked (ppb)	Intra-day Assays (n=6)		Inter-day Assays (n=6)	
	Percentage Recovery±CV	RSD (%)	Percentage Recovery±CV	RSD (%)
45	91.70±4.55	4.95	91.49±3.88	4.24
90	94.36±4.52	4.80	90.66±3.89	4.29
135	95.59±3.03	3.17	89.22±5.25	5.89

The results of the study exhibited that the highest level of tylosin was detected at the second milking with a mean concentration of 1625.1±358.97 µg/kg (Fig. 3). Also, the level of tylosin in milk was decreased after the second milking continuously and its level was lower than the maximum residue limit (50 µg/kg) at ninth milking with a mean concentration of 38.26±5.29 µg/kg (Fig. 3).

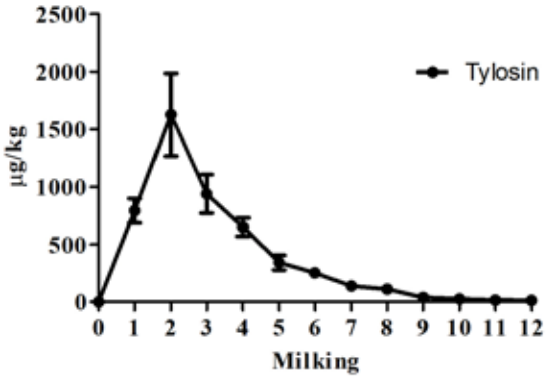

Figure 3. Passage of tylosin into the milk of buffaloes.

Also, 40 buffalo milk samples offered for consumption in Afyonkarahisar were analysed for the presence of tylosin. According to results of the analysis, all of the milk samples were found to be tylosin-free.

DISCUSSION

Macrolides are widely used in veterinary medicine based on their broad-spectrum. They show activity activity against Gram-positive and some of Gram-negative bacteria (Rigos et al., 2003; Sugawara et al., 2017).

Some quality parameters including the limit of detection and determination values and also recovery rates of tylosin in similar studies for milk samples were presented in Table 2. The LOD values of the selected methods were ranged from 0.024 µg/kg (Şanli et al., 2011) to 1 µg/kg (Aguilera-Luiz et al., 2008; Bogialli et al., 2007) whereas LOQ values were reported between 0.007 µg/kg (Şanli et al., 2011) and 3 µg/kg (Aguilera-Luiz et al., 2008). In addition, recovery rates of tylosin in milk samples were found to be between 88% and 109 % (Dubois et al., 2001; Wang et al., 2006; Wang and Leung, 2007). The results of the previous reports are in line with the result of the employed study for which LOD and LOQ values were determined as 0.19 µg/kg and 0.64 µg/kg, respectively while recovery rates ranged from 89 % to 95 %.

Table 2. Selected chromatographic methods for the residue analysis of tylosin in milk samples.

Method Type	Matrix	LOD µg/kg	LOQ µg/kg	Recovery (%)	Reference
LC-MS/MS	Buffalo Milk	0.19	0.64	89-95	Current Study
HPLC-DAD	Sheep Milk	0.024	0.007	90	Şanli et al. (2011)
LC-MS/MS	Bovine Milk	NA	NA	97-100	Dubois et al. (2001)
LC-MS/MS	Bovine Milk	0.02	NA	96-109	Wang and Leung (2007)
LC-MS/MS	Bovine Milk	0.06	NA	99-105	Wang et al. (2006)
LC-MS/MS	Bovine Milk	1	2	88-97	Bogialli et al. (2007)
UPLC-MS/MS	Bovine Milk	1	3	90-95	Aguilera-Luiz et al. (2008)

NA: Not available.

Several studies investigated the passage of tylosin in the milk of livestock animals. Al-Wabel (2008) evaluated the milk passage of tylosin in lactating Najdi ewes after a single intramuscular injection of this antibiotic (10 mg/kg) by microbiological agar plate assay and measurable residual levels of tylosin were reported in all animals up to 72 h following the treatment. In another study conducted by Avci and Elmas (2014), pharmacokinetic and amount of residue in milk of tylosin for healthy Holstein breed cows were determined after a single intramuscular injection at the dose of 17.5 mg/kg. The mean tylosin concentration was reported as 0.20±0.09 µg/ml (96 h after administration) which was higher than the established MRL of 50 µg/kg and they concluded the withdrawal period was inadequate to ensure the elimination of this drug based on the determined half-life of 26.36 ± 5.55 h in milk for the related study. Quintanilla et al. (2018) administered macrolide antibiotics including tylosin (0.5 ml/10 kg bw.), spiramycin (0.5 ml/10 kg bw.) and erythromycin (1 ml/10 kg bw.) three consecutive days to dairy goats for an *in vivo* experiment. Then, they produced ripened cheeses from contaminated milk samples. After 24 hours of injection, milk residues of the related antibiotics were found to be relative higher than the respective MRL for erythromycin 234.9 ± 52.7 µg/kg; tylosin 198.7 ± 57.8 µg/kg and spiramycin 1539.8 ± 469.4 µg/kg and making the cheese from these milk were not possible. However, the seven-day period was enough to clear tylosin and erythromycin from goat milk, only spiramycin was found to be at the concentration of 79.6 ± 19.2 µg/kg. In addition, no antibiotic residues were determined in the cheeses after these time period. They recommended seven days days of withdrawal time to ensure milk safety regarding these antibiotics. Our results are compatible with the above mentioned studies, and the results of the present study showed that highest levels of tylosin were detected at the second milking (1625.1±358.97 µg/kg) and residue level decreased under the maximum residue limit of 50 µg/kg at ninth milking (38.26±5.29).

CONCLUSIONS

This study determined the milk passage of tylosin for the Anatolian buffaloes by employing a precise, reliable, and accurate LC-MS/MS method. The method was able to determine tylosin within the related MRL for milk due to its low LOD and LOQ values. The present study gives information on the withdrawal period of tylosin in the milk of Anatolian buffaloes. Also, real milk samples were evaluated for the presence of tylosin and determined as safe regarding residue risk of this antibiotic in Afyonkarahisar Province.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

None declared.

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