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Research Article

Determination of Kanamycin Residue in Anatolian Buffalo Milk by LC-MS/MS

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Abstract

The present study aimed to evaluate the persistence of kanamycin in lactating Anatolian buffalo milk followed by an intramuscular injection of kanamycin. The collection of milk samples was performed twice daily up to the 10th milking followed by kanamycin injection and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was employed for the residue analysis. The detection limit of the method was determined as 3.56 µg/kg. The highest concentrations of kanamycin were determined in the first milking after injection and mean concentration of this milking was found to be as 1473 µg/kg. Kanamycin residue in all buffalo milk samples was lower than the maximum residue limit (150 µg/kg) with the fifth milking. In addition, a monitoring study was conducted to determine whether buffalo milk marketed in Afyonkarahisar pose a public health risk regarding kanamycin residue. The results of the monitoring study showed that one (2%) of the 50 buffalo milk samples contained kanamycin at the concentration of 22.36 µg/kg. In conclusion, this study determined the persistence of kanamycin in Anatolian buffalo milks based on an LC-MS/MS method. In addition, results of the study showed that buffalo milks marketed in Afyonkarahisar Province pose a very low risk regarding kanamycin residue.

Keywords: *Kanamycin, Aminoglycosides, Anatolian buffaloes, Milk, LC-MS/MS*

Anadolu Manda Sütlerinde Kanamisin Kalıntısının LC-MS/MS İle Belirlenmesi

Öz

Bu çalışmada, laktasyondaki Anadolu mandalarına intramüsküler olarak kanamisin enjekte edilmesini takiben sütlerindeki kanamisin kalıntısının belirlenmesi amaçlandı. Süt numunelerinin toplanması, kanamisin enjeksiyonunu takiben günde iki kez 10'uncu sağıma kadar gerçekleştirildi ve kalıntı analizi için likit kromatografisi tandem kütle spektrometresi (LC-MS/MS) kullanıldı. Metodun tespit limiti 3.56 µg/kg olarak belirlendi. En yüksek kanamisin konsantrasyonları enjeksiyondan sonraki ilk sağımda tespit edildi ve sağımdaki ortalama konsantrasyon 1473 µg/kg olarak belirlendi. Manda sütü örneklerinde kanamisin kalıntı düzeyinin beşinci sağımdan itibaren maksimum kalıntı sınırından (150 µg/kg) daha düşük olduğu belirlendi. Ayrıca, Afyonkarahisar'da pazarlanan manda sütlerinin kanamisin kalıntısı konusunda halk sağlığı açısından risk oluşturup oluşturmadığını belirlemek amacıyla bir saha taraması yapıldı. Saha taraması sonucunda 50 manda sütü örneğinden birinin (%2) 22.36 µg/kg konsantrasyonunda kanamisin içerdiği saptandı. Sonuç olarak bu çalışmada kanamisinin Anadolu manda sütlerinde kalıcılığı LC-MS/MS metodu ile belirlendi. Ayrıca, çalışmanın sonuçları Afyonkarahisar'da tüketime sunulan manda sütlerinin kanamisin kalıntısı açından çok düşük risk taşıdığını gösterdi.

Anahtar sözcükler: *Kanamisin, Aminoglikozidler, Anadolu mandası, Süt, LC-MS/MS*

INTRODUCTION

Milk is an important source for human diet due to its major nutrients including fat, proteins, and carbohydrates. Also, it contains essential vitamins and minerals such as calcium, selenium, magnesium, riboflavin, pantothenic acid and

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vitamin B_{12} [1,2]. Buffalo milk also possesses rich nutrient content and it is the most produced milk source after the cow milk worldwide. In addition, this valuable milk source is employed in the production of many dairy products including cheese, cream, butter, and yoghurt [3,4].

Antibiotics are potent chemicals used for the treatment of various diseases in livestock animals [5]. Kanamycin as a broad-spectrum aminoglycoside antibiotic is widely used for the treatment of pneumonia, mastitis, and diarrhoea in veterinary medicine. This antibiotic exhibits its antimicrobial effect by interfering with ribosomal RNA of gram-negative bacteria [6]. Nevertheless, kanamycin may also induce several side effects such as allergic reactions, ototoxicity, nephrotoxicity, hematopoietic system toxicity, and neuromuscular blocking in human and animals [7]. Improper use of antibiotics can cause residue risk in foods of animal origin, which may cause food safety concerns [8,9]. To protect public health and food safety, the maximum residue limit for kanamycin in milk was established by the European Union as 150 µg/kg [10].

Several detection methods including immunoassays, capillary electrophoresis, high-performance liquid chromatography, gas chromatography, and liquid chromatography-tandem mass spectrometry (LC-MS/MS) were developed for aminoglycoside residue analyses with different sensitivities. However, LC-MS/MS is accepted as the most reliable confirmatory method based on its high sensitivity and accuracy [11,12].

Several studies were reported on pharmacokinetic features of aminoglycosides for farm animals [13-15]. However, the information about the persistence of this antibiotic in the milk of Anatolian buffaloes is lacking. The present work determined kanamycin persistence in Anatolian buffalo milk by means of LC-MS/MS method. Also, the developed method was employed to analyze 50 buffalo milk samples marketed in Afyonkarahisar, Turkey.

MATERIAL and METHODS

Material

Kanamycin sulfate and formic acid (LC-MS grade) obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and water (LC-MS grade) were purchased from Merck (Darmstadt, Germany). All other reagents and chemicals were analytical grade provided by commercial sources.

Five clinically healthy female Anatolian buffaloes, weighing 400-500 kg were selected for this study. The experimental animals were obtained from Afyon Kocatepe University, Veterinary Faculty Research and Application Farm. This study was approved by Afyon Kocatepe University Animal Experiments Local Ethics Board (Approval no. 49533702/77). All animals were kept under similar conditions having standard ration and free access to water. Each experimental animal was intramuscularly given a single dose of kanamycin 10 mg/kg with a commercial product (Kanovet, Vetaş, İstanbul, Turkey). Milk samples were collected during 5 days at the 0 (blank sample), 12, 24, 36, 48, 60, 72, 84, 96, 108, and 120 h. A blank sample was taken prior to drug administration from each animal. Collected milk samples were directly stored at -20°C for further analysis.

From August to December 2017, a total of 50 buffalo milk samples were collected in Afyonkarahisar province, Turkey. Milk samples were provided by producers and local markets. All samples were transported to the laboratory immediately after sampling under cold conditions and stored at -20°C in a deep freezer for further analysis.

The stock solution of kanamycin was prepared in distilled water (1 mg/mL) and stored at -20°C prior to use. Working solutions of kanamycin were also prepared in distilled water by serial dilution. To generate eight-point concentrations (0.5, 1, 2, 5, 10, 20, 50, 100 ng/mL) of the calibration curve, calibration standard samples were prepared in milk by spiking with an appropriate volume of serially diluted stock solution.

Methods

The extraction of milk samples was performed as previously described by Jank et al.[16] with some modifications. Briefly, each milk sample (2 mL) was transferred into a polypropylene centrifuge tube and then mixed with acetonitrile (4 mL). The purification of milk samples was completed by three centrifugation steps for 10 min at 4000 rpm and 4°C (except last one which was performed at 2°C). Then, the supernatant was kept in a water bath (≤45°C) that was evaporated under an N_2 stream until the reduction of the volume solvent to approximately 500 µL and the volume was adjusted to 1ml before transferred to HPLC vials.

The LC/MS/MS analysis of Anatolian Buffalo milk samples was carried out via Agilent Technologies 1200 series (Waldbronn, Germany), attached with a binary high-pressure gradient pump. Atlantis HILIC column (150 × 2.1 mm, 3 µm; Waters, Milford, MA, USA) was employed for LC separation at 40°C. The mobile phases consisted of solvent A (0.1% formic acid solution) and solvent B (acetonitrile containing 0.1% formic acid). The gradient of LC separation was as follows: 0.0 min, A/B (80/20); 3.0 min, A/B (10/90); 4.0 min, A/B (10/90); 4.10 min, A/B (80/20). The flow rate of the mobile phase was set at 0.4 ml/min and the injection volume of the sample was 10 µL.

Agilent 6460 LC/MS Triple Quadrupole instrument equipped with an ESI (Waldbronn, Germany) was used for mass spectrometry analysis. A nitrogen generator (Balston, Haverhill, MA, USA) was employed to produce nebulizer and drying gas (350°C). All MS parameters including sheath gas flow, nebulizer gas, capillary voltage sheath gas temperature, and Collision Energy were as 10 L/min, 40 p.s.i., 4000 V, 400°C, and 15 eV, respectively. Positive ion

mode was chosen for all MS analysis. Kanamycin retention time was found to be as 2.65. Its molecular weights, precursor ions (m/z), and product ions (m/z) were 485.0, 324.1, 162.8 respectively.

The method was validated by spiking raw milk samples. The quality parameters established were linearity range, limit of detection (LOD), limit of quantification (LOQ), recovery, and intra- and inter-day precisions. The limit of detection (LOD) was defined as the lowest concentration of kanamycin that the analytical process can reliably differentiate from background levels (signal-to-noise ratio≥3), while the limit of quantification (LOQ) was defined as the lowest concentration of kanamycin that can be quantified (a signal-to-noise ratio ≥10).

RESULTS

The typical chromatogram of kanamycin was shown in *Fig. 1*. The method was validated by determining linearity, recovery, precision and accuracy, LOD, and LOQ. The quantification of kanamycin in buffalo milk samples was performed by LC-MS/MS. Chromatographic separation was also performed using an LC technique in line with Kim et al.[17]. The linearity of the calibration curve (y=282.5x-192.03) showed an appropriate correlation (r^2=0.999) in the range from 0.5 to 100 µg/kg *(Fig. 2)*. Relative standard deviation (RSD%) was used to express the overall precision of the method and they were less than 5.79%. The accuracy expressed regarding intra-day and inter-day recoveries at three different levels of 80, 160, 400 µg/kg. Intra-day recoveries ranged from 102.55±5.20 to 108.70±5.96 µg/kg while inter-day recoveries ranged from 100.50±4.80 to 107.04±6.19 µg/kg, which confirms the method has a good recovery and precision *(Table 1)*. Some researchers also developed LC-MS methods for the detection of kanamycin residue in milk and their results were summarized in *Table 2*.

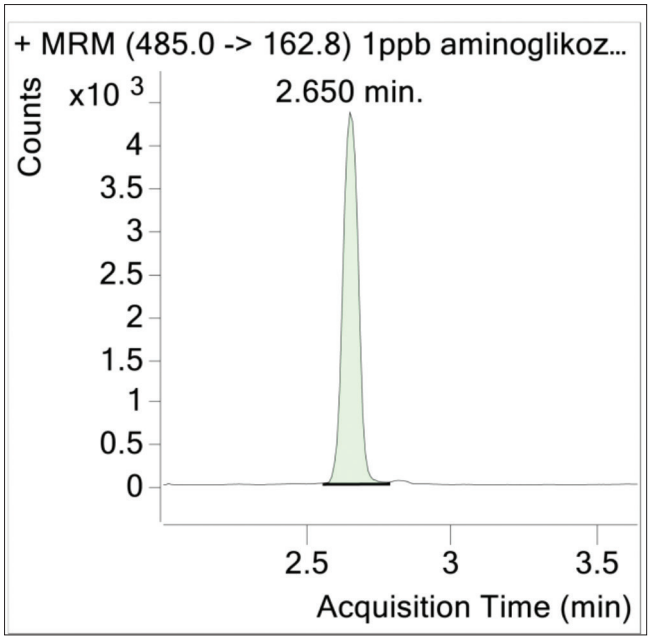

Fig 1. Chromatogram of kanamycin standard

For the experimental study, the highest concentrations of kanamycin were detected at the first milking ranging from 1231 to 1877 µg/kg with a mean concentration of 1473 µg/kg. Also, kanamycin milk concentration decreased consequently during milking period and kanamycin concentration was observed under the maximum residue limit (150 µg/kg) at the fifth milking. The mean kanamycin milk residual concentration measured at the 10 milking post-treatment was as high as 4.96 µg/kg *(Fig. 3)*.

For the monitoring study, a total of 50 buffalo milk obtained from Afyonkarahisar and analyzed for the presence of kanamycin residues. Kanamycin was not detected in 49 samples but only 1 buffalo milk sample contained 22.36 µg/kg kanamycin.

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

Spiked (ppb)	Intra-day Assays (n=8)		Inter-day Assays (n=8)	
	Percentage Recovery±CV	RSD (%)	Percentage Recovery±CV	RSD (%)
80	108.70±5.96	5.49	107.04±6.19	5.79
160	102.55±5.20	5.07	100.50±4.80	4.78
400	103.80±4.27	4.11	102.02±2.79	2.73

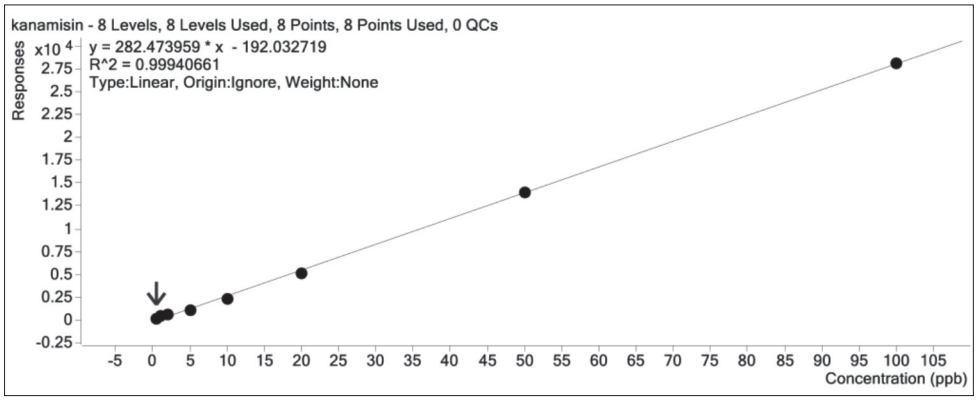

Fig 2. Calibration curve for kanamycin

Table 2. *Selective methods for the quantification of kanamycin in milk*

Method Type	Matrix	LOD µg/kg	LOQ µg/kg	Recovery (%)	Reference
LC-MS	Buffalo Milk	3.56	11.85	100-108	Current Study
LC-MS	Bovine Milk	15	37.5	105-106	Arsand et al.[13]
LC-MS	Bovine Milk	0.7	1.0	NA	Goutalier et al.[18]
LC-MS/MS	Bovine Milk	14	45	78-104	Bousova et al.[19]
LC-MS	Bovine Milk	33	33	92	Saluti et al.[20]
LC-MS	Bovine Milk	<11.5	<18.5	81–91	Tao et al.[21]
LC-MS	Bovine Milk	11	36	78-86	Yang et al.[22]

NA: *Not available*

	Kanamycin ± SD (µg/kg)
Control t=0	<LOD
Milking 1	1473.46±271.16
Milking 2	728.92±155.17
Milking 3	287.33±87.73
Milking 4	153.89±30.14
Milking 5	53.56±11.87
Milking 6	31.51±4.04
Milking 7	25.82±5.51
Milking 8	18.37±3.63
Milking 9	10.16±3.38
Milking 10	4.96±0.8

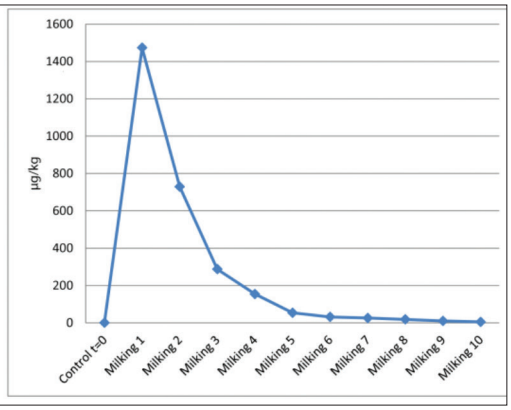

Fig 3. Persistence of kanamycin in milk of buffaloes

DISCUSSION

Acetonitrile was chosen for the extraction of kanamycin from milk matrix due to its protein precipitation capacity. Aminoglycosides have good stability under cold circumstances. Therefore, kanamycin was prevented from degradation by centrifugations at low temperatures (4°C). The LOD of kanamycin was determined as 3.56 µg/kg while its LOQ value was determined as 11.85 µg/kg. The validation parameters including LOD, LOQ, and recovery values were compatible with other studies [18-22].

In this experimental study, for the first time, the passage of kanamycin into buffalo milk was followed and its milk excretion kinetics was determined by LC-MS/MS. Kanamycin was detected in milk between the 0 and 10 milkings after intramuscular administration to dairy buffaloes. The highest concentrations of kanamycin were detected at the first milking and its milk concentration decreased under the maximum residue limit of 150 µg/kg [10] at the fifth milking. In this study, variations in kanamycin levels of each animal may be caused due to individual differences. There are limited studies evaluating the persistence of kanamycin in the milk of livestock animals. In one of these studies, it was reported that kanamycin was intramammary administered to four healthy Holstein lactating dairy cows. In this purpose, kanamycin monosulphate was infused to each quarter of cow with a single dose of 100.000 IU (=133 mg). The maximum concentration of kanamycin in the glandular tissue was determined as 44.3±10.7 µg/g at 2 h and kanamycin was still present in the udder at the concentration of 12.9±5.6 µg/g at 24 h. At the end of the experiment, they showed that large concentrations of this drug could be reached in the glandular tissue [18]. In another study, it was reported that withdrawal period of kanamycin determined as 4 milking after the intramuscular injection of kanamycin to ewes at the dose of 12 mg/kg twice daily for 3 days [23].

Milk is one of the most consumed foods of animal origin worldwide. However, misuse of antibiotics during treatment of milk-producing animals may cause antibiotic residues in their milk [7]. Aminoglycosides are potent antibiotics used in veterinary medicine [24]. This study also evaluated the food safety risk of buffalo milk marketed in Afyonkarahisar, Turkey regarding the presence of kanamycin residues. According to the results of the study, only 1 of 50 (2%) buffalo milk samples contained kanamycin at the concentration of 22.36 µg/kg. The level of the kanamycin residue in milk was under the established MRL level of 150 µg/kg for kanamycin in milk and this result can be considered as a positive sign regarding food safety. Similarly, Unusan [25] performed an field monitoring study based on an ELISA method specific for aminoglycosides (streptomycin) in Turkey and reported that only one (1.7%) of 60 ultra-heat-treatment milk samples contained aminoglycosides. de Oliveira et al.[26] analyzed a total of 299 pasteurized bovine milk samples collected from retail

markets in Brazil by ELISA kit and an LC–APCI–MS/MS QToF method and reported that none of the samples contained streptomycin and dihydrostreptomycin residues. Also, 192 raw milk samples were analyzed in Hebei Province of China for the presence of twenty-eight veterinary drug residues including kanamycin tested by ultraperformance liquid chromatography with tandem mass spectrometry method and they reported none of the milk samples was contaminated with kanamycin [27]. In a study from Italy, milk samples from 45 dairy farms were screened for the presence of antimicrobials during 12 months and reported that none of the milk samples contained aminoglycosides residues [28]. Vragovic et al.[29] reported that aminoglycoside (streptomycin) contamination ranged from 0 to 73.82 µg/kg for 75 milk samples collected from Croatian markets. In addition, Du et al.[30] analyzed a total of 198 milk samples collected from China and reported 15.1% of samples contained aminoglycoside antibiotic streptomycin with the highest value of 7.69 µg/kg. de Novaes et al.[31] surveyed a total of 961 milk samples for veterinary drug residues in Brazil and the results of their study showed that lower levels of neomycin and gentamicin were detected while no residues of streptomycin were detected in milk samples.

Consequently, we report here the milk kinetic of kanamycin for Anatolian buffaloes by a precise, reliable, and accurate LC-MS/MS method with simple sample preparation. The LOD and LOQ of the method were sufficient to detect kanamycin in milk under the maximum residue limits set by EU. The results of the study provide novel information for veterinarians regarding the withdrawal period of kanamycin in buffalo milk that is in line with the withdrawal time set for cattle by the EU. Also, the analysis of real milk samples indicated a very low residue risk regarding aminoglycoside antibiotic kanamycin in Afyonkarahisar Province of Turkey.

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